


## RESEARCH ARTICLE

# *Toxoplasma gondii* autophagy-related protein ATG9 is crucial for the survival of parasites in their host

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**Abstract**

Autophagy is a conserved, life-promoting, catabolic process involved in the recycling of nonessential cellular components in response to stress. The parasite *Toxoplasma gondii* is an early-diverging eukaryote in which part of the autophagy machinery is not exclusively involved in a catabolic process but instead has been repurposed for an original function in organelle inheritance during cell division. This function, depending essentially on protein TgATG8 and its membrane conjugation system, is crucial for parasite survival and prevented an in depth study of autophagy in the mutants generated so far in *Toxoplasma*. Thus, in order to decipher the primary function of canonical autophagy in the parasites, we generated a cell line deficient for TgATG9, a protein thought to be involved in the early steps of the autophagy process. Although the protein proved to be dispensable for the development of these obligate intracellular parasites *in vitro*, the absence of TgATG9 led to a reduced ability to sustain prolonged extracellular stress. Importantly, depletion of the protein significantly reduced parasites survival in macrophages and markedly attenuated their virulence in mice. Altogether, this shows TgATG9 is important for the fate of *Toxoplasma* in immune cells and contributes to the overall virulence of the parasite, possibly through an involvement in a canonical autophagy pathway.

**KEYWORDS**

ATG9, autophagy, stress response, toxoplasma, virulence

## 1 | INTRODUCTION

Macroautophagy (simply referred to as “autophagy” thereafter) is a self-degradation process conserved among eukaryotes. It allows cells to eliminate undesired cytosolic materials such as misfolded proteins or damaged organelles under normal growth conditions and recycle their own components under stress conditions such as starvation. During the autophagy process, cytosolic components are sequestered within a double membrane vesicle, called the autophagosome, and then delivered into a lysosomal compartment for degradation (Shibutani & Yoshimori, 2014). Autophagosome formation requires autophagy-related (ATG) proteins; many of which were originally identified in the yeast *Saccharomyces cerevisiae* (Devenish & Klionsky, 2012). One of the primary functions of autophagy is participating in the maintenance of basal cellular homeostasis in normal growth conditions. Usually, lack of basal autophagy leads to no apparent

abnormalities, like for yeast cells growing in nutrient-rich medium (Tsukada and Ohsumi, 1993), although this housekeeping function is indispensable for some mammalian cell types such as neural cells (Hara et al., 2006). Starvation-induced autophagy, on the other hand, is essential for stress management in almost all eukaryotes. The lack of oxygen or nutrients such as amino acids or glucose, are known to trigger autophagy, which plays a key part in mobilizing diverse cellular energy and nutrient stores (Kaur & Debnath, 2015; Russell, Yuan, & Guan, 2014) to maintain normal functions of the cells. Autophagy can also be induced by other stress, reactive oxygen species and reactive nitrogen species in particular, which are harmful for cellular components, as they generate damaged proteins and DNA (Filomeni, De Zio, & Cecconi, 2015).

The protist *Toxoplasma gondii* (*T. gondii*) is an obligate intracellular parasite whose survival is based on scavenging essential nutrients from its host cell (Blader & Koshy, 2014). Infection can range from asymptomatic to severe, depending on the virulence of the parasite cell lines and the immune status of the host (Hunter & Sibley, 2012). The

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majority of infection cases in humans are asymptomatic, as the parasite is controlled by the immune system and remains in a dormant state in the brain and other tissues. However, *T. gondii* can provoke severe diseases that may be fatal for immunocompromised patients and for developing fetuses of primary-infected pregnant women (Montoya & Liesenfeld, 2004). Tachyzoites, the invasive and fast-replicating forms of the parasite, which are responsible for the symptoms of the acute form of the disease, undergo multiple rounds of division within their host cells, leading to significant tissue damage. Immunological studies have shown that the elimination of the parasite involves interferon- $\gamma$  (IFN- $\gamma$ )-dependent mechanisms, implicating both innate and adaptive immune responses (Sturge & Yarovinsky, 2014).

*T. gondii* possesses an apparently reduced autophagy machinery but is nevertheless able to generate autophagosome-like structures under acute starvation condition or following drug treatment (Besteiro, Brooks, Striepen, & Dubremetz, 2011; Ghosh, Walton, Roepe, & Sinai, 2012; Lavine & Arrizabalaga, 2012). These vesicles resemble bona fide autophagosomes: they appear as double-membrane compartments containing more or less intact cytosolic materials and are decorated with protein ATG8, which is the most widely used autophagosomal marker (Slobodkin & Elazar, 2013). However, there is no clear evidence so far that these vesicles, once generated, are being degraded and their content is recycled as expected in a canonical autophagy pathway. In a very unusual fashion though, during normal intracellular growth, TgATG8 also localizes at the outermost membrane of the apicoplast, a relict plastid found in apicomplexan parasites (Kong-Hap et al., 2013), where it plays a noncanonical role in organelle inheritance during cell division (Lévêque et al., 2015). The apicoplast is involved in several key metabolic pathways, so it is essential for the survival of the parasites (Sheiner, Vaidya, & McFadden, 2013). Accordingly, interfering with the function of TgATG8, or of proteins regulating its membrane association such as TgATG3 and TgATG4, is lethal for the parasites (Besteiro et al., 2011; Kong-Hap et al., 2013; Lévêque et al., 2015). These mutants are thus essentially unsuitable for functional studies of the canonical autophagy pathway.

There is still a great deal of uncertainty concerning the origin of the membranes required for autophagosome formation. They seem to originate from multiple compartments, such as the endoplasmic reticulum, Golgi, mitochondria, plasma membrane, and early and recycling endosomes (Lamb, Yoshimori, & Tooze, 2013). ATG9 is a transmembrane ATG protein whose precise function is elusive but is thought to deliver membranes to the preautophagosomal structures and autophagosomes (Mari et al., 2010). It is an essential component of the autophagy initiation machinery, which has been shown to be necessary for optimal autophagy in mammalian cells and in budding yeast (Noda et al., 2000; Young et al., 2006). With the aim of interfering only with autophagy-related functions in the parasites, we thus sought to study and to disrupt the function of the single *T. gondii* ATG9 homolog.

In contrast with TgATG8 and its related machinery, in *Toxoplasma* ATG9 depletion had no effect on apicoplast homeostasis. *In vitro*, TgATG9-depleted parasites only displayed a mild phenotype, when experiencing stress due to long extracellular incubation. They also showed a reduced proteolytic capacity, suggestive of an impaired autophagy pathway, although direct implication of the protein in the

biogenesis of autophagosomes could not be demonstrated. Importantly, however, further characterization of the mutant *in vivo* revealed that TgATG9 is crucial for the virulence of the parasites in mice. Thus, our study highlights that there might be important roles for canonical autophagy in the context of the host organism.

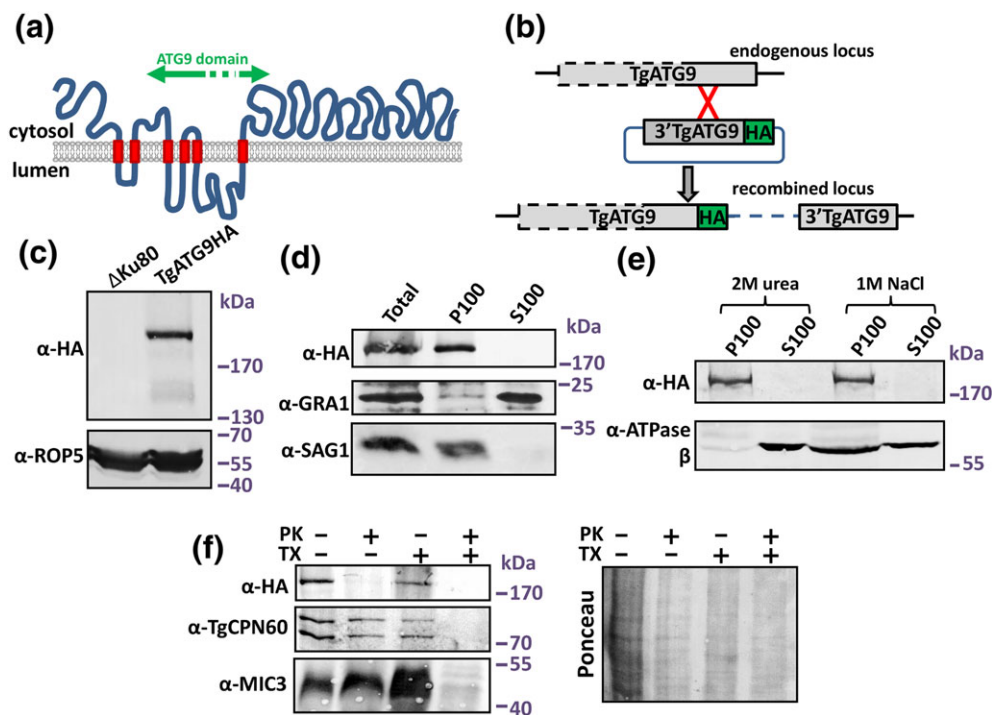
## 2 | RESULTS

### 2.1 | *T. gondii* contains a single putative ATG9 homolog which partially localizes to the trans-Golgi network and endolysosomal compartments

ATG9 is the only known multipass transmembrane autophagy protein among about 40 ATG proteins identified to date. It has six conserved transmembrane domains (He et al., 2006; Noda et al., 2000; Young et al., 2006). We performed homology searches in the *T. gondii* genomic database ([www.toxodb.org](http://www.toxodb.org)) and identified a single putative homolog for ATG9 (TGME49\_260640). TgATG9 is predicted to be a 1655 amino acids (aa)-long protein, with a generally well-conserved "ATG9 domain" (accession number: PF04109 in the Pfam database <http://pfam.xfam.org/>) extending from aa 370 to aa 870 (Figure 1a, Figure S1a,c). However, other parts of the proteins have less overall homology. This is quite common in ATG9 from other eukaryotes: for instance, the cytosolic N- and C-terminal domains of mammalian ATG9 have no significant homology to the respective yeast domains (Webber & Tooze, 2010).

Another characteristic of ATG9 is the presence of six transmembrane domains (Figure S1c). We generated a C-terminally-HA-tagged version of the native protein (TgATG9-HA) by endogenous locus recombination (Figure 1b), and found after gel electrophoresis and immunoblot analysis that TgATG9 migrates close to its predicted molecular mass of 184 kDa (Figure 1c). After parasite fractionation, the protein was found to be exclusively associated with the membrane fraction (Figure 1d). Moreover, we found this association of TgATG9 with the insoluble fraction is resistant to chaotropic agents such as urea or high salt concentrations, as expected for a transmembrane protein (Figure 1e). We also analyzed the exposure of the C-terminal HA tag, by proteinase K digestion assays and found it is exposed to the cytosolic face of the membranes (Figure 1f). Thus, combining bioinformatic predictions of the transmembrane domains, together with biochemical analysis, we found the overall topological model of TgATG9 (Figure 1a) to be similar to its counterparts in budding yeast (He et al., 2006) and mammals (Young et al., 2006) (Figure S1c).

We then assessed the subcellular localization of TgATG9 by immunofluorescence assay (IFA) using an anti-HA antibody. The protein was found to localize to vesicular structures, usually in the apical part of the parasites (Figure 2a). Contrarily to TgATG8, TgATG9 does not seem to be associated with the apicoplast (Figure 2a). We tested for colocalization with a number of organelles and found no particular association of TgATG9 with the rhoptries, micronemes and mitochondrial network either (Figure S2). On the other hand, we could detect partial colocalization with several markers, which are, from the highest to the lowest correlation coefficient, the vacuolar compartment (costained with the TgCRT marker [Warring, Dou, Carruthers,



**FIGURE 1** TgATG9 is a membrane-associated protein. (a) Topological model for TgATG9 based on bioinformatics predictions and biochemical analyses displayed in (e). The conserved ATG9 domain (Pfam PF04109) is delineated in green. (b) Strategy for adding a C-terminal triple hemagglutinin (HA) tag to TgATG9 by single homologous recombination at the endogenous locus of the corresponding gene. (c) Detection by immunoblot of the HA-tagged TgATG9-HA protein in parasite extracts. ROP5 has been used as a loading control. (d) Immunoblot analysis of parasite lysates after fractionation shows TgATG9-HA is associated with membrane fractions (P100 and S100 are the pellet and soluble fractions, respectively, after a 100,000 g centrifugation). GRA1 is a soluble protein control and SAG1 is a membrane-associated protein control. (e) Immunoblot analysis shows TgATG9-HA present in the P100 fraction described in (d) is resistant to high salt or urea extraction, in contrast with the  $\beta$  subunit of the F<sub>1</sub> ATPase, which was used as a peripheral membrane protein control. (f) Proteinase K digestion assay, in the presence or absence of detergent, shows that the C-terminus of TgATG9-HA is exposed on the cytoplasmic side. CPN60 and MIC3, which are proteins contained within the lumen of membrane-bound organelles (the apicoplast and micronemes, respectively) are protected from Proteinase K digestion in the absence of detergent

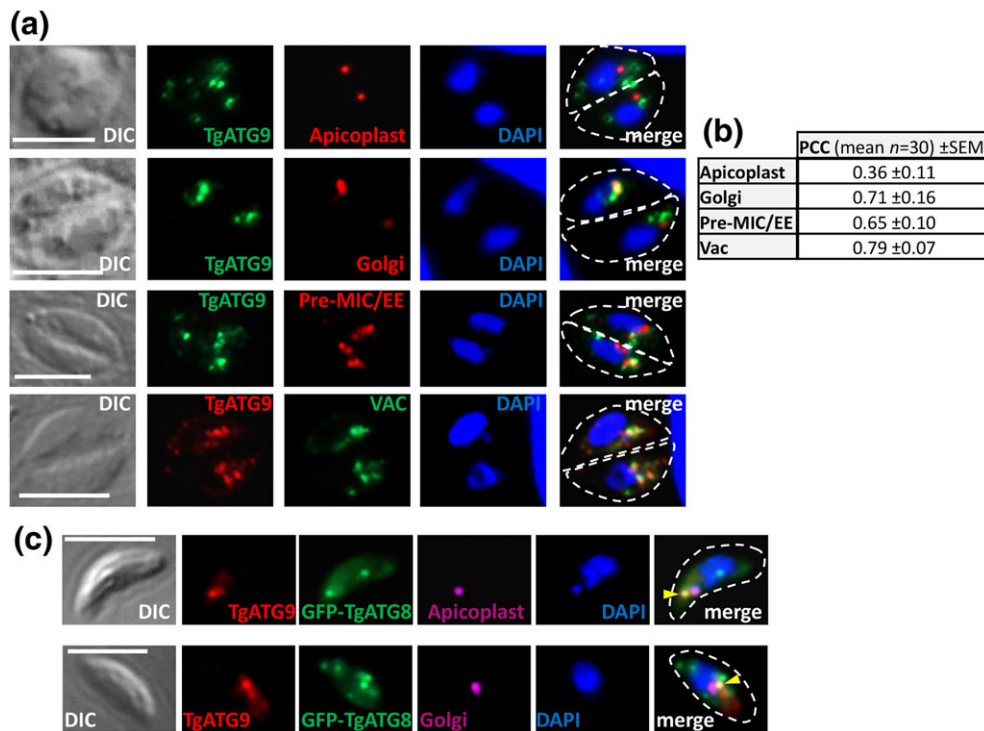
McFadden, & van Dooren, 2014)), the Golgi apparatus (costained with the GRASP marker [Lorestani et al., 2010]), as well as vesicles of the endolysosomal pathway (early endosomes were costained with the pro-M2AP marker [Harper et al., 2006]; Figure 2a,b). We also evaluated the localization of the protein in autophagy-inducing conditions and observed an occasional colocalization of the TgATG9 signal with some TgATG8-positive vesicles, which are distinct from the apicoplast or Golgi apparatus signals, and thus likely represent autophagic vesicles (Figure 2c). ATG9 is an early marker of autophagosome biogenesis and is only transiently associated with these vesicles (Orsi et al., 2012); it is thus unsurprising to see only a partial colocalization of the two markers. Overall, these localization data are consistent with previous studies on other eukaryotic ATG9: in mammalian cells, for instance, ATG9 is found on vesicles trafficking between the Golgi, diverse endocytic vesicles, and autophagosomes (Longatti et al., 2012; Orsi et al., 2012; Takahashi et al., 2011).

## 2.2 | Intracellular growth of ATG9-depleted parasites is unaffected in nutrient-rich conditions in vitro

In order to functionally characterize TgATG9, we sought to generate a TgATG9-depleted cell line. All autophagy-related genes previously characterized in our laboratory (TgATG3, TgATG4 and TgATG8) are

indispensable for intracellular growth of the parasite, likely due to their role in apicoplast homeostasis. In case TgATG9 would also be essential, we generated an inducible knock-down TgATG9 (KDTgATG9) cell line (Figure 3a). The endogenous promoter of TgATG9 was replaced by an inducible TetO7Sag4 promoter, which can be reversibly turned on or off thanks to a tetracycline-based transactivator system (Meissner, Brecht, Bujard, & Soldati, 2001). Replacement of the endogenous promoter by the inducible promoter system in KDTgATG9 was confirmed by polymerase chain reaction (PCR; Figure 3b). The promoter replacement was done in the TgATG9-HA background cell line and did not affect extensively TgATG9 expression levels (Figure S3a), or its localization (Figure S3b). Also, this way we could monitor the down-regulation of the protein by immunoblot using an anti-HA antibody. TgATG9 was no longer detectable after 2 days of anhydrotetracycline (ATc) treatment (Figure 3c, Figure S3b). However, in contrast with the apicoplast-related TgATG mutants, ATc-driven depletion of TgATG9 in this cell line did not affect the parasite lytic cycle in vitro (Figure S4a). Consistent with this, we found that conditional depletion of TgATG9 had no particular impact on the apicoplast or on the mitochondrial network (Figure S5a). Loss of TgATG9 had also no apparent effect on TgATG8 localization at the apicoplast either (Figure S5b).

Overall, this suggested that TgATG9 is a dispensable gene, but to rule out an absence of phenotype that could be due to some residual



**FIGURE 2** TgATG9 localizes to multiple membrane-bound subcellular compartments. (a) Co-labeling, in intracellular parasites, of TgATG9-HA with markers from the apicoplast (TgATRX1), Golgi apparatus (GRASP-RFP), premicronemal or early endosomal compartment (“Pre-MIC/EE,” proTgM2AP), and vacuolar compartment (“VAC,” TgCRT-GFP). (b) Pearson’s correlation coefficient values for costainings between TgATG9 and indicated markers; values are mean  $\pm$  standard error calculated from  $n = 30$  cells. (c) Colabeling, in starved extracellular parasites, of TgATG9-HA, with GFP-TgATG8-decorated vesicles (yellow arrowhead) distinct from the apicoplast (labelled with TgATRX1) or the Golgi apparatus (stained for the GRASP marker), and thus likely representing autophagic vesicles. Differential interference contrast images are shown on the left. DNA is stained in blue with DAPI. Scale bar = 5  $\mu$ m

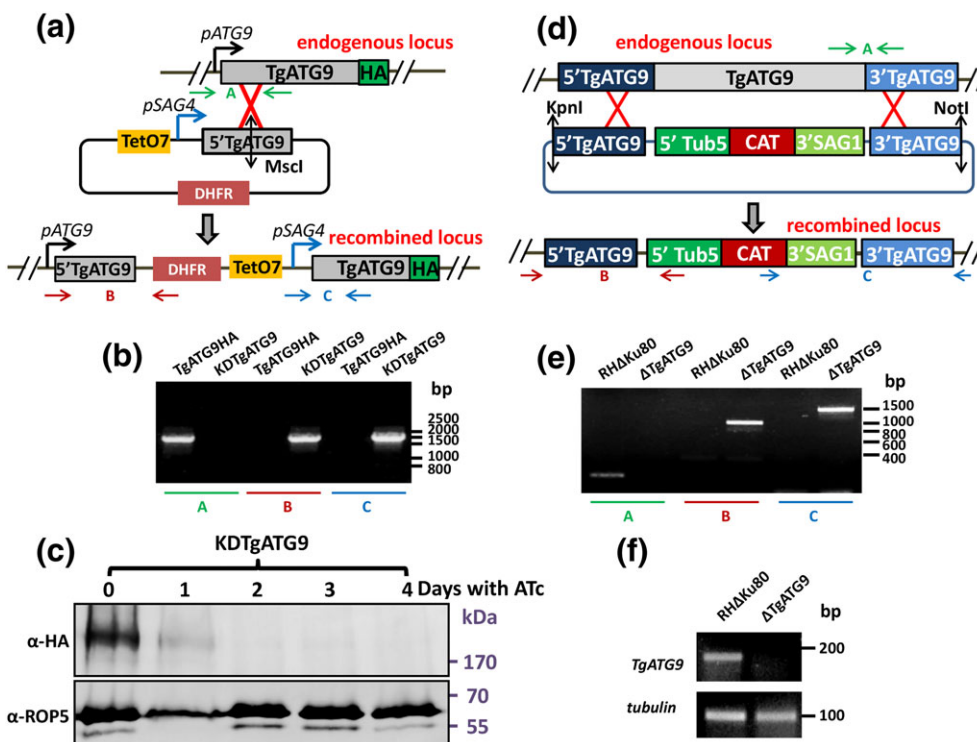
proteins, we went on to completely delete TgATG9. A TgATG9 knock-out cell line ( $\Delta$ TgATG9) was generated by double homologous recombination (Figure 3d). Two independent clones were selected and were found to be similar in subsequent phenotypic analyses (not shown). The replacement of the entire TgATG9 coding region by a chloramphenicol acyltransferase (CAT) selection cassette was confirmed by PCR (Figure 3e). Accordingly, semiquantitative RT-PCR analyses using TgATG9-specific primers showed that there was no more TgATG9 transcript in the  $\Delta$ TgATG9 cell line (Figure 3f). Similar to the inducible TgATG9 knock-down cell line,  $\Delta$ TgATG9 parasites showed no intracellular growth defect in vitro (Figure S4b). Real-time quantitative PCR analysis of parasite growth in human foreskin fibroblasts (HFF) confirmed the mutant parasites were not impaired in growth, even suggesting a slightly increased growth rate (Figure S4c).

The absence of an apicoplast-related phenotype and a preserved viability in nutrient-rich conditions for TgATG9-depleted parasites make this protein a suitable candidate to assess its putative involvement in canonical autophagy in *T. gondii*.

### 2.3 | Assessing alterations of the autophagy pathway due to the loss of TgATG9

*T. gondii* tachyzoites spend most of their lytic cycle inside their intracellular replicative niche. Hence, the extracellular milieu is very likely for them a source of a significant environmental stress. In particular, the ability to respond to nutrient stress is important for the fitness of

extracellular parasites (Konrad, Wek, & Sullivan, 2011; Konrad, Wek, & Sullivan, 2014). Our previous studies have shown that extracellular parasites, even incubated in Dulbecco’s modified eagle medium (DMEM) medium with a full complement of amino acids, already display a basal level of TgATG8-decorated autophagosomes significantly higher than intracellular parasites (Besteiro et al., 2011). Autophagosomes numbers are further increased when extracellular parasites are incubated in an amino acids-depleted medium such as host-based security system (HBSS; Besteiro et al., 2011; Kong-Hap et al., 2013). To investigate the involvement of TgATG9 in extracellular survival and its possible role in autophagy, we first used extracellular  $\Delta$ TgATG9 parasites transiently expressing the GFP-TgATG8 autophagosomal marker to quantify the formation of autophagosome-like structures. Surprisingly, the  $\Delta$ TgATG9 cell line was still able to generate multiple GFP-TgATG8-decorated vesicles (Figure 4a). Quantification of the percentage of puncta-bearing parasites, or the number of puncta per parasite, showed no significant difference between extracellular parasites of the  $\Delta$ TgATG9 and parental cell line, both in amino acids starvation (HBSS) and complete (DMEM) media (Figure 4b,c). As shown in previous studies in other eukaryotic models, depletion of ATG9 does not lead to a complete inhibition of GFP-ATG8 puncta appearance following starvation (Bader, Shandala, Ng, Johnson, & Brooks, 2015; Young et al., 2006). It was suggested in the original description of the yeast ATG9 mutant that, instead of being needed for the initiation of autophagy vesicle formation, the protein is more likely needed for the completion of the sequestering

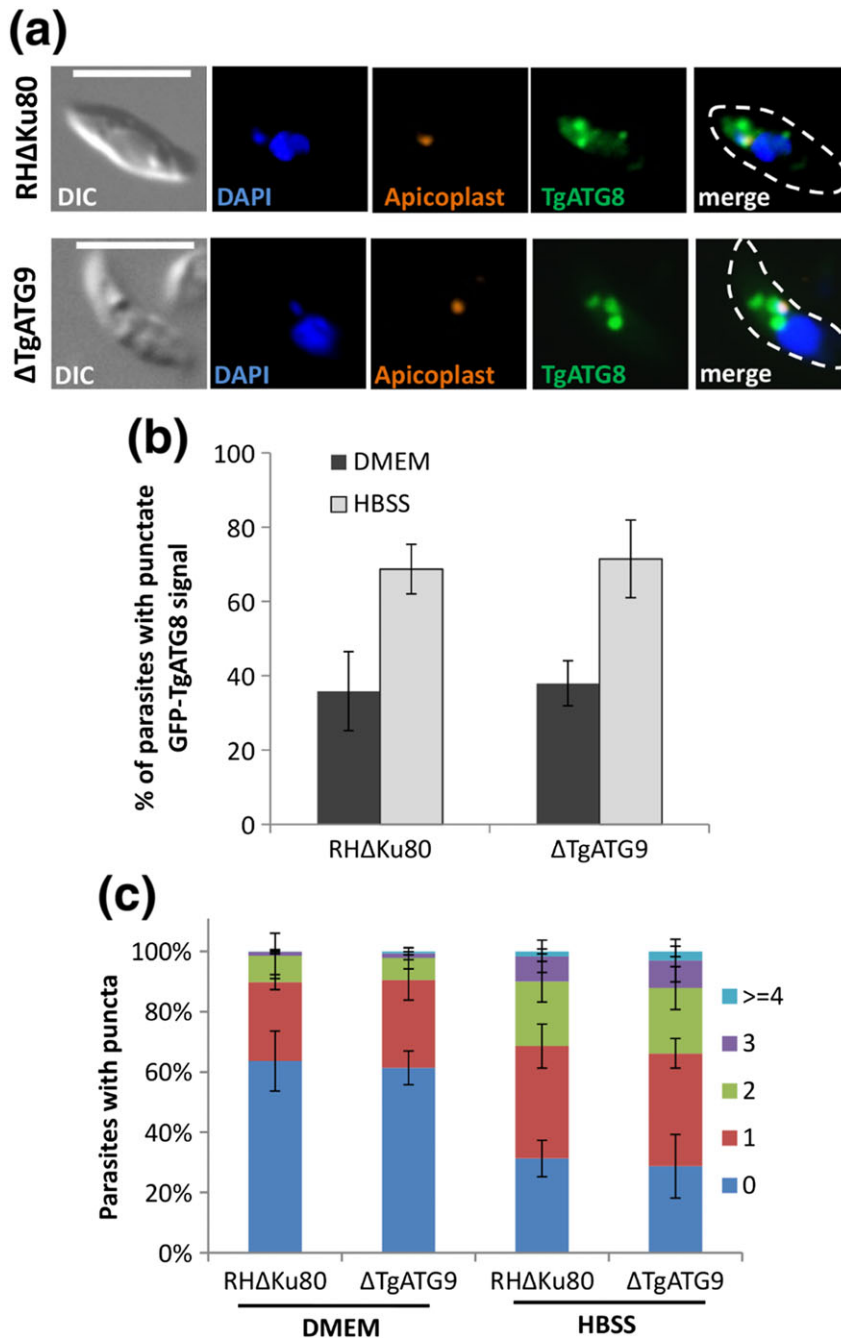


**FIGURE 3** Two parallel strategies were implemented to generate *TgATG9*-depleted parasites. (a) A conditional knock-down cell line was generated in the *TgATG9*-HA background by replacing the endogenous promoter with an ATc-regulated promoter. Clones were obtained after pyrimethamine selection. Arrows represent primers used to verify integration by the PCR shown in (b). TetO7 = tet operator, DHFR = dihydrofolate reductase selection marker, *pSAG4* = SAG4 minimal promoter. (b) Genomic DNA regions from *TgATG9*-HA and KDTgATG9 cell lines were amplified with primers couples depicted in (a) for PCR detections of the endogenous and recombined loci. (c) Immunoblot assessment of *TgATG9*-HA depletion in protein lysates from parasites incubated in absence or presence of Atc for up to 4 days. ROP5 was used as a loading control. (d) A *TgATG9* knock-out cell line was generated in the RH $\Delta$ Ku80 background by double homologous recombination. Clones were obtained after chloramphenicol selection. Arrows represent primers used to verify integration by the polymerase chain reaction (PCR) shown in (e). CAT = chloramphenicol acetyl transferase; *5'*Tub5 and *3'*SAG1: untranslated regions from the *tubulin* and *SAG1* genes, respectively, for driving chloramphenicol acetyl transferase expression. (e) Genomic DNA regions from RH $\Delta$ Ku80 and  $\Delta$ TgATG9 cell lines were amplified with primers couples depicted in (d) for PCR detections of the endogenous and recombined loci. (f) Semi-quantitative RT-PCR analysis of *TgATG9* expression in the  $\Delta$ TgATG9 mutant and parental cell lines. Specific  $\beta$ -*tubulin* primers were used as controls

membrane of the autophagosome (Noda et al., 2000). Of course, because of the limit of optical microscopy, we could not distinguish between functional autophagosomes or incomplete GFP-*TgATG8*-positive structures. We tried to assess morphological differences by electron microscopy, but these structures are particularly difficult to identify, and we could not obtain quantitative data (not shown).

One major hindrance to a more complete analysis of the mutant was the lack of functional assay available for measuring the autophagic flux in *T. gondii*. One way to monitor the autophagic flux is to measure the turnover rate of long-lived proteins, which are normally degraded by autophagy, while short-lived proteins are thought to be degraded by the proteasome (Bauvy, Meijer, & Codogno, 2009). We thus sought to adapt to *Toxoplasma* a protocol using a “clickable” methionine analog (L-azidohomoalanine or AHA) for global protein labeling, followed by quantification by flow cytometry after autophagy-inducing conditions for measuring autophagy-dependent proteolysis (Zhang, Wang, Ng, Lin, & Shen, 2014). After trying different concentrations and incubation times, we found conditions where the incorporation of AHA is suitable for protein labeling without affecting parasite viability (Figure 5a,b). To avoid labeling of short-lived proteins (whose turnover depends on the proteasome),

extracellular parasites were submitted to a 1 hr chase in methionine-rich DMEM after AHA incorporation, and they were subsequently incubated for 4 hrs in either starvation (HBSS) or complete (DMEM) media. A viability dye was also added in the end of autophagy-inducing treatment with the aim of labeling dead parasites, which would be subsequently excluded from our flow cytometry analysis (Figure 5c). After incubation in HBSS, the majority of the parasite population was dead and this rendered the quantification difficult (not shown). Although extracellular parasites kept in complete medium are likely experiencing a significant nutrient stress (Besteiro et al., 2011; Konrad et al., 2011; Konrad et al., 2014), they remained essentially viable (Figure 5c), and thus much more suitable to measure proteolysis and the involvement of autophagy. Interestingly, after 4 hrs incubation in complete DMEM, flow cytometry analyses showed a moderate, but statistically significant, reduction of proteolysis in  $\Delta$ TgATG9 mutant compared to the parental cell line (Figure 5d). As a control, we used a cathepsin L mutant cell line ( $\Delta$ TgCPL), deficient for a major vacuolar protease (Parussini, Coppens, Shah, Diamond, & Carruthers, 2010), thus likely impaired in its overall proteolytic capacity, and it showed the same trend as the  $\Delta$ TgATG9 mutant (Figure 5d).

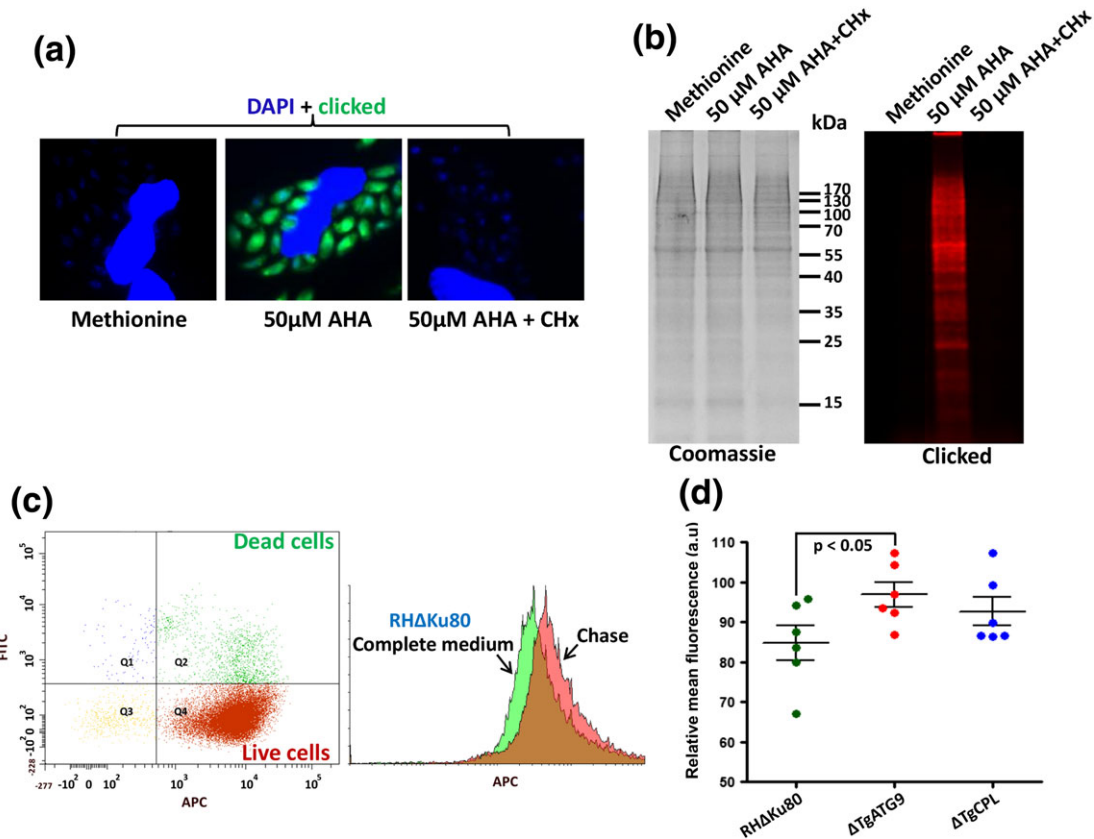


**FIGURE 4** The formation of TgATG8-decorated vesicles is unaffected in TgATG9-depleted parasites. (a) Examples of extracellular RHΔKu80 and ΔTgATG9 parasites transiently expressing GFP-TgATG8 that were starved for 6 hrs in HBSS and found to display autophagosome-like punctate signal (distinct from the apicoplast, which was stained with TgATR1). DIC = differential interference contrast. DNA is stained in blue with DAPI. Scale bar = 5 μm. (b) Quantification of ΔTgATG9 parasites transiently expressing GFP-TgATG8 that displayed a punctate GFP-TgATG8 signal (other than the apicoplast) following a 6-hr starvation in HBSS or incubation in DMEM as a control. Mean values from three independent experiments were represented ± SEM. (c) Quantification of GFP-TgATG8 puncta (other than the apicoplast), in ΔTgATG9 parasites transiently expressing GFP-TgATG8 starved for 6 hrs in HBSS or kept in DMEM. Mean values from three independent experiments were represented ± SEM

## 2.4 | Depletion of TgATG9 reduces extracellular survival of *T.gondii*

To assess precisely whether the viability of extracellular parasites was differently affected in the mutant, we incubated freshly egressed parasites in amino acid-free or in complete media. The use of a membrane impermeable fluorescent viability dye, coupled with quantification by microscopy, allowed us to measure the mortality rate at any given time during the incubation period (Figure 6a, Figure S6a). In the absence of amino acids, both the parental and the TgATG9 mutant cell lines showed a rapidly increasing mortality rate that began to plateau (or even decrease, possibly due fluorescence loss after parasite degradation), after 2 hrs of treatment (Figure S6a). Parasites incubated in complete DMEM, however, displayed generally a much more reduced mortality rate. Interestingly though, ΔTgATG9 mutant parasites

displayed a systematically higher mortality rate than the control, and it increased drastically after 16 hrs of treatment (Figure 6a). To assess the overall viability of these parasites kept in extracellular conditions, we used their ability to invade and establish a parasitophorous vacuole inside host cells as a readout. This confirmed that, for both cell lines, an incubation in HBSS for several hours dramatically decreased parasite viability (Figure S6b). As for the proteolysis assay described above, the high mortality rate due to these particularly harsh conditions was not suitable to observe any significant difference between the two cell lines. However, ΔTgATG9 parasites kept in DMEM medium for 16 hrs showed a reduced viability in comparison to the parental cell line kept in the same conditions (Figure 6b). This is in accordance with the increased death rate observed for the mutant parasites in these conditions, and this suggests TgATG9 is important for viability in the extracellular state.



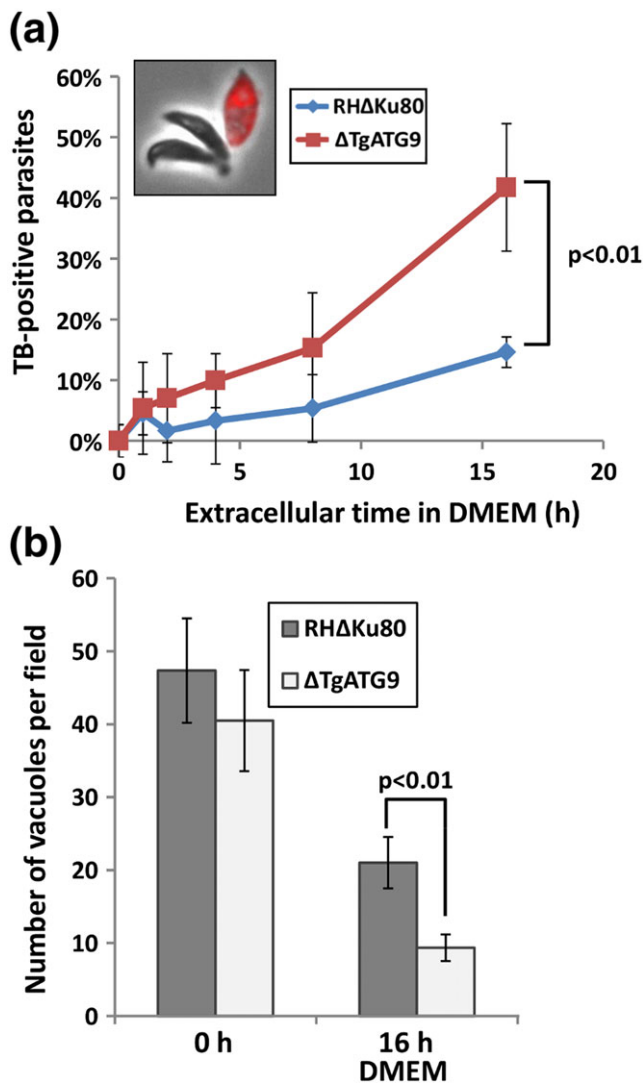
**FIGURE 5** Quantitative measurement of the proteolysis from long-lived proteins using AHA metabolic labeling. (a) Fluorescence microscopy after the click reaction shows methionine analog AHA was successfully incorporated into intracellular tachyzoites following a 6-hr incubation. The use of protein synthesis inhibitor cycloheximide (Chx) together with AHA prevented its incorporation. DNA is stained in blue with DAPI. (b) Protein-labeling profile of parasites incubated in the conditions described in (a) was assessed by in gel fluorescence imaging. (c) Example of flow cytometry analysis of AHA-labeled extracellular parasites. Selected parasites were negative for the viability exclusion dye eFluor520 (FITC channel) and positive for clicked AHA labeling (APC channel). Decrease of AHA labeling in these parasites after extracellular incubation in complete medium likely reflects proteolysis (right). (d) Quantification of AHA fluorescence values after incubation of RHAΔKu80, ΔTgATG9 and ΔTgCPL extracellular parasites kept in DMEM for 4 hrs suggests that the ΔTgATG9 and ΔTgCPL mutants have a lesser ability to degrade proteins that the RHAΔKu80 control cell line. Mean values from six independent experiments were represented ± SEM. *p* value was calculated using unpaired Student's *T* test with two-tailed equal variance

## 2.5 | Depletion of TgATG9 strongly diminishes parasite virulence in vivo

During acute infection of intermediate hosts, *T. gondii* tachyzoites multiply rapidly following complete cycles of invasion, intracellular replication, and lytic egress. Being obligate intracellular parasites, their survival depends primarily on their ability to invade their host cells, which provide for their nutritional needs, because if they persist in the long term as an extracellular form, they are more prone to external stresses (Khan, Behnke, Dunay, White, & Sibley, 2009). On the other hand, the parasites also have to face a significant pressure from their hosts' immune system and, in particular, activated effector cells (Yarovinsky, 2014). Therefore, we sought to investigate how the loss of TgATG9 would impact parasite virulence in vivo. BALB/c mice were infected by intraperitoneal injection of 100 ΔTgATG9 parasites, and their survival was monitored on a daily basis (Figure 7b). Compared with mice infected with the RHAΔKu80 parental cell line control that died after 11 days, most ΔTgATG9-infected mice showed no fatality throughout the whole duration of the experiment. To verify that the lack of virulence was due to the specific absence of TgATG9, we also

used the KDTgATG9 cell line in the presence or absence of ATc (Figure 7b). In the absence of ATc, KDTgATG9 parasites were found to be slightly less efficient in killing mice than the parental cell line, which might be due to a subtle alteration in TgATG9 basal protein levels, or timing of expression during the cell cycle, resulting from the promoter change strategy we used for generating this conditional knock-down cell line (Figure S3a). Also, it should be noted that in the presence of ATc, the survival of mice infected with TATI1-ΔKu80 control parasites was slightly prolonged, which might be attributed to a direct effect of the drug present in the drinking water of mice throughout the experiment. More importantly, mice infected with KDTgATG9 parasites and kept in the presence of ATc survived throughout the whole duration of the experiment. Overall, this shows TgATG9 is crucial for parasite fitness in the host.

Parasite burden in peritoneal exudates was evaluated at day 4 post-infection, using real time quantitative PCR to measure levels of transcripts of the tachyzoite-specific surface antigen SAG1, and showed an apparently reduced number of ΔTgATG9 parasites compared with control (Figure 7c). Moreover, parasites were not detected in spleen extracts (not shown), which is consistent with an early



**FIGURE 6** TgATG9 mutant parasites are less able to sustain extracellular stress. (a) Mortality rate of RHΔKu80 and ΔTgATG9 extracellular parasites kept in DMEM medium for increased periods of time was assessed by their permeability to trypan blue, which is impermeable to intact membranes and becomes fluorescent when complexed with intracellular proteins (inset). Shown are mean values  $\pm$  SEM from three independent experiments. The  $p$  value was calculated using unpaired Student's  $T$ -test with two-tailed equal variance. (b) Viability of RHΔKu80 and ΔTgATG9 parasites at the end of the experiment described in (a) was assessed by their ability to invade host cells. Shown are the mean values  $\pm$  SEM from one representative experiment out of three. The  $p$  value was calculated using unpaired Student's  $T$  test with two-tailed equal variance

clearance of ΔTgATG9 parasites in the peritoneum. When injected in the peritoneal cavity, the parasite is able to invade macrophages and other cells of innate immunity, which have been suggested to be instrumental in disseminating the parasites in the host (Da Gama, Ribeiro-Gomes, Guimarães, & Arnholdt, 2004). Thus, we next assessed the ability of TgATG9-depleted parasites to survive within macrophages. We isolated thioglycolate-elicited peritoneal macrophages from BALB/c mice, and they were infected with freshly egressed parasites of the ΔTgATG9 or the parental control cell lines. Parasite growth was quantified after 48 hrs by real time quantitative PCR using the SAG1 marker (Figure 7d). In sharp contrast with experiments where

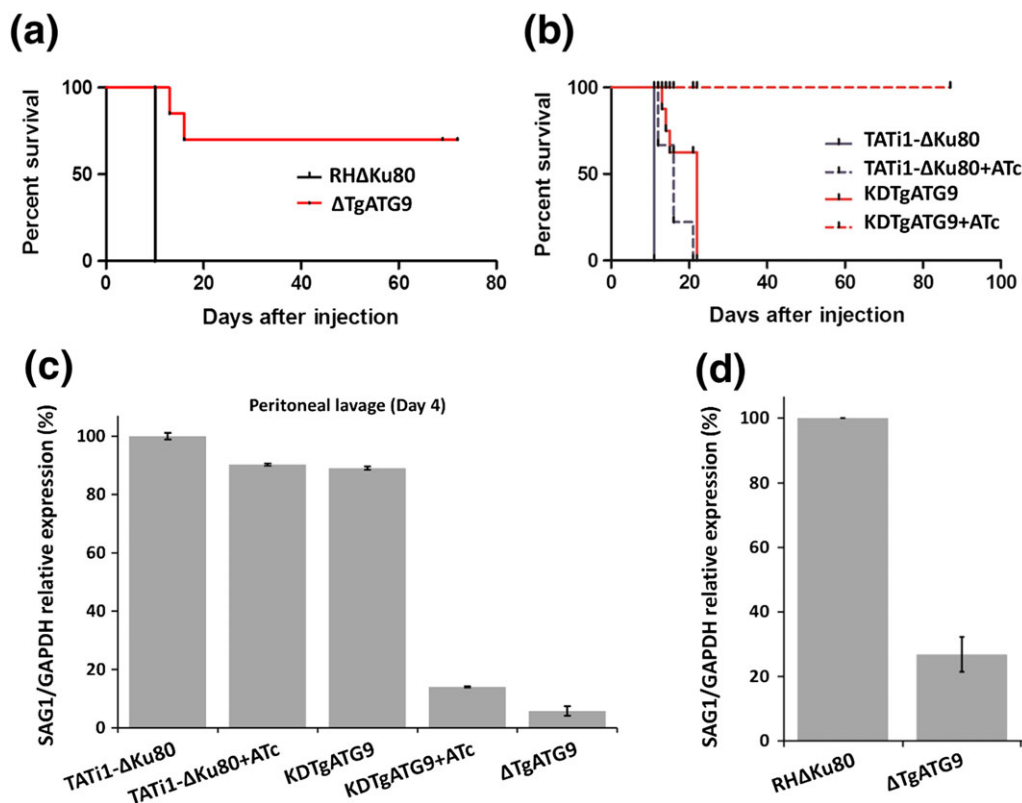
the same technique was used to assess parasite growth in HFFs (Figure S4c), ΔTgATG9 parasites in macrophages were drastically affected two days after invasion. This suggests a reduced growth and/or an increased clearance for TgATG9-depleted parasites, specifically in the context of the macrophages. This is likely the explanation for the absence of parasitemia we found in the spleen and the overall reduced virulence of these mutant parasites for their host.

### 3 | DISCUSSION

Autophagy is an important mechanism for maintaining cell homeostasis in eukaryotes. This biological process has only recently started to be characterized in apicomplexan parasites, and it appears that these early-branching eukaryotes bear a number of original features compared with classic eukaryotic models. In response to starvation, *Toxoplasma* tachyzoites are able to generate subcellular structures resembling autophagosomes and decorated with autophagosomal marker TgATG8. Electron microscopic studies also brought morphological evidences of degraded cellular material in these autophagic vesicles (Besteiro et al., 2011; Ghosh et al., 2012). However, there has been no clear demonstration yet that the autophagosomes formed under stress conditions and their content are actually recycled within tachyzoites. The main reason for this is the lack quantitative assay for measuring the autophagic flux in the parasites. Moreover, all autophagy-related mutants that were generated so far in *Toxoplasma* (TgATG3, TgATG4, and TgATG8 [Besteiro et al., 2011; Kong-Hap et al., 2013; Lévêque et al., 2015]) are affected for TgATG8 function, and this protein happens to also have a peculiar noncatabolic role at the apicoplast membrane (Lévêque et al., 2015). This role being essential for the survival of the parasites, these mutants are unsuitable for studying canonical autophagy.

In the present study, we describe TgATG9, which is the *Toxoplasma* homolog of a protein known to be important in the early steps of autophagosome formation in other eukaryotes. The predicted topology of TgATG9 and its localization, both during normal growth and in starvation conditions, are very similar to what has been described for its other eukaryotic counterparts (Noda et al., 2000; Orsi et al., 2012), suggesting it might also be involved in canonical autophagy in the parasites. Depletion of TgATG9 did not affect parasite growth and apicoplast homeostasis, confirming that the lethal phenotype of other previously generated TgATG mutants was probably due to the impairment of an apicoplast-related function, and not to a lack of canonical autophagy. It also suggests autophagy is not essential for parasite growth in normal in vitro culture conditions, as observed in several other eukaryotic cell types when grown with a full complement of nutrients. In the yeast *Saccharomyces cerevisiae*, for example, the autophagy machinery is dispensable for vegetative growth in rich medium but essential to survive nitrogen starvation, which was at the basis of the strategy originally used to decipher the molecular components of the machinery in this model eukaryote (Devenish & Klionsky, 2012; Tsukada and Ohsumi, 1993).

When we next investigated the fate of the parasites upon prolonged absence of amino acids, we could observe that these conditions led to an equally detrimental phenotype for mutant and control



**FIGURE 7** TgATG9 is crucial for parasite survival inside macrophages and for virulence in vivo. (a) Survival curve of BALB/c mice ( $n = 10$ ) infected intraperitoneally with 100 parasites from the  $\Delta$ TgATG9 and RH $\Delta$ Ku80 cell lines. Data are representative from three independent experiments. (b) Survival curve of BALB/c mice ( $n = 10$ ) infected intraperitoneally with 100 parasites from the KDTgATG9 and TATI1- $\Delta$ Ku80 cell lines, and kept, or not, in the presence of ATc. (c) Real-time quantitative PCR detection of parasites in peritoneal extracts of mice ( $n = 5$ ), four days post-infection with the cell lines described in (a) and (b). *T. gondii* SAG1 and mouse *Glyceraldehyde-3-Phosphate Dehydrogenase* were used to quantify relative abundance of parasite and host messenger RNAs, respectively. Data presented are mean value from triplicate  $\pm$ SEM. (d) In vitro parasite growth in peritoneal macrophages was evaluated by real-time quantitative PCR. Peritoneal macrophages isolated from mice were infected at a ratio of 1:1 with parasites, and 48 hrs later relative abundance of parasite and host messenger RNAs was quantified as described in (c). Mean values from three independent experiments were represented  $\pm$  SEM

cell lines. Harsh starvation conditions, although useful to induce and observe the appearance of autophagy vesicles (Besteiro et al., 2011; Ghosh et al., 2012), are rapidly inducing death in *Toxoplasma* tachyzoites. It has been previously suggested that autophagy is actually a key actor of this starvation-induced death (Ghosh et al., 2012). However, our study shows that the mortality rate and the overall loss of viability in the population are similar between TgATG9-depleted and control parasites. Not only this would argue against the occurrence of an autophagy-mediated cell death (Ghosh et al., 2012), but it also suggests the inability of the autophagy pathway to efficiently protect extracellular parasites from the detrimental consequences of a prolonged amino acids starvation.

Assessing the formation of TgATG8-decorated vesicles upon starvation in TgATG9-depleted parasites, however, could not give us a definitive answer on the involvement of TgATG9 in autophagosome biogenesis. It has been shown in other eukaryotes that the absence of ATG9 does not completely abolish the formation of autophagosomes (Bader et al., 2015; Young et al., 2006), driving researchers towards alternative methods, like measuring the proteolytic capacity of mutant cells (Young et al., 2006), to show autophagy was impaired. We thus implemented this approach in our model, by using a metabolic labeling technique involving a methionine analog

that can be linked chemically to a fluorophore for quantification (Zhang et al., 2014). Once again, the high mortality in the parasite population following harsh starvation conditions prevented the analysis of the mutant. However, using this technique, we could detect a proteolysis defect in the  $\Delta$ TgATG9 extracellular parasites incubated in complete DMEM, compared with parental parasites kept in the same conditions. This would suggest TgATG9 is indeed involved in the proteolytic capacity of tachyzoites through its function in the autophagy pathway.

One should note that in spite of these inferred evidences, TgATG9 might also have another function which is beyond canonical autophagy. Descriptions of nonautophagy-related functions for ATG proteins have been increasing in the recent literature (Bestebroer, V'kovski, Mauthe, & Reggiori, 2013; Mauthe et al., 2016; Subramani & Malhotra, 2013). Another possibility is that there might be a fully functional autophagy pathway in *Toxoplasma*, but TgATG9 might not be necessary for it. For instance, it is surprising to see that while ATG9 homologs are clearly present in coccidia (Figure S1a,b), they seem to be absent from other apicomplexan species. This is also true for *Plasmodium*, which has been nevertheless suggested to contain a functional catabolic autophagy pathway on the basis of colocalization studies between autophagosomal vesicles and PfRAB7-endosomes (Tomlins et al., 2013). Whether this suggests canonical autophagy might not,

after all, be functional in these apicomplexan parasites or they have evolved a different way of initiating early steps of autophagosome biogenesis remains to be investigated further.

As they invade, tachyzoites establish themselves inside a parasitophorous vacuole, from where they will get access to nutrient sources from the host (Blader & Koshy, 2014). On the other hand, at the end of the lytic cycle, when searching for a new host cell to reinvade, the parasites are under the stress of the extracellular environment. Extracellular tachyzoites can use their own internal carbon sources to power the ATP demanding gliding motility initially (Lin, Blume, Ahsen, Gross, & Bohne, 2011), but a prolonged extracellular state leads to a significant decrease in viability (Khan et al., 2009 and this study). In these conditions, tachyzoites seem to be able to sense stress and initiate a response, like for instance, triggering conversion into bradyzoites (Yahiaoui et al., 1999; a slow-growing form more resistant to metabolic, immunological, or chemical stress) upon reentry into a host cell. Thus, even in complete DMEM, the extracellular environment probably lacks essential nutrients, or extracellular tachyzoites might not be able to uptake efficiently these nutrients. A conserved eukaryotic translational control pathway in *Toxoplasma*, relying on the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2 $\alpha$ ) has been characterized in *Toxoplasma* (Sullivan, Narasimhan, Bhatti, & Wek, 2004) and is important to sustain the viability of extracellular tachyzoites (Joyce, Queener, Wek, & Sullivan, 2010; Konrad et al., 2011; Konrad et al., 2014). Interestingly, the eIF2 $\alpha$  pathway is an upstream regulator of autophagy in response to nutrient deprivation and other stresses in mammalian cells (Kroemer, Mariño, & Levine, 2010). The fact that TgATG9-depleted parasites are less able to survive long extracellular incubations would suggest that autophagy could be an important player of the eIF2 $\alpha$ -controlled integrated stress response in *Toxoplasma*, similarly to what has been observed for other eukaryotic models and certainly deserves further investigations.

The ability to survive in the extracellular environment following lysis of a host cell is the key to drive successfully the invasion of a new host and subsequent propagation of the tachyzoites. However, to what extent this really is important in the *in vivo* context is not known. Once the parasites are established within a tissue, they probably have an easy access to potential host cells and might not remain extracellular for extended periods of time. In sharp contrast with the moderate phenotype we observed *in vitro*, TgATG9-depleted parasites were clearly not able to propagate and to generate a sustained infection following intra-peritoneal injection into BALB/c mice. During infection, macrophages are important regulatory and effector cells, as they can both be infected by *T. gondii* and contribute to its propagation within tissues, or they can limit parasite replication and produce cytokines that contribute to resistance (Da Gama et al., 2004). Interestingly, we demonstrated *in vitro* that the survival of TgATG9-depleted parasites in macrophages is compromised, hinting the protein plays a key role in the interaction between parasites and these immune cells.

Host control of *Toxoplasma* depends on the production of pro-inflammatory cytokines and is largely dependent on IFN- $\gamma$  for activating effector mechanisms to eliminate intracellular parasites (Hunter & Sibley, 2012). Interestingly, the IFN- $\gamma$ -dependent response in innate immune cells may include depletion of essential amino acids such as tryptophan, arginine, and generation of nitric oxide (Yarovinsky,

2014). Both amino acids starvation and oxidative stress are potent autophagy inducers (Kroemer et al., 2010), suggesting an involvement of this pathway in surviving these stresses. It should also be noted that response to both types of stress can involve the eIF2 $\alpha$  pathway (Harding et al., 2003). However, populations of *T. gondii* in North America and Europe are dominated by three clonal lineages (known as types I, II, and III) which are differentially able to modulate the host immune response. Present work was performed with parasites of a type I strain, which are normally highly virulent for mice, and are known to be able to disrupt the IFN- $\gamma$ -dependent response to a significant extent (Hunter & Sibley, 2012). Thus, it would now be particularly interesting to investigate TgATG9 function in type II parasites to assess the full contribution of host IFN- $\gamma$ -dependent stress on the elimination of TgATG9 mutants, as well as the ability of these parasites to enter the chronic dormant bradyzoite stage.

More work is needed to definitely demonstrate that there is a fully functional catabolic autophagy pathway in *Toxoplasma*. However, our data suggest that autophagy could be part of an integrated stress response pathway in the parasite. This response might be of particular importance for tachyzoites in the context of the host for surviving either as extracellular parasites, or inside the hostile environment of immune cells. This can be put in parallel with studies performed on TgCPL, a protease functioning as a classic degradative enzyme within the parasite endolysosomal system, both for extracellular and intracellular parasites (Dou, McGovern, Di Cristina, & Carruthers, 2014; Parussini et al., 2010). Strikingly, TgCPL-depleted parasites have a relatively mild growth phenotype *in vitro*, while they are attenuated for their virulence in mice (Dou et al., 2014). These data, together with our own results on TgATG9, are thus suggesting a potential implication of components of both the autophagic and proteolytic machineries in the survival of *Toxoplasma* tachyzoites in the context of the host and certainly calls for further investigations.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Ethics statement

All murine protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB; IACUC Permit Number #15-08-346). All animals were housed in specific pathogen-free facilities. Humane endpoints were used as requested by the AUB IACUC according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines and guide of animal care use book (Guide, NRC 2011). Mice were sacrificed for any of the following reasons: (a) impaired mobility (the inability to reach food and water), (b) inability to remain upright, (c) clinical dehydration and/or prolonged decreased food intake, (d) weight loss of 15–20%, (e) self-mutilation, (f) lack of grooming behavior or rough or unkempt hair coat for more than 48 hours, (g) significant abdominal distension, and (h) unconsciousness with no response to external stimuli. Animals were deeply anesthetized before cervical dislocation. All animals were housed in approved pathogen-free housing. Eye pricks were done following deep anesthesia with isoflurane.

## 4.2 | Parasites and cells culture

Tachyzoites of the RH $\Delta$ Ku80 (Huynh & Carruthers, 2009a), TATI1- $\Delta$ Ku80 (Sheiner et al., 2011) *T. gondii* strains, as well as derived transgenic parasites generated in this study were maintained by serial passage in human foreskin fibroblast (HFF, American Type Culture Collection, CRL 1634) cell monolayer grown in DMEM (Gibco) supplemented with 5% decomplexed fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and a cocktail of penicillin-streptomycin (Gibco) at 100  $\mu$ g/mL.

## 4.3 | Bioinformatic analyses

Sequence alignments were performed using the multiple sequence comparison by log-expectation algorithm of the Geneious software suite ([www.genious.com](http://www.genious.com)). The phylogenetic tree was built using the neighbor-joining method included in the same software suite. Topology and TgATG9 transmembrane helices prediction was performed using the TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and the Phobius prediction software (<http://phobius.sbc.su.se/>). Domain searches were performed in the Pfam database (<http://pfam.xfam.org/>).

## 4.4 | Generation of an HA-tagged TgATG9 cell line

The ligation independent strategy (Huynh & Carruthers, 2009b) was used for C-terminal HA<sub>3</sub>-tagging of TgATG9. A 1.66 kbp fragment corresponding to the 3' end of TgATG9 was amplified by PCR from genomic DNA, with the Phusion polymerase (New England Biolabs) using primers ML1946/ML1947 (primers used in this study are listed on **S1Table**) and inserted in frame with the sequence coding for a triple HA tag, present in the pLIC-HA<sub>3</sub>-CAT plasmid. The resulting vector was linearized with MluI and 40  $\mu$ g of DNA were transfected into the TATI1- $\Delta$ Ku80 cell line to allow integration by single homologous recombination.

## 4.5 | Generation of conditional knock-down and knock-out TgATG9 cell lines

The conditional knock-down TgATG9 cell line (KDTgATG9) was generated based on the Tet-off system using the plasmid DHFR-TetO7Sag4-TgATG9. In brief, the 5' region of TgATG9 starting with the initiation codon was amplified from genomic DNA by PCR using Q5 polymerase (New England Biolabs) with ML1892/ML1893 primers containing BglII and NotI restriction sites, respectively. The 1.7 kb fragment was then inserted into BglII/NotI site of the DHFR-TetO7Sag4 plasmid (Morlon-Guyot et al., 2014), downstream of the ATc-inducible TetO7Sag4 promoter, obtaining the DHFR-TetO7Sag4-TgATG9 plasmid. The plasmid was then linearized by MscI digestion and transfected into the TATI1- $\Delta$ Ku80 cell line expressing 3  $\times$  HA-tagged TgATG9. Transfected parasites were selected with g at a concentration of 1 ng/mL and cloned by serial limiting dilutions. Correct integration in the positive clones was verified by PCR (Figure 3b) using primers ML2530/ML2531 (a), ML1774/ML2531 (b), and ML1771/ML2530 (c).

The TgATG9 knock-out cell line ( $\Delta$ TgATG9) was generated by replacing the endogenous TgATG9 locus with CAT cassette using double homologous recombination strategy. Briefly, 0.95 kb and 1.1 kb DNA fragments corresponding to the upstream of the initiation codon and downstream of the stop codon of TgATG9, respectively, were amplified from genomic DNA using ML2072/ML2073 and ML2074/ML2075 primers. These fragments were respectively cloned into KpnI/HindIII and BamHI/NotI site of pTub5/CAT plasmid (Soldati & Boothroyd, 1993), containing in between the CAT coding region under *tubulin promoter*. The resulting pTub5/CAT-TgATG9 plasmid was then digested with KpnI/NotI and transfected into the RH $\Delta$ Ku80 cell line. Transfected parasites were selected with chloramphenicol at concentration of 20 ng/mL and cloned by limit dilutions. Correct integration in the positive clones was verified by PCR (Figure 3e) using primers ML1851/ML2223 (a), ML2232/ML2233 (b) and ML388/ML2222 (c). Semi-quantitative rt-PCR was used to check for specific TgATG9 mRNA depletion (see below).

## 4.6 | TgATG9 mRNA analysis

Total mRNAs of freshly egressed extracellular parasites from the TATI1- $\Delta$ Ku80, KDTgATG9 (incubated with or without ATc at 1.5  $\mu$ g/mL for 3 days), RH $\Delta$ Ku80, and  $\Delta$ TgATG9 cell lines were extracted using Nucleospin RNA II Kit (Macherey-Nagel). The cDNAs were synthesized with 660 ng of total RNA per RT-PCR reaction using Superscript II first-strand synthesis system (Invitrogen). Specific TgATG9 ML2535/ML2536 primers and, as a control, *Tubulin  $\beta$*  ML841/ML842 primers were used to amplify specific transcripts with the GoTaq DNA polymerase (Promega). PCR was performed with 22 cycles of denaturation (30 s, 95  $^{\circ}$ C), annealing (20 s, 56  $^{\circ}$ C), and elongation (20 s, 72  $^{\circ}$ C).

## 4.7 | Subcellular fractionation

3.10<sup>7</sup> TgATG9-HA tachyzoites were solubilized in 1 ml of Tris HCl 50 mM pH 7.5 and sonicated twice for 30 seconds. Cellular debris were removed by centrifugation at 500 g for 10 minutes. The supernatant was submitted to an ultracentrifugation at 100,000 g for 30 minutes to yield a membrane-enriched high speed pellet and high speed supernatant soluble fractions, respectively. The supernatant fraction was TCA-precipitated and extracts were resuspended in SDS-PAGE loading buffer prior to immunoblot analysis.

Alternatively, the high speed pellet was further extracted by 1 M NaCl or 2 M urea for 2 hrs at 4  $^{\circ}$ C and submitted to another round of ultracentrifugation to yield a pellet and supernatant fraction for subsequent immunoblot analysis.

## 4.8 | Proteinase K digestion assay

Parasites were lysed by sonication in homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2, 2 mM dithiothreitol) and centrifuged at 1,500 g for 10 minutes to remove intact cells. An organellar fraction was obtained by centrifugation at 15,000 g for 30 minutes. The pellet was resuspended in homogenization buffer. Proteinase K (Sigma) and Triton X-100 were optionally added at

0.1 mg/ml and 0.5% v/v, respectively and incubated for 30 minutes at 4 °C before analysis by SDS-PAGE and immunoblotting.

#### 4.9 | Immunoblot analysis

Protein extracts from  $10^7$  freshly egressed tachyzoites or from fractionation and the proteinase K digestion assays described above were separated by SDS-PAGE. Rat monoclonal anti-HA (3F10, Roche) was used to detect tagged TgATG9. Mouse anti-SAG1 (Couvreux, Sadak, Fortier, & Dubremetz, 1988), mouse anti-ROP5 (Leriche & Dubremetz, 1991), rabbit anti-TgCPN60 (Agrawal, van Dooren, Beatty, & Striepen, 2009), rabbit anti-MIC3 (Garcia-Réguet et al., 2000), or mouse anti-GRA1 (Lecordier, Mercier, Sibley, & Cesbron-Delauw, 1999) antibodies were used as controls.

#### 4.10 | Plaque assays

Confluent monolayers of HFFs grown in 24-well plates were infected with  $2 \times 10^5$  freshly egressed tachyzoites and incubated with or without ATc (at 1.5 µg/ml) for 6 days. Infected cell layer was then fixed in cold methanol (for 1 min) and stained with Giemsa (for 1 min). Images were acquired with an Olympus MVX10 macro zoom microscope equipped with an Olympus XC50 camera. Plaque area measurements were done using Zen software (Zeiss).

#### 4.11 | Parasite viability assays

Freshly egressed extracellular tachyzoites of the RHΔKu80 and ΔTgATG9 cell lines were incubated in complete (DMEM) or amino acids starvation (HBSS) media at 37 °C with 5% CO<sub>2</sub>. The experiment was performed in batch and at 0 h, 1 h, 2 h, 4 h, 8 h, and 16 h after incubation; live extracellular parasites were collected and stained with Trypan Blue (TB, Sigma) to determine the mortality rate by counting TB-positive parasites under a fluorescence microscope (excitation: 550/25 nm, emission: 605/70 nm): indeed, dead parasites take up TB into the cytoplasm because of loss of their membrane selectivity, and there TB becomes fluorescent as it is complexed to proteins (Harrison, Callebaut, & Vakaet, 1981). An assay for assessing parasite viability was also conducted by evaluating their invasive capacity. A total of  $2 \times 10^5$  of freshly lysed tachyzoites of RHΔKu80 and ΔTgATG9 cell lines was used to infect confluent monolayer of HFFs grown on coverslips for 18 hrs. The number of parasitophorous vacuoles per field was visualized by IFA using anti-ROP1 antibody, with 100× objective lens.

#### 4.12 | Immunofluorescence microscopy

For immunofluorescence assays IFA, intracellular tachyzoites grown on coverslips containing HFF monolayers were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS, permeabilized for 10 min with 0.3% Triton X-100 in PBS and blocked with 0.1% (w/v) BSA in PBS. Primary antibodies used (at 1/1000, unless specified) for detection of the organelles were as follows: for the apicoplast, anti-ATRX1 (DeRocher et al., 2008) and for the mitochondrial network, anti-F<sub>1</sub> β ATPase (P. Bradley, unpublished); for rhoptries, anti-ROP1 (J.F. Dubremetz, unpublished); for micronemes, anti-MIC3 (Garcia-Réguet et al., 2000);

for early endosomes or pre-micronemal compartment, anti-proM2AP (Harper et al., 2006). Rat monoclonal anti-HA antibody (Roche) was used at 1/500 to detect epitope-tagged TgATG9. For colabeling with autofluorescent protein markers of sub-cellular compartments, constructs allowing the transient expression of vacuolar marker TgCRT-GFP (Warring et al., 2014) and Golgi marker GRASP-RFP (Nishi, Hu, Murray, & Roos, 2008) were transfected in HA-tagged TgATG9-expressing tachyzoites. Staining of DNAs was performed on fixed cells incubated for 5 min in a 1 µg/ml DAPI solution. All images were acquired at the Montpellier RIO imaging facility from a Zeiss AXIO Imager Z2 epifluorescence microscope equipped with a Camera ORCA-flash 4.0 camera (Hamamatsu) and driven by the ZEN software. Adjustments for brightness and contrast were applied uniformly on the entire image. Pearson's correlation coefficient was calculated for costainings on at least 30 individual images of parasites using the "Coloc 2" plugin from the ImageJ/Fiji software ([http://imagej.net/Coloc\\_2](http://imagej.net/Coloc_2)).

#### 4.13 | Analysis of autophagosome-like structures

To visualize autophagosome-like structures, *T. gondii* tachyzoites were transfected to express transiently GFP-TgATG8 (Besteiro et al., 2011) and costained with antibodies for the TgATRX1 apicoplast marker (DeRocher et al., 2008) to discard the apicoplast-related signal.

#### 4.14 | Virulence assays in mice

10 week-old female BALB/c mice (Jackson laboratories) were infected by intraperitoneal (i.p.) injection of 20 or 100 tachyzoites freshly harvested from cell culture. In order to monitor an equal viability of tachyzoites from the KDTgATG9, ΔTgATG9, and control cell lines, invasiveness of the parasites was evaluated by simultaneous plaque assay with a similar dose of parasites on HFFs. The immune response of surviving animals was tested following eye pricks performed on day 7 postinfection: sera were tested by immunoblotting against tachyzoites lysates (not shown). For inducible knock-down strains survival experiments, ATc (Sigma-Aldrich) was dissolved in water at a concentration of 0.2 mg/ml. The drinking water bottle was wrapped in aluminum foil to prevent precipitation of ATc due to light and the solution changed every 36 hrs. Survival experiments were done on groups of 10 mice per parasite cell line. Mice survival was checked on a daily basis until their death, endpoint of all experiments. Data were represented as Kaplan and Meier plots using Prism software (Graphpad).

#### 4.15 | Analysis of parasite survival in macrophage

Thioglycolate-elicited macrophages were obtained from peritoneal fluids 3 days after i.p. injection of 2 ml thioglycolate at 3.85% into BALB/c mice, and were cultured in RPMI medium (Invitrogen) supplemented with 10% of fetal calf serum, 1% penicillin-streptomycin, 1% kanamycin, 20 mM Hepes pH 7, and 1% glutamine (Invitrogen).

For the determination of parasite replication *in vivo*, 10–12 week-old female BALB/c mice (groups of 5 mice per condition) were infected intraperitoneally with 100 tachyzoites freshly harvested from cell culture. On day 4 post-infection, mice infected with either TATI1-ΔKu80, KDTgATG9 (incubated with or without ATc), RHΔKu80 and ΔTgATG9

cell lines, were deeply anesthetized, and then sacrificed by cervical dislocation. The peritoneal cavity of each mouse was washed with 10 ml of PBS. The i.p. wash was spun at 1000 rpm for 5 min to pellet cells. Cells were processed to extract total RNA to study the capacity of parasite replication. Liver and spleen were harvested for homogenization followed by total RNA extraction and quantification of parasites by real time PCR.

#### 4.16 | Determination of parasitemia by quantitative PCR

Total RNA from infected HFF cells, primary murine macrophages, or from mouse tissue (peritoneum, spleen) was extracted using TRIzol (Thermo Scientific), and experiments were performed from 5 µg of RNA. cDNA was prepared using the Revert Aid First cDNA synthesis Kit (Thermo Scientific). SYBR green-based quantitative PCR was performed using the CFX96 Real-Time PCR Detection System (BioRad, Serial No. 785BR04788). Individual reactions were prepared with 0.25 µM of each primer (HH-34/HH-35 for human glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), HH-36/HH-37 for mouse *GAPDH*; HH-21/HH-23 for *TgSAG1*), 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 µl. PCR reaction consisted of a DNA denaturation step at 95 °C for 3 min, followed by 35 cycles (denaturation at 95 °C for 15 s, annealing at 56 °C for 60 s, extension at 72 °C for 30 s). For each experiment, reactions were performed in triplicates and expression of individual genes was normalized to *GAPDH* Threshold cycle (Ct) values and the percentage of expression was calculated according to the Livak method (Schmittgen & Livak, 2008).

#### 4.17 | Proteolysis assay using AHA labeling

In order to quantify the degradation of proteins by autophagy, we adapted a proteolysis assay based on an existing method described for mammalian cells (Zhang et al., 2014), using a methionine analog, AHA, that can be chemo-selectively linked to a fluorescent moiety for labeling proteins (Click-iT AHA, ThermoFisher Scientific). Freshly egressed parasites were first incubated in methionine-free DMEM for 30 min to deplete intracellular methionine pools. They were then labeled with AHA in methionine-free DMEM for 6 hours, followed by a 1-hr incubation in DMEM containing an excess of methionine (2 mM) to chase out labeled short-lived proteins. Extracellular parasites were then incubated in full (DMEM) or starvation (HBSS) medium for 4 hrs. To label the dead population of parasites prior to flow cytometry analysis, fixable viability dye eFluor520 (eBioscience) was used following the protocol supplied by the manufacturer. Cells were then fixed in 2% (w/v) PFA and permeabilized in 0.5% Triton X-100. Click reaction was performed as previously described (Zhang et al., 2014) with the appropriate fluorophore. AHA-labeled proteins were then visualized in gel with an Odyssey Fc imaging system (LI-COR Biosciences), or whole parasites were analyzed by flow cytometry on a Facsanto flow cytometer (BD Biosciences) from the MRI platform.

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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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