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Autophagy in Apicomplexa: a life sustaining death mechanism?

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Abstract

Programmed cell death (PCD) pathways remain understudied in parasitic protozoa in spite of the fact that they provide potential targets for the development of new therapy. The best understood PCD pathway in higher eukaryotes is apoptosis although emerging evidence also points to autophagy as a mediator of death in certain physiological contexts. Bioinformatic analyses coupled with biochemical and cell biologic studies suggest that parasitic protozoa possess the capacity for PCD including a primordial form of apoptosis. Recent work in *Toxoplasma* and emerging data from *Plasmodium* suggest that autophagy-related processes may serve as an additional death promoting pathway in Apicomplexa. Detailed mechanistic studies into the molecular basis for PCD in parasitic protozoa represent a fertile area for investigation and drug development.

Keywords

apicomplexa; autophagy; programmed cell death (PCD); *Toxoplasma*; *Plasmodium*

Cell death pathways in unicellular eukaryotes

Whether programmed cell death (PCD) pathways exist in unicellular organisms has been the subject of considerable debate [1, 2]. The presence of PCD pathways in unicellular organisms including protozoa is at first glance paradoxical, as cell death would not appear to be selected for evolutionarily [2–4]. In recent years our understanding of PCD in higher eukaryotes has expanded considerably, to include non – apoptotic pathways, including autophagic mechanisms that under ambient physiologic conditions are often ‘pro survival’, not ‘pro death’ [5, 6]. By today’s nomenclature, apoptosis and autophagy are usually called type I and type II death pathways (Box 1) to signify the demise of the cell by distinct

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cellular mechanisms [7, 8]. Interestingly, In spite of their mechanistic divergence, the coup-de-grace for both pathways often involves the mitochondrion and / or the loss of mitochondrial function [9, 10]. Perhaps consistent with this point of convergence in the execution of PCD, emerging evidence points to a considerable level of crosstalk between the apoptotic and autophagic pathways [5, 11].

Apoptosis-like programmed cell death in unicellular eukaryotes

In spite of conventional wisdom arguing against PCD pathways in unicellular eukaryotes, an expanding literature shows that both caspase dependent and independent apoptotic pathways do indeed exist in yeast [1, 12, 13]. This has opened up the intriguing possibility that PCD pathways exist in other unicellular eukaryotes, including the parasitic protozoa [2, 4, 14]. Molecular studies of PCD pathways in early branching eukaryotes, including parasitic protozoa are in their infancy. Apoptotic death in higher eukaryotes is mediated by specific effectors that include the caspase family of proteases [15]. The presence of related molecules, the metacaspases, in yeast [12] and parasitic protozoa, including *Plasmodium* [16, 17], *Toxoplasma* (annotated in the genome), and the kinetoplastids [18, 19] suggest a primordial form of apoptosis probably exists in unicellular eukaryotes (reviewed in [2, 4, 14, 20]. This view is further supported by morphological and biochemical features consistent with progression of the apoptotic death pathway defined for higher eukaryotes (Box 1). Studies on apoptotic death mechanisms in Plasmodia and kinetoplastids suggest activation occurs primarily within the insect vectors [4, 14], although some features have been noted upon cytotoxic drug treatment of mammalian life cycle stages, at least in some studies [21–23]. Involvement of apoptosis-related death in Plasmodia is, however, not universally accepted [24, 25]. Further complicating analysis of death for Plasmodia and *Toxoplasma* is that it can sometimes be ‘delayed’ (see [3, 4] and references within) meaning damage to organelles such as the apicoplast does not induce death until after the cell has divided and entered a new cell cycle. Although extensive work has been done on the impact of *Toxoplasma gondii* on the host apoptotic cascade [26], investigations into apoptosis within *Toxoplasma* have not been conducted. Thus, while tantalizing evidence for the existence of a primitive apoptotic cascade has been found, whether this is the primary PCD pathway under all conditions, or whether alternative pathways also exist, remains to be established.

Autophagy as a non-apoptotic programmed cell death pathway

A non-apoptotic PCD pathway that has gathered much recent attention is autophagy [6, 27–29]. There are dual functions for autophagy within the cell. While typically viewed as life sustaining under conditions of nutrient limitation, autophagy can also be a death pathway under specific metabolic and physiological circumstances [6, 27]. As the term implies, autophagy is a mechanism by which either cytoplasmic or organellar components of the cell are selectively degraded, providing a housekeeping function for the turnover of aged or defunct cellular components (reviewed in [30, 31]). This pathway assumes a life sustaining function under conditions of nutrient limitation, as excess cellular components are degraded to recycle their building blocks into new macromolecule synthesis [30, 31]. While these housekeeping functions are vital, the role of autophagy in programmed cell death is proving

to be increasingly important [29]. Autophagy also has additional functions in development and differentiation in diverse organisms from mammals [32] to parasitic protozoa [33, 34].

Most molecular studies of autophagy have been done in the yeast *Saccharomyces cerevisiae* and have primarily examined the response to nutrient limitation [30]. These studies, which have many parallels in the study of higher eukaryotes [35] have revealed a highly structured set of events which when activated trigger the cytosolic formation of a unique organelle, the autophagosome (Box 2) [36].

Autophagy-associated death linked to nutrient limitation in Apicomplexa

In inspecting autophagy in *Toxoplasma*, we recently reported on the consequences of nutrient limitation on actively growing intracellular parasites [37]. The key findings were somewhat surprising: in as little as 4 hours, nutrient limitation was sufficient to trigger starvation induced translational repression (evidenced by increased phospho-TgIF2 levels [38]), that was accompanied by systematic fragmentation of the parasite mitochondrion (Figure 1) [37]. The latter phenotype was selective in that the morphology of other organelles (rhoptries, micronemes and apicoplast) were not affected. Despite the secretory organelles being unaffected, there was a marked inhibition of parasite invasion and also a delay in replication for parasites that were invasion competent [37]. Notably, only parasites with an intact mitochondrion appeared invasion competent, indicating that the loss of mitochondrial integrity represents a lethal event [37]. Starvation induced fragmentation of the single mitochondrion as well as inhibition of invasion were completely blocked by S3-methyladenine (3-MA) (Figure 1), a classical autophagy inhibitor [37]. In addition, the *T. gondii* homolog of the widely used intrinsic reporter for autophagy, TgATG8 (Atg8 in yeast and LC3 in mammals [Box 2]) exhibited a marked shift to a punctate distribution (Figure 1) as well as a shift in electrophoretic mobility consistent with lipid modification seen in yeast and higher eukaryotes (Box 2). Similar changes in TgATG8 distribution and electrophoretic mobility are also seen in extracellular parasites under nutrient limiting conditions [39]. Together these changes parallel what is observed in yeast and higher eukaryotes, but with one notable difference; in *Toxoplasma*, mitophagy is accompanied by the pharmacologically inhibitable demise of the parasite, suggesting that the decision between a life-sustaining and death-promoting role for autophagy rests on a razor's edge. What specifically influences the decision in *Toxoplasma* to switch autophagy between life-sustaining vs death-promoting activities is not known [40].

Nutrient limitation in these experiments, a condition that intracellular parasites are not likely to encounter, may have fortuitously revealed an Achilles heel that is a surrogate for an as yet to be identified PCD stimulus. A death stimulus functionally intertwined with nutrient sensing would highlight the importance of intermediary metabolism in regulating parasite death. Interestingly then, the signal responsible for this loss of mitochondrial integrity in response to starvation was shown to be amino acid depletion as opposed to the loss of energy metabolites (glucose and pyruvate) [37]. Similar to higher eukaryotes (including *S. cerevisiae*) [41], the induction of autophagy was also achieved by targeting the *Toxoplasma* homolog of TOR (target of rapamycin) [37]. Rapamycin treatment in nutrient replete media mimicked amino acid starvation effects on the integrity of the mitochondrion and was

similarly blocked by 3-methyladenine [37]. Interestingly, starvation of *Plasmodium* also induces events associated with autophagy, despite the absence of a clear TOR homolog for this parasite (see below). Perhaps the recently described signaling mediated by PfeIK1 in response to amino acid starvation [42] supplements for lack of an obvious TOR orthologue, but clearly more work on how *P. falciparum* senses amino acid starvation is needed.

Bioinformatic analyses now completed by several groups indicate that these relatively early branching eukaryotes clearly possess the core machinery for macro-autophagy but lack clear orthologues of several autophagy genes seen in higher eukaryotes [34, 43]. There are 35 gene products (designated 'ATG') directly involved in the initiation, progression and completion of macro-autophagy in yeast with additional factors feeding into the system [44]. The possible absence of certain key autophagy genes in the genomes of parasitic protozoa is not entirely surprising in light of their evolutionary antiquity. On a functional level, the fact that the ATG genes are largely dispensable in yeast under nutrient replete conditions, but are required when nutrients are limiting, indicates that autophagy plays an important role under specific physiological conditions (reviewed in [31, 35]) that could well be less common for intracellular parasites. The fact that viable mice can be generated following the systemic knockout of several ATG orthologs indicates that some steps in autophagy are also non essential for mammals [32].

By contrast, TgATG3 has been shown to be essential in *T. gondii* with conditional mutants exhibiting severe defects in both the maintenance of mitochondrial integrity as well as replication [39]. Our ongoing work with TgATG1 (a kinase required for the activation of autophagy) and TgATG8 (required for autophagosome formation; see Box 2) indicates that these proteins are also essential (D. Ghosh, B. Eller and A. Sinai, unpublished), suggesting that a core autophagic cascade is absolutely required in *Toxoplasma*. However, in response to altered physiological conditions triggered by amino acid limitation (and potentially other yet to be defined stimuli), the hyper-activation of autophagy crosses a yet to be defined threshold resulting in a systematic progression to death [37]. In this regard autophagy in *Toxoplasma* is fundamentally distinct from the processes seen in higher eukaryotes, including yeast- where the pathway itself is non-essential under nutrient replete conditions [45].

Activation of autophagy in *Plasmodium*

Similarly, the unique replicative and developmental strategies employed by Plasmodia suggest a requirement for unique autophagic processes. Recent work from the Coppens laboratory reveals involvement of autophagy in the differentiation of sporozoites to merosomes within hepatocytes [46, 47]. The involvement of autophagy in Plasmodia infected red blood cells (iRBC) is yet to be studied. Specific questions arise: among them, does autophagy play a role in the maintenance and thus inheritance of Plasmodia organelles, most notably a sole mitochondrion? In addition, the fact that autophagy represents a PCD mechanism in *Toxoplasma* [37] suggests that in some cases it could control PCD in *Plasmodium* as well.

To test whether autophagy is present in the iRBC forms of *P. falciparum*, we recently probed for the one universally well accepted molecular signal of autophagy, punctate redistribution of the PfATG8 protein upon application of one routine, universally well accepted inducer of autophagy, cell starvation [31]. Although additional work remains to be done, Figure 2 shows exceedingly intriguing results (D. Gaviria, A.P. Sinai, and P.D. Roepe, unpublished). Since ATG8 proteins from *T. gondii* and *P. falciparum* are ~ 70 % identical with long stretches where the sequence is identical, we were able to use anti *Tg*ATG8 antisera to easily detect *Pf*ATG8 protein at the expected mass in western blot analysis of trophozoite proteins (D. Gaviria., A.P. Sinai, and P.D. Roepe, unpublished). Not surprisingly then, staining control *P. falciparum* trophozoites within intact iRBC for *Pf* ATG8 reveals expected ATG8 protein localized within the cytosol of the parasite (Figure 2 top). Antibody controls indicate staining is specific for *Pf* ATG8 protein (see caption, Figure 2). Consistent with the induction of an autophagy cascade, when these same trophozoites are starved for six hours by placing in them in starvation media without both glucose or amino acids, *Pf* ATG8 is redistributed in the expected wider, more punctate pattern (Figure 2 bottom), with some protein (and presumably associated vesicles) near the RBC membrane. To a first approximation, this pattern is reminiscent of autophagy cascades for many other cell types; however, in these other examples, we know that the ATG8 protein puncta are localizing to developing cytosolic autophagosomes [31]. In light of the absence of organelles in the RBC cytoplasm, the 'target' of these putative *Pf*ATG8 autophagosome puncta is unknown. RBC hemoglobin, or perhaps even extra-RBC (i.e., blood) nutrients, appear to us to be logical possible targets. Remarkably then, for *P. falciparum* within iRBC, *Pf* ATG8 positive vesicles may be exported out of the parasite, to the periphery of the iRBC during starvation induced death. Presumably the vesicles become attached to the RBC plasma membrane, or perhaps junctions between the RBC membrane and the parasite parasitophorous membrane. We suggest that for iRBC stages of *P. falciparum*, a highly specialized autophagy – endocytosis is induced by starvation to recover food either from the red cell cytosol or perhaps even from outside the red cell.

In sum, it appears that a bare bones autophagy cascade does exist for *P. falciparum*, that it is active in liver stages to clear unneeded organelles [46, 47], and that highly specialized, but essential features of the induction of autophagy are also present for iRBC stages. Analogous to many other examples, the iRBC *P. falciparum* cascade is induced in response to nutrient starvation, yet, curiously, no obvious TOR ortholog that in other eukaryotes couples nutrient starvation to activation of autophagy appears to be encoded by the *P. falciparum* genome (A.P. Sinai and P.D. Roepe, unpublished). This then leads us to conclude that under normal growth conditions iRBC *P. falciparum* autophagy is probably a constitutively activated 'pro survival' process that assists in nutrient acquisition and that upregulating the process is unusual. If this process is either inhibited or upregulated by drugs, this would be deleterious to the intracellular survival of the parasite.

Autophagy mediated death: a potential consequence of drug treatment?

Growth inhibitory concentrations of chloroquine (CQ) (i.e., IC₅₀-levels of 10 – 100 nM) would not be predicted to directly inhibit either basal autophagosome traffic or increased traffic related to starvation – induced *Pf*ATG8 redistribution (Figure 2). However, cytosolic

concentrations of CQ as recently defined [48] would be predicted to do this, via the well known lysosomotropic behavior of CQ at these higher concentrations. In fact, an interesting but somewhat overlooked paper presents rather dramatic accumulation of unidentified vesicles upon administration of cytotoxic dosages of CQ [49] that could be due to inhibition of vesicle – lysosome fusion. It would be interesting to inspect whether these vesicles harbour PfATG8 protein.

Almost nothing is known about how cytotoxic antimalarial drugs kill parasites within the iRBC, but this information is central for new antimalarial drug design (Box 3). While possibly not triggered by growth inhibitory (cytostatic) drug concentrations, increased autophagy might be induced when cytotoxic drug concentrations are employed, due to protein misfolding or organellar damage (targets for autophagy) caused by the higher drug dose. This may particularly be the case for quinolines (e.g., CQ) which by interfering with hemoglobin digestion within the digestive vacuole also effectively starves the parasite for hemoglobin derived amino acids (recall amino acid starvation induces autophagy for *Toxoplasma* [37]). The point is, either drug inhibition of basal autophagy, or drug induced upregulation of autophagy, is easily envisioned to affect PCD for intra erythrocytic *P. falciparum*.

Our initial inspection of these possibilities (D. Gaviria, , M. Paguio, , L. Turnbull, , M. Ferdig, ,A.P Sinai and P.D. Roepe, unpublished) takes advantage of well characterized CQ resistant (CQR) vs sensitive (CQS) strains of *P. falciparum*. We find that CQ treatment at cytotoxic (but not cytostatic) dose does indeed instigate redistribution of PfATG8 similar to Figure 2, and that the putative autophagy cascade revealed by PfATG8 is altered in important ways for CQR *P. falciparum*. Again, although much work remains to be done, it is possible that, for at least some drugs, apicomplexan cell death is linked in some fashion to autophagy.

Unraveling the specific involvement of autophagy as a PCD mechanism in Apicomplexa has immediate implications for our understanding of drug sensitivity and resistance. Relatedly, emerging studies suggest that activation of autophagy and the accompanying redistribution of TgATG8 and fragmentation of the mitochondrion is associated with the cytotoxic effects of monensin (G. Arrizabalaga, unpublished). By contrast, parasite death triggered by pyrimethamine does not appear to involve the loss of mitochondrial integrity [39] suggesting that not all drug induced death involves autophagy. As the need for the development of new antimalarial drugs becomes increasingly dire, detailed studies of antimalarial drug action should perhaps now be expanded to include a pathway that is already a defined target for the development of a broad range of drugs against diseases as diverse as diabetes and cancer [50, 51]. The repurposing of existing drugs directed at the TOR/PI3K pathways has already revealed several leads against kinetoplastid parasites [52]. As mechanistic studies focused on dissecting the death pathways of these parasites expand, new insight into both basic biology and novel, unique drug targets is certain to emerge.

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Box 1. Common Classification of PCD Pathways

PCD causes irreparable DNA damage, arrests energy production from the mitochondria, and shuts down anabolic activities, ensuring the demise of the cell. Apoptosis (type I PCD) is a primary mechanism of PCD in higher eukaryotes (reviewed in [8]). A third PCD pathway, 'necroptosis' is believed to be a programmed form of necrosis, but is much less well understood [53]. Apoptotic stimuli can be generated within the cell, or be introduced by external stimuli. Two versions of type I PCD occur, one characterized by release of a mitochondrial NADH oxidase called apoptosis inducing factor (AIF), and the other by a cascade of proteases (initiator caspases casp2,8,9 or 10) that activate executioner caspases (casp3,6 or 7) [15]. The targets of either AIF or executioner caspases are needed for cell integrity, thus release of AIF or executioner caspase results in a multitude of 'lesions' from which a cell cannot recover. A central player in both forms of apoptosis is the mitochondrial network since release of cytochrome c or AIF from the intermembrane space to the cytoplasm either triggers and amplifies caspase activation [9], or allows nuclear localization of AIF where it then catalyzes chromatin condensation, respectively. A balance between pro- and anti-apoptotic Bcl2 family protein activities governs the threshold at which mitochondrial events commit the cell to death [9]. Also in type I PCD, activation of specific nucleases cleaves DNA into discrete nucleosomal units that appear as a ladder following electrophoresis [8]. Experimental readouts for apoptosis [8] thus include the presence of nuclear AIF, evidence of caspase activation based on substrate cleavage, and laddering of nuclear DNA or in situ DNA fragmentation (TUNEL assay). Other signs of type I PCD include externalization of phosphatidylserine and reduced mitochondrial membrane potential. The identification of metacaspases [12] coupled with observation of several of these biochemical features linked to apoptosis suggests that apoptosis-like pathways are also present in unicellular organisms, including parasitic protozoa (reviewed in [2, 4]).

The involvement of autophagy (type II PCD) as a bona fide death pathway has been the subject of some controversy [6, 54], since in higher eukaryotes it has also been found to promote increased survival in otherwise dying cells. Autophagy is a degradative pathway normally employed in cellular homeostasis to turn over bulk cytoplasm and / or recycle damaged organelles [35]. Under specific physiological conditions, the selective targeting of organelles, particularly mitochondria, can culminate in a PCD pathway functionally distinct from apoptosis [28]. Morphological features including the development of autophagosomes (Box 2) in the absence of chromatin fragmentation are currently used to define type II PCD. However, there can also be considerable crosstalk between the apoptotic and autophagic pathways that then modifies progression to cell death [5]. Evidence for autophagy in yeast PCD [27] as well as recent studies with parasitic protozoa [14, 37] suggest type II PCD may be particularly important for unicellular eukaryotes under unusual physiological conditions.

Box 2. Autophagosome formation: a signature for induction of autophagy

Activation of autophagy can be defined as a poorly understood maturation of membrane structures known as phagophores into double - membraned cytosolic vesicles called autophagosomes. Once formed, these autophagosomes deliver various types of cargo for proteolysis or digestion by fusing with lysosomes (see [30, 31] and references within). The origins of the phagophore are currently debated, and may include Golgi, mitochondrial, endoplasmic reticulum, or plasma membranes. A core set of genes (designated 'ATG#' in yeast [44]) involved in the stimulus dependent induction of phagophore formation (Atg1, Atg13), vesicle nucleation (Atg6, Atg14, and the PI3 Kinases Vps34 and Vps15), autophagosome maturation (Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12, and Atg16) and membrane recycling (Atg9) have been described in addition to other accessory factors. The genomes of parasitic protozoa reveal orthologs of many but not all of the core autophagy associated genes [34, 37, 43]. Molecular events that control maturation of the autophagosome are being elucidated, and include two key conjugations, one between Atg12 and Atg5 proteins to expand the phagophore, and another between Atg8 protein and phosphatidylethanolamine mediated by the Atg7-Atg3 conjugation systems. Lipidated ATG8 in higher eukaryotes is also known as LC3II, and lipidation usually requires prior cleavage of C terminal arginine to reveal the terminal acceptor glycine, via the Atg4 protease. Interestingly however, neither *T. gondii* or *P. falciparum* genomes harbor an obvious Atg4 protein, and both TgATG8 and PfATG8 end in glycine [37]. This suggests some level of constitutive autophagosome formation in the two parasites, which is further supported by the constitutive presence of both lipidated and non lipidated TgATG8 in *Toxoplasma* ([39] and *Plasmodium* (P.D. Roepe and A.P. Sinai, unpublished). The continuous presence of a pool of lipidated ATG8 in *Toxoplasma* and *Plasmodium* suggests that basal autophagy is required for normal homeostasis. The activation of autophagy above this basal level in response to starvation is associated with an increase in the levels of lipidated-ATG8 and / or its redistribution to puncta that delimit putative autophagosomes. This makes cellular ATG8 staining a convenient marker for mature autophagosomes from yeast to mammalian cells as well as *Toxoplasma* (Figure 1) and *Plasmodium* (Figure 2).

Box 3. 'New directions': cytostatic (IC₅₀) vs cytotoxic (LD₅₀) drug activities

Central to a description of any antimicrobial drug is distinction between growth inhibitory (cytostatic) vs cell kill (cytotoxic) drug activity. This can be complicated or ambiguous. Cytostatic activity is typically calculated by quantifying growing cells in the constant presence of various drug concentrations, plotting that growth vs concentration of drug, and then determining the midpoint of the effect, denoted 'IC₅₀' (inhibitory concentration that yields 50 percent growth relative to control). Similar approaches have been used for decades to calculate IC₅₀ for intracellular *P. falciparum* and *T. gondii* parasites. By contrast, the midpoint of cytotoxic effect that kills 50 % of cells (e.g., 'LD₅₀', for 'lethal dose') is usually calculated from a clonogenic assay wherein cells are plated and colonies that form in an appropriate amount of time are counted to deduce the number of live cells prior to plating. Importantly, no such assays are possible for *P. falciparum*, and although soft agar overlays of *T. gondii*-infected monolayers do permit plaque assays, overtly lethal (LD₅₀) drug concentrations could also adversely impact the host cells, so IC₅₀ data are emphasized. Yet cytotoxic activity of antiparasitic drugs is arguably more important in the clinic than is the cytostatic activity [55]. Only one recent paper has described an approach to conveniently tabulate LD₅₀ for known antimalarial drugs [48], and only one other approach for measuring cytotoxic activity (e.g., a 'limiting dilution' approach) has been described [56, 57]. In further describing LD₅₀ phenomena, and in optimizing the cytotoxic activity of additional candidate antiparasitic drugs, molecular level knowledge of parasite PCD pathways is crucial. Vice versa, study of parasite PCD pathways would benefit enormously from additional convenient LD₅₀ assays.

Figure 1.

The effect of starvation on *Toxoplasma* mitochondrial morphology and distribution of TgATG8. *Toxoplasma* tachyzoites (Type I-RH) were used to infect an HFF monolayer for 24 h in complete tissue culture media (CM) [37]. The infected cells were either maintained in complete media or treated with starvation media (SM-(6%CM in HBSS)) for an additional 8 hours prior to fixation with methanol. **Top panels:** In CM mitochondria (F1β) exhibit typical ring structure. By contrast, exposure to SM results in the progressive fragmentation of the mitochondria. The surface antigen SAG1 outlines the margins of the parasites (red). **Bottom panels:** Parasites grown in CM exhibit normal mitochondrial morphology (F1β) with TgATG8 appearing as diffuse cytoplasmic staining with occasional puncta. By contrast, following exposure to SM the mitochondria assume a punctate morphology (F1β-green) with the TgATG8 signal (red) becoming considerably more punctate.

Figure 2.

Redistribution of PfATG8 protein (green spots) upon starvation – induced cell death. Top three panels are strain HB3 *P. falciparum* grown in red blood cell culture under normal conditions; the bottom is a parasite from the same culture after being placed in starvation media (no sugar, no amino acids) for 6 hr. For imaging, cells were washed 3 times with 25 mM HEPES, fixed with 4% formaldehyde/0.0075% glutaraldehyde in PBS for 30 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, reduced with 0.3mg/mL sodium triacetoxyborohydride for 10 minutes, blocked with 5% goat serum for 1 hour, and then treated with antibody (1:1000) diluted in 5% goat serum/PBS Tween-20 for 1 hr at 37°C in the dark. The primary antibody was rabbit anti TgATG8; the secondary was goat anti rabbit IgG conjugated to DyLight488 fluorophore. Parasite nuclei were also stained with DAPI (blue) and mounted using Fluorogel mounting media. Samples were imaged using a spinning disk confocal microscope and 405 and 491nm laser lines at 200ms exposure and 35% laser power. Images were iteratively deconvolved using an experimental point spread function and AutoQuantX2 software and displayed using Imaris 7.4.2. Controls (titrating antibodies, no primary antibody vs no secondary antibody) reveal staining is specific for PfATG8.