Title: Identification of TgAtg8-TgAtg3 interaction in *Toxoplasma gondii*

Author: Di Chen Jiaxin Lin Yangyang Liu Xiangzhi Li Gaozhi Chen Qianqian Hua Qinqin Nie Xin Hu Feng Tan

PII: S0001-706X(15)30110-8
DOI: http://dx.doi.org/doi:10.1016/j.actatropica.2015.09.013
Reference: ACTROP 3731

To appear in: *Acta Tropica*

Received date: 2-7-2015
Revised date: 2-9-2015
Accepted date: 21-9-2015

Please cite this article as: Chen, Di, Lin, Jiaxin, Liu, Yangyang, Li, Xiangzhi, Chen, Gaozhi, Hua, Qianqian, Nie, Qinqin, Hu, Xin, Tan, Feng, Identification of TgAtg8-TgAtg3 interaction in Toxoplasma gondii.Acta Tropica http://dx.doi.org/10.1016/j.actatropica.2015.09.013

# Identification of TgAtg8-TgAtg3 interaction in *Toxoplasma gondii*

Di Chen[a],JiaxinLin[b], YangyangLiu[b],XiangzhiLi[c], GaozhiChen[d],QianqianHua[e], QinqinNie[f], Xin Hu[b]*, Feng Tan[g]*

[a]School of the First Clinical Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, PR China

[b] School of Medical laboratory science and school of life science, Wenzhou Medical University, Wenzhou, Zhejiang 325035, PR China

[c] Guangxi Entry-Exit Inspection and Quarantine Bureau, Nannin, Guangxi 530000, PR China

[d]Chemical Biology Research Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035,PR China

[e] Clinical Laboratory, Dongyang People's Hospital, Jinhua, Zhejiang 322100, PR China

[f]School of the Second Clinical Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, PR China

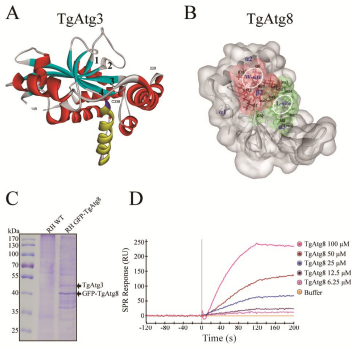
[g]Department of Parasitology, School of Basic Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, PR China

* Corresponding Authors.

Xin Hu. Tel.: +86 57786699195; E-mail address: gail529@163.com;

Feng Tan. +86 57786689860; E-mail address:tanfengsong@163.com

## Graphical abstract




## Highlights

- TgAtg8-TgAtg3 interaction occurs in *Toxoplasma gondii.*
- The TgAtg8 can bind directly TgAtg3 *in vitro*.
- There is higher affinity between TgAtg8 and TgAtg3.
- TgAtg8 possesses the conserved structure to interact with other TgAtg containing AIM.
- TgAtg3 contains core E2 enzymatic activity structure and a truncated handle region.


## ABSTRACT

Autophagy is a catabolic process in eukaryotic cells involved in the targeted degradation of cellular organelles and the cytoplasm. Recent works in *Toxoplasmagondii* suggest that the autophagy processes may serve as an important pathway in modulating parasite survival or death. As an important modulator of Atg8 lipidation and autophagy, Atg8-Atg3 interaction has been attracting increasing attention. However, there is no direct evidence that TgAtg8-TgAtg3 interaction occurs in the parasite. In this study, we firstly found TgAtg8 partially colocalized with TgAtg3 in GFP-TgAtg8 transgenic strains using IFA. Then, lysates from GFP-TgAtg8 tachyzoites were directly subject to large-scale tandem affinity purification with anti-GFP antibody. Western blot and tandem mass spectrometry (MS/MS) analysis determined the interaction between TgAtg8 and TgAtg3. Additionally, we performed

real-time interaction analysis with a surface plasmon resonance biosensor using BIAcore system. As expected, the result demonstrated a concentration-dependent increases in resonance signals and indicated the TgAtg8 could bind directly TgAtg3*in vitro*.Noteworthily, A KD of 34.9nMobtained from TgAtg8-TgAtg3 interaction indicate a high-affinity between Atg8-Atg3 in *Toxoplasma*.Furthermore, homology modeling and sequence alignment showed that TgAtg8 has greatest sequence and structural conservation. Within TgAtg3, this protein possesses the core E2 enzymatic activity structure and a truncated handle region which may contain AIM sequence. Taken together, our findings would help elucidate the formation mechanism of autophagosome in *Toxoplasma* and provide a possibility for looking into parasitic drug targets.

**Keywords:***Toxoplasma gondii*; autophagy; Atg8; Atg3; Surface plasmon resonance; Protein-Protein interaction

## 1. Introduction

Toxoplasmosis is caused by infection with the protozoan *Toxoplasma gondii*, an obligate intracellular parasite. It can cause substantial morbidity and mortality in humans and animals and is a major public health burden in the developing world(Dubey et al. 2005; Hide et al. 2009; Dubey 2010).Existing treatment drugs have associated toxicities and do not eliminated parasite(Weiss et al. 2009; Canon-Franco et al. 2014). Discovery of novel compounds in anti-toxoplasmosis drug development is essential for future intervention strategies.

In recent years, programmed cell death (PCD) pathways have shed a light on providing potential targets for the development of new therapy(van Zandbergen et al. 2010). The best-known PCD pathway in higher eukaryotic cells is apoptosis, type I PCD. However, whether there is apoptosis pathway within *T. gondii* remains to be demonstrated in spite of the fact that a primitive apoptotic cascade does indeed exist in *Toxoplasma* genome(Sinai et al. 2012). Recent emerging data indicate that autophagy-related process, a type II PCD, may play a vital role in life sustaining and death promoting in *T. gondii* whilethe decision-making mechanismsin *Toxoplasma* to shift autophagybetween these two activitiesis not known(Besteiro et al. 2011; Ghosh et al. 2012). Consequently, as a unique and essential

programmedcell death occurring in *Toxoplasma gondii*,the discovery of autophagy offers novel therapeutic tools to treat toxoplasmosis.

Macroautophagy, usually referred to as autophagy, is a major intracellular degradation process in eukaryotes(Eisenstein 2014).In autophagy process, the autophagosome is a transient but key structure, by which the cytosolic or organellar materials are sequestered for degradation(Yang et al. 2010).As the central event of autophagy, the formation of autophagosome is governed by a series of autophagy-related (Atg) proteins, which were originally identified in the budding yeast *Saccharomyces cerevisiae*(Nakatogawa et al. 2009; Mizushima et al. 2011). Of these, two ubiquitin-like conjugation systems, Atg8-phosphatidylethanolamine (PE) and Atg12-Atg5, have been identified in yeast and most of eukaryotes(Hurley et al. 2014). The Atg12-Atg5 conjugation system is able to accelerate Atg8 lipidation but may not be essential in some eukaryotes and several protozoa, including *T. gondii*(Ichimura et al. 2004; Mizushima et al. 2014).

In most eukaryotes, nascent Atg8 is proteolytically processed by Atg4 protease to expose C-terminal Gly residue(Kirisako et al. 2000). The exposed Gly of Atg8 is bound to Atg7, an E1-like activating enzymethrough a thioester bond(Tanida et al. 1999). Then the activated Atg8 is transferred to Atg3, an E2-like conjugating enzyme and to form Atg8-Atg3 complex through another thioester bond(Ichimura et al. 2000). Subsequently, Atg8 is covalently attachedto the lipid PE moiety in the phagophore membrane by Atg3(Yamaguchi et al. 2010).

To date, database searches and bioinformatics have illustrated thatalthough lack of the clear orthologs of the Atg12-Atg5-Atg16 complex,*T. gondii*appears to contain the core conjugation system involved in the attachment of Atg8 to PE, including TgAtg3, TgAtg4, TgAtg7 and TgAtg8. Moreover, when TgAtg3 function was interfered with conditional gene knockdown strategy, not only conjugation of TgAtg8 to PE was obstructed, but also the tachyzoites were severely disturbed in growth and showed obvious organellar defects(Besteiro et al. 2011).These findings suggest that both TgAtg8 and TgAtg3 have a function in survival of the parasite, and especially, the mechanismof TgAtg8-TgAtg3 interaction may be conserved in *T. gondii*as seen in other species. But, even so, there is no direct evidence that TgAtg8-TgAtg3 interaction occurs in *Toxoplasma*.

Herein, we detect the binding interaction between TgAtg8 and TgAtg3 by*in vitro* and *in vivo* studies. Our data reveal TgAtg8 directly interact with TgAtg3, which provides an attractive candidate

for anti-toxoplasmosis drug design.

## 2. Materials and methods

### *2.1. Host cells and Parasite culture*

Tachyzoites of the RH wild type strain or GFP-TgAtg8 strain(Besteiro et al. 2011)were routinely propagated in human foreskin fibroblast cells (hTERT) monolayers(Xue et al. 2013) cultivated in normal culture media, which consists of Dulbecco's modified Eagle medium (DMEM, pH 7.2) supplemented with 1% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and 5 mg/ml gentamicin antibiotic (Sigma). Cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$.

### *2.2. Indirect Immunofluorescent assay (IFA)*

Induction of*Toxoplasma* autophagy was performed in starvation conditions as described previously(Besteiro et al. 2011). Briefly, extracellular tachyzoites of GFP-TgAtg8 strain were collected from freshly lysed hTERT, sedimented by centrifugation and washed twice in Hank's Balanced Salt Solution (HBSS, Invitrogen). The parasites were resuspended in HBSS and incubated for 8 h at 37 °C. Then, they were fixed in 4% paraformaldehyde/PBS for 20 min and then permeablized in PBS containing 0.2% Triton X-100 plus 3% BSA for 10 min. After blocked in PBS with 3% BSA for 1 h,the parasites were first probed with rabbit polyclonal anti-TgAtg3 (1:1 000). Secondary antibodies were Alexa Fluor® 568 goat anti-rabbit IgG (1:1000, Invitrogen). Fluorescent images were obtained with a Nikon ECLIPSE Ci-L epifluorescence microscope. Non-induced extracellular tachyzoites were used as control.

### *2.3. Protein Extraction andImmunoprecipitation of TgAtg8*

Two 150 $cm^2$ flasks of hTERT were cultured in DMEM supplemented with 1% FBS for 24 h. Confluent hTERTs were then infected with GFP-TgAtg8 strain tachyzoites. When approximately 95% of the infected cells had been lysed, the extracellular tachyzoites were harvested and purified from host cell debris using 3.0-µm Nuclepore filters (Whatman, GE healthcare).After ~8 hours of induction of autophagyin HBSS, theparasite pellet was resuspended and lysed in 0.5 ml of lysis buffer composed of 50 mMTris-HCl (pH7.4), 150 mMNaCl, 1% Nonidet 40 (NP40) and a protease inhibitor cocktail (Roche) and then sonicated with 18 short bursts of 15 s followed by intervals of 30 s on ice. Unbroken debris was removed by centrifugation for 20 min at 4°C and 16 000 *g* and protein concentration in the

supernatant was confirmed with the 2-D Quant kit (GE Healthcare) in accordance with the manufacturer's protocol. As a control, RH wild type tachyzoites were treated in a same manner.

0.5 ml of parasite lysate was incubated with 20 µl of anti-GFP beads (Abcam) for 2 h at 4°C under gentle agitation, and then washed five times with lysis buffer. Remaining buffer was discarded and the beads were resuspended in 100 µl of lysis buffer and 30 µl of SDS-PAGE loading buffer and denatured for 10 minutes at 95°C followed by SDS-PAGE.

*2.4. Western blot analysis*

Western blot assays were performed using immunoprecipitated proteins by 12% SDS-PAGE. After transferred to methanol-activated polyvinylidene fluoride membranes (Millipore Corp), the membranes were incubated in blocking buffer containing 0.05% Tween 20 and 5% nonfat milk powder. For identification of both TgAtg3 and TgAtg8, rabbit-derived polyclonal antibodies against anti-TgAtg3 (1:1 000) and anti-TgAtg8 (1:1 000) that have been raised in our laboratory were used as the primary antibodies and incubated overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Sigma) diluted at 1:2000 and chemiluminescence substrate for detection (Sigma).

*2.5. Mass Spectrometry*

Parasite lysates were immunoprecipitated and then separated by SDS-PAGE and visualized with Novex Colloidal Blue Stain Kit (Invitrogen). Bands of interest were in-gel tryptically digested following destainand send to Shanghai Sangon Biotech company (Shanghai, China) for tandem mass spectrometry (MS/MS) analysis.

*2.6. Prokaryotic expression and purification*

To express the N-terminal His-tagged TgAtg3 or TgAtg8 fusion protein, either full length TgAtg3(ToxoDB: TGGT1_236110) or TgAtg8 (ToxoDB: TGGT1_254120) was amplified from RH strain cDNA using primers A3F: GGAATTCCATATGcatcatcatcatcacAGCAGCGGCATGACGAAGCCTCAAGAGACACC andA3R:CCC AAG CTT CTA CTT CTC GTT TTT TGT ACT GCG G or A8F:GGAATTCCATATGcatcatcatcatcacAGCAGCGGCATGCCATCGATTCGCGACand A8R: CCCAAGCTTTTACCCCAGAGTGTTCTC, respectively. Afterwards, the amplified fragments were

ligated into pCold™III expression vector (TaKaRa) usingNdeI and HindIII restriction sites.Each constructs was transformedinto *Escherichia coli*BL21 (DE3) and sequenced to confirm their identities, theneither $His_6$-TgAtg3 or $His_6$-TgAtg8 was induced with 1 mM IPTG for 22 h at 15°C under vigorousshaking.

After induction, the cells were harvested by centrifugation at 4°C 10,000 g for10 min and the pellet were resuspended in PBS buffer (pH 7.4) with a Complete EDTA-free protease inhibitorcocktail tablet (Roche) and lysed by sonication. The solublefraction was obtained by centrifugation at 4°C 12,000 g for 10 min and dialyzed in BufferA(500mMNacl,20mMTris-HCl,pH 7.9) overnight. The resulting lysate was loaded onto a Ni-NTA Agarose affinity column (QIAGEN) pre-equilibrated with Buffer A. Washing column use BufferA after lysate loading. Approximately 8 column volumes of BufferA containing 60 mMimidizole were used for washing. Afterwards, the fusion protein of interest was eluted with 250 mMimidazoleand dialyzed in PBS buffer overnight. All purification steps were conducted at 4 °C.The quality of the purified proteins was examined by SDS-PAGE.The concentration of proteins was detected by BCA method.

*2.7. BIAcore analysis*

The binding affinity of $His_6$-TgAtg8 was determined using a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) with a HTE sensor chip(ProteOn™, #176-5033). 0.4mg of purified $His_6$-TgAtg3 (in acetate acid buffer pH 5.5) was loaded to sensors whichactivated with 10 mM $NiSO_4$ pre-conditioned with nickel according to the manufacturer's instruction. Various concentrations of $His_6$-TgAtg8 were injected onto the flow cell in running buffer (PBS, 0.1% SDS, 5% DMSO) at a flow rate of 30μM /min for 120s of association phase,followed with 120s of dissociation phase at 25°C. The final graphs were obtained by subtracting blank sensor grams from the duplex or quadruplex sensor grams. Data were analyzed with ProteOn manager software. The association rate (Ka), the dissociation rate (Kd) and the dissociation constant (KD = Kd / Ka), the data were calculated using the global fitting of the kinetic data from various concentrations of $His_6$-TgAtg8 using one to one Langmuir binding model.

*2.8. Secondary Structure-based Sequence Alignment and Homology Modeling*

A multiple sequence alignment was generated using ClustalX. Homology models of TgAtg8 and

the TgAtg3 were calculated using both programs I-TASSER(Roy et al. 2010) and MODELLER(Eswar et al. 2006).The structure of TgAtg8 was modeled onto both known crystal structure of*S.cerevisiae* (Sc) Atg8 (PDB ID 2zpn) and *Plasmodium falciparum*(Pf) Atg8 (PDB ID 4EOY)crystal structures. Both of ScAtg3 (PDB ID 2DYT) and human Ubc9 (PDB ID 1U9A) were used as TgAtg3 template proteins. All calculations were carried out under default conditions unless otherwise stated. To build the homology models, Visualization of the structures was done using UCSF Chimera (Pettersen et al. 2004) and Accelrys Discovery Studio Visualizer commercial software.

## 3. Results

### *3.1. The TgAtg8-TgAtg3 interaction occurs in tachyzoite of Toxoplasma in vivo*

To study the interaction between TgAtg8 and TgAtg3, we firstly observedthe colocalization of TgAtg8 and TgAtg3 in tachyzoite stage using IFA. As shown in Fig. 1, whether with the induction of autophagy or not, TgAtg8 partially colocalized with TgAtg3 in GFP-TgAtg8 parasites.It has been discovered that a significant proportion of TgAtg8 present as a lapidated protein in basal condition, suggesting the TgAtg8-TgAtg3 interaction appears to be already present prior to the induction of autophagy(Besteiro et al. 2011; Kong-Hap et al. 2013).

Furthermore, lysates from GFP-TgAtg8 transgenic strain tachyzoites were directly subject to large-scale tandem affinity purification with anti-GFP antibody.Eluted proteins were resolved using SDS-PAGE and immunoblottedwith antibodies against either TgAtg8 or TgAtg3, confirming that this protein corresponded to ectopic GFP-TgAtg8 conjugated to endogenous TgAtg3. In contrast, both proteins were not observed in wild-type controls (Fig. 1B and C).At the same time, the gel was stained with Coomassie brilliant blue and the protein bands of interest were trypsin-digested and subject to tandem mass spectrometry (MS/MS) analysis. The results obtained revealedtwo unique peptides that match the amino acid sequenceof TgAtg3, ten unique peptides that match the aminoacid sequence of TgAtg8. This result confirmed thatTgAtg8 interacts with TgAtg3.

### *3.2. TgAtg8 can interact with TgAtg3 in vitro*

To characterize the interaction kinetics between the TgAtg8 and TgAtg3, we performed real-time interaction analysis with a surface plasmon resonance biosensorusing BIAcore system. Here, we immobilized the higher molecular weight protein, $His_6$-TgAtg3, on the surface of

a HTE biosensor chipvia His tag followed by injection of purified $His_6$-TgAtg8 at five 2-fold serial dilutions and response versus time was measured. As expected, the result demonstrated a concentration-dependent increases in resonance signals and indicated the TgAtg8 could bind directly TgAtg3*in vitro*(Fig. 1D). Furthermore, A KD of 34.6 nM was determined, indicating a strong affinity and interaction between TgAtg8 andTgAtg3.

*3.3. The structure of TgAtg8 is well conserved across Atg8 homologues*

Thus far, most of evidences have shown that Atg8 and its conjugation system are evolutionarily conserved. In autophagy process, both autophagic receptors and enzymes modifying Atg8 utilize a common Atg8-family interacting motif (AIM) for direct interaction withW-site and L-site in Atg8-family proteins(Ichimura et al. 2008; Noda et al. 2008; Kirkin et al. 2009; Okamoto et al. 2009).To predict AIM binding site in TgAtg8, the homology model of the structure of TgAtg8 was generated based on the known crystal structure of ScAtg8(Noda et al. 2008) and the similarity of TgAtg8 with other Atg8 family members was compared using a structure-based multiple sequence alignment.As shown in Fig.2A, the predicted structure of TgAtg8 maps onto the structure of ScAtg8 quite well, with the conserved ubiquitin-like domain at C terminus and helical domain consisting of tandem α helices, α1 and α2, at N terminus. Like all Atg8-family proteins, TgAtg8 possesses an exposed β2 strand and two distinguishing hydrophobic pockets, W-site and L-site. The W-site is located between α2 and β2 and comprised of Glu16, Ile20, Pro29, Ile31, Lys47, Leu49 and Phe112, and the L-site is located between β2 and α3 and comprised of Phe48, Val50, Pro51, Met54, Phe59 and Ile 62. According to the multiple sequence alignment, these residues are highly conserved among Atg8-family proteins, particularly those composing the W-site (Fig. 2B). These results clearly indicate that the TgAtg8 possesses the structure foundation to interact with other TgAtg containing AIM.

*3.4. TgAtg3 possesses a conserved E2 core region and the handle region*

Although TgAtg3 is vital for the normal development of *T. gondii* tachyzoites and thought to be an E2-like enzyme, it is not clear if TgAtg3 has structural similarity with canonical E2 enzymes because they share litter sequence homology. So far, All E2 enzymes reported possess a conserved E2 core consisting of four α-helices surrounding a central four-stranded β-sheet(Tong et al. 1997). For this reason, we firstly made a structural comparison of TgAtg3 with human Ubc9, a canonical E2 enzyme for small ubiquitin-like modifier (SUMO). Apparently, the structure of TgAtg3 possesses a conserved

E2 core region although it seems to be significantly dissimilarto that of Ubc9 (Fig. 3).

It has been reported that Atg8 protein interact with Atg3 and most Atg8-binding proteins involved in cytoplasm to vacuole targeting pathway (Cvt) in yeast and selective autophagy in mammals via the AIM, W/Y/F-X-X-L/I/V, where X is an acidic residue preferred(Noda et al. 2008).To search the AIM in TgAtg3, we compared further the structure of TgAtg3 with that of ScAtg3. In addition to a conserved E2 core region, ScAtg3 has two unique insertions, the handle region (HR) and the flexible region (FR). It was identified that ScAtg3 HR directly interact with the AIM binding site on ScAtg8 using the WEDL sequence(Yamada et al. 2007).Likewise, the overall structure of TgAtg3 contains the truncatedHR (Fig. 3). Taken together, these results suggest that the AIM in TgAtg3 is probably similar toknown motifs in yeast, humansor*Plasmodium falciparum*.

## 4. Discussion

As an important modulator of Atg8 lipidation and autophagy, Atg8-Atg3 interaction has been attracting increasing attention.For instance, it is reported that several small molecule inhibitors disrupting the *Plasmodium falciparum* Atg8-Atg3 interaction are able to prevent PfAtg8 lipidation with PE, and suppress the growth and development in liver and blood stage parasites(Hain et al. 2012; Hain et al. 2014).Recently, it is revealed that *Toxoplasma*TgAtg3 could be involved in the conjugation of TgAtg8 and the ability of TgAtg8 lipidation and autophagosome formation was seriously impaired in TgAtg3-depleted strains(Besteiro et al. 2011). However, there is unclear whether the TgAtg8-TgAtg3 interaction occurs in the parasite. In the present study, we provided direct evidence for the interaction between TgAtg8 and TgAtg3 by *in vitro* and *in vivo* studies.Noteworthily, in BIAcore assay, we calculated the KD value which is commonly used to describe the affinity between a ligand and a protein. The smaller the KD value, the more tightly bound the ligand is, or the higher the affinity between ligand and protein.Compared with A KD of 290nM of PfAtg8-PfAtg3 interaction(Hain et al. 2012),the KD value obtained from TgAtg8-TgAtg3 interaction is lower (KD=34.9 nM), implying a higher affinity between Atg8-Atg3 in *Toxoplasma*than in*Plasmodium*. Thus, we speculate the interaction is probably more important in *Toxoplasma gondii* although both of them belong to the phylum *Apicomplexa*.

Atg8 is an ubiquitin-like protein and usually recognized as the most reliable marker for

autophagosomes since it is a key and conserved protein for the autophagy, functioning in cargo recruitment into autophagosomes(Weidberg et al. 2011)and in autophagosomal biogenesis(Nakatogawa et al. 2007; Xie et al. 2008).In apicomplexan parasites,intriguingly, Atg8 also localizes on the apicoplast(Kitamura et al. 2012; Kong-Hap et al. 2013; Mizushima et al. 2014) that is a unique and essential organelle for parasites and is associated with the synthesis of fatty acids, isoprenoids, and heme(van Dooren et al. 2013). Moreover, the ultrastructure observation determines further that the apicoplast is not degraded with colocalization of Atg8(Kitamura et al. 2012; Jayabalasingham et al. 2014). Importantly, Atg8 can no longer localize on the apicoplasts when the C-terminal glycine of Atg8 is replaced with alanine(Eickel et al. 2013; Kong-Hap et al. 2013).Although there is unclear the function of Atg8 at the apicoplast membrane, these findings implicate that it is lipidated and thus still requires Atg8-Atg3 interaction.

In this study, we predicted the tertiarystructure through homology modeling and found TgAtg8 contains conserved cargo receptor sites, W- site and L- site, which are responsible for interaction between Atg8 and Atg8-binding proteins. Alignment analysis demonstrated further the amino acid residues constructing both site are well conserved across Atg8 homologues. To date, the crystal structure of *Plasmodium falciparum* Atg8 has been elucidated(Hain et al. 2012a).Therefore, we compared TgAtg8 with PfAtg8. Like the PfAtg8, TgAtg8 also contains an Apicomplexan-specific loop, residue 68-76, which has been confirmed play the important role in mediating the interaction of PfAtg8 with PfAtg3(Hain et al. 2012a).Furthermore, we found all amino acid residues composing W- and L-site in TgAtg8 are completely consistent with PfAtg8 except for the residue Phe112. Taken together, it strongly suggests that TgAtg8 possesses conserved function and biological activity as identified PfAtg8, andTgAtg8-TgAtg3 interaction is not only associated with parasite autophagy,may also contributed to the formation and maintenance of the apicoplast.

Thus far, the three-dimensional structure of Atg3 has only been reported in yeast(Yamada et al. 2007).Therefore, we predicted also the tertiarystructure of TgAtg3 using the ScAtg3 as template.However, the structure showed that although it possesses the conserved the E2 core region,the HR containing AIM motif is severely truncated in TgAtg3, similarly as in *P. falciparum* homologue. Within PfAtg3 protein,the AIM sequence is different from known motifs in yeast and humans due to the existence of Pro, rather than Leu, Ile or Val at the fourth position of the motif. Further experiments are required to identify AIM in TgAtg3 and if it is contributed to mediate the TgAtg8-TgAtg3

interaction. Meanwhile, further crystallization of full length TgAtg8 and TgAtg3 will help elucidate their structural characterization and the interaction model between TgAtg8-TgAtg3.

In conclusion, our studies of the *Toxoplasma gondii* TgAtg8-TgAtg3 interaction provide a possibility for looking into parasitic drug targets. Especially, besides its therapeutic application, a drug disrupting the interaction between TgAtg8 and TgAtg3 would help elucidate the role of TgAtg8 in the different stages of *Toxoplasma* life cycle.

**Acknowledgment**

We are grateful to Dr. William J. Sullivan, Jr. (Indiana University School of Medicine, IN) forproviding the human foreskin fibroblast cells, and Dr. SébastienBesteiro (Universites de Montpellier, France) for the RH GFP-TgAtg8 parasite strain as well as Amarjit Singh for refining the language. This work was supported by grants from the Commonwealth Technology Application Project of Zhejiang Province (2014C33161) to FT and the Natural Science Foundation of Zhejiang Province (LQ14H190003) to XH, as well asStudent Scientific Research Project of Wenzhou Medical University (wyx201401012)to DC.

**References**

Besteiro, S., Brooks, C.F., Striepen, B., Dubremetz, J.F., 2011. Autophagy protein Atg3 is essential for maintaining mitochondrial integrity and for normal intracellular development of Toxoplasma gondiitachyzoites. PLoSPathog 7, e1002416.

Canon-Franco, W.A., Lopez-Orozco, N., Gomez-Marin, J.E., Dubey, J.P., 2014. An overview of seventy years of research (1944-2014) on toxoplasmosis in Colombia, South America. Parasit Vectors 7, 427.

Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans. 2nd edn. CRC Press Inc, Boca Raton, New York.

Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L., Lehmann, T., Vianna, M.C., Miska, K., Sreekumar, C., Kwok, O.C., Shen, S.K., Gamble, H.R., 2005. Prevalence of viable Toxoplasma gondii in beef, chicken, and pork from retail meat stores in the

United States: risk assessment to consumers. J Parasitol 91, 1082-1093.

Eickel, N., Kaiser, G., Prado, M., Burda, P.C., Roelli, M., Stanway, R.R., Heussler, V.T., 2013. Features of autophagic cell death in Plasmodium liver-stage parasites. Autophagy 9, 568-580.

Eisenstein, M., 2014. Molecular biology: Remove, reuse, recycle. Nature 514, S2-S4.

Eswar, N., Webb, B., Marti-Renom, M.A., Madhusudhan, M.S., Eramian, D., Shen, M.Y., Pieper, U., Sali, A., 2006. Comparative protein structure modeling using Modeller. CurrProtoc Bioinformatics Chapter 5, 5-6.

Ghosh, D., Walton, J.L., Roepe, P.D., Sinai, A.P., 2012. Autophagy is a cell death mechanism in Toxoplasma gondii. Cell Microbiol 14, 589-607.

Hain, A.U., Bartee, D., Sanders, N.G., Miller, A.S., Sullivan, D.J., Levitskaya, J., Meyers, C.F., Bosch, J., 2014. Identification of an Atg8-Atg3 protein-protein interaction inhibitor from the medicines for Malaria Venture Malaria Box active in blood and liver stage Plasmodium falciparum parasites. J Med Chem 57, 4521-4531.

Hain, A.U., Weltzer, R.R., Hammond, H., Jayabalasingham, B., Dinglasan, R.R., Graham, D.R., Colquhoun, D.R., Coppens, I., Bosch, J., 2012a. Structural characterization and inhibition of the Plasmodium Atg8-Atg3 interaction. J StructBiol 180, 551-562.

Hain, A.U., Weltzer, R.R., Hammond, H., Jayabalasingham, B., Dinglasan, R.R., Graham, D.R., Colquhoun, D.R., Coppens, I., Bosch, J., 2012b. Structural characterization and inhibition of the Plasmodium Atg8-Atg3 interaction. J StructBiol 180, 551-562.

Hide, G., Morley, E.K., Hughes, J.M., Gerwash, O., Elmahaishi, M.S., Elmahaishi, K.H., Thomasson, D., Wright, E.A., Williams, R.H., Murphy, R.G., Smith, J.E., 2009. Evidence for high levels of vertical transmission in Toxoplasma gondii. Parasitology 136, 1877-1885.

Hurley, J.H., Schulman, B.A., 2014. Atomistic autophagy: the structures of cellular self-digestion. Cell 157, 300-311.

Ichimura, Y., Imamura, Y., Emoto, K., Umeda, M., Noda, T., Ohsumi, Y., 2004. In vivo and in vitro reconstitution of Atg8 conjugation essential for autophagy. J BiolChem 279, 40584-40592.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida,

I., Kominami, E., Ohsumi, M., Noda, T., Ohsumi, Y., 2000. A ubiquitin-like system mediates protein lipidation. Nature 408, 488-492.

Ichimura, Y., Kumanomidou, T., Sou, Y.S., Mizushima, T., Ezaki, J., Ueno, T., Kominami, E., Yamane, T., Tanaka, K., Komatsu, M., 2008. Structural basis for sorting mechanism of p62 in selective autophagy. J BiolChem 283, 22847-22857.

Jayabalasingham, B., Voss, C., Ehrenman, K., Romano, J.D., Smith, M.E., Fidock, D.A., Bosch, J., Coppens, I., 2014. Characterization of the ATG8-conjugation system in 2 Plasmodium species with special focus on the liver stage: possible linkage between the apicoplastic and autophagic systems? Autophagy 10, 269-284.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., Ohsumi, Y., 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 151, 263-276.

Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., Bilusic, I., Theurillat, J.P., Overvatn, A., Ishii, T., Elazar, Z., Komatsu, M., Dikic, I., Johansen, T., 2009. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell 33, 505-516.

Kitamura, K., Kishi-Itakura, C., Tsuboi, T., Sato, S., Kita, K., Ohta, N., Mizushima, N., 2012. Autophagy-related Atg8 localizes to the apicoplast of the human malaria parasite Plasmodium falciparum. PLoS One 7, e42977.

Kong-Hap, M.A., Mouammine, A., Daher, W., Berry, L., Lebrun, M., Dubremetz, J.F., Besteiro, S., 2013. Regulation of ATG8 membrane association by ATG4 in the parasitic protist Toxoplasma gondii. Autophagy 9, 1334-1348.

Mizushima, N., Sahani, M.H., 2014. ATG8 localization in apicomplexan parasites: apicoplast and more? Autophagy 10, 1487-1494.

Mizushima, N., Yoshimori, T., Ohsumi, Y., 2011. The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol 27, 107-132.

387 Nakatogawa, H., Ichimura, Y., Ohsumi, Y., 2007. Atg8, a ubiquitin-like protein required for
388 autophagosome formation, mediates membrane tethering and hemifusion. Cell 130, 165-178.

389 Nakatogawa, H., Suzuki, K., Kamada, Y., Ohsumi, Y., 2009. Dynamics and diversity in autophagy
390 mechanisms: lessons from yeast. Nat Rev Mol Cell Biol 10, 458-467.

391 Noda, N.N., Kumeta, H., Nakatogawa, H., Satoo, K., Adachi, W., Ishii, J., Fujioka, Y., Ohsumi, Y.,
392 Inagaki, F., 2008. Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes
393 Cells 13, 1211-1218.

394 Noda, N.N., Ohsumi, Y., Inagaki, F., 2010. Atg8-family interacting motif crucial for selective
395 autophagy. FEBS letters 584, 1379-1385.

396 Okamoto, K., Kondo-Okamoto, N., Ohsumi, Y., 2009. Mitochondria-anchored receptor Atg32
397 mediates degradation of mitochondria via selective autophagy. Dev Cell 17, 87-97.

398 Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E.,
399 2004. UCSF Chimera--a visualization system for exploratory research and analysis. J ComputChem 25,
400 1605-1612.

401 Roy, A., Kucukural, A., Zhang, Y., 2010. I-TASSER: a unified platform for automated protein
402 structure and function prediction. Nat Protoc 5, 725-738.

403 Sinai, A.P., Roepe, P.D., 2012. Autophagy in Apicomplexa: a life sustaining death mechanism? Trends
404 Parasitol 28, 358-364.

405 Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., Kominami, E., 1999.
406 Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. MolBiol Cell 10, 1367-
407 1379.

408 Tong, H., Hateboer, G., Perrakis, A., Bernards, R., Sixma, T.K., 1997. Crystal structure of
409 murine/human Ubc9 provides insight into the variability of the ubiquitin-conjugating system. J
410 BiolChem 272, 21381-21387.

411 van Dooren, G.G., Striepen, B., 2013. The algal past and parasite present of the apicoplast. Annu Rev
412 Microbiol 67, 271-289.

413 van Zandbergen, G., Luder, C.G., Heussler, V., Duszenko, M., 2010. Programmed cell death in

414 unicellular parasites: a prerequisite for sustained infection? Trends Parasitol 26, 477-483.

415 Weidberg, H., Shvets, E., Elazar, Z., 2011. Biogenesis and cargo selectivity of autophagosomes. Annu
416 Rev Biochem 80, 125-156.

417 Weiss, L.M., Dubey, J.P., 2009. Toxoplasmosis: A history of clinical observations. Int J Parasitol 39,
418 895-901.

419 Xie, Z., Nair, U., Klionsky, D.J., 2008. Atg8 controls phagophore expansion during autophagosome
420 formation. MolBiol Cell 19, 3290-3298.

421 Xue, B., Jeffers, V., Sullivan, W.J., Uversky, V.N., 2013. Protein intrinsic disorder in the acetylome of
422 intracellular and extracellular Toxoplasma gondii. MolBiosyst 9, 645-657.

423 Yamada, Y., Suzuki, N.N., Hanada, T., Ichimura, Y., Kumeta, H., Fujioka, Y., Ohsumi, Y., Inagaki, F.,
424 2007. The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that
425 mediates Atg8 lipidation. J BiolChem 282, 8036-8043.

426 Yamaguchi, M., Noda, N.N., Nakatogawa, H., Kumeta, H., Ohsumi, Y., Inagaki, F., 2010. Autophagy-
427 related protein 8 (Atg8) family interacting motif in Atg3 mediates the Atg3-Atg8 interaction and is
428 crucial for the cytoplasm-to-vacuole targeting pathway. J BiolChem 285, 29599-29607.

429 Yang, Z., Klionsky, D.J., 2010. Eaten alive: a history of macroautophagy. Nat Cell Biol 12, 814-822.

430

431 **Figure captions**

432 **Fig. 1.** The interaction between TgAtg8 and TgAtg3 was identified by *in vivo* and *in vitro* studies.(A)
433 IFA assays showed whether with the induction of autophagy or not, TgAtg8 partially colocalized with
434 TgAtg3 in GFP-TgAtg8 parasites. (B) After ~8 hours of starvation,lysate from tachyzoites of both RH
435 wild type (WT) and RH GFP-TgAtg8 were immunoprecipitated with anti-GFP antibody beads and
436 detected by SDS-PAGE. Anticipated GFP-TgAtg8 and TgAtg3 were indicated with arrows. (C)
437 Immunoprecipitated samples were detected using Western blot with anti-TgAtg8 and anti-TgAtg3,
438 respectively. (D) BIAcore analysis. Increasing concentrations of $His_6$-TgAtg8 wasinjected over an
439 $His_6$-TgAtg3 conjugated chip and the binding response was measured.

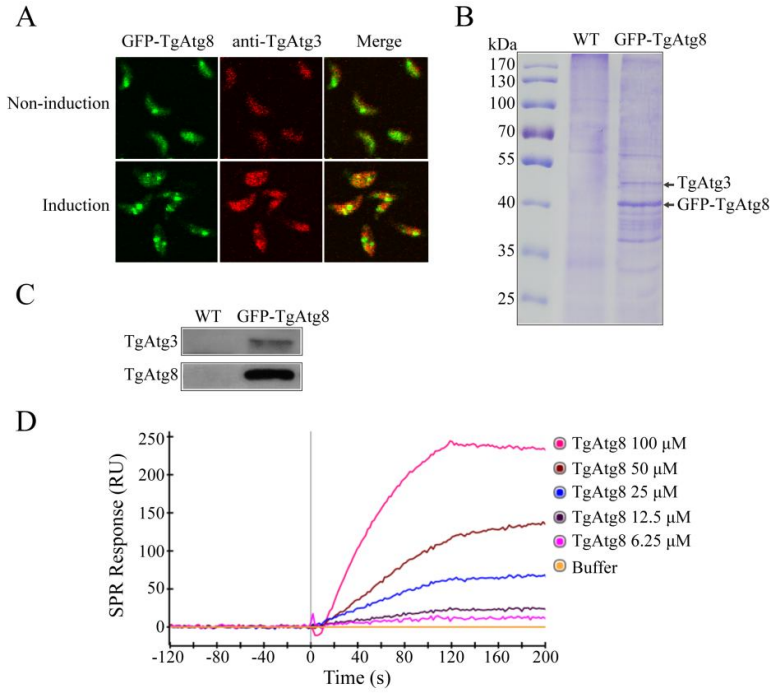
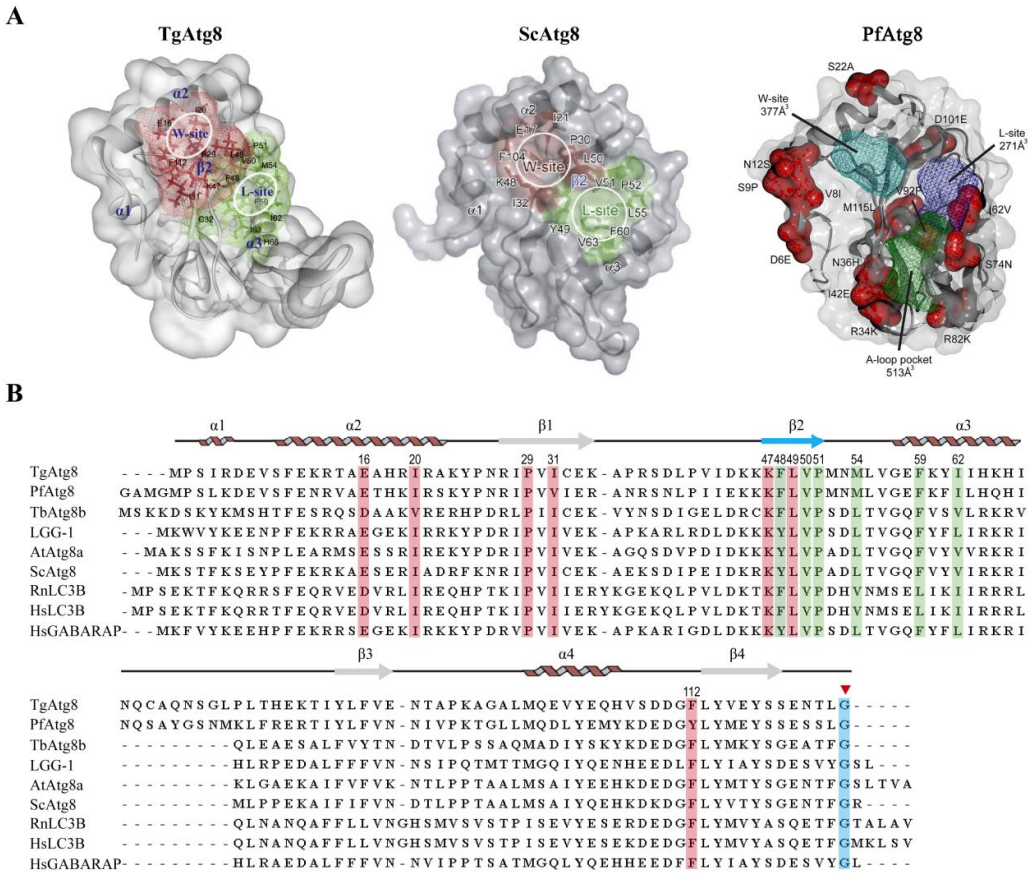



**Fig. 2.**The structure and sequence of TgAtg8 is evolutionarily conserved across Atg8-family proteins. (A) Predicted tertiarystructure of TgAtg8 (left), the structure of*S. cerevisiae*Atg8 (middle; PDB ID 2zpn)(Noda et al. 2010) and the structure of *P. falciparum* Atg8 (right; PDB ID 4EOY)(Hain et al. 2014). Two hydrophobic pockets responsible for the recognition of Trp and Leu in the AIM (WXXL) motif are labeledW-site and L-site, respectively, and circled. Residues constituting these pockets are colored red and green, respectively, and their side chains are shown with a stick model. (B)Sequence alignment of TgAtg8. The secondary structural elements of TgAtg8 are shown above the alignment. Residues constituting W- and L-sites arecolored red and green, respectively. Basic residues responsible for ionic interaction with acidic residues in AIM are enclosed with a blue square. Residue numbers of TgAtg8 are shown above the alignment. Pf, *Plasmodium falciparum*; Tb, *T. brucei*; At, Arabidopsis thaliana; Sc, *S. cerevisiae*; Rn, Rattusnorvegicus; Hs, *Homo sapiens.*

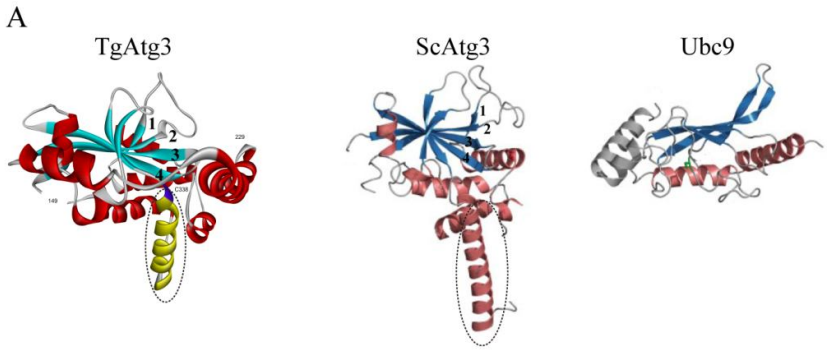



453

454

455

456 **Fig. 3.** Stereo view of the ribbon diagram of TgAtg3. Predicted tertiarystructure of TgAtg3 (left), the
457 structure of*S. cerevisiae*Atg3 (middle, PDB ID 2DYT), Ubc9 (Protein Data Bank code 1U9A)(Yamada
458 et al. 2007).Conservedα-helices are lettered and indicated with red helical ribbons, conservedβ-strands
459 are numbered and indicated withcyan arrows, and the handle region is indicated with golden helical
460 ribbons. Residues adjacent to the disordered regions are numbered.




461