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Research Letter

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Autophagy participates in the unfolded protein response in *Toxoplasma gondii*

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One sentence summary: In Toxoplasma, ER stress and starvation are potent activators of the eIF2α pathway that, in turn, regulates autophagy.
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ABSTRACT

Environmental and genetic perturbations of endoplasmic reticulum (ER) function can lead to the accumulation of unfolded proteins. In these conditions, eukaryotic cells can activate a complex signaling network called the unfolded protein response (UPR) to reduce ER stress and restore cellular homeostasis. Autophagy, a degradation and recycling process, is part of this response. The parasitic protist *Toxoplasma gondii* is known to be able to activate the UPR upon ER stress, and we now show that this pathway leads to autophagy activation, supporting the idea of a regulated function for canonical autophagy as part of an integrated stress response in the parasites.

Keywords: Autophagy; endoplasmic reticulum stress; unfolded protein response; integrated stress response; *Toxoplasma*; eIF2α

INTRODUCTION

The endoplasmic reticulum (ER) is a multifunctional organelle that is particularly important for lipid biosynthesis, calcium homeostasis and protein folding. Perturbations that disrupt the ER capacity to process and fold proteins can lead to an accumulation of unfolded or misfolded proteins in the ER lumen, and generate proteotoxicity. Eukaryotic cells can sense and counter ER stress through the upregulation of a signaling pathway called the unfolded protein response (UPR) (Hetz 2012). The UPR is acting at several levels to reduce protein load in the ER, increase protein-folding capacity and eliminate abnormal proteins. For instance, it induces chaperones, promotes degradation of misfolded proteins and also represses protein translation. To achieve this, there is a complex signaling network integrating information transmitted through several UPR sensors and their downstream effectors that are rather well characterized in metazoan and in yeast (Wu, Ng and Thibault 2014). For instance, the α-subunit of eukaryotic initiation factor 2 (eIF2) is instrumental for controlling protein translation. More generally, eIF2α is central to the integrated stress response (ISR), which is activated not only after intrinsic stresses such as in the ER, but also extrinsic stresses such as nutrient shortage (Pakos-Zebrucka *et al.* 2016). In the context of ER stress, phosphorylation of eIF2α by the protein kinase R (PKR)-like ER kinase (PERK) (Fig. 1A), results in a rapid decline in de novo protein synthesis, thus lowering the influx of nascent proteins in the ER. Nutrient deprivation, on the other hand, will lead to eIF2α

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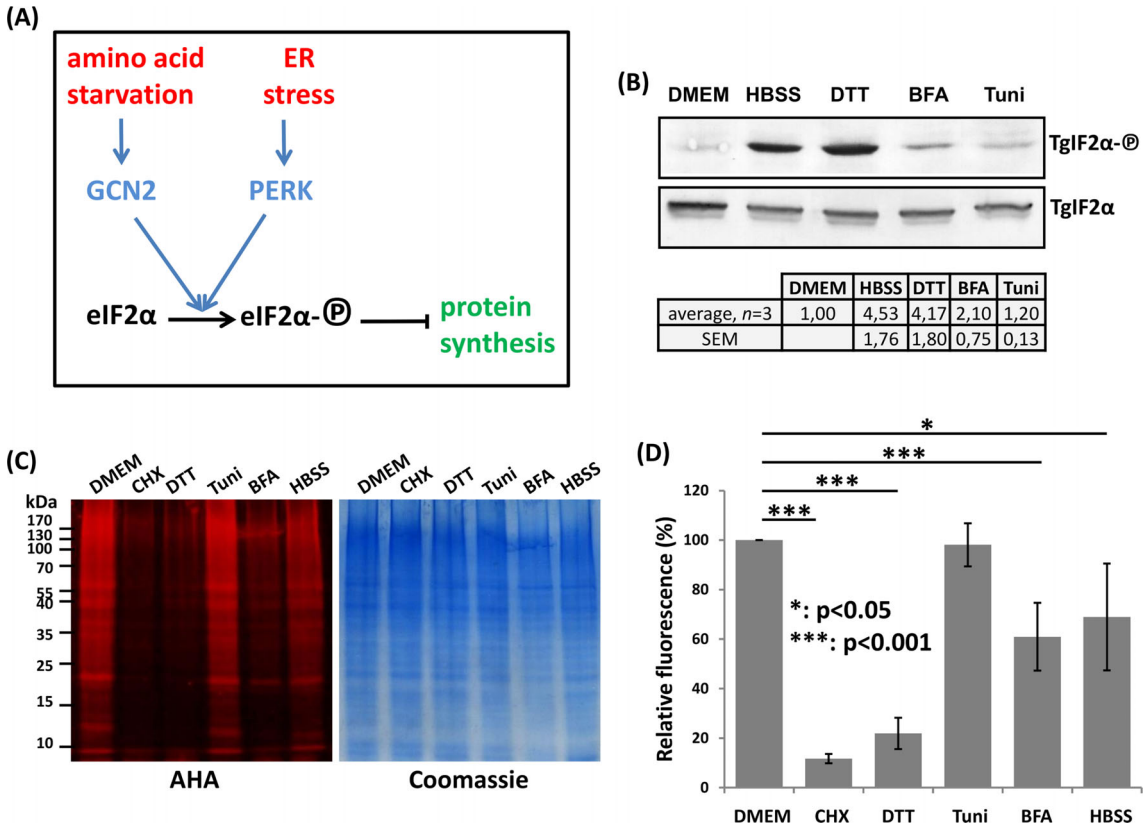

	DMEM	HBSS	DTT	BFA	Tuni
average, *n*=3	1,00	4,53	4,17	2,10	1,20
SEM		1,76	1,80	0,75	0,13

Figure 1. Modulation of TgIF2α phosphorylation and subsequent inhibition of protein synthesis by compounds triggering ER stress and by nutrient starvation. (**A**) Schematic representation of the eIF2α pathway. (**B**) Immunoblot analysis of protein extracts from freshly egressed *T. gondii* tachyzoites kept extracellular for 6 hours in HBSS (for amino acids starvation), in DMEM or in DMEM supplemented with various compounds triggering ER stress: 5 mM dithiothreitol (DTT), 10 μM brefeldin A (BFA) and 10 μg/ml tunicamycin (Tuni). Total or phosphorylated TgIF2α were revealed with specific antibodies. The displayed immunoblots are representative of three independent experiments. The table below shows densitometry analysis that was carried out with values collected from the three experiments: phosphorylated TgIF2α vs total TgIF2α ratio was calculated and values were set to 1 for the DMEM control in each independent experiment, relative enrichment was then calculated for the different experimental conditions. Values displayed are mean ± standard error of the mean (SEM). (**C**) Extracellular parasites were subjected to ER stress treatment or starvation as described above, in the presence of 12.5 μM L-azidohomoalanine (AHA) for 5 hours to label newly synthesized proteins in each sample. Click reaction was performed and fluorescent protein labeling was visualized in gel (left). Coomassie staining of total proteins is shown as a control (right). Protein synthesis inhibitor cycloheximide (CHX) was used as a negative control. (**D**) Whole parasite AHA fluorescence was quantified and analyzed by flow cytometry. Values are mean from $n = 3$ experiments ± SEM. Asterisks denote statistical significance as determined by Student's T test.

phosphorylation through the general control nonderepressible 2 (GCN2) kinase (Fig. 1A).

Toxoplasma gondii is an obligate intracellular parasite that is responsible for toxoplasmosis, a potentially severe and life-threatening disease for developing fetuses and immunocompromised patients, yet otherwise benign in most immunocompetent individuals (Montoya and Liesenfeld 2004). These parasitic protists, which belong to the phylum Apicomplexa, have a complex life cycle, alternating between different hosts and developmental forms, and changes in their environment are thus likely exposing them to a variety of stress stimuli during their development. In addition to parasite-specific strategies for long-term adaptation to stress, leading to the differentiation into dormant resistance forms, these early-diverging eukaryotes also possess part of the molecular machinery involved in the evolutionarily conserved response to acute stress. For example, phosphorylation of *Toxoplasma* eIF2α (TgIF2α) was previously shown to repress protein translation as part of the parasite stress response and developmental changes (Sullivan *et al.* 2004; Narasimhan *et al.* 2008; Joyce *et al.* 2010; Konrad, Wek and Sullivan 2011, 2014).

Another pathway potentially playing an important role in the parasite response to stress is autophagy. This self-degradative pathway promotes the salvage of cellular components by segregating them in multi-membrane vesicles called autophagosomes, which will fuse with lysosomes for digestion and recycling of their content. It is stimulated by multiple cellular stresses, including nutrient deprivation, but also hypoxia, reactive oxygen species and protein aggregation (Kroemer, Mariño and Levine 2010). The molecular machinery for autophagy is only partly conserved in *Toxoplasma*, and part of it is involved in a non-canonical function related to the apicoplast, the apicomplexan plastid (Lévêque *et al.* 2015). However, the parasites are also able to generate autophagosomes in response to nutrient shortage (Besteiro *et al.* 2011; Ghosh *et al.* 2012). These vesicles are decorated with AuTophaGy-related protein 8 (ATG8), a widely used autophagosomal marker (Shpilka *et al.* 2011). Although there is no clear demonstration of a fully functional catabolic autophagy pathway in the parasite yet, recent functional investigation of a *Toxoplasma* ATG9 homologue (a protein that is potentially important for the early steps of autophagosome formation), revealed a possible role for canonical autophagy in the parasites for surviving stress conditions as extracellular parasites or within host immune cells (Nguyen *et al.* 2017).

Nutrient starvation and the UPR, both able to modulate eIF2α phosphorylation, are also potent stimuli for autophagy

(Kroemer, Mariño and Levine 2010). We thus aimed at investigating, in the context of *Toxoplasma*, the involvement of autophagy in the UPR, and its potential interplay with the eIF2α regulatory pathway.

MATERIALS AND METHODS

Parasites and cells culture

Parasites were maintained by serial passage in human foreskin fibroblast (American Type Culture Collection, Manassas, USA, CRL 1634) cell monolayers grown in Dulbecco's modified Eagle medium (DMEM, Gibco, Villebon sur Yvette, France) supplemented with 5% decomplemented fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and a cocktail of penicillin–streptomycin (Gibco) at 100 μg/ml.

Stress induction and immunoblot analyses

Freshly egressed parasites were collected by centrifugation and incubated for 6 hours in Hank's Balanced Salt Solution (HBSS, Gibco), or DMEM in the presence of ER stress-inducing agents: 5 mM dithiothreitol (DTT), 10 μM brefeldin A (BFA) and 10 μg/ml tunicamycin (Tuni) (all purchased from Sigma-Aldrich, Saint Quentin Fallavier, France). Parasites were then centrifuged, and the pellet was resuspended in loading buffer before being separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblot. The primary antibodies used for detection are specific for TgIF2α or its phosphorylated form, and were used as described before (Narasimhan *et al.* 2008). Immunoblot signal quantification was performed using the ImageJ software v1.51 (US National Institutes of Health, Bethesda, USA).

Analysis of autophagosome-like structures

To visualize autophagosome-like structures, we used *Toxoplasma gondii* tachyzoites expressing green fluorescent protein (GFP)-tagged TgATG8, or C-terminal glycine mutant TgATG8^{G124A} (Besteiro *et al.* 2011). Alternatively, TgIF2α mutant parasites (TgIF2α^{S71A}) (Joyce *et al.* 2010) were transfected to express transiently GFP-TgATG8. Inhibition of TgIF2α dephosphorylation was achieved with guanabenz acetate (GA) (Santa Cruz Biotechnology, Dallas, USA). Immunofluorescence analyses were performed either on extracellular or intracellular parasites after they were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline solution, with the modification that the extracellular parasites were made to adhere onto poly-L-lysine slides (VWR, Fontenay-sous-Bois, France) for 20 minutes prior to processing for immunofluorescent labeling. All parasites were co-stained with antibodies for the Cpn60 apicoplast marker (a kind gift from Boris Striepen) (Agrawal *et al.* 2009) to discard the apicoplast-related TgATG8 signal.

Protein labeling with L-azidohomoalanine

To label neo-synthesized proteins, we used a methionine analog, L-azidohomoalanine (AHA), that can be chemoselectively linked to a fluorescent moiety for labeling proteins (Click-iT AHA, Thermo Fisher Scientific, Villebon sur Yvette, France). Extracellular parasites were subjected to ER stress treatment or starvation as described above, in the presence of 12.5 μM AHA for 5 hours to label newly synthesized proteins in each sample. The click reaction was performed as described before (Nguyen *et al.* 2017). Protein synthesis inhibitor cycloheximide was used as a negative control. AHA-labeled proteins were then visualized in gel with an Odyssey Fc imaging system (LI-COR Biosciences, Lincoln, USA), or whole parasites were analyzed by flow cytometry on a Facscanto flow cytometer (BD Biosciences, Le Pont de Claix, France) from the Montpellier resources in imaging (MRI) platform.

Electron microscopy

Electron microscopy was performed as described previously (Lévêque *et al.* 2015).

RESULTS

Both nutrient starvation and endoplasmic reticulum stress lead to the activation if the Toxoplasma eukaryotic initiation factor 2 pathway

First, we examined the impact on *Toxoplasma* tachyzoites (the fast replicating form of the parasite) of a variety of pharmacological compounds that are known to induce ER stress. These compounds act by different mechanisms, namely inhibition of N-glycosylation in the ER (Tuni), inhibition of ER/Golgi transport (BFA) and reduction of disulfide bonds (DTT). Freshly egressed extracellular parasites were incubated for 6 hours in complete medium (DMEM) with these compounds, or in a medium devoid of amino acid (HBSS), and the phosphorylation status of TgIF2α was assessed by immunoblot (Fig. 1B). In accordance with previous reports (Ghosh *et al.* 2012; Konrad, Wek and Sullivan 2014), amino acids starvation led to a strong increase in TgIF2α phosphorylation. ER stress inducers also all led to an increase in TgIF2α phosphorylation, albeit with different efficiency. DTT treatment was clearly the most potent, while Tuni was the least. One should note that commercial Tuni is a mix of four different isoforms and some batches are sometimes less efficient than others. We next evaluated the impact of these compounds on translation by measuring de novo protein synthesis in parasites kept in similar conditions, with a clickable amino acid analog (L-azidohomoalanine, AHA). Both in-gel (Fig. 1C) and cytometry-based fluorescence measurements (Fig. 1D) confirmed starvation and ER stress inducers DTT and BFA were able to repress protein synthesis. Again, DTT appeared to be the most potent compound for this.

Endoplasmic reticulum stress induces the biogenesis of autophagosomes in *Toxoplasma*

To assess the impact of ER stress on autophagy, we then tested these compounds on parasites expressing GFP-fused TgATG8, a protein that relocalizes to the membrane of autophagosomes upon starvation (Besteiro *et al.* 2011). Because TgATG8 is also constitutively associated with the apicoplast in an autophagy-independent fashion, primarily in replicating intracellular tachyzoites (Kong-Hap *et al.* 2013; Lévêque *et al.* 2015), we performed a co-staining with an apicoplast marker to discriminate GFP-TgATG8-decorated autophagic vesicles. We could see that both for extracellular (Fig. 2A) and intracellular (Fig. 2C) parasites, treatment with ER stress-inducing compounds was generating GFP-TgATG8-decorated autophagic vesicles akin to those induced by amino acid starvation. Quantification of GFP-TgATG8 puncta in extracellular (Fig. 2B) and

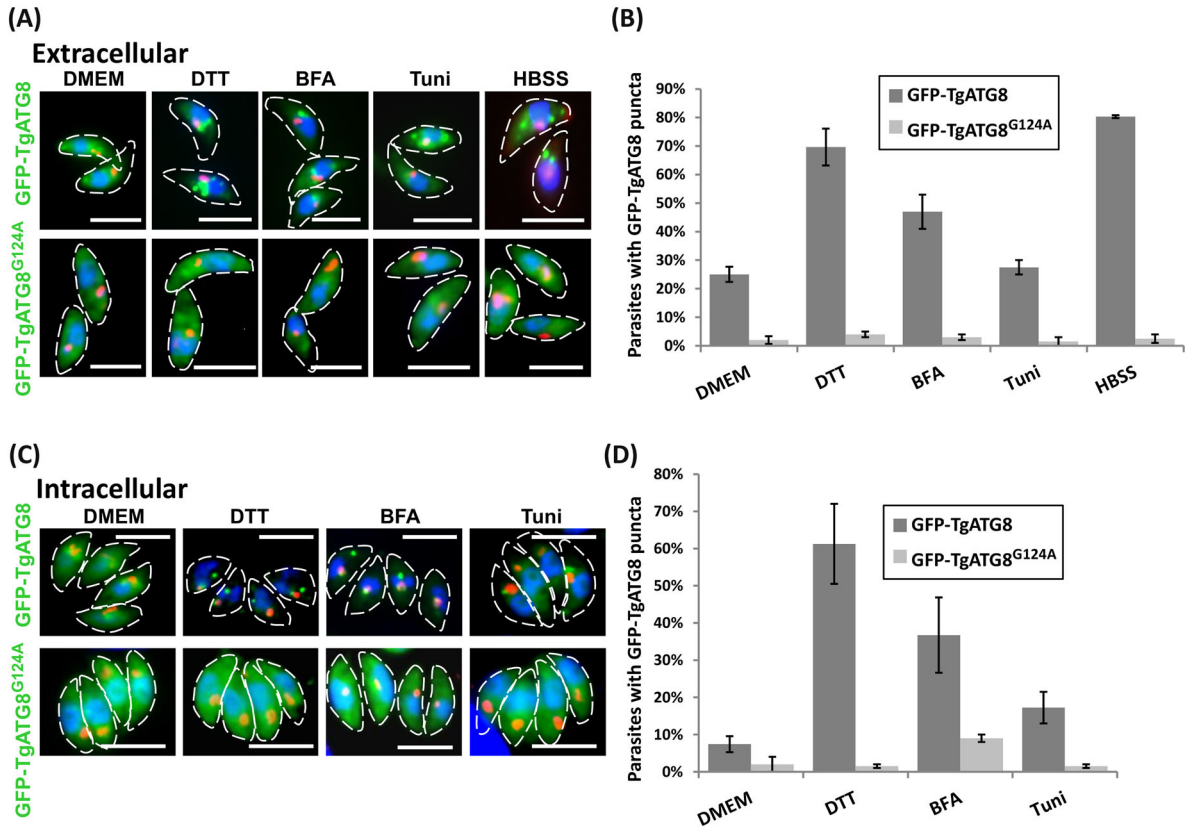

Figure 2. Compounds triggering ER stress induce the formation of autophagosomes in both intracellular and extracellular *Toxoplasma*. (**A**) Examples of extracellular GFP-TgATG8-expressing parasites displaying punctate signal distinct from the apicoplast upon ER stress treatment or starvation, as described in Fig. 1B legend. Parasites expressing GFP-TgATG8^{G124A} were used as a negative control. The apicoplast was labeled with an anti-Cpn60 antibody (red). DNA was labeled with DAPI (blue). Scale bars: 5μm. (**B**) Quantification of the proportion of extracellular parasites presenting a punctate GFP-TgATG8 signal distinct from the apicoplast upon treatment. GFP-TgATG8 mutated on its C-terminal glycine (GFP-TgATG8^{G124A}) that fails to conjugate to autophagosomal membranes was used as a control. (**C**) Examples of intracellular GFP-TgATG8-expressing parasites displaying punctate GFP-TgATG8 signal distinct from the apicoplast upon ER stress treatment, as described in Fig. 1B legend. Scale bars: 5 μm. (**D**) Quantification of the proportion of intracellular parasites presenting a punctate GFP-TgATG8 signal distinct from the apicoplast upon treatment. GFP-TgATG8 mutated on its C-terminal glycine (GFP-TgATG8^{G124A}) that fails to conjugate to autophagosomal membranes was used as a control.

intracellular (Fig. 2D) parasites showed that DTT led to the most potent effect, in accordance with what we observed for TgIF2α phosphorylation or translation repression (Fig. 1B–D). It should be noted that DTT treatment of intracellular parasites led to some egress, as previously described (Stommel *et al.* 1997), although enough parasites remained to be counted. As a control, we used a cell line expressing GFP-TgATG8 mutated on its C-terminal glycine (GFP-TgATG8^{G124A}) that fails to conjugate to autophagosomal and apicoplast membranes (Kong-Hap *et al.* 2013), and showed that treatment with the compounds did not lead to the appearance of GFP-TgATG8^{G124A}-decorated puncta (Fig. 2). This confirmed that the puncta we observed are likely to be of autophagosomal nature and are not protein aggregates generated by the ER-stressing compounds. To further verify this, we also performed morphological observations by electron microscopy on DTT-treated extracellular parasites. Similar to the previously described amino acid starvation conditions (Besteiro *et al.* 2011), we identified large vacuoles and occasional cytoplasm-containing multimembrane structures resembling autophagosomes (Fig. 3). Besides, we also observed some lipid bodies (Fig. 3) that were essentially absent from the control. This might not be surprising, as there is a close relationship between lipid bodies formation and the cellular response to ER stress (Hapala, Marza and Ferreira 2011).

The Toxoplasma eukaryotic initiation factor 2 pathway regulates stress-induced autophagosome formation

To assess whether the TgIF2α pathway is acting upstream of the autophagic pathway, we sought to modulate the phosphorylation state of TgIF2α and see the effect on stress-generated autophagosomes. We first treated intracellular parasites with GA, an inhibitor of eIF2α dephosphorylation which has been shown to have activity against *Toxoplasma in vitro* (Narasimhan *et al.* 2008; Konrad *et al.* 2013). Treatment with GA alone was sufficient to induce the appearance of GFP-TgATG8-positive autophagic vesicles in the parasites, and it also significantly potentiated the effect of DTT on autophagosomes biogenesis (Fig. 4A). Conversely, we sought to check for autophagosome biogenesis when TgIF2α phosphorylation is inhibited. To do so, we expressed GFP-TgATG8 in parasites genetically altered to express TgIF2α in which the phosphorylated serine is definitely substituted by an alanine (TgIF2α^{S71A}) (Joyce *et al.* 2010), and quantified autophagosome formation after DTT-induced ER stress, or amino acids starvation, in extracellular parasites. We could see that in the TgIF2α phosphorylation mutant, the induction of autophagosome formation by nutrient or ER stresses was largely abolished (Fig. 4B). Altogether, these data indicate that the stress-induced formation of autophagosomes is depending on the TgIF2α pathway.

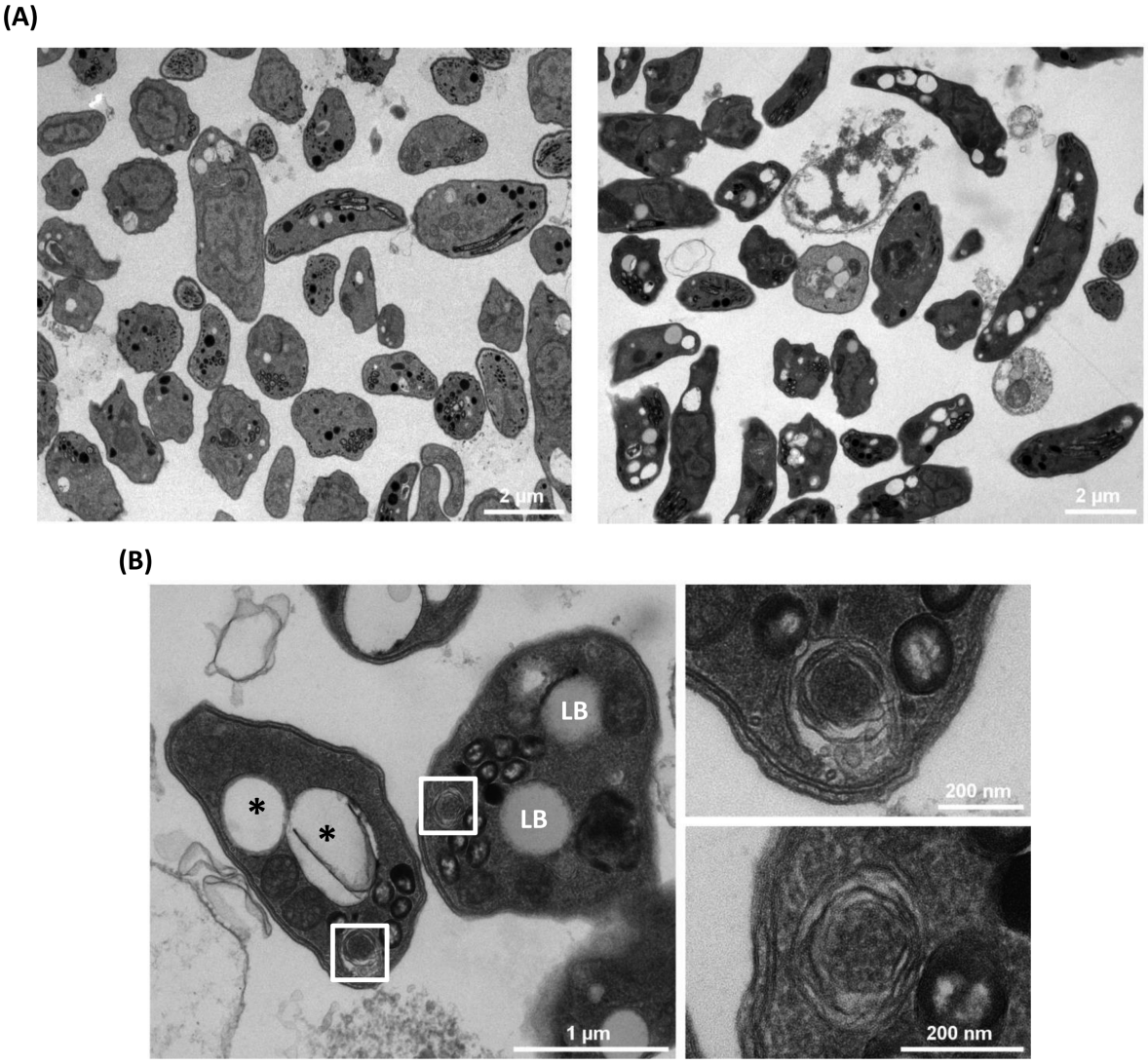

Figure 3. Morphological observation of autophagic vesicles in DTT-treated parasites by electron microscopy. (**A**) Large field view, showing a large number of vacuoles and some lipid bodies in parasites incubated in DMEM for 6 hours with 5 mM DTT (right) compared with the control incubated for 6 hours in DMEM without DTT (left). (**B**) Focus on two DTT-treated parasites displaying putative degradative vacuoles (asterisk), lipid bodies (LB) and cytoplasm-containing multi-membrane structures resembling autophagosomes (squared). Right: higher magnification of the squared areas.

DISCUSSION

eIF2, and in particular its regulation by phosphorylation, is central to the ISR, which is activated in response to a variety of stresses including nutrient deprivation or perturbation of ER homeostasis. This regulator of protein translation is part of a cytoprotective pathway, which is a highly conserved adaptation to stress in eukaryotes. Although *T. gondii* is an early diverging eukaryote, it possesses a well-conserved eIF2 and four eIF2α kinases (TgIF2Ks) that potentially regulate its phosphorylation state. TgIF2K-A is similar to PERK, it localizes to the ER and is likely involved in sensing accumulation of misfolded proteins in this compartment (Narasimhan *et al.* 2008). TgIF2K-B has no clear homologue in other species but was suggested to sense stresses that disrupt cytosolic homeostasis, such as oxidative stress (Narasimhan *et al.* 2008). Finally, TgIF2K-C and TgIF2K-D share the highest sequence identity with GCN2, the mammalian eIF2α kinase that is activated in response to nutrient limitation (Konrad, Wek and Sullivan, 2011, 2014).

Autophagy is one of the pro-survival pathways activated downstream of eIF2 (Tallóczy *et al.* 2002; Kouroku *et al.* 2007). However, how eIF2α phosphorylation contributes to autophagy initiation is not completely elucidated. In mammalian cells, although eIF2α phosphorylation shuts off general translation, it seems to stimulate the selective translation of the activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) transcription factors, which can upregulate the expression of autophagy proteins light chain 3 (LC3, the mammalian homologue of ATG8) and ATG5, respectively (Milani *et al.* 2009; Rouschop *et al.* 2010). These transcription factors have no homologues in *Toxoplasma*, which is not surprising knowing that these parasites rely largely on a plant-derived family of transcription factors instead (Suvorova and White 2014). Thus, we do not know how TgIF2α phosphorylation controls the activation of the autophagy pathway in *Toxoplasma*. Because the ER also constitutes a major source or scaffold for the autophagic isolation membrane (Hayashi-Nishino *et al.* 2009), it has been

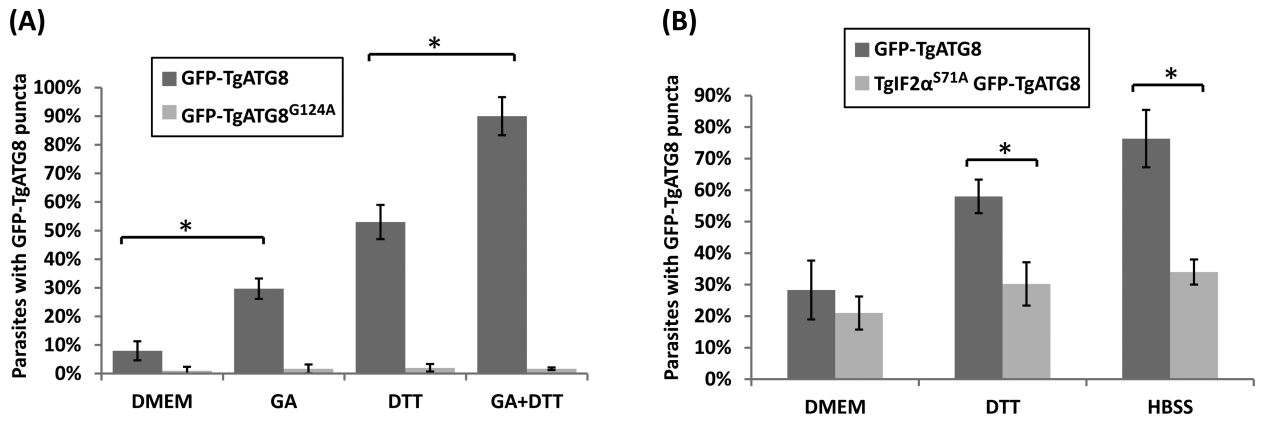

Figure 4. The biogenesis of autophagosomes upon stress is dependent on the TgIF2α pathway. (**A**) Intracellular parasites were incubated in DMEM for 6 hours with 15 μM guanabenz acetate (GA), 5 mM DTT, or both together, and the percentage of parasites bearing GFP-TgATG8 puncta was assessed. Values are mean from $n = 3$ experiments ± SEM. Asterisk denotes a statistical significance with $P < 0.05$ as determined by Student's T test. (**B**) Wild-type or TgIF2α phosphorylation mutant (TgIF2α^{S71A}) parasites were transiently transfected to express GFP-TgATG8, and 24 hours post transfection they were released from their host cells and incubated for 6 hours in DMEM, with or without 5 mM DTT, or in HBSS. The percentage of parasites bearing GFP-TgATG8 puncta was then assessed. Values are mean from $n = 3$ experiments ± SEM. Asterisk denotes a statistical significance with $P < 0.05$ as determined by Student's T test.

suggested that eIF2α phosphorylation might somehow affect the ER in a manner that promotes the physical formation of the isolation membrane (Kroemer, Mariño and Levine 2010). However, the site of autophagosomes formation is currently unknown in *Toxoplasma* and this hypothesis could not be verified.

In any case, our data show that the biogenesis of autophagosomes in the parasites is a highly regulated process that depends on the eIF2α pathway. It also demonstrates for the first time in *T. gondii* that ER stress is a stimulus that can robustly trigger autophagosome formation, in addition to the previously described use of amino acids starvation (Besteiro *et al.* 2011; Ghosh *et al.* 2012), or the use of monensin, a potent inducer of oxidative stress (Lavine and Arrizabalaga 2012). To bring the proof of a fully functional degradative pathway in *Toxoplasma*, the fate of autophagosomal contents still remains to be determined, as the interactions between autophagosomes and a lytic compartment have not yet been described in details. However, the recent involvement of autophagy-related protein TgATG9 in surviving stress-generating environments as extracellular parasites or inside host immune cells (Nguyen *et al.* 2017), brings further evidence that *T. gondii* autophagy machinery is part of an ISR pathway.

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Conflict of interest. None declared.

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