Toxoplasma Gondii ROP17 Promotes Autophagy via the Bcl-2–Beclin 1 Pathway

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Abstract

Background

Toxoplasmosis caused by *Toxoplasma gondii* (*T. gondii*) has been and continues to be a major threat to public health worldwide. Proteins with serine/threonine (S/T) kinase activity, such as rhoptry organelle protein (ROP)16 and ROP18, are secreted by *T. gondii* rhoptries organelles that play important roles in modulating the survival and proliferation of *Toxoplasma*. ROP17 is another important effector kinase protein. Nevertheless, little is known about its function in regulating the interplay between *T. gondii* and host cells during infection. In this study, we investigated the function and the underlying mechanisms of ROP17 *in vitro* and *in vivo*.

Methods

The autophagy of cells in the small intestines of mice infected with *T. gondii* tachyzoite (RH strain) was detected by assessing the LC3B, Beclin 1 and p62 levels using immunohistochemical staining. ROP17 overexpression augmented starvation-induced autophagy in HEK 293T cells as measured by MDC staining, transmission electron microscopy (TEM), fluorescence microscopy and Western blot analysis. The interaction of ROP17 and Bcl-2 was confirmed using co-immunoprecipitation (co-IP) analysis. Moreover, the levels of phosphorylation of Bcl-2 and total Bcl-2 in the small intestines of mice infected with *T. gondii* tachyzoite were detected using immunohistochemical staining. In the end, Pearson coefficient was used to analyse correlations between the expression of ROP17 with the expression of autophagy markers LC3B, beclin 1 and p62, and with phosphorylation of Bcl-2 and Bcl-2.

Results

Infection with *T. gondii* resulted in autophagy of mouse small intestine cells accompanied by increased levels of LC3B and Beclin 1 and decreased levels of p62, which were measured by immunohistochemistry. Meantime, the elevated levels of Phosphorylated Bcl-2 and declined Bcl-2 in *T. gondii*-infected mouse small intestine tissue and in ROP17 overexpressing HEK 293T cells were detected by immunohistochemistry and Western blotting. ROP17 overexpression promoted serum starvation-induced cellular autophagy in the HEK293T cells, reflecting the augmentation of autophagosomes and GFP-LC3B puncta accompanied by increased levels of LC3B and Beclin 1 and decreased levels of p62. Moreover, we found that ROP17 interacted with Bcl-2 and phosphorylated and degraded it to liberate Beclin 1, thus promoting autophagy. Pearson coefficient analysis showed that there existed strong positive correlations between the expression of ROP17 with the expression of LC3B, Beclin 1 and phosphorylation of Bcl-2, while strong negative correlations between the expression of ROP17 and the expression of p62 and Bcl-2.
Conclusions

Our findings indicate that ROP17 plays a pivotal role in maintaining *Toxoplasma* survival and proliferation in host cells by promoting cellular autophagy, which depends on the Beclin 1-Bcl-2 pathway.

Introduction

*Toxoplasma gondii* (*T. gondii*) is an obligate intracellular parasite capable of infecting all warm-blooded animals, including avian and mammalian species, with approximately one-third of the global human population infected [1]. *T. gondii* infections are asymptomatic in most people; however, in immunocompromised patients, this infection can cause severe diseases such as encephalitis [2]. *T. gondii* has high proliferation and mobile potential and can spread among different host tissues and body fluids [3]. *T. gondii* invades the central nervous system (CNS) by hijacking leukocytes to use as shuttles that enable extravasation across the blood-brain barrier (BBB)[4].

*T. gondii* invades all nucleated cells, residing and growing in cells through the formation of parasitophorous vacuoles (PVs). In addition, *T. gondii* tachyzoites introduce a large set of proteins, including dense granule proteins (GRAs) and rhoptry organelle proteins (ROPs), to co-opt host cell functions. These proteins reach the host cytosol through either direct injection or translocation across the parasitophorous vacuolar membrane (PVM) [5]. Most known ROPs are phosphokinases that belong to the serine/threonine (S/T) kinase family, ROP2 [6]. The best-characterized active ROP2-like kinase is ROP18, which has been extensively studied for its defence mechanism against immunity-related GTPases (IRGs) that are generated in response to interferon gamma upregulation to breach the PVM and kill the internal parasites [7]. ROP17, another serine/threonine protein kinase, resides on the host cytosol side of the PVM in infected cells and has previously been shown to phosphorylate and thereby inactivate host immunity-related GTPase in collaboration with ROP18 and ROP5 [8]. However, the details of ROP17 function remain unknown.

Autophagy is a conserved cell-autonomous catabolic stress response pathway dedicated to the breakdown of cellular material and cell content recycling. Apart from its homeostatic role, autophagy is actively involved in both the clearance of pathogens and the provision of nutrients for pathogen survival [9]. *T. gondii* infects host cells and induces autophagy, leading to parasite death in autophagosomes; however, this pathogen can also take advantage of autophagy in host cells to promote their proliferation [10]. The different roles of autophagy in *T. gondii* have not been fully elucidated. In this study, autophagy in the small intestine of *T. gondii*-infected mice was assayed. To this end, an ROP17-containing eukaryotic expression plasmid was transfected into HEK 293T cells, and the autophagy of the transfected cells was assessed. Furthermore, we identified the intracellular signalling pathways involved in the ROP17-mediated activation of autophagy.

Materials And Methods
Animals, parasites, reagents and ethics statement

Inbred mice used in this study were 8-week-old male BALB/c mice obtained from the Center of Laboratory Animals, Shanxi Medical University, Taiyuan. The mice were maintained in housing with a 12:12 h light-dark cycle with free access to food and water. Two New Zealand rabbits, approximately 60 days old, were kept in individual cages. All procedures were performed in accordance with the laws and conditions of the Guide for the Care and Use of Laboratory Animals and Institutional Animal Ethics Committee guidelines of Shanxi Medical University. *T. gondii* tachyzoites (RH strain) were kindly provided by the Peking University Health Science Center and were maintained via serial intraperitoneal passage in BALB/c mice [11].

The following antibodies used in this study: rabbit monoclonal anti-LC3B (No. 3868, Cell Signaling), rabbit monoclonal anti-Beclin-1 (No. 3495, Cell Signaling), rabbit monoclonal anti-p62 (No. 39749, Cell Signaling), rabbit monoclonal anti-Flag tag (No. 14793, Cell Signaling), rabbit monoclonal anti-phospho-Bcl-2 (Ser70) (No. 2827 Cell Signaling), rabbit polyclonal anti-JNK (No. 9252, Cell Signaling), rabbit monoclonal anti-phospho-SAPK/JNK (Thr183/Tyr185) (No. 4668, Cell Signaling) and rabbit monoclonal β-actin (No. 4970, Cell Signaling). Horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibodies (No. 7074, Cell Signaling) were also used. Anti-FLAG M2 affinity gel (No. A2220) was obtained from Sigma-Aldrich.

Preparation of rabbit anti-*T. gondii* ROP17 serum

Purified *T. gondii* ROP17 protein (TgROP17) was prepared according to our previous study [12]. Antiserum against TgROP17 was produced in two male New Zealand rabbits (body weight: 2.0–2.5 kg). Before challenge, a 5 ml peripheral blood sample was collected from each rabbit ear vein for the preparation of a preimmune serum. The TgROP17 protein (500 μg) was hypodermically injected into the backs of the rabbits, and the same dose was used in four boosters administered every 10 days thereafter. Blood was taken from the marginal ear vein 10, 20, 30 and 40 days after the first challenge and on the 10th day after the final challenge. In addition, a blood sample was obtained from the jugular vein for establishing the generation of a specific antibody with enzyme-linked immunosorbent assay (ELISA) with TgROP17 used as a coating antigen. The antiserum phase was obtained by centrifugation separation at 2300 g for 10 minutes at 4 °C. After removing the precipitated residues, 0.02% NaN₃ was added to the blood serum, which was stored at -80 °C.

Titre determination of rabbit anti-ROP17 serum

The titre of the ROP17 antiserum was detected by direct coating ELISA according to the published method [13]. First, a polystyrene microtitre plate (Corning, USA) was coated with 100 ng/100 μl of purified GST-ROP17, and the liquid was discarded after overnight incubation at 4 °C. By incubating the plate with PBST containing 1% bovine serum albumin (BSA) for 2 h at 37 °C, the nonspecific binding sites were blocked. The anti-ROP17 polyclonal antiserum was diluted to different concentrations (1: 1,000; 1: 2,000; 1: 4,000; 1: 8,000; 1: 16,000; 1: 32,000; 1: 64,000; 1: 128,000 ; 1: 256,000; and 1: 512,000) with 1% BSA and
incubated for 2 h at 37 °C. After being washed with PBST, the plate was incubated for 1 h with goat anti-rabbit IgG-HRP (Sigma, USA) diluted to 1:2,000 with 1% BSA. Tetramethylbenzidine (TMB) chromogenic reagent (Boster, Wuhan, China) was added to the plate that had been washed with TBST. After 50 ml of 2 M H₂SO₄ was added to plates to stop the reaction, the absorbance was measured at 450 nm by an ELISA plate reader (Epoch Multi-Volume Spectrophotometer System, BioTek, USA).

**Immunohistochemistry**

At the indicated time after intraperitoneal injection of 10³ T. gondii tachyzoites, the mice were sacrificed by intraperitoneal injection of sodium pentobarbital (100 mg/kg). The small intestine was dissected, and the faeces inside was removed by washing with cold PBS. Then, the small intestine was stored overnight in 4% paraformaldehyde at 4 °C. Tissues that were 5 μm thick were dewaxed in xylene and rehydrated in a series of ethanol solutions. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The sections were heated in a microwave in citrate buffer solution to retrieve antigens, and non-specific binding was prevented with goat serum blocking for 1 h. The sections were incubated overnight at 4 °C with ROP17 (dilution 1:200), LC3B, Beclin 1, p62, Bcl-2 and phospho-Bcl-2 (Ser70) antibody (dilution 1:100), followed by incubation with horseradish peroxidase-coupled goat anti-rabbit secondary antibody at 37 °C for 1 h and staining with 3,3′-diaminobenzidine. The nucleus was counterstained blue with haematoxylin. The samples incubated with rabbit serum instead of indicated primary antibodies were used as negative control. The experimental procedure was performed strictly following the manufacturer’s instructions. One hundred cells were captured randomly in a field of view under the microscope, and the percentage of the positive cells was calculated.

**Cell culture and treatment**

HEK 293T cells were purchased from the Cell Culture Center of the Chinese Academy of Medical Sciences (Beijing, China) and cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA). The medium was supplemented with 10% (v/v) foetal bovine serum (FBS; Gibco, USA). The cells were grown at 37 °C in an 5% incubator with CO₂. The cells were passaged every 2–3 days when they reached 70–80% confluency.

HEK 293T cells cultured in a 6-well plate (2.5 × 10⁵ cells/well, 1 mL medium/well) were transfected with p3×Flag-CMV-14 and p3×Flag-CMV-14-7gROP17 using Lipofectamine 3000 (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. Forty-eight hours later, the cells were treated with serum-free medium and cultured for another 4 h and 8 h to induce autophagy.

**Monodansylcadaverine (MDC) staining**

The transfected HEK 293T cells were treated by serum starvation for 0, 4 and 8 h, and then, the cells were incubated with MDC (50 μmol/L) at 37 °C for 30 minutes. Then, the cells were washed two times with pre-cooled PBS and immediately observed and imaged under a fluorescence microscope (EVOS M5000,
Thermo Fisher Scientific). One hundred cells per sample were analysed, and the percentage of autophagic cells was calculated.

**Transmission electron microscopy (TEM)**

After serum starvation treatment, the HEK 293T cells were collected and washed with cold PBS, fixed overnight in 3% glutaraldehyde at 4 °C and subsequently post-fixed in 1% osmium tetroxide for 30 minutes. The cells were gradient dehydrated with ethanol solutions ranging from 50% to 100% in a 10% graded series and then embedded in Eponate 12 resin (Ted Pella, Redding, CA, USA). The blocks were cut into ultrathin sections and then doubly stained with uranyl acetate and lead citrate. Autophagosomes were observed under a transmission electron microscope (JEOL-100CX, Japan).

**Examination of GFP-LC3B puncta**

HEK 293T cells were co-transfected with p3×Flag-CMV-14 or p3×Flag-CMV-14-\textit{TgROP17} and pCMV-GFP-LC3 (GenePharma Co., Ltd, China). After 48 h, the cells were cultured in serum-free medium for 4 h and 8 h. GFP-LC3 puncta were quantified by observation with a fluorescence microscope (EVOS M5000, Thermo Fisher Scientific) and counting at least 300 cells. The results are plotted as the mean ± SD of three independent experiments.

**Co-immunoprecipitation**

Co-immunoprecipitation analysis was performed in accordance with the manufacturer’s instructions. Briefly, HEK 293T cells were transfected with p3×Flag-CMV-14 or p3×Flag-CMV-14-\textit{TgROP17} for 48 h, and then, the cells were harvested and lysed using sonication in cell lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; and a 1% protease inhibitor cocktail (Roche, Switzerland)). The cell lysates were centrifuged, and the supernatant was incubated overnight with FLAG M2 Affinity Gel with gentle agitation at 4 °C. The following day, the beads were washed six times with cell lysis buffer and heated in 2 × sample buffer. After brief centrifugation, the supernatant was used for Western blotting.

**Western blotting**

The protein from 1×10^6 tachyzoites of \textit{T. gondii} was prepared using 1×SDS loading buffer heated for 10 minutes. Total protein from the HEK 293T cells was extracted using RIPA buffer with protease inhibitors and quantified using BCA protein assay reagent (Thermo Scientific, USA). Equal amounts of protein samples were separated by 12% SDS-PAGE and transferred to a 0.2 μm PVDF membrane (Millipore, USA). The membrane was blocked in TBST buffer with 5% non-fat milk for 1 h before overnight incubation at 4 °C with different primary antibodies. Primary antibodies were detected with their corresponding HRP-IgGs using an ECL blot detection system (Transgene, China) with a Bio-Rad Universal Hood II Gel Doc XR system. β-actin on the same membrane was used as an internal control.

**Statistical analysis**
Data are presented as means ± standard deviation (SD). Differences between two groups were analysed using Student’s t-test. Pearson coefficient was used to analyse correlations between the expression of ROP17 with the expression of autophagy markers LC3B, beclin 1 and p62, and with phosphorylation of Bcl-2 and Bcl-2. An r value ≥ 0.5 was considered as strong correlation, and an r value < 0.5 was considered as poor correlation [14]. A p-value < 0.05 was considered as statistically significant. All data were analysed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, U.S.A.).

Results

Rabbit anti-ROP17 antiserum was efficient for detecting ROP17 expression

The direct-coated ELISA showed that the final tire of rabbit anti-ROP17 polyclonal antiserum was 1:128,000 (Fig. 1a). To confirm the immunoreactivity of the ROP17 polyclonal antiserum, Western blotting was employed to detect the purified GST-ROP17 and tachyzoite protein of T. gondii using rabbit anti-ROP17 polyclonal antiserum as the primary antibody. As illustrated in Fig. 1b, specific bands of approximately 96 KDa (GST-ROP17) and 69 KDa (ROP17, from tachyzoite of T. gondii) were detectable, indicating that the rabbit anti-ROP17 antibody was prepared successfully.

Intra-abdominal mouse inoculation with tachyzoites of T. gondii induces autophagy in small intestine cells

Under natural conditions, following host oral ingestion of tissue cysts or oocysts, the parasites invade the intestinal tissue and rapidly develop into tachyzoites [15, 16]. However, in laboratory research, the tachyzoites of T. gondii are typically maintained and cultured through serial passaging in mice upon intraperitoneal infection of the tachyzoites, which emerge within 2 to 4 days. To determine whether autophagy occurs in tachyzoite-infected murine cell models, paraffin-embedded sections of the small intestine were prepared for the immunohistochemical detection of autophagy markers. Beclin 1 and LC3B are markers of autophagy since beclin1 is involved in the initial step of autophagosome formation and LC3 forms puncta on the autophagosome membrane [17]. In addition, the ubiquitin-binding protein p62, which functions as a molecular adaptor for autophagic machinery and its substrates, has been widely used as a biochemical marker for general autophagy detection [18]. As shown in Fig. 2, the levels of LC3B and beclin 1 increased while that of p62 decreased gradually with prolonged time of infection by the tachyzoites of T. gondii. Notably, the number of tachyzoites of T. gondii was also augmented upon enhanced autophagy, as illustrated by ROP17-positive staining. These data clearly showed that the infection by the tachyzoites of T. gondii via the intraperitoneal injection triggered autophagy and promoted parasite proliferation in mouse small intestine cells. However, the underlying mechanism of this phenomenon is unclear and needs to be revealed.

The overexpression of ROP17 promotes the autophagy induced by serum starvation

To evaluate the putative roles of ROP17 in T. gondii tachyzoite and host cell interplay, we used the HEK 293T cell line as model since this cell line allows highly efficient transfection and expression of
exogenous proteins. Transient transfection of the p3×Flag-CMV-14 or p3×Flag-CMV-14-\textit{Tg}ROP17
eukaryotic expression plasmid for 48 h and the expression of ROP17-Flag was confirmed by Western
blotting (Fig. 1b). Given that autophagy has dual roles in \textit{T. gondii} infection, either clearing this protozoan
or providing nutrients for its proliferation [19, 20], we examined the initiation of autophagy in HEK 293T
cells with or without ROP17 after serum starvation for 0 h, 6 h and 12 h by MDC staining, which is a
classical method to detect autophagy [21]. To distinguish the role of ROP17 in autophagy, the HEK 293T
cells were incubated with MDC (1 mM) after serum starvation. The staining results indicated that the
number of acidic vacuoles was increased in the ROP17-overexpressing HEK 293T cells compared with
control cells (Fig. 3a and b), suggesting that ROP17 possibly induced autophagy. Morphologically,
autophagy is characterized by the formation of double-membrane vesicles called autophagosomes,
which are essential for the isolation and degradation of cytoplasmic components [22]. To confirm that
ROP17 induced autophagy upon serum starvation, transmission electron microscopy (TEM) was used to
investigate autophagosome formation. As illustrated in Fig. 3c and d, the number of autophagosomes in
the ROP17-overexpressing cells was greater than that in control cells. In addition, the number of GFP-
LC3B puncta is a good indicator of autophagosome presence and is closely related to the number of
autophagosomes [23]. ROP17-overexpressing HEK 293T cells and parental cells transfected with GFP-
LC3 were subjected to serum starvation. As shown in Fig. 3e and f, a marked accumulation of GFP-LC3
puncta was observed in the ROP17-overexpressing HEK 293T cells, suggesting that ROP17 led to
autophagosome accumulation. To confirm the role of ROP17 in autophagy induction, the autophagy
markers LC-3B, Beclin 1 and p62 were analysed by Western blotting. As illustrated in Fig. 4, the levels of
Beclin 1 and LC3B in the serum-starved ROP17-overexpressing HEK293T cells were greater than those in
the control cells, while p62 showed the opposite trend at the same indicated times. All of these data
suggest that ROP17 promotes the autophagy induced by serum starvation.

\textit{Tg}ROP17 phosphorylates Bcl-2 to induce the dissociation of the Beclin 1-Bcl-2 complex and promote
autophagy

Autophagy is tightly regulated by Beclin 1 activity, which is inhibited by Bcl-2 via the formation of the
Beclin 1-Bcl-2 complex. Dissociation of the Beclin 1-Bcl-2 complex stimulates autophagy in response to
conditions of nutrient deprivation and other cellular stresses [24]. Previous reports have demonstrated
that Bcl-2 is phosphorylated at multiple sites, including threonine 69, serine 70 and serine 87, by JNK,
causing the dissociation of Beclin 1 and the induction of autophagy [25]. Because ROP17 contains
conserved ATP-binding residues and catalytic triads that are essential for S/T kinases, similar to JNK [26],
we sought to determine whether ROP17 can phosphorylate Bcl-2 and then liberate Beclin 1 to trigger
autophagy. Western blotting showed that ROP17 enhanced the phosphorylation of Bcl-2 (Ser 70) and led
to its degradation (Fig. 5). To determine whether phosphorylation is ROP17- or JNK-dependent, the
phosphorylation of JNK was also measured. As shown in Fig. 5, ROP17 did not change the levels of JNK
or its phosphorylation. To exploit the function of ROP17 in Bcl-2 phosphorylation, co-immunoprecipitation
experiments were performed, and the data showed that ROP17 interacts with Bcl-2 (Fig. 6). Collectively,
these data demonstrate that ROP17 interacts with Bcl-2 and phosphorylates it and then promotes the
dissociation of the Beclin 1-Bcl-2 complex to activate autophagy.
Infection with *T. gondii* tachyzoites facilitates the phosphorylation and degradation of Bcl-2 in the mouse small intestine cells

Based on the results from the *in vitro* assay, we wanted to determine whether ROP17 functions as an autophagy inducer via the Beclin 1-Bcl-2 pathway *in vivo*. Mouse small intestine slices that had been used to analyse autophagy (Fig. 2) were collected to evaluate the levels of Bcl-2 and its phosphorylation. As illustrated in Fig. 7, the phosphorylation of Bcl-2 increased; however, the Bcl-2 level was attenuated gradually with prolonged time of infection with the tachyzoites of *T. gondii*. Combined with the data shown in Fig. 2, the Rop17 modulation of autophagy via the Beclin 1-Bcl-2 pathway was confirmed *in vivo*.

To further confirm ROP17 as a cellular autophagy modulator, correlations between the expression of ROP17 with the expression of autophagy markers LC3B, beclin 1 and p62, as well as phosphorylation of Bcl-2 and Bcl-2 were evaluated using Pearson’s correlation coefficient. As shown in Fig. 8, a strong positive correlation was illustrated between the expression of ROP17 with the expression of LC3B (Fig. 8a, $r = 0.9826, p = 0.0443$), Beclin 1 (Fig. 8b, $r = 0.9945, p = 0.0334$), and phosphorylation of Bcl-2 (Fig. 8d, $r = 0.9908, p = 0.0229$), while a strong negative correlation was revealed between the expression of ROP17 and the expression of p62 (Fig. 8c, $r = -0.9735, p = 0.0364$), and Bcl-2 (Fig. 8e, $r = -0.9762, p = 0.0301$). These data together indicate that the increase of LC3B, Beclin 1 and phosphorylation of Bcl-2, the decrease of p62 and Bcl-2 are ROP17-modulated.

**Discussion**

Autophagy is an evolutionarily conserved cell survival process that removes damaged organelles, toxic metabolites and pathogens and enhances energy production to maintain cell survival under nutrient-deficient conditions. On the other hand, excessive self-digestion and degradation of important intracellular components can lead to cell death. Whether autophagy plays a pro-survival or pro-death role depends on the type and extent of the stress, cell context, etc.[27]. For intracellular pathogens, host cells provide a replicative niche but also combat intruders via different responses. Among these responses, host autophagy is a significant hindrance to the intracellular growth of pathogens but can also be subverted by pathogens to capture nutrients that support their survival. However, the role of autophagy during infection is complex; some pathogens rely on the induction of host autophagy to survive within host cells, while others are destroyed by it. The different roles of autophagy in eukaryotic pathogens rely on the host cell types they infect, infective stage and response strategies[28].

Tachyzoites of *T. gondii* infect virtually all nucleated cells and survive by residing in a compartment called the parasitophorous vacuole (PV), which is formed during the active invasion of host cells and is dependent on the parasite actin-myosin motor and sequential secretion of proteins from micronemes and rhoptries [29]. Rhoptries are key secretory organelles that synthesize rhoptry organelle proteins (ROPs) and play essential roles in parasite survival [30]. ROP16, ROP17 and ROP18 are serine/threonine (S/T) protein kinases secreted by rhoptries [31]. ROP16 phosphorylates the host transcriptional factors STAT3
and STAT6 to negatively regulate the production of IL-12 and Th1 inflammatory responses, which are essential for the host to survive infection with *T. gondii* [32, 33]. ROP18 phosphorylates immunity-related GTPases (IRGs) to avoid their accumulation in the PV and to protect the parasite from clearance [34]. ROP17 has a C-terminal kinase catalytic domain, and this kinase region contains a divergent RAH region that interacts with the parasitophorous vacuole membrane (PVM) of *T. gondii* [35]. In infected cells, ROP17 resides on the cytosol side of the PVM and protects the parasite from clearance by forming complexes with ROP18 kinases that inhibit IRG recruitment to the intracellular *T. gondii*-containing vacuole [8]. ROP17 inhibits the innate immune response by reprogramming host gene expression to promote *T. gondii* survival [36]. In addition, ROP17 has also been found to participate in the translocation of dense granule effectors across the PVM and promote *T. gondii* dissemination by hijacking monocyte tissue migration [5, 37].

Although some functions of ROP17 have been revealed, there are only a few reports on its function in parasite survival. In the present work, autophagy level was measured in *Toxoplasma*-infected murine models. Specifically, a ROP17-containing eukaryotic expression plasmid was transfected into HEK 293T cells, and the regulatory role of ROP17 on autophagy was studied. In the mouse model infected via intraperitoneal injection of tachyzoites of *T. gondii*, the levels of autophagy markers (LC3B, Beclin 1 and P62) were measured in small intestine tissues since *T. gondii* infects individual humans through the ingestion of contaminated feline faecal material, food, and water and first invades digestive tracts. The immunohistochemistry data showed that LC3B and Beclin1 were increased while p62 was decreased with the prolonged infection time. These data demonstrated that autophagy was initiated after *T. gondii* infection. Increasing evidence indicates that autophagy is essential for the elimination of *Toxoplasma* via canonical [38] or noncanonical autophagy [39]. To reveal the role of autophagy on the tachyzoites of *T. gondii* in the mouse intestine, immunohistochemistry was performed using rabbit anti-ROP17 serum, and the data showed that the number of parasites increased accompanied with the increase in autophagy markers expression. These results demonstrate that autophagy promotes the survival of *T. gondii* in the murine intestine.

To determine the role and mechanism of ROP17 in autophagy, the p3×Flag-VMV-14/ROP17 eukaryotic expression plasmid was transfected into HEK 293T cells, and autophagy was measured under serum starvation conditions since nutrient status is closely associated with autophagy activation. Our results showed that ROP17 markedly increased the number of autophagosomes in the ROP17-transfected cells, as demonstrated by the visualization of MDC staining, number of GFP-LC3B puncta and TEM assessment. Western blotting also showed that the autophagy markers LC3B and Beclin 1 were increased in the ROP17-transfected cells compared to their levels in the control cells. Importantly, convincing evidence of autophagy activation with decreased p62 levels was detected by immunoblotting, which leads to the exclusion of failed autophagosome clearance as an explanation.

Beclin 1 plays an important role in autophagosome formation by interacting with Vps34 (also named class III-type phosphoinositide 3-kinase) [40]. The ability of Beclin 1 to activate Vps34 is tightly regulated via both transcriptional regulation and interaction with Beclin 1-binding proteins [41]. Emerging evidence
demonstrates that the binding of Bcl-2 to Beclin 1 negatively regulates autophagosome formation and then inhibits autophagy [42]. Beclin 1 activates autophagy upon its dissociation from Bcl-2, which is phosphorylated by JNK [43]. Because ROP17 contains conserved ATP-binding residues and catalytic triads that are essential for S/T kinases, similar to JNK [26], we assayed the role of ROP17 in the phosphorylation of Bcl-2. Our results showed that ROP17 can interact with Bcl-2 and then phosphorylate it at Ser70 in the ROP17-transfected cells under starvation conditions. However, the levels of JNK expression and phosphorylation were unchanged under the same conditions.

Currently, the underlying molecular mechanisms for the effect of ROP17 on the virulence of T. gondii remain to be further investigated. Considering the information presented above, we speculate that ROP17 induces autophagy to provide nutrients for T. gondii survival via the Beclin 1-Bcl-2 pathway.

Conclusion

Collectively, our findings suggest that ROP17 is advantageous to the survival and proliferation of the intracellular protozoan T. gondii via Beclin 1-Bcl-2-dependent autophagy activation. Bcl-2 is also involved in the regulation of apoptosis, which is essential for the fate of T. gondii. Additional studies are necessary to investigate the role of ROP17 in the crosstalk between components of autophagy and apoptosis to uncover its virulence function.

Abbreviations

T. gondii: Toxoplasma gondii; ROP: rhoptry organelle protein; LC3: Microtubule-associated protein light chain 3; Bcl-2: B-cell lymphoma-2; MDC: Monodansylcadaverine; TEM: transmission electron microscopy; co-IP: co-immunoprecipitation; GFP: green fluorescent protein; CNS: central nervous system; BBB: blood-brain barrier; PVs: parasitophorous vacuoles; PVM: parasitophorous vacuolar membrane; IRGs: immunity-related GTPases; HRP: horseradish peroxidase; GST: Glutathione S-Transferase; ELISA: enzyme-linked immunosorbent assay; KDa: kilodalton; JNK: Jun N-terminal Kinase; ATP: adenosine-triphosphate

Declarations

Ethics approval and consent to participate

All procedures of animal experiment were performed in accordance with the laws and conditions of the Guide for the Care and Use of Laboratory Animals and Institutional Animal Ethics Committee guidelines of Shanxi Medical University.

Consent for publication

Not applicable.

Availability of data and materials
Data supporting the conclusions of this article are included within the article.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

HL W, ZY C and CF W conceived and designed experiments. JS, YH L, XL M and KR Q performed cells experiments including Western blotting and MDC; MG, WT W, WW and HX G performed animal immunohistochemistry assay. JR H, TT M and BX H performed TEM; HL L and JJ L performed data analysis; MG and JS performed co-IP assay; HL W and WT W wrote the manuscript. All authors read and approved the manuscript.

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Figures
The rabbit anti-ROP17 antiserum was evaluated using direct-coated ELISA (a) and Western blotting (b). The rabbit serum added before immunization served as the negative control, and 1% BSA was used as the blank control, as shown in Fig. 1a. The lysate from the HEK 293T cells which were transfected with p3×Flag-CMV-14-ROP17 for 48 h and 1 μg of purified GST-ROP17 were used as positive controls to assess the expression of ROP17 in the T. gondii tachyzoites, as shown in Fig. 1b.
Tachyzoites of *T. gondii* induced autophagy in the mouse small intestine cells. A total of 103 tachyzoites of *T. gondii* were injected into the mouse abdominal cavity, and the small intestine was collected 24 h and 48 h later for immunohistochemistry assays using the indicated antibodies. Normal mouse small intestine served as the control. The positive expression rate of the target proteins was calculated, and the data are expressed as the means ± SD (n=3). Red arrows indicate positive cells. The scale bar is 50 μm.
for all slices. * p < 0.05 compared with the normal mouse group, and # p < 0.05 compared with the T. gondii infection group for 24 h group.

Figure 3

ROP17 enhanced the autophagy of HEK 293T cells subjected to serum starvation. (a and b) Autophagic vacuoles of the ROP17-overexpressing 293T cells and control cells were visualized by MDC staining after serum starvation for 0, 6 and 12 h. The scale bar represents 20 µm. (c and d) Transmission electron microscopy analysis of the serum-starved ROP17-overexpressing 293T cells and control cells. ROP17-overexpressing cells displayed more autophagosomes (indicated by red arrows) than the controls. The relative numbers of autophagosomes in the different groups are shown (d). The scale bar represents 500 nm. (e and f) The 293T cells were co-transfected with p3×Flag-CMV-14 or p3×Flag-CMV-14-TgROP17 and GFP-LC3 and subjected to serum starvation for 0, 6 and 12 h. GFP-LC3B puncta were observed by confocal laser scanning microscopy (e), and showed a punctuated pattern (f), indicating the induction of
autophagy. The scale bar represents 20 µm. The data are expressed as the mean±S.D. of three independent experiments. *Indicates a significant difference between groups as shown, *p < 0.05.

| serum starvation time | control | ROP17 | LC3B/β-actin | Beclin 1/β-actin | p62/β-actin |
|-----------------------|---------|-------|-------------|-----------------|-------------|
| 0h                    | +       | -     | 0.001       | 0.073           | 0.432       |
| 6h                    | +       | -     | 0.042       | 0.174           | 0.374       |
| 12h                   | +       | +     | 0.081       | 0.268           | 0.306       |
| 0h                    | -       | +     | 0.001       | 0.089           | 0.427       |
| 6h                    | -       | +     | 0.106       | 0.275           | 0.291       |
| 12h                   | -       | +     | 0.238       | 0.624           | 0.162       |

Figure 4

ROP17 induced autophagy in serum-free ROP17-overexpressing HEK 293T cells. ROP17-induced autophagy, detected by the presence of Beclin 1, LC3B and p62, was analysed by Western blotting. β-actin served as a loading control. One representative of three independent experiments is shown. The quantitative results are presented as the ratio of LC3B to β-actin, Beclin 1 to β-actin and p62 to β-actin (n = 3). *p < 0.05 indicates a significant difference compared with the difference calculated for the previous time point, and #p < 0.05 indicates a significant difference compared with the value calculated for same time point in the control group.
ROP17 augmented autophagy via the beclin 1-Bcl-2 pathway. HEK 293T cells were transfected with p3×Flag-CMV-14 or p3×Flag-CMV-14-TgROP17 and then subjected to serum starvation for 0 h, 6 h and 12 h. The phosphorylation of Bcl-2 and Bcl-2, and the phosphorylation of JNK and JNK were tested by Western blotting. β-actin served as a loading control. One representative of three independent experiments is shown. The quantitative results are presented as the ratio of phosphorylation of Bcl-2 to β-actin and Bcl-2 to β-actin (n = 3); *p < 0.05 indicates a significant difference compared with the value calculated for the previous time point, and #p < 0.05 indicates a significant difference compared with the value calculated for the same time point in the control group.
A co-IP assay was employed to investigate the interaction of ROP17 and Bcl-2. HEK 293T cells were transfected with p3×Flag-CMV-14 or p3×Flag-CMV-14-TgROP17, and the cell lysates were incubated with FLAG M2 Affinity Gel. The precipitate was assayed using Western blotting with Flag and Bcl-2 antibodies.
Tachyzoites of *T. gondii* boosted the phosphorylation and degradation of Bcl-2 in the mouse small intestine. A total of 103 tachyzoites of *T. gondii* were injected into the mouse abdominal cavity, and the small intestine was collected 24 h and 48 h later for immunohistochemical analysis using Bcl-2 and phospho-Bcl-2 (Ser70) antibodies. The normal mouse small intestine served as the control. The positive expression rate of the target proteins was calculated. Data are expressed as the means ± SD (n=3). Red arrows indicate positive cells. The scale bar represented 50 μm for all slices; *p < 0.05 indicates a
significant difference compared with the normal mouse group, and \(^\# p < 0.05\) indicates a significant difference compared with the T. gondii infection for the 24 h group.

![Graphs](image)

**Figure 8**

ROP17 expression correlates with autophagy markers and Bcl-2. Immunohistochemistry assay were performed in small intestine tissue obtained from tachyzoites of T. gondii-infected mouse at 24 h and 48 h after the infection. ROP17, LC3B, Bcelin 1, p62, Bcl-2 and phosto-Bcl-2 (Ser70) were stained with corresponding antibodies. Normal mouse small intestine served as the control. The positive expression rate of target proteins was calculated, and the correlations between ROP17 expression and LC3B, Bcelin 1, p62, Bcl-2 and phosto-Bcl-2 were analysed using Pearson's correlation coefficient. \( r \geq 0.5 \) was considered as strong correlation, and an \( r < 0.5 \) was as poor correlation, \( p < 0.05 \) was considered as statistically significant.