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RESEARCH ARTICLE

Plasmodium falciparum OTU-like cysteine protease (PfOTU) is essential for apicoplast homeostasis and associates with noncanonical role of Atg8

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Summary

The metabolic pathways associated with the mitochondrion and the apicoplast in *Plasmodium*, 2 parasite organelles of prokaryotic origin, are considered as suitable drug targets. In the present study, we have identified functional role of a novel ovarian tumour unit (OTU) domain-containing cysteine protease of *Plasmodium falciparum* (PfOTU). A C-terminal regulatable fluorescent affinity tag on native protein was utilised for its localization and functional characterization. Detailed studies showed vesicular localization of PfOTU and its association with the apicoplast. Degradation-tag mediated knockdown of PfOTU resulted in abnormal apicoplast development and blocked development of parasites beyond early-schizont stages in subsequent cell cycle; downregulation of PfOTU hindered apicoplast protein import. Further, the isoprenoid precursor-mediated parasite growth-rescue experiments confirmed that PfOTU knockdown specifically effect development of functional apicoplast. We also provide evidence for a possible biological function of PfOTU in membrane deconjugation of Atg8, which may be linked with the apicoplast protein import. Overall, our results show that the PfOTU is involved in apicoplast homeostasis and associates with the noncanonical function of Atg8 in maintenance of parasite apicoplast.

KEYWORDS

apicoplast, autophagy-linked protein Atg8, malaria, OTU-like protease, *Plasmodium*, regulatable fluorescent affinity tag

1 | INTRODUCTION

Malaria remains to be the most important parasitic disease in tropical and subtropical areas, caused by the protozoan parasite *Plasmodium spp*. Out of the five species of *Plasmodium* that infect humans, *Plasmodium falciparum* causes the most severe form of malaria. About 450,000 people died of malaria in 2015, with another 1.2 billion people are at high risk (WHO, 2015). Under these circumstances, characterization of new drug targets and designing of new pharmacophores become imperative. The apicoplast and the mitochondrion, two important organelles in the parasite, play essential roles in parasite survival, growth, and development; the metabolic pathways in these two parasite organelles are considered as potential drug targets due to their possible prokaryotic origin. Indeed, a number of antibiotics, which target prokaryotic transcription/translation machinery, possess antiparasitic efficacies (Goodman et al., 2014; Rosenthal, 2008; Schlitzer, 2007). Understanding of unique metabolic pathways, which are functionally important for maintenance and homeostasis of these organelles, may help us to identify novel drug targets. Organelle proteases have been shown to play essential roles in growth and segregation of parasite organelles, and these are suggested to be potential drug targets (Jain et al., 2013; Rathore et al., 2010; Tanveer et al., 2013). The apicoplast, relict plastid in the parasite, was acquired through secondary endosymbiosis during parasite evolution. Elucidation of the apicoplast metabolic networks has revealed the biosynthetic pathways essential for parasite survival, including haem biosynthesis and isoprenoid precursor (IPP) biosynthesis. In fact, IPP biosynthesis is shown to be the essential and critical role of the parasite apicoplast; IPP supplementation can rescue the parasite after treatment with antibiotics that cause loss of the apicoplast (Yeh & deRisi, 2011). Most of the genes encoded on the plastid genome have been transferred to the nucleus during evolution; proteins encoded by these nuclear genes are targeted to

the organelle. This trafficking pathway is essential for apicomplexan parasites, and its disruption can hamper growth and development of the apicoplast (Lindner, Meissner, Schettert, & Wrenger, 2013; Nair et al., 2011; Ramya, Karmodiya, Surolia, & Surolia, 2007a; Ramya, Mishra, Karmodiya, Surolia, & Surolia, 2007b; Spork et al., 2009; van Dooren, Tomova, Agrawal, Humbel, & Striepen, 2008).

Autophagy is an important catabolic process in which cytoplasm and damaged organelles are delivered to lysosomes for degradation and recycling. Autophagy is known to be involved in maintenance of cellular homeostasis, helps the cell to overcome stress, and acts as an immune evasion mechanism (Choy et al., 2012; Han et al., 2015; Mesquita et al., 2012). A number of autophagy-linked proteins (Atgs) are identified in eukaryotes that associate in different functional cascades. One of the most characterised cascades is the Atg8–Atg3 cascade, which is involved in autophagosome formation; the Atg8 is an ubiquitin-like (Ubl) protein, and during autophagy, it gets conjugated to phosphatidylethanolamine (PE) with the help of Atg7 and Atg3 (Nakatogawa, Ishii, Asai, & Ohsumi, 2012; Tanida et al., 2004). In higher eukaryotes, it has been suggested that deconjugation of Atg8 from PE plays an important role in regulation of autophagy. The *P. falciparum* homologue of Atg8 protein was characterised recently; however, it is suggested that it has adapted to play noncanonical role in trafficking of proteins to the apicoplast and organelle maintenance (Eickel et al., 2013; Tomlins et al., 2013). In addition, the canonical role of *Plasmodium* Atg8 in cellular processes such as ribophagy and piecemeal microautophagy of the nucleus has been also suggested (Cervantes et al., 2014). Further, it is also suggested that Atg8 plays a role in microneme clearance as well as autophagosome fusion similar to mammalian systems (Voss et al., 2016).

The ovarian tumour unit (OTU) domain-containing cysteine proteases belong to deubiquitinating enzyme (DUB) family, which have been reported to play nonclassical roles in cellular trafficking, DNA damage, and host–pathogen interactions (Hu et al., 2013; Millard & Wood, 2006; Reuven Wiener, Wang, & Wolberger, 2012; Seki et al., 2013; Sun & Dai, 2014). In this study, we have characterised a *P. falciparum* OTU-like cysteine protease (PfOTU) and studied its role during the asexual-stage parasite cycle. Functional characterization was carried out in transgenic parasites having regulatable fluorescent affinity (RFA) tag fused to the native PfOTU; we show that PfOTU is localised to the vesicular structures juxtaposed to the apicoplast during the asexual stages of the parasites. Further, using targeted degradation strategy, we show that PfOTU associates with apicoplast protein import and homeostasis. Our study also deciphers the cellular function of PfOTU in regulating the membrane-conjugated state of Atg8, thus its involvement in apicoplast maintenance.

2 | RESULTS

2.1 | Identification and sequence analysis of an OTU homologue in *P. falciparum*, PfOTU

In our attempt to understand functional role of parasite proteases, we identified a novel member of DUB family of protein in malaria parasite. The *P. falciparum* genome (PlasmoDB) harbours two genes, PF3D7_0923100 and PF3D7_1031400, which encode OTU domain (Pfam Accession No. PF02338) containing proteins; among these, PF3D7_1031400 is predicted to harbour an N-terminal apicoplast targeting sequence (five out of five tests are positive by PlasmoAP, including an enrichment for serine, threonine residues). This protein was chosen for further characterization and named as PfOTU; like other DUBs, PfOTU has a conserved DGNC motif and the OTU domain (56–167 aa) with the cysteine protease active site triad: cysteine, histidine, and aspartic acid residues (Figure S1a). Sequence alignment analysis showed that PfOTU shared high homology with OTU homologue in other protozoa, *Eimeria tenella* (45% identity, E-value $2.0e^{-17}$) and unicellular organism, *Chlamydomonas reinhardtii* (37% identity; E-value $2.0e^{-18}$), whereas it showed about 28% identity with homologue in *Drosophila melanogaster* (E-value $6.0e^{-6}$; Figure S1b). However, PfOTU lacks other motifs necessary for deubiquitinase activity such as ubiquitin-interacting motif (UIM) and ubiquitin-associated domain compared to homologues in other organisms (Figure S1c).

2.2 | Generation of transgenic parasites with C-terminal RFA tag in PfOTU

To understand the functional role of PfOTU in the parasite, we used a recently developed RFA tag system for C-terminal tagging of the native gene using the pGDB vector (Muralidharan, Oksman, Iwamoto, Wandless, & Goldberg, 2011). The RFA tag comprises of green fluorescent protein (GFP) in frame with *Escherichia coli* DHFR degradation domain (DDD) and hemagglutinin acid. The DDD system enables the folate analogue trimethoprim (TMP) to stabilise the fusion protein, whereas removal of TMP results in degradation of the fusion protein by proteasome machinery. The C-terminal fragment of *pfotu* (last 745 bp region except the stop codon) was cloned into the pGDB vector to generate the construct PfOTU-pGDB. Plasmepsin-1 knockout (PM1-KO) parasites (with hDHFR integrated into the genome; Liu, Gluzman, Drew, & Goldberg, 2005) were transfected with PfOTU-pGDB and selected over blasticidin. Successful integrants were obtained by on/off cycling of drug selection, and single parasite clones were isolated by serial dilution. The individual clones were assessed for integration of plasmid DNA at the *pfotu* locus by polymerase chain reaction (PCR) using total DNA with three sets of primers: (a) 1016A and 1017A; (b) 834A and 1007A; and (c) 882A and 1017A for the integration locus as shown in Figure 1a. The PCR amplification by primer set 882A-1017A showed a band of ~2.8 kb only in transgenic parasites suggesting the presence of a *pfotu* gene with RFA tag (Figure 1b). The expression and stabilisation of the fusion protein PfOTU-RFA were further assessed by Western blot analysis with both anti-GFP and anti-OTU antisera. A band of ~130 kDa corresponding to the fusion protein was detected in the transgenic parasites, whereas only the native protein of ~110 kDa was present in the PM1-KO parasites (Figure 1c).

2.3 | Localization of PfOTU in transgenic parasites by GFP targeting

Confocal microscopy of these transgenic parasites showed vesicular localization of the fusion protein; at ring and trophozoite stages,

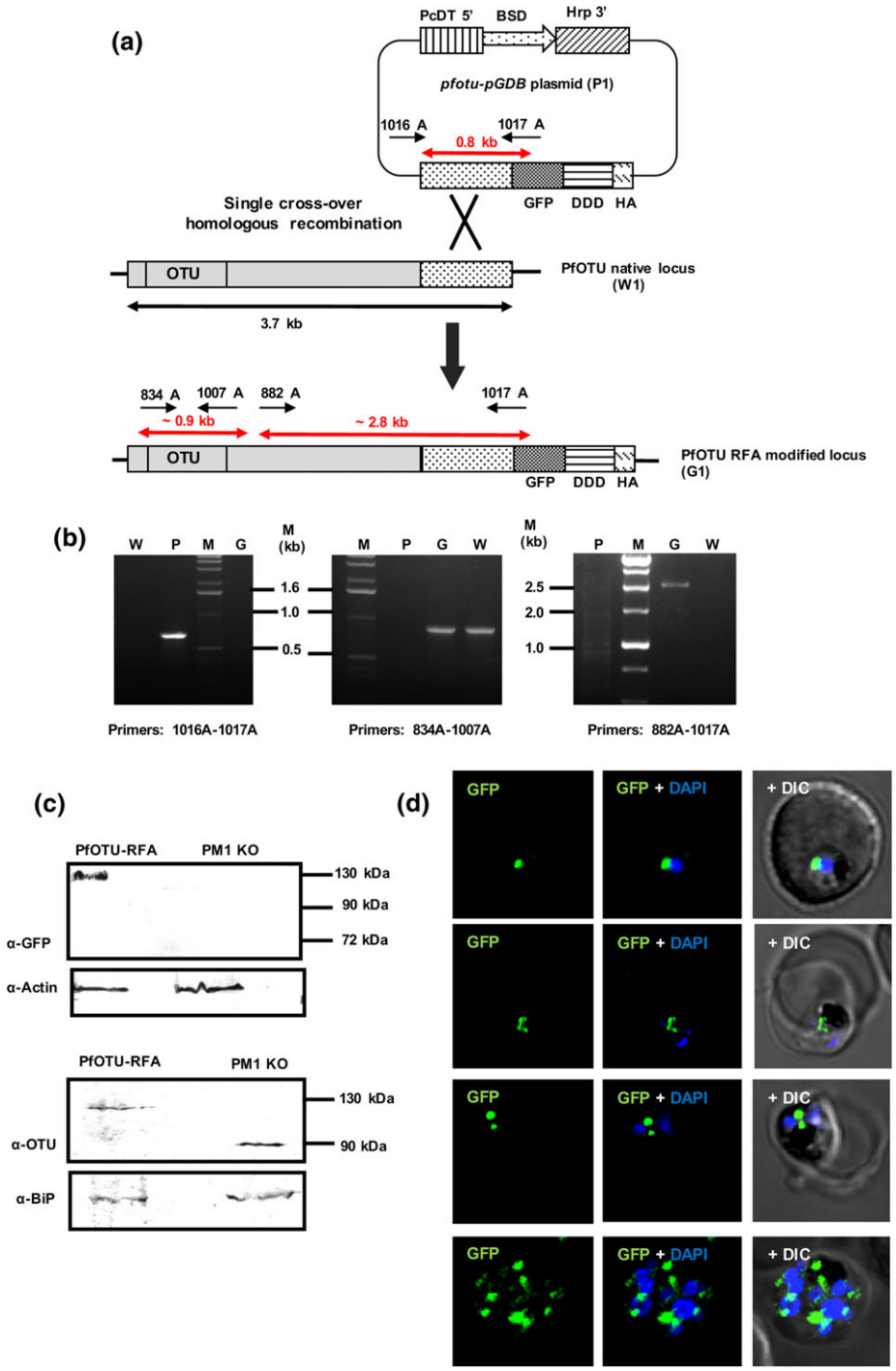

FIGURE 1 Integration at RFA-tag (DDD-GFP) with *pfotu* gene locus and localization of PfOTU-RFA fusion protein in transgenic parasites. (a) Schematic diagram showing predicted homologous recombination between pGDB-PfOTU construct and *pfotu* gene locus; primer locations along with their expected amplicon sizes in the gene loci or vector construct are indicated by solid red arrows. (b) PCR-based analysis of transgenic parasites using different primer combinations: Primers 1016A and 1017A; primers 834A and 1007A; primers 882A and 1017A. Mitochondrial DNA molecular weight ladder. Lanes P, G, and W represent PCR amplification using the *pfotu-pGDB* plasmid DNA, genomic DNA of transgenic RFA parasites, and genomic DNA of wild-type parasites as template. (c) Immunoblot analysis of transgenic PfOTU-RFA and untransfected PM1-KO parasite cultures. A single band of ~130 kDa corresponding to the size of the fusion protein was detected with anti-GFP antibodies only in PfOTU-RFA parasites as compared to untransfected PM1-KO parasites, whereas a band of ~130 and ~110 kDa was detected in PfOTU-RFA and PM1-KO parasites respectively using anti-PfOTU antibodies. Parallel blots probed with anti-actin or anti-BiP as loading controls. (d) Confocal microscopy images of live PfOTU-RFA parasites grown in the presence of TMP at early-, mid-, and late-trophozoite and schizont stages. The nucleus is stained with DAPI (blue). GFP = green fluorescent protein; DDD = DHFR degradation domain; HA = hemagglutinin acid; OTU = ovarian tumour unit; RFA = regulatable fluorescent affinity; PM1-KO = Plasmepsin-1 knockout; PCR = polymerase chain reaction

two or more GFP-labelled vesicle-like structures were present in the parasite cytosol (Figures 1D and S2). In schizonts, each of the developing merozoites contained one fluorescent vesicle. The specificity of the GFP fluorescence was confirmed by immunostaining of these parasites with anti-OTU antisera; the Pearson's coefficient was 0.9–1.0, suggesting complete colocalization (Figure S3). To further analyse the localization of PfOTU-RFA in the parasite, we carried out costaining studies of the apicoplast and the mitochondrion. The apicoplast was immunostained for both nuclear-encoded apicoplast proteins (PfClpP protease and PfoTpT [outer triose phosphate transporter]) and an apicoplast genome-encoded protein (PfEF-Tu, encoded by *tufA*; Chaubey, Kumar, Singh, & Habib, 2005; Mullin et al., 2006; Rathore et al., 2010). In all the cases, the PfOTU-GFP-labelled vesicles were observed to be in proximity or partially overlapping with apicoplast staining (Figures 2a,b and S4a,b). The 3-D image reconstruction based upon confocal Z-stacks showed these vesicles to merge into the apicoplast in some cases (Figures 2a,b and S4c). To further confirm spatial association of PfOTU with the apicoplast, we carried out immunoelectron microscopy using monoclonal anti-GFP antibodies. In trophozoite-stage parasites, the gold labelling was observed in membrane-bound vesicles of 100–200 nm diameter. In some sections, these labelled vesicles were present near the apicoplast, and in some cases, the immunostaining was observed at the apicoplast surface overlapping with its membrane (Figure 2c). Staining of transgenic parasites with MitoTacker Red CMXRos showed elongated/branched mitochondria whereas the GFP-labelled vesicles were present distinct from this organelle. The Pearson's correlation coefficient was calculated to be between 0.1 and 0.3 suggesting that there was no colocalization (Figures 2d and S5a). The confocal microscopy-acquired Z-stacks were reconstructed into 3-D images; this also showed that the vesicles were indeed distinct from the mitochondria (Figure 2d).

2.4 | Selective degradation of C-terminally tagged PfOTU inhibits parasite growth and disrupts intraerythrocytic parasite cycle

The presence of the *E. coli* DDD in the fusion protein allows selective degradation of the native protein upon removal of the stabilising ligand TMP. Synchronised ring-stage parasites were grown in the absence of TMP for 24 hr and analysed by flow cytometry as well as Western blot analysis; there was more than 90% reduction in GFP-labelled parasite population in this set as compared to parasites grown in the presence of TMP (Figure 3a). Western blot analysis of parasite lysates from the two sets (+TMP and −TMP) using anti-OTU antibodies showed reduction in levels of PfOTU-RFA fusion protein, thus confirming the selective degradation of RFA-tagged proteins in the absence of TMP. To study the effect of downregulation of PfOTU on parasite development, transgenic parasites were grown in the presence or absence of TMP (+TMP and −TMP set) and parasite growth and development was analysed over three development cycles. Parasite growth analysis, based upon the percentage of new rings formed, showed no significant effect on parasite growth at 48 hr (p = 0.39); the percentage of new ring-stage parasites were nearly the same in both the cultures. However, at 96 and 144 hpi, there was a major reduction (~40% and ~50% respectively; p = .003) in −TMP set as compared +TMP set (Figure 3b). Analysis of the developmental stage profile at different time points by Giemsa staining showed that in −TMP set, the parasites developed normally during the first cycle (48 hr) and formed new rings as in the control set (+TMP). These ring-stage parasites developed into trophozoites (72 hr) in both sets; however, in −TMP set, all of these trophozoites were not able to form mature schizonts (90 hr) and some of these parasites formed densely stained structures. These stressed parasites (~30%) remained as pyknotic structures and were not able to develop merozoites or form new rings in the next cycle (110 hpi; Figure 3c,d). This led to a significant reduction in overall parasitaemia (~35%–40%). This effect continued over the next cycle, and there was a further reduction in parasitaemia; by the end of the third cycle (144 hpi), parasites grown in the absence of TMP showed ~50% reduction as compared to the control set grown in the presence of TMP (Figure 3c,d).

2.5 | Reduction in PfOTU levels affects growth and segregation of the parasite apicoplast

The parasite growth reduction in the second cycle in −TMP set is reminiscent of the "delayed-death" phenotype, which is associated with developmental abnormalities in the apicoplast. Therefore, we assessed the effect of downregulation on PfOTU on apicoplast development. Parasites from +TMP and −TMP sets were immunostained for the apicoplast-encoded protein EF-Tu at different time points (36, 48, 72, and 90 hpi). The growth and development of the apicoplast in the −TMP culture was indistinguishable from that of +TMP during the first cell cycle; the apicoplast showed elongated morphology in trophozoite stages, branched pattern in the early-schizont-stage parasites and divided normally. However, during the second cell cycle, the growth and development of apicoplast was severely affected in −TMP set, the EF-Tu staining was observed to be faint, and apicoplast was observed as a single punctate structure even in late stages (Figure 4a). In a parallel set of experiment, staining with MitoTracker Red suggested no developmental abnormalities of mitochondria in both +TMP and −TMP sets (Figure S5b); these results suggested specific effect of downregulation of PfOTU on parasite apicoplast.

To quantitatively show this effect on apicoplast development after selective degradation of PfOTU in the parasite, we assessed replication of apicoplast genome as compared to the replication of nuclear genome in the +TMP and −TMP parasite sets. Quantitative PCR-based analysis was carried out to estimate the genomic equivalents of *tufA* gene of the apicoplast genome; in addition, genomic equivalents of *cox-3* gene of the mitochondrial genome were also estimated. The genomic equivalents of *tufA* and *cox-3* from −TMP set showed very little change as compared to control +TMP set in the first cycle (Figure 4b). However, in the second cycle, the apicoplast genomic equivalents showed about threefold reduction in −TMP set as compared to the +TMP set (Figure 4b). There was no significant effect on mitochondrial genome as the genome equivalents for *cox-3* between −TMP and +TMP set in these parasites (Figure 4b). To further confirm that the effect on parasite growth after downregulation of PfOTU is specific to the apicoplast, we

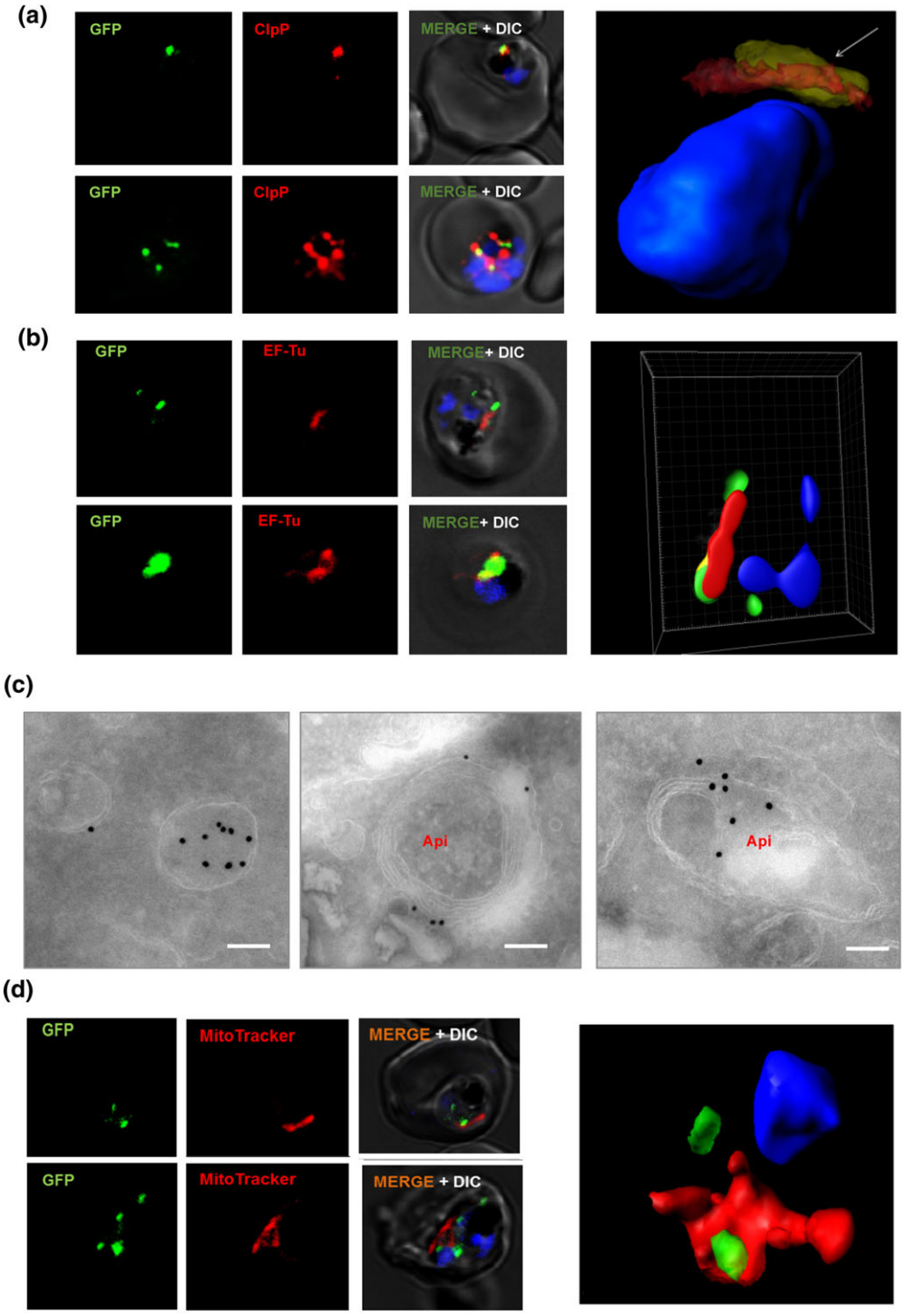

FIGURE 2 Subcellular localization studies of PfOTU in transgenic parasites. (a–b) Confocal microscopy images of transgenic parasites PfOTU-RFA (green) and immune stained for apicoplast-targeted protein ClpP (a) and apicoplast-resident protein EF-Tu (b). For each set, a three-dimensional reconstruction was developed using series of Z-stacks from confocal images with Imaris software. ClpP and EF-Tu staining are shown in red; the ClpP/EF-Tu and GFP colocalising area is seen as yellow staining. (c) Localization of PfOTU by immuneelectron microscopy. Ultra-thin sections of transgenic PfOTU-RFA parasites were labelled with anti-GFP antibody and gold-labelled secondary antibody. The labelling was seen in the cytosolic vesicles or associated with the apicoplast having characteristic four membranes. Scale bar: 100 nm. (d) Confocal microscopic images showing the MitoTracker staining (red) in PfOTU-RFA transgenic parasites; corresponding three-dimensional reconstruction using series of Z-stack images showing GFP-labelled vesicles (green) distinct from the mitochondria (red). GFP = green fluorescent protein; OTU = ovarian tumour unit; RFA = regulatable fluorescent affinity

carried out parasite rescue assays by IPP (isopentenyl pyrophosphate) supplementation. The −TMP parasite culture, when supplemented with IPP in the second growth cycle, showed nearly 80% recovery in growth (Figure 4c). These results clearly show that the selective downregulation of PfOTU disrupts apicoplast development and segregation.

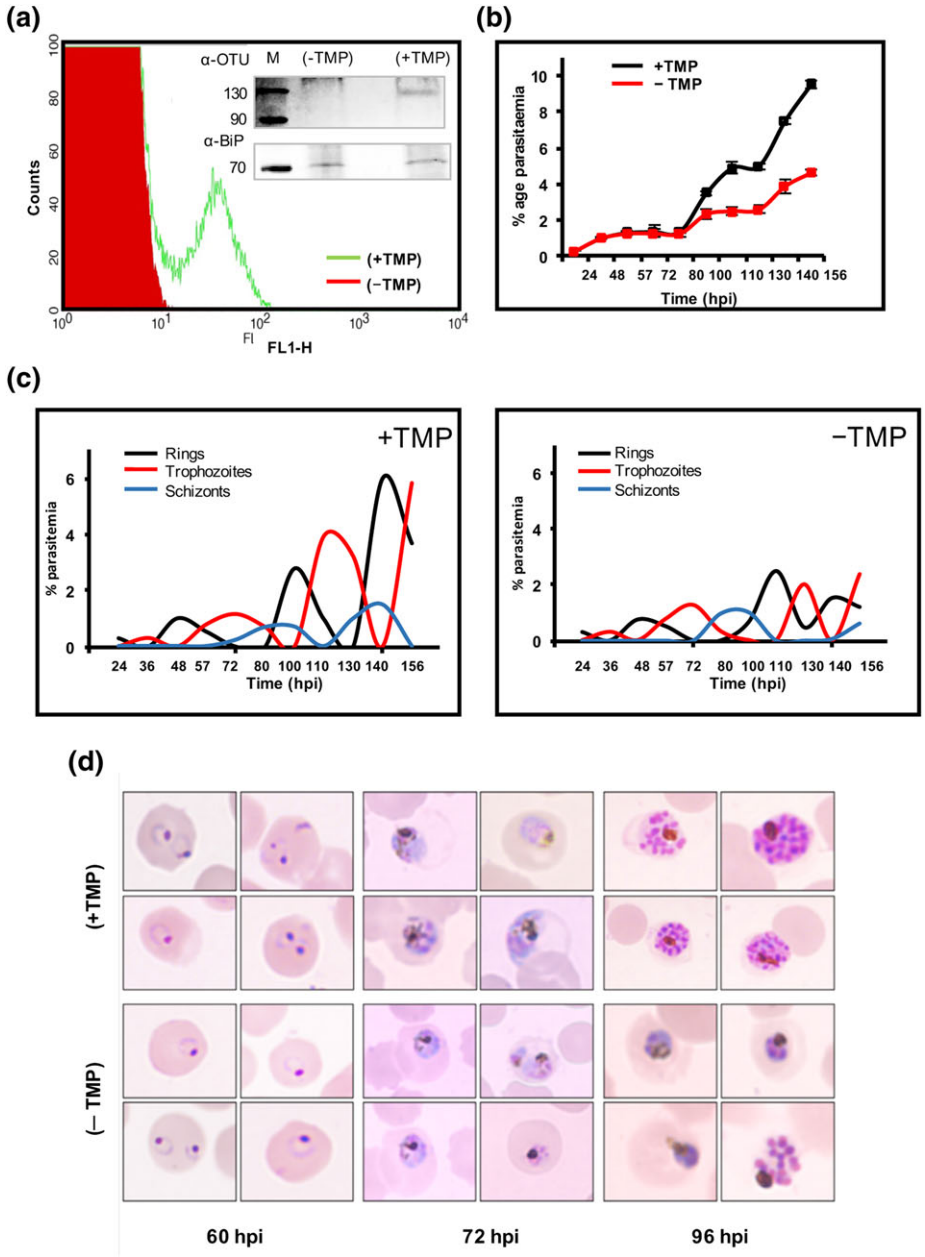

FIGURE 3 Selective degradation of PfOTU-RFA in the parasite leads to growth inhibition and disrupts asexual developmental cycle. (a) Downregulation of PfOTU-RFA protein in the parasites. Flow cytometry histogram showing GFP-labelled parasites populations for PfOTU-RFA parasite cultures grown with or without TMP (+TMP or −TMP). Immunoblot analysis of PfOTU-RFA parasite lysates (+TMP and −TMP sets) using anti-OTU antibodies is shown in the inset; equal loading shown by probing parallel blot with anti-BiP antibodies. M, molecular weight ladder (kDa). (b) Parasite growth shown as parasitaemia over three developmental cycles for PfOTU-RFA parasite cultures grown in the presence or absence of TMP (+ and −TMP sets). (c) Parasite stage composition at different time points (0–156 hr) for PfOTU-RFA transgenic parasite cultures grown with or without TMP (+TMP and −TMP sets). (d) Giemsa-stained parasites showing the effect on morphology for +TMP and −TMP sets at different time points. RFA = regulatable fluorescent affinity; TMP = trimethoprim; OTU = ovarian tumour unit

2.6 | PfOTU downregulation affects protein trafficking to the apicoplast

A number of apicoplast proteins are encoded by nuclear genome, which subsequently get trafficked and imported into the organelle (Foth et al., 2003; Ramya, Mishra et al., 2007b; Rathore et al., 2010). To assess any effect of PfOTU downregulation on this apicoplast import pathway, we first carried out immunostaining for a nuclear-encoded apicoplast protein, PfClpP, in both parasite sets (+TMP and −TMP) at trophozoite–schizont stages (36–44 hpi). In +TMP set, the parasites showed an apicoplast staining as an elongated structure in trophozoite stages, which segregated during the schizont stages with each developing merozoite having ClpP-labelled apicoplast; however in the −TMP set, a number of parasites (~70%) showed abnormal PfClpP staining pattern in the trophozoite stages, which appeared as either diffused or punctuate (Figure 5a,c); and in large number of schizonts (~80%), the staining was not associated with each developing merozoite (Figure 5a,b,d).

The PfClpP is known to get processed at the N-terminal during its import into the apicoplast; the full-length PfClpP protein is of ~40 kDa (precursor form), which gets processed into a ~23-kDa mature form in

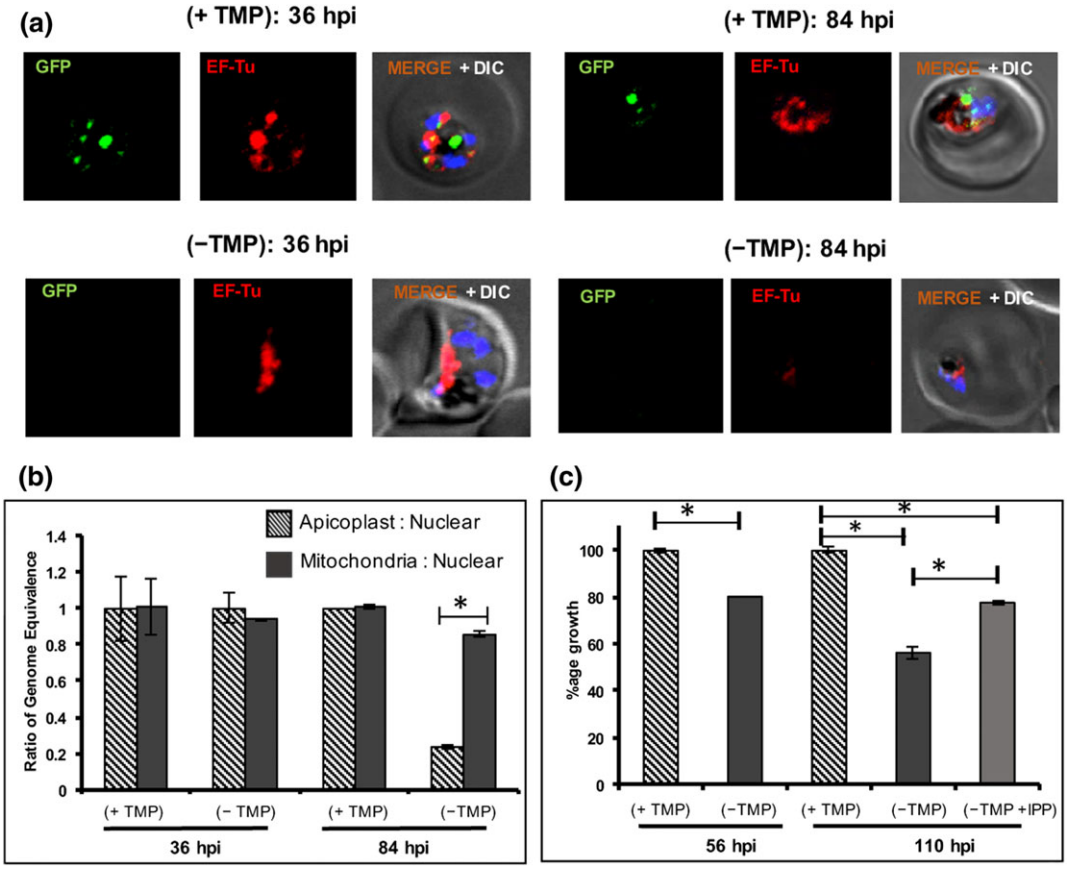

FIGURE 4 Downregulation of PfOTU effects apicoplast development and segregation. (a) Fluorescent microscopy images of PfOTU-RFA parasites immunostained with anti-EF-Tu (red) antibodies at different developmental stages (ring, tropozoite, and schizonts) during first and second growth cycle for +TMP and −TMP set. (b) Quantitative PCR-based analysis using total DNA isolated from PfOTU-RFA parasites grown in the presence or absence of TMP (+TMP and −TMP sets) during the first and second cycle (36 and 84 hpi); graph showing normalised genomic equivalents calculated for *clpP* gene present on nuclear genome, *tufA* present on apicoplast genome, and *cox-3* present on mitochondrial genome. Apicoplast to nuclear genome ratio was reduced in the second cycle, whereas mitochondrial to nuclear genome ratio is unaffected. (c) IPP supplementation is able to rescue parasite growth inhibition due to downregulation of PfOTU in −TMP set (−TMP + IPP). IPP was supplemented at 48 hpi, and parasite growth was assessed for two growth cycles. The *p* values were calculated by Student's *t* test: $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$. PCR = polymerase chain reaction; TMP = trimethoprim; IPP = isopentenyl pyrophosphate; OTU = ovarian tumour unit; RFA = regulatable fluorescent affinity; GFP = green fluorescent protein

the apicoplast (Chaubey et al., 2005; Rathore et al., 2010). We followed this processing of PfClpP as a measure of protein trafficking/import into the apicoplast. Western blot analysis of parasites in the +TMP set using anti-PfClpP antibodies detected only a single band of ~23 kDa corresponding to mature protein (Figure 5e). However, in the −TMP set, two bands of ~40 and ~23 kDa corresponding to the precursor and mature protein respectively (pClpP and mClpP) were detected (Figure 5e). This implies that downregulation of PfOTU in turn results in reduced apicoplast protein import drug the first cycle. As a control, the same parasite lysates were probed with anti-PfClpQ antisera, a nuclear-encoded mitochondrial protein. In both the +TMP and −TMP sets, two bands of ~23 and ~18 kDa (precursor and mature forms of PfClpQ, respectively) were detected (Figure 5f), suggesting that there is no defect in mitochondrial protein import.

2.7 | PfOTU is involved in Atg8-mediated apicoplast maintenance

Our data showed that selective degradation of PfOTU disrupts protein import into apicoplast and its segregation in subsequent cycle. The autophagy-related protein (Atg8) in *P. falciparum* localises to vesicular structures and also associates with the apicoplast; it has been suggested that it plays a role in protein trafficking to the apicoplast in *Plasmodium* as well as in *Toxplasma* (Cervantes et al., 2014; Lévêque et al., 2015; Tomlins et al., 2013). We assessed any functional association of PfAtg8 and PfOTU in maintenance of the apicoplast. We first carried out immunostaining of PfOTU-RFA parasites with anti-Atg8 antibodies. The immunostaining pattern showed that the anti-Atg8 antibodies recognised a number of closely associated vesicular structures and a large organellar structure, the apicoplast, as shown in earlier studies (Kitamura et al., 2012; Tomlins et al., 2013). The GFP-labelled PfOTU vesicles were found to be closely associated with these vesicular structures and apicoplast having Atg8 labelling (Figure 6a). An OTU-like cysteine protease in *Legionella*, RavZ protease, is shown to be play role in regulating levels of Atg8 on PAS (pre-autophagosomal structure) surfaces; this RavZ protease cleaves the amide bond between C-terminal glycine and an adjacent residue to delipidate it from PAS membrane (Choy et al., 2012b). We explored the possible role of PfOTU in regulating levels of lipidated Atg8 in *Plasmodium*. The level of lipidated and

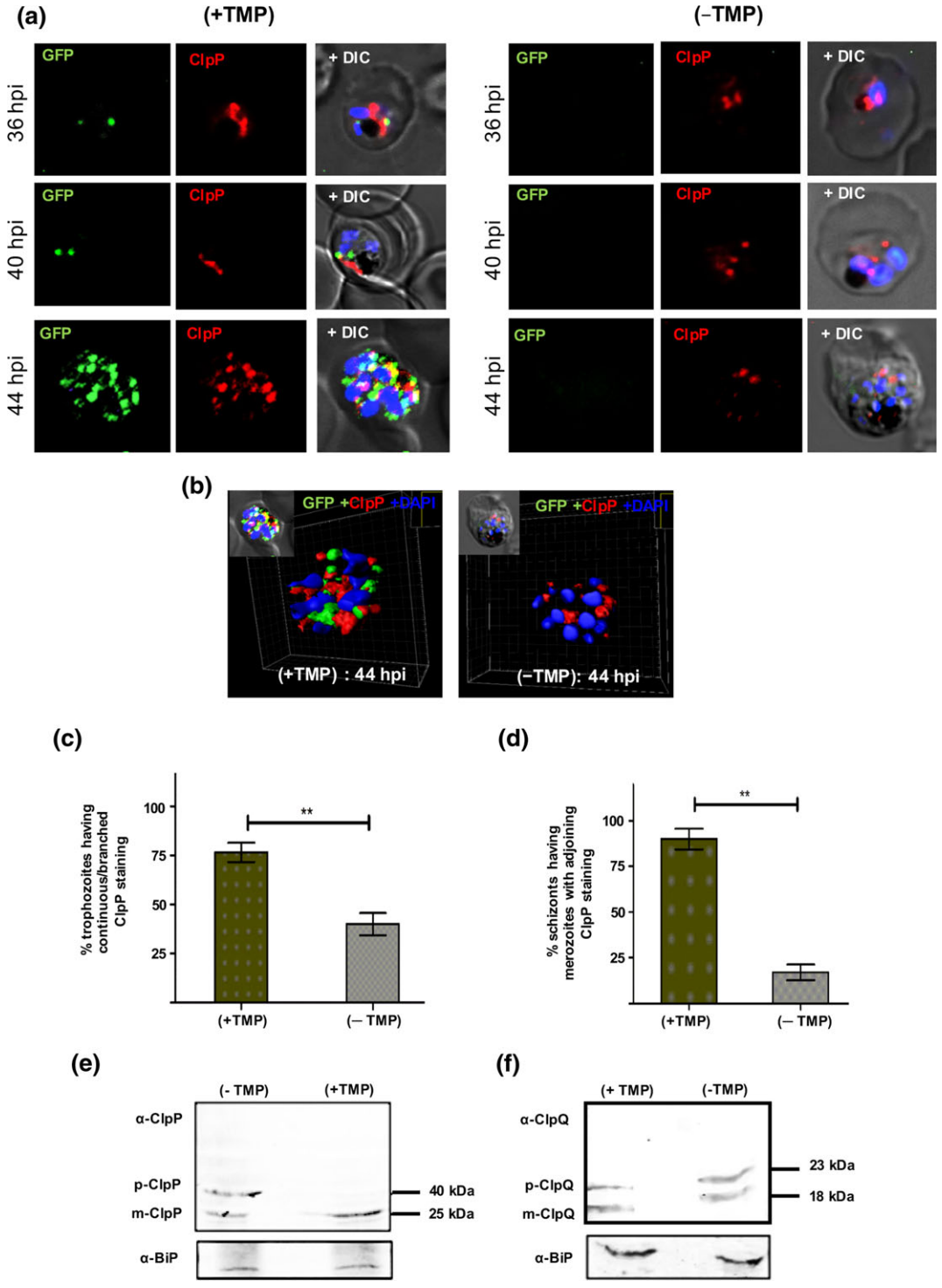

FIGURE 5 Apicoplast-targeted protein import is disrupted after PfOTU downregulation. (a) Fluorescent images of PfOTU-RFA parasites grown in the presence or absence of TMP (+TMP and −TMP sets) and immunostained for apicoplast-targeted protein ClpP (red) during the first growth cycle showing disrupted apicoplast staining for −TMP set. (b) Three-dimensional reconstruction using series of Z-stack images of schizont-stage parasites immunostained for ClpP. (c) Bar graph showing percentage of trophozoites with complete/branched ClpP staining in +TMP and −TMP sets (p value: .0019; n = 20). (d) Bar graph showing percentage of schizont-stage parasites having ClpP staining associated with each developing merozoite in +TMP and −TMP sets (p value: .0036; n = 20). (e–f) Immunoblot analyses of PfOTU-RFA parasites grown with or without TMP (+TMP and −TMP sets), using antibodies for apicoplast-targeted ClpP protein (anti-PfClpP antibody; e) and mitochondrial targeted ClpQ protein (anti-PfClpQ antibody; f). Bands representing the precursor and mature forms for each protein are labelled with p- and m-, respectively. The p values were calculated by Student's t test: $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$. OTU = ovarian tumour unit; RFA = regulatable fluorescent affinity; TMP=trimethoprim

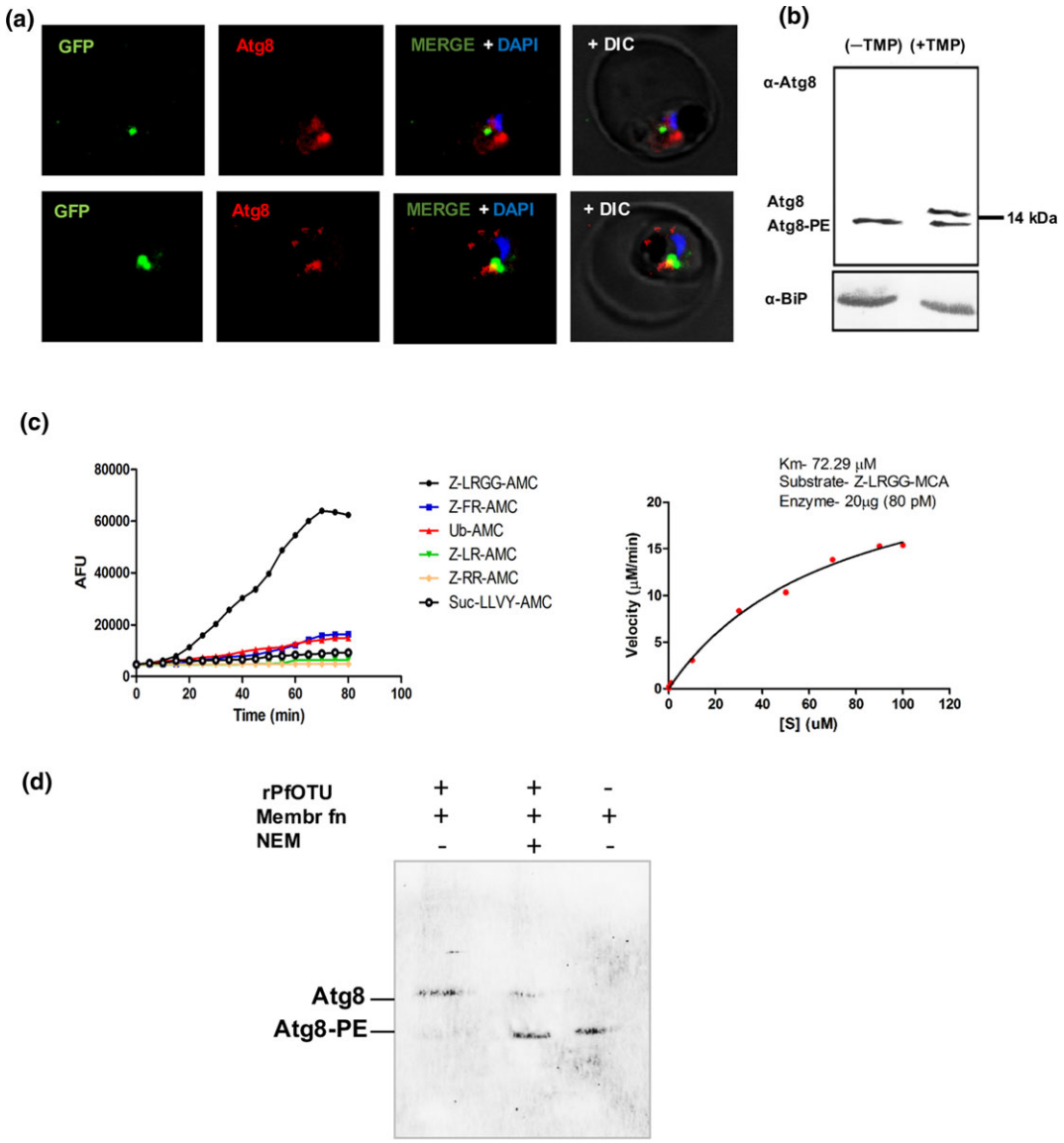

FIGURE 6 PfOTU is involved in Atg8-mediated apicoplast maintenance. (a) Confocal microscopy images of PfOTU-RFA parasites immunostained with anti-Atg8 antibodies (red); PfOTU and Atg8 labelling showed close association in the parasite. (b) Immunoblot analysis of PfOTU-RFA parasite lysates from +TMP and −TMP sets, separated on 13.5% urea SDS-PAGE and probed with anti-Atg8 antibodies; both lipidated (Atg8-PE) and delipidated (Atg8) forms of the protein were detected in +TMP set, whereas only lipidated form is detected in −TMP set. (c) Protease activity of recombinant PfOTU with different fluorogenic peptide substrates, the change in fluorescence (arbitrary fluorescence units [AFU]) due to release of free AMC over time is shown. The recombinant protein demonstrated maximum activity against Z-LRGG-AMC, with a *K*m of 72.29 μM, and this activity could be inhibited by the cysteine-alkylating agent N-ethylmaleimide. However, no activity could be detected with deubiquitination specific substrates (Ub-AMC) or other cysteine protease substrates (Z-FR-AMC, Z-LR-AMC, and Z-RR-AMC). Michaelis–Menten fit for PfOTU proteolytic activity with Z-LRGG-AMC is plotted for 30 min. (e) Delipidation of Atg8-PE from purified parasite membrane fraction (Membr fr) using recombinant PfOTU. Parasite membrane fraction treated with recombinant PfOTU with or without NEM, reaction mixture was subsequently analysed by immunoblotting using urea SDS-PAGE and anti-Atg8 antibodies to detect lipidated (Atg8-PE) and delipidated (Atg8) forms as described above. PE = phosphatidylethanolamine; GFP = green fluorescent protein; OTU = ovarian tumour unit; RFA = regulatable fluorescent affinity; TMP = trimethoprim

nonlipidated Atg8 forms were analysed in +TMP and −TMP sets; parasite lysates were separated on urea PAGE and probed with anti-Atg8 antibodies. The Atg8 was found to be nearly equally present as both lipidated (Atg8-PE, a faster migrating band) and nonlipidated (a slower migrating band) in the parasites from the +TMP set as shown earlier for wild-type parasites (Tomlins et al., 2013); however, in the −TMP parasites, the Atg8 was majorly detected as a single band (Atg8-PE; Figure 6b). This shows that downregulation of PfOTU reduces levels of delipidated Atg8 in the parasite.

To further confirm the role of PfOTU in Atg8 delipidation, we designed an in vitro activity assay using PfOTU protease and membrane-associated native Atg8 isolated from the parasite. The recombinant PfOTU protein was expressed using *E. coli* system and purified by affinity chromatography (Figure S6a), the protease activity of recombinant PfOTU was established using small peptide substrates, which could be inhibited by the cysteine alkylating agent N-ethylmaleimide (NEM; see Data S1, Figures 6c and S6d). The parasite membrane fraction was purified, which contains the lipidated form of Atg8; this was

used as substrate in PfOTU activity assay with or without the NEM inhibitor; Western blot analysis using urea PAGE and anti-Atg8 antibodies detected delipidated Atg8 as predominant in the reaction mixture after PfOTU activity (Figure 6d, lane 2), whereas the control reaction, without PfOTU, showed only the lipidated form of Atg8 (Figure 6d, lane 4). Further, NEM was able to inhibit the activity of recombinant PfOTU and both lipidated and delipidated Atg8 could be detected in the reaction mixture (Figure 6d, lane 3). These results show that PfOTU is directly involved in the delipidation of PfAtg8.

3 | DISCUSSION

One of the key steps towards development of new antimalarials is identification of unique and essential metabolic pathways in the parasites. In this respect, the metabolic pathways in the relict plastid of *Plasmodium*, the apicoplast, are considered as attractive drug targets. Here, we report the functional characterization of a novel cysteine protease, PfOTU, which is associated with the parasite apicoplast. The OTU domain-containing proteases belong to family of DUBs and Ubl-targeting protease (Balakirev, Tcherniuk, Jaquinod, & Chroboczek, 2003; Borodovsky et al., 2002). These are cysteine proteases that play a role in DNA repair and modulation of signalling pathways (Wang et al., 2009). Multiple reports have suggested that human OTU1 interacts with components of the ERAD (endoplasmic reticulum-associated degradation) pathway and serves to modulate substrate degradation (Sowa, Bennett, Gygi, & Harper, 2009, Wickliffe et al., 2011). It is also shown that the yeast OTU1 interacts with Cdc48, a component of the ERAD pathway (Messick et al., 2008; Rumpf & Jentsch, 2006). Components of the ERAD pathway are shown to be localised in the apicoplast of both *Plasmodium* and *Toxoplasma* (Spork et al., 2009; Park et al., 2015).

An *in silico* analysis of various components of the ubiquitination pathway among Apicomplexans predicted members of the OTU family to be present in the *P. falciparum* (Ponts et al., 2011). In order to study the role of this enzymes family in the parasite's life cycle, we characterised a putative OTU protease in *P. falciparum* (Gene ID: PF3D7_1031400). Sequence analysis showed the presence of an OTU-like protease domain with conserved active site residues—Cys, His, and Asp—and also contains a putative apicoplast transit peptide (Foth et al., 2003). However, it lacks motifs necessary for deubiquitinase activity including ubiquitin-interacting motif and ubiquitin-associated domain. Indeed, the in vitro activity analysis using recombinant PfOTU protein showed that it harbours cysteine protease activity but lacks deubiquitinase activity.

To study the localization and functional role of PfOTU in the parasite, we used the RFA-tag system for C-terminal tagging of the native gene, so that the transgenic parasites express PfOTU fused with a GFP and DDD (Muralidharan et al., 2011). The RFA tag can be utilised for localization of the native protein as well as to downregulate the protein levels in the parasite. Because the *P. falciparum* DHFR is sensitive to TMP, the background parasite line should contain hDHFR gene that is resistant to it; therefore, PM1-KO parasites (with hDHFR integrated into the genome, but without any deleterious effect; Liu et al., 2005) were used for transfection of vector constructs. We obtained successful integrants after two drug cycles and confirmed genome integration at the *pfotu* locus. Confocal microscopy showed vesicular localization of PfOTU-GFP; these vesicles were observed near the nucleus during the early stages and distributed in the cytosol during the late stages. Detailed costaining studies showed these vesicular structures associate with the apicoplast in mature trophozoite stages. The immunoelectron microscopy studies confirmed association of PfOTU-GFP with cytosolic vesicles as well as with the apicoplast. As in case of plant plastids, most of the apicoplast resident proteins in *Plasmodium* as well as in *Toxoplasma* are encoded by nuclear genome; these proteins gets targeted to the apicoplast via the secretory pathway using a bipartite N-terminal sequence. These proteins are trafficked into the apicoplast by a two-step process: First, the signal peptide directs entry into the endomembrane system and is then cleaved off; these "endomembrane derived vesicles" are then routed to the apicoplast in a Golgi-independent way where they dock at the apicoplast surface (Tonkin et al., 2006). The apicoplast is surrounded by four membranes, and import process for these protein represents a major cell biological challenge. A protein translocon, endoplasmic reticulum associated protein degradation (ERAD), is capable of transporting proteins across a biological membrane, and it is typically associated with retranslocation of incorrectly folded protein substrates to the cell cytosol for degradation by the ubiquitin (Ub)-proteasome system. In *Plasmodium*, components of ERAD have been localised to the apicoplast and suggested to be involved in apicoplast protein import through the outer membranes (Agrawal et al., 2013; Kalanon, Tonkin, & McFadden, 2009; Spork et al., 2009). Further entry into the apicoplast lumen through the inner membranes of apicoplast is mediated by the Tic/Toc protein complex followed by subsequent cleavage of the transit peptide (Tonkin et al., 2008; Agrawal, van Dooren, Beatty, & Striepen, 2009; Glaser et al., 2012). The localization of PfOTU-RFA vesicle-like structures resembles with the apicoplast-associated vesicular localization of a thioredoxin family protein, Atrx1, which show a circumplastid localization in *Toxoplasma* (DeRocher et al., 2008). We could not detect two bands of PfOTU proteins in the Western blot, which may suggest its N-terminal processing; one reason could be that the PfOTU is not trafficked inside the apicoplast matrix, and thus, it is not exposed to the protein-uptake/processing machinery in the apicoplast inner membranes.

The selective degradation of PfOTU-RFA confirmed functional association of PfOTU with the apicoplast; these parasites showed defects in the development of apicoplast and delayed death. The apicoplast cannot be synthesised *de novo* and must be inherited from the parental generation, and therefore, the delayed death effect is inherent of apicoplast-specific drugs. Detailed morphological and growth analysis showed that in subsequent cycle after downregulation of PfOTU, the trophozoites were not able to develop into mature schizonts and appeared as densely stained pyknotic structures. Similarly, growth and development of the apicoplast in the −TMP culture was indistinguishable from that of +TMP during the first cell cycle; however, during the second cell cycle, the growth and development of apicoplast was severely affected in −TMP set, and apicoplast was observed as a single punctate structure even in late stages. These effects in turn result in loss in nuclear to apicoplast genome ratio. These effects are similar to the one showed by apicoplast-targeting

drugs such as doxycycline and clindamycin (Dahl & Rosenthal, 2007; Dahl et al., 2006; Goodman & McFadden, 2007). Parasites treated with doxycycline or clindamycin grow through the first cycle normally without any effect on apicoplast, which divide normally and canonical to the nuclear division; however, in the subsequent cycle, the apicoplast elongation branching and segregation gets disrupted, causing parasite death in that cycle (Dahl et al., 2006; Goodman & McFadden, 2007). Similarly, inhibition of apicoplast-associated ClpP protease using specific inhibitor caused hindrance in growth and segregation of apicoplast in subsequent cycle resulting parasite growth inhibition (Rathore et al., 2010).

Immunostaining for apicoplast-encoded (EF-Tu) and nuclear-encoded apicoplast protein (ClpP) suggested hindrance in trafficking/import of nuclear-encoded proteins, as also confirmed by assessment of processing of ClpP protease, during the first cycle. Indeed, interference in protein trafficking to the organelle has been shown to cause defect in apicoplast growth (Ramya, Karmodiya et al., 2007a; Ramya, Mishra et al., 2007b). This effect of selective PfOTU downregulation was ascertained to be specific for the apicoplast by IPP supplementation experiments, which was able to rescue the parasites from growth inhibition even when IPP was added 48 hr after TMP removal. This is in agreement with an earlier report that addition of IPP, a product of the nonmevalonate IPP biosynthesis pathway, is sufficient to rescue ~80% of parasite viability after treatment with doxycycline, which effects the development of functional apicoplast (Yeh & DeRisi, 2011).

The OTU is a thiol protease belonging to DUB/Ubl-specific protease (ULP) family; members of this family are involved in cleavage of ubiquitin or Ubls from substrate protein as well as processing of their C-terminally extended precursors forms (Ronau et al., 2016). It is suggested that it is difficult to predict their specificity to cleave ubiquitin or Ubls based upon primary sequence of the enzyme. Recently, an OTU homologue in *Toxoplasma gondii*, TgOTUD3, is shown harbour deubiquitination property and it was able to cleave monoubiquitinated targets (Dhara & Sinai, 2016). Our biochemical analysis showed that PfOTU is not a DUB; however, it demonstrated protease activity using a small peptide substrate (LRGG). This peptide corresponds to the conserved C-terminal sequence in ubiquitin and many Ubls, which is involved in interaction of ubiquitin/Ubl with the active site cleft of the DUB/ULP protease (Nicholson et al., 2008; Welchman, Gordon, & Mayer, 2009). One of the Ubl proteins, Ubl-LC3/Atg8 protein, contains this conserved C-terminal sequence and it is known to be involved in autophagosome formation in eukaryotic cells. A recent study reported the role of an OTU protease (RavZ) in inhibiting host autophagy by regulating the levels of LC3/Atg8 on PAS (Choy et al., 2012). This protease cleaves the peptide bond between Atg8 C-terminal glycine and the adjacent aromatic amino acid residue, which renders Atg8 incapable of further conjugation to PE. In *P. falciparum*, the Atg8 protein has been reported to be associated with the apicoplast and suggested to be involved in maintenance of this organelle (Kitamura et al., 2012; Tomlins et al., 2013). We also found that the Atg8 localises to both the vesicle-like structures as well as with the apicoplast in *P. falciparum* parasites. In contrast to LC3/Atg8 system in other eukaryotes, the *Plasmodium* Atg8 already has a C-terminal-exposed glycine and does not require cleavage by Atg4 to get attached to PE. The OTU-like RavZ protease is also shown to associate with the phosphatidylinositol 3-monophosphate (PI3P) rich membrane regions in PAS (Horenkamp, Kauffman, Kohler, Sherwood, et al., 2015). In *P. falciparum*, the PI3P is shown to be localised in the food vacuole membrane and the apicoplast membrane (Tawk et al., 2010). Indeed, the PfOTU labelling is observed in proximity to the apicoplast and IEM studies also suggested its association with the apicoplast membrane; however, some of the PfOTU-labelled vesicles were also present close to the food vacuole region. The *Legionella* protein RavZ has been shown to interact with PI3P through a binding pocket in its C-terminal that is rich in basic residues (Horenkamp et al., 2015). Sequence analysis of the of PfOTU revealed a C-terminal region rich in basic amino acid residues (lysine and arginine); an electrostatic potential map of this region of PfOTU (812–920 aa) shows that it forms a positively charged pocked as a putative PI3P binding site (Figure S7).

We assessed any association of PfOTU levels and Atg8 lipidation status; downregulation of PfOTU reduced the delipidation of Atg8 in the parasite. Further, we also showed direct activity of PfOTU to delipidate the membrane-associated native Atg8. This data suggest that the PfOTU plays a RavZ-like role in the parasite and regulate the levels of free Atg8 and Atg8-PE. Our results correlate with the predicted function of Atg8 in trafficking of apicoplast-bound vesicles to the developing apicoplast (Tomlins et al., 2013); fusion of these Atg8-labelled vesicles with the apicoplast may depend upon deconjugation of Atg8 from vesicles/apicoplast. Downregulation of PfOTU disrupts the Atg8 deconjugation process, which in turn hinders the import of apicoplast proteins. Indeed, it is not uncommon for some *Plasmodium* proteins to gain new functions suited to specific requirements of an intracellular parasite. Overall, our data show that PfOTU plays an essential role in apicoplast homeostasis by regulating of the noncanonical function of Atg8 in protein trafficking/import.

4 | EXPERIMENTAL PROCEDURES

4.1 | Parasite culture, plasmid construct, and parasite transfection

P. falciparum 3D7, PM1-KO (Liu et al., 2005), and transgenic parasites were cultured with 4% haematocrit in RPMI media (Invitrogen Corp., San Diego, CA, USA) supplemented with 0.5% albumax and 0.01% hypoxanthine, using a protocol described previously (Trager & Jensen, 1976). Parasite cultures were synchronised by repeated sorbitol treatment following Lambros and Vanderberg (1979). To generate a transfection vector construct, the C-terminal fragment of *pfotu* (PF3D7_1031400.1, last 745 BP except the stop codon) was cloned into pGDB vector at the *Xho*I and *Avr*II restriction sites to give the construct PfOTU-RFA. Synchronised ring-stage PM1 KO parasites were transfected with 100 μg of PfOTU-RFA plasmid by electroporation (310 V, 950 μF; Crabb et al., 2004); transfected parasites were selected over blasticidin (BSD, Calbiochem; 2.5 μg/ml) and TMP (5 μM, Sigma-Aldrich). Cycling of selection drug (BSD; 3 weeks on and 3 weeks off) was carried out for selection of parasites with plasmid integrated in the main genome. After the second cycle, parasites were analysed for plasmid integration and positive clones were isolated by limiting dilution method.

4.2 | *P. falciparum* growth analysis and transient downregulation of PfOTU-RFA

For growth assay, tightly synchronised ring-stage parasites were washed thrice with incomplete media, diluted to 0.3%–0.4% parasitaemia in 2% haematocrit and allowed to grow in media with (+TMP) or without (−TMP) for three developmental cycles. Thin smears were made from each well at different time points and stained with Giemsa for microscopic analysis; parasite growth and morphology was monitored at the indicated time points. To assess the effect of IPP on the parasite growth after downregulation of PfOTU, the IPP (Isoprenoids LC) was added to the assay at a final concentration of 200 μM.

The expression of fusion protein in *P. falciparum* transgenic PfOTU-RFA parasites was assessed by flow cytometry. Synchronised parasites at trophozoite stages (28–32 hpi) were cultured in the presence or absence of TMP for 4 hr. These parasites were then pelleted down, washed thrice with 1× phosphate-buffered saline (PBS; pH 7.4) and stained with ethidium bromide (1.0 μg/ml) for 30 min at 37 °C in dark, followed by washing with 1× PBS. The stained samples were analysed by flow cytometry on FACS Calibur (Becton Dickinson, San Jose, CA, USA) and fluorescence staining (Em-525/Ex-488 nm; Em-590/Ex-570 nm) of infected red blood cells was assessed using CellQuestPro software.

4.3 | Isolation of total DNA, RNA, complementary DNA synthesis, and quantitative real-time PCR and Western blot analysis

Total genomic DNA was isolated from PfOTU-RFA parasites (+TMP and −TMP sets) at different stages, along with untransfected PM1-KO parasites as control following standard protocol (Kirsten, Inger, Hedvig, Artur, & Wahlgren, 2015). Gene-specific primer sets were designed using Beacon Designer 4.0 software for each organelle and nuclear genome: *tufA* (apicoplast, PF3D7_1348300), *cox3* (mitochondria, mal_mito_1), *clpP* (nuclear, PF3D7_0307400), as well as for 18S *rRNA* (housekeeping gene as control; primer details are given in Table S1). The amplification reaction contained 100-ng template genomic DNA, 2× Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and 300-nM gene-specific primers. Real-time PCR was performed in Micro Amp optical 96-well plates in automated ABI Step one Plus Version. Threshold cycle (Ct) values were calculated using SDS 2.4 Software (Applied Biosystem). Standard curves were used to determine genome equivalents of Ct values for respective gene and 18S ribosomal RNA for each sample. Genome equivalents of each gene was normalised using that of 18S ribosomal RNA for all the samples. For +TMP and −TMP parasite sets, the organelle:nuclear genome ratio was calculated relative to that of control sample.

For Western blot analyses, parasites were isolated from different experimental sets by saponin lysis (0.15%). Total parasite lysates were separated by SDS-PAGE under reducing conditions before proteins were transferred to PVDF membrane using a Transblot Wet transfer system (Bio-Rad) according to manufacturer's instructions. The membranes were blocked in blocking buffer (1× PBS, 0.1% Tween 20, 5% milk powder) for 2 hr. The blots were washed and incubated for 1 hr with a primary antibody (rabbit anti-ClpP, Rathore et al., 2010); rabbit anti-ClpQ (Jain et al., 2013); rabbit anti-PfoTpT (Mullin et al., 2006); and mouse anti-Atg8 (developed against conserved region of *Plasmodium* Atg8, Eickel et al., 2013) each used at 1:1000 dilutions. The secondary anti-mouse-HRP conjugate or anti-rabbit-HRP conjugate (Promega, Madison, Wisconsin, USA) antibodies were used at 1:3000 dilutions for the respective blots. The protein bands reacting with the antibodies were detected using the Amersham ECL detection kit and visualised by exposing blots to autoradiography films (Kodak). The lipidation status of Atg8 was assessed following Zens, Sawa-Makarska, and Martens (2015). Briefly, the parasite lysates were separated on 13.5% SDS-PAGE with 6 M urea and the lipidated and delipidated forms of Atg8 were detected by standard Western blot analysis as described above.

4.4 | Expression of recombinant protein and generation of polyclonal antisera

A fragment of the *pfotu* gene containing the protease domain (39–236 aa) was cloned in pET28a expression vector (Novagen) to express corresponding recombinant protein. Detailed method of expression and purification of recombinant proteins is given in the Supporting Information.

To generate polyclonal sera against PfOTU, female BALB/c mice were immunised (on day 0) with the purified recombinant protein (25 μg) formulated in complete Freund's adjuvant (Sigma, USA). The mice were administered two booster doses (days 14 and 28) of the proteins formulated in Freund's incomplete adjuvant. The mice serum was collected 10 days after the second boost. Two New Zealand white rabbits (3 months old) were immunised in the same way with 250 μg of recombinant protein (on day 0) and administered two booster doses (on days 28 and 49). The rabbit sera were collected on day 56. All animal experiments were conducted in accordance with the guidelines approved by the Institutional Animals Ethics Committee of ICGEB, New Delhi, India.

4.5 | Protease activity assays and enzyme kinetics

Fluorometric assays for protease activity of recombinant PfOTU were carried out using different fluorogenic peptide substrates in 200-μl reaction volume in a black 96-well plate (Nunc, USA) containing recombinant protein in assay buffer in the absence or presence of an inhibitor. Detailed method for enzyme activity assay is given in the Supporting Information. The kinetic constant K_M and V_{max} were determined using the Graph Pad Prism V5.0 software package.

4.6 | Organelle staining, immunostaining, and fluorescence microscopy

To visualise mitochondrion morphology, MitoTracker Red CMXRos (Life Technologies) was added at a final concentration of 50 nm, directly to parasite suspensions in complete culture medium, and incubated with shaking at 37 °C for 20 min; subsequently, 40,6-diamidino-2-phenylindole (DAPI, Sigma) was added to final concentration of 2 μg/ml, and parasites were incubated for further 10 min. Following

three washes with 1× PBS (pH 7.4), the parasites were mounted on glass slides.

Indirect immunofluorescence assays were performed on *P. falciparum* 3D7 or transgenic parasite lines as described earlier (Jain et al., 2013; Rathore, Datta, Kaur, Malhotra, & Mohmmed, 2015). Briefly, the parasite samples were fixed, incubated with primary antibody (rabbit anti-ClpP, rabbit anti-EF-Tu, rabbit anti-PfOTU, or mouse anti-Atg8 diluted in 3% bovine serum albumin, 1× PBS) and subsequently with appropriate secondary antibody (Alexa Fluor 594, Life Technologies) with intermittent washing. The parasite nuclei were stained with DAPI (2 μg/ml).

The GFP-expressing parasites, organelle stained, and immunostained parasites were viewed using a Nikon A1 Microscope with N-SIM (Structured Illumination Microscopy, for superresolution). For confocal microscopy, images were acquired with Plan Apochromat 100×/1.40 NA oil immersion objective lens in NIS Elements, and Z-stacks were taken for 21 steps at 100-nm intervals. All images were captured and analysed using NIS-Elements AR. Individual Z-stacks were adjusted for brightness and contrast and exported as 8-bit RGB TIFF formats. All images were captured and analysed using NIS-Elements C/NIS-Elements AR. Individual Z-stacks were exported as 8-bit RGB TIFF formats and selected confocal Z-stacks were further reconstructed in Imaris (Bitplane, Zurich) as 3-D models.

4.7 | Cryoimmunoelectron microscopy

The transgenic *P. falciparum* parasites expressing PfOTU-RFA at trophozoite stages were fixed with 4% paraformaldehyde, 0.04% glutaraldehyde in 1× PBS at RT for 25 min, embedded in LR white, and infiltrated with a cryopreservative and plasticizer (2.3 M sucrose, 20% polyvinyl pyrrolidone). After freezing in liquid nitrogen, samples were sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems Inc., Bannockburn, IL) at −60 °C. Ultra-thin sections were blocked with 5% fetal bovine serum and 5% normal goat serum in PBS for 30 min, incubated with mouse monoclonal anti-GFP antibodies (Roche) or preimmune mouse sera diluted 1:100 in blocking buffer, washed thoroughly, and incubated with 18-nm colloidal gold-conjugated goat anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, USA) for 1 hr. Sections were stained with 0.3% uranyl acetate, 1.7% methyl cellulose and visualised under JEOL 1200EX transmission electron microscope (JEOL, USA). All labelling experiments were conducted in parallel with controls omitting the primary antibody or using preimmune sera as primary antibodies.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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