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Original Article

Potential role of autophagy in proteolysis in *Trichomonas vaginalis*

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Abstract *Background:* Autophagy has been shown to be involved in the pathogenesis of several protists, offering prospects for the developments of new drugs targeting autophagy. However, there is no evidence illustrating functional autophagy in the deep-branching trichomonads. The human parasitic protist *Trichomonas vaginalis* has been predicted to possess reduced autophagic machinery, with only autophagy-related protein 8 (Atg8) conjugation system required for autophagosome formation.
Methods: The recombinant protein of TvAtg8 (rTvAtg8) and the polyclonal antibody against rTvAtg8 were generated. The expression and localization of TvAtg8 was monitored upon autophagy induction by glucose restriction (GR) compared with glucose-rich cultivation. The role of TvAtg8 in proteolysis was clarified.
Results: Here, we report that *T. vaginalis* Atg8 (TvAtg8) is upregulated and conjugated to autophagosome-like vesicles upon autophagy induction by GR. Moreover, we investigate, for

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the first time, the role of autophagy in *T. vaginalis*. Proteasome inhibition (PI)-induced autophagy compensates for the removal of polyubiquitinated proteins under glucose-rich condition. GR-induced autophagy is a major proteolytic system in *T. vaginalis*. These results suggest that autophagy is vital for proteolysis in *T. vaginalis* with an impaired ubiquitin-proteasome system or under glucose-limited environment.
Conclusion: Our findings unveiled previously unidentified functions of autophagy in proteostasis in trichomonads, advancing our understanding of this highly conserved process in the ancient eukaryote.

Introduction

Autophagy is involved in turnover of intracellular macromolecules and organelles under physiological conditions. Autophagy also plays a key role in cell fate decisions under various stress conditions.[1] In most cases, autophagy is an adaptive response for cell survival upon nutrient starvation.[2] Increasing evidence indicates that autophagy participates in multiple biological functions, including development and differentiation,[3] immunity,[4] and regulation of lifespan.[5] Autophagy has recently been considered as an alternative protein degradation pathway that compensates for the compromised ubiquitin-proteasome system (UPS).[6] The autophagic process involves the formation of autophagosomes, which is mediated by the Atg12-Atg5 and Atg8 conjugation systems. Atg8 is widely used as an autophagosome marker in various organisms.[7] During autophagy, the cytosolic form of Atg8 is cleaved by the protease Atg4, conjugated to Atg7 and Atg3, and finally formed an amide bond with phosphatidylethaolamine (Atg8-PE), the lipidated form of Atg8 on the autophagosomal membrane.[8]

Autophagy has been suggested to correlate with the pathogenesis of several protists.[9] For example, autophagy participates in the development of *Trypanosoma cruzi*[10] and is essential for the differentiation and virulence in *Leishmania major*.[11] Additionally, autophagy is crucial for the proliferation and encystation in *Entamoeba invadens*.[12] Autophagy has been proposed as a cell death mechanism in *Blastocystis hominis*,[13] *Trypanosoma brucei*,[14] *Toxoplasma gondii*[15] and *Plasmodium berghei*.[16] However, it remains debated whether autophagy is an alternative form of programmed cell death or a survival response to death stimuli.[17] Intriguingly, only a limited repertoire of Atg orthologues has been identified in several protists, drawing our attention to the noncanonical autophagic machinery in the ancient eukaryotes.[9]

Human trichomoniasis caused by *Trichomonas vaginalis* is the most widespread non-viral sexually transmitted infection, with approximately 276 million cases reported annually worldwide.[18] *T. vaginalis* colonizes the urogenital tract of humans and leads to serious health outcomes for women, including vaginitis, preterm delivery, infertility, low birth weight infants, and susceptibility to cervical cancer.[19] Trichomoniasis is also implicated as a risk factor for HIV transmission[20] and lethal prostate cancer.[21] Bioinformatics analysis of the *T. vaginalis* genome reveals that the parasite possesses the genes encoding proteins of the Atg8 conjugation system but lacks those encoding proteins of the Atg12-Atg5 system.[22] We previously reported that glucose restriction (GR) could induce an autophagy-like response in *T. vaginalis*,[22] providing a great opportunity to study the molecular mechanisms of autophagy in trichomonads. Herein, we aim to unravel the biological roles of autophagy in *T. vaginalis*. These findings significantly enhance our understanding of the functions of autophagy in *T. vaginalis*, paving the way for future interrogations of the detailed molecular events mediating autophagy in trichomonads.

Methods

Parasite and culture conditions

The *T. vaginalis* (JH31A#4) trophozoites were maintained in YIS medium,[23] pH 5.8, containing 10% heat-inactivated horse serum and 1% glucose (high-glucose medium, HGM) at 37 °C. For glucose-restricted cultivation, parasites were maintained in the same medium without glucose supplement at 37 °C as previously described.[22]

Drugs and treatments

Proteasome activity was inhibited after treatment of cells with the proteasome inhibitor MG132 (5 μM for 3 h) (Sigma, MO, USA). Proteasome activity was determined by the Proteasome Activity Fluorometric Assay kit (Biovision, CA, USA) according to the manufacturer's instructions. Autophagy was inhibited following treatment of cells with the autophagic inhibitor wortmannin (50, and 100 μM for 3 h) (Sigma, Missouri, USA).

Production of the TvAtg8 antiserum

The recombinant proteins were generated by pTrcHis and pTrcHis2 TOPO® TA expression Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, the full-length coding sequence of *Atg8* (TVAG_486080) was amplified and cloned

Table 1 Identification of putative TvAtg8 orthologs in *T. vaginalis*.

ATG proteins	*T. vaginalis* orthologus (Domains)[a]	*Saccharomyces cerevisiae*			*Mus musculus*			*Homo sapiens*		
		Protein (Accession no.)[b]	E-value	Identity (%)	Protein (Accession no.)	E-value	Identity (%)	Protein (Accession no.)	E-value	Identity (%)
Atg8a	**TVAG_486080** (GABARAP)	Atg8p (NP_009475.1)	2.E−13	46	MAP1LC3a (NP_080011.1)	2.E−18	35	GABARAPL1 (NP_113600.1)	2.E−28	46
					MAP1LC3b (NP_080436.1)	5.E−16	32	GABARAPL2 (NP_009216.1)	4.E−28	49
								MAP1LC3c (NP_001004343.1)	7.E−22	44
Atg8b	**TVAG_239800** (UBQ superfamily)	ND[c]			MAP1LC3a (NP_080011.1)	7.E−12	33	GABARAPL1 (NP_113600.1)	3.E−19	43
								GABARAPL2 (NP_009216.1)	2.E−17	41
								MAP1LC3c (NP_001004343.1)	6.E−13	34

[a] Domains were predicted by NCBI Conserved Domain Database.
[b] NCBI reference sequence numbers.
[c] ND, without an ortholog.

into the pTrcHis vector to produce the recombinant plasmid construct. The plasmid was introduced into *E. coli* (TOP10) for the expression of the recombinant protein. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the expression of the histidine (HIS)-tagged recombinant protein was analyzed by 15% SDS-PAGE and western blotting using mouse anti-His antibody (GeneTex, Texas, USA). The recombinant protein was purified by His-bound resin (Novagen, USA) column chromatography and the rabbit polyclonal antibody was commercially produced (AbKing, Taiwan).

Immunoblot analysis

Immunoblot analysis was performed as previously described.[24] For separation of the TvAtg8 lipidated form, whole cell lysates were separated by SDS-PAGE containing 6 M urea.[25] The primary antibodies were used: mouse anti-His antibody, (1: 2000 dilution), mouse anti-ubiquitin antibody (1:500 dilution) (ENZO Life Sciences), rabbit anti-TvAtg8 (1: 1000 dilution) antibody. The bound antibody was detected by anti-mouse or anti-rabbit IgG secondary antibodies (1:5000 dilution) (GeneTex, Texas, USA).

Immunofluorescence assay

Cells were fixed onto microscopic slides with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature (RT). Cells were permeabilized with 0.1% Triton X-100/PBS for 10 min at RT and then blocked with 3% bovine serum albumin (BSA)/PBS for 1 h at RT. Cells were incubated with the indicated primary antibodies in 3% BSA-0.1% Triton X-100/PBS for 1 h at RT. The primary antibodies were used: rabbit polyclonal anti-TvAtg8 (1:500 dilution) and mouse anti-ubiquitin antibody (1:500 dilution). After being washed, cells were incubated with Alexa fluor 488 or 594 anti-rabbit or anti-mouse IgG antibody (1:500 dilution) (Thermo Fisher Scientific) for 1 h at RT. Cells were washed

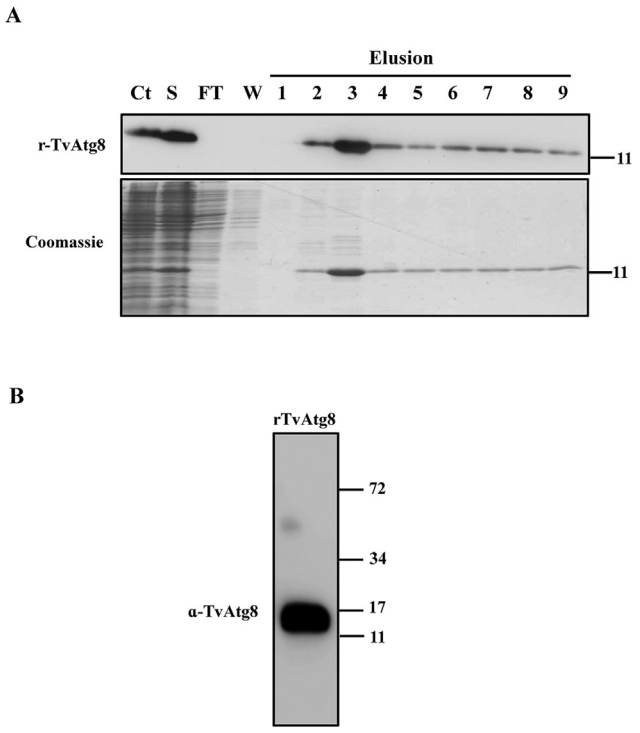

Figure 1. Cloning and purification of the recombinant TvAtg8 protein. (A) The recombinant TvAtg8 protein was purified by His-bound resin column chromatography and analyzed by western blotting using anti-His antibody. Lane Ct, control recombinant protein; Lane S, soluble fraction; Lane FT, flow-through fraction; Lane W, wash fraction. The numbers represent each fraction collected from elution. (B) The TvAtg8 polyclonal antibody was used to recognize the recombinant protein.

and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, Merck, Darmstadt, Germany) to label the nuclei for 15 min. Slides were examined using confocal microscopy (Zeiss LSM510).

Statistical analysis

Quantitative data were expressed as mean ± SD. of three independent experiments unless otherwise indicated. A Student's *t*-test (two-tailed) was used to evaluate the significant differences between groups. $P < 0.05$ was considered statistically significant.

Results

Monitoring autophagy in *T. vaginalis* under different glucose concentrations

Although two Atg8 paralogues have been identified,[22] TvAtg8a (TVAG_486080) was the only orthologue of yeast

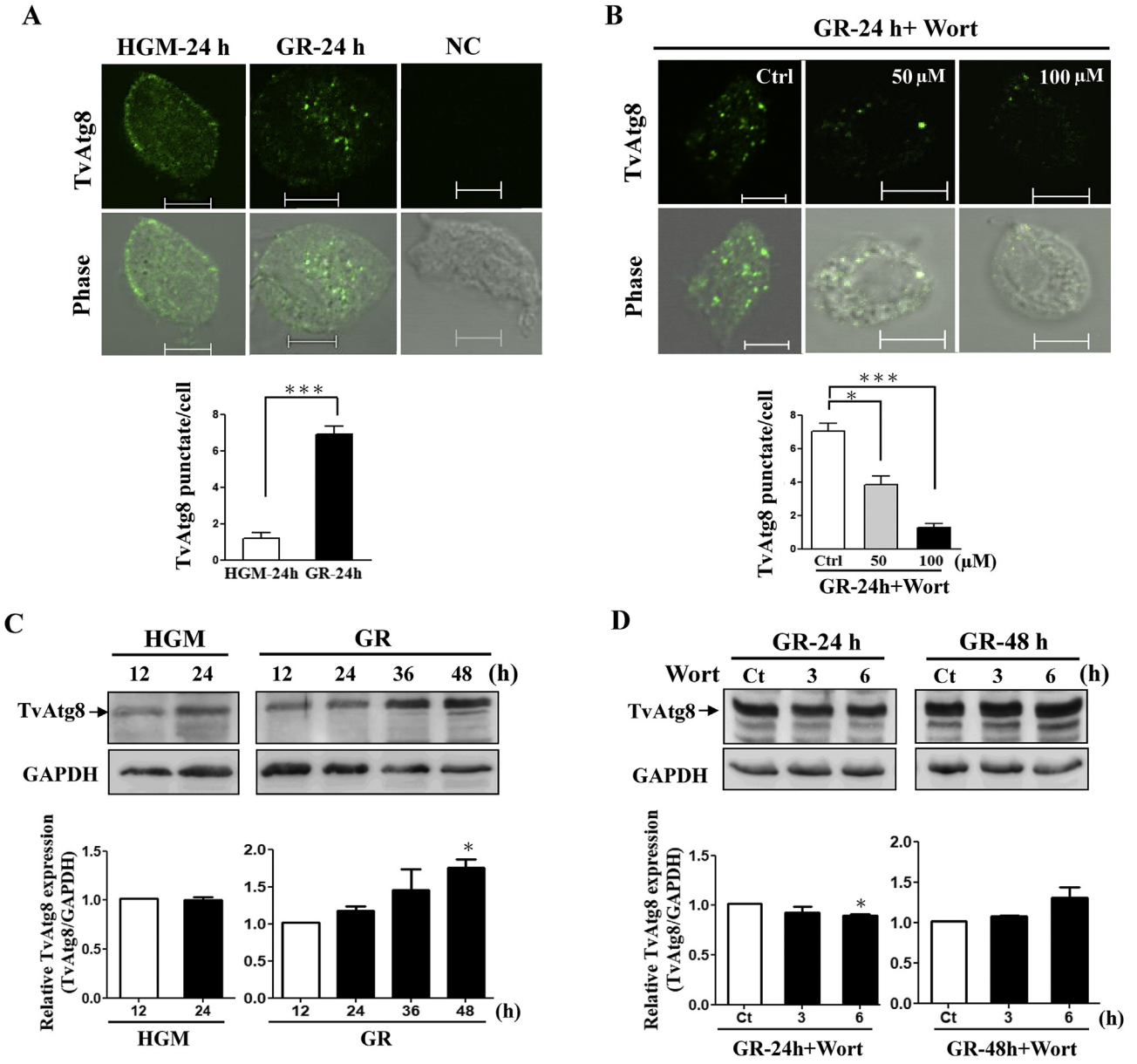

Figure 2. TvAtg8 is conjugated to autophagosome-like vacuoles during autophagy induced by GR. (A) The distribution of TvAtg8 in HGM and GR-cultivated cells (24 h) was detected. Scale bar = 5 μm. NC: the negative control probed with a secondary antibody. (B) GR-cultivated cells (24 h) were treated with Wort (50 and 100 μM) for 3 h and the distribution of TvAtg8 was monitored compared with that of control (Ctrl). The average number of TvAtg8 puncta was determined in different microscopic fields (30 cells, 10 cells/group). (C) Cell lysates were fractionated by 15% SDS-PAGE containing 6 M urea and the expression of TvAtg8 were analyzed. (D) Trophozoites cultured under GR (24 and 48 h) were treated with 100 μM Wort and the expression of TvAtg8 was determined. *$p < 0.05$, ***$p < 0.001$.

Atg8 and showed a higher sequence identity to human Atg8 proteins (44—49%) than that of TvAtg8b (TVAG_239800) (34—43%) (Table 1). To clarify whether TvAtg8 is a potential autophagosome marker in *T. vaginalis*, the TvAtg8a coding sequence was cloned into an expression vector and the recombinant protein (rTvAtg8) was generated via a prokaryotic expression system (Fig. 1A, B). A polyclonal TvAtg8 antibody was raised against rTvAtg8, showing a major protein band consistent with the predicted molecular weight (MW) (~14 kDa) (Fig. 1C). The formation of Atg8 puncta is a hallmark of autophagy.[8] As GR has been shown to induce autophagic vacuoles (AVs) formation in *T. vaginalis*,[22] we thus determined the distribution of TvAtg8 in GR-cultured cells compared with that in high-glucose medium (1% glucose, HGM)-cultured cells by immunofluorescence assay. TvAtg8 mainly exhibited diffuse fluorescence over the cytoplasm of HGM-cultured cells, whereas it displayed a punctate pattern in GR-cultivated cells (the puncta per cell for 24 h of HGM and GR cultivation was 1.2 ± 0.35 and 6.93 ± 0.45, respectively, $P < 0.05$) (Fig. 2A). Additionally, GR-cultured cells treated with the autophagy inhibitor wortmannin (Wort)[22] suppressed the formation of TvAtg8 puncta in a dose-dependent manner (the puncta per cell for the DMSO-treated control and cells treated with 50 and 100 μM Wort for 3 h decreased from 7.03 ± 0.50 to 3.83 ± 0.55 and 1.3 ± 0.21, respectively, $P < 0.05$), suggesting that TvAtg8 localized to autophagosome-like vacuoles under GR condition (Fig. 2B).

To further assess whether TvAtg8 was processed to bind to the autophagosomes upon autophagy induction, cell lysates from the GR cultures with different incubation time (12 h, 24 h, 36 h, and 48 h) were fractionated on SDS-PAGE containing 6M urea,[25] allowing for separation of the lipidated TvAtg8. Immunoblot analysis of whole cell lysates using anti-TvAtg8 antibody revealed that the major protein band was progressively increased in abundance during GR cultivation for 48 h (Fig. 2C). However, the MW of the GR-enriched protein (~34 kDa) was higher than that of rTvAtg8. To evaluate whether the GR-enriched TvAtg8 could reflect the extent of autophagy, its expression level

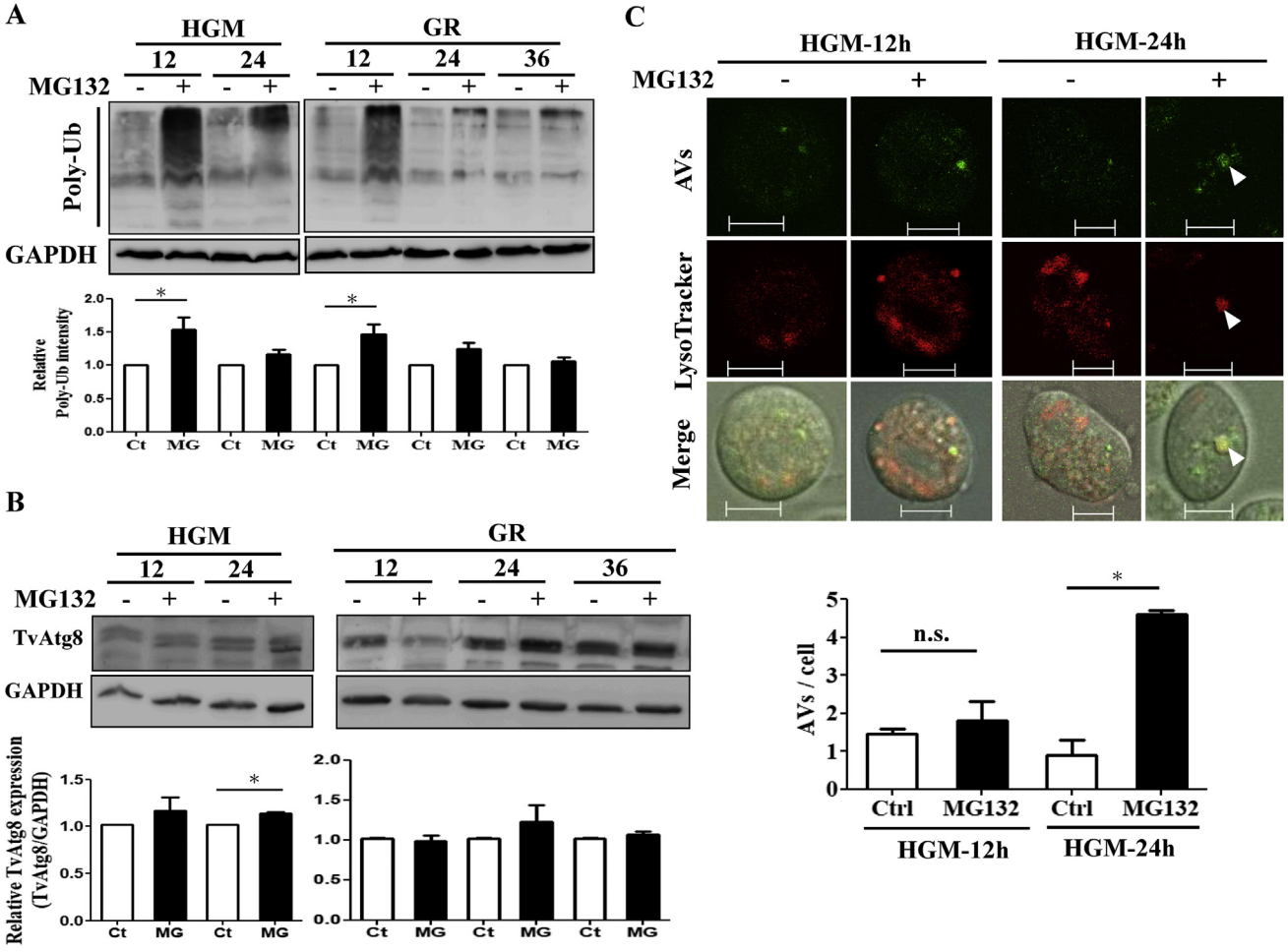

Figure 3. Proteasome inhibition activates autophagy in *T. vaginalis* under HGM cultivation. (A) HGM- and GR-cultured cells were treated with the proteasome inhibitor MG132 (5 μM) for 3 h and the accumulation of polyubiquitinated proteins was determined. (B) HGM- and GR-cultured cells were treated with MG132 (5 μM) for 3 h and the protein expression of TvAtg8 was determined. (C) To track AVs and lysosomes, HGM-cultured cells (12 and 24 h) treated with MG132 (5 μM) for 3 h were co-stained with autophagic green fluorescent dye and Lysotracker Red. Scale bar = 5 μm. The average number of AVs was determined by counting the autophagic green fluorescent signals in cells (n = 20). *$p < 0.05$. n.s., not significant.

was detected in the early- and late-stationary phase of GR cultures (GR-24 and GR-48 h) following Wort treatment. Wort treatment only reduced the expression of GR-enriched TvAtg8 in the early-stationary phase of GR culture (Fig. 2D), suggesting that Wort inhibits the initial stage of autophagy.

Proteasome inhibition (PI) activates autophagy as a compensatory proteolytic system in the stationary phase of HGM culture

To verify the function of UPS in *T. vaginalis*, the accumulation of ubiquitinated proteins was detected in both HGM and GR-cultured cells treated with the proteasome inhibitor MG132. MG132 treatment significantly increased the levels of ubiquitinated proteins in the log phase of HGM and GR cultures (HGM-12 h and GR-12 h, $P < 0.05$) compared with those of the DMSO-treated control; however, the effect of MG132 treatment was attenuated in the stationary phase of HGM culture (HGM-24 h) and after longer incubation in GR cultures (GR-24 h and 36 h) (Fig. 3A). This data suggests that the UPS is critical for the degradation of polyubiquitinated proteins during cell proliferation in HGM and GR cultivation. The diminished polyubiquitinated proteins in the stationary phase of HGM culture after PI implicated that autophagy was activated to compensate for protein degradation. We thus examined whether PI could induce autophagy in *T. vaginalis*. After MG132 treatment, TvAtg8 accumulated in the stationary phase of HGM-cultured cells (Fig. 3B). This data was supported by monitoring the autophagic flux in live HGM-cultured cells after PI. Apparent formation of AVs and subsequent fusion with lysosomes in stationary-phase cells of HGM culture were observed (Fig. 3C).

To verify PI-induced autophagy could compensate for the suppressed UPS, the ubiquitinated proteins were analyzed in the stationary-phase HGM culture after treatment with MG132 and/or Wort (MG132/Wort). The levels of ubiquitinated proteins increased in cells treated with Wort or MG132 compared with the untreated control. Two

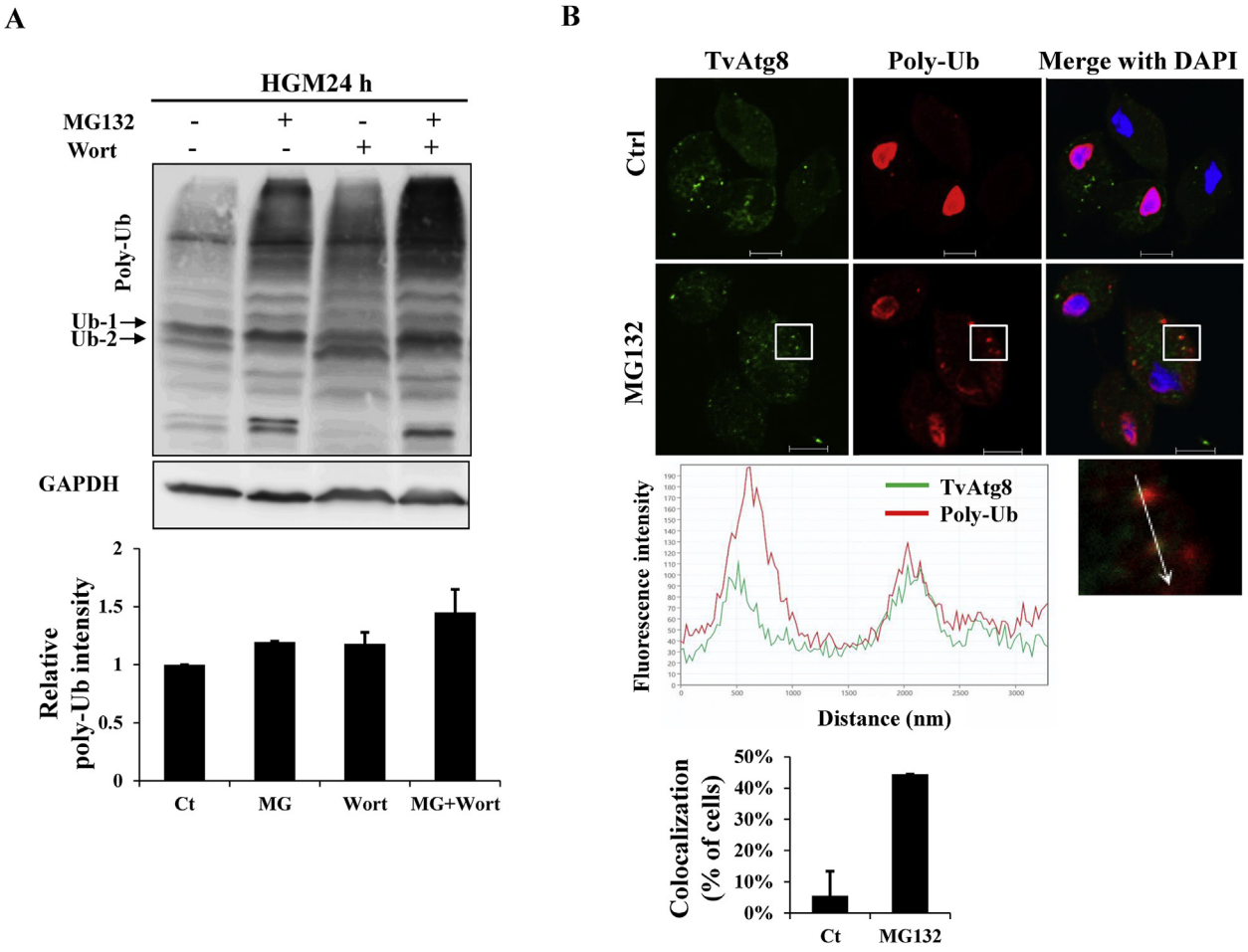

Figure 4. PI-induced autophagy removes polyubiquitinated proteins in *T. vaginalis* under HGM cultivation. (A) The accumulation of poly-Ub-tagged proteins was monitored in HGM-cultured cells (24 h) treated with DMSO, MG132 (5 μM), Wort (50 μM), or MG132 in combination with Wort for 3 h. Ub-1 and Ub-2 are representative ubiquitinated proteins largely accumulated in cells treated with MG-132 and Wort, respectively. (B) The distribution of TvAtg8 and Ub-tagged proteins in HGM-cultured cells (24 h) treated with MG132 (5 μM) were monitored. Scale bar = 5 μm. The colocalization of TvAtg8 and poly-Ub-tagged proteins was expressed as the overlap of two fluorescence intensity along the white arrow. The percentage of colocalization in the MG132-treated group (n = 18) compared to the untreated group (n = 18) was analyzed.

specific Ub-positive proteins, Ub-1 and Ub-2, preferentially accumulated in the UPS-suppressed (lane +/-) and autophagy-suppressed (lane -/+) cells, respectively (Fig. 4A). Compared with the untreated control (lane -/-), PI-induced autophagy strikingly enhanced the removal of Ub-2 protein, suggesting that autophagy induced by the compromised UPS compensates for protein degradation. Combined treatment with MG132 and Wort (lane +/+) displayed similar poly-Ub pattern to the MG132-treared cells (lane +/-). PI-induced autophagy was therefore not suppressed by Wort. Immunofluorescence analysis revealed that the TvAtg8 puncta were partially colocalized with the

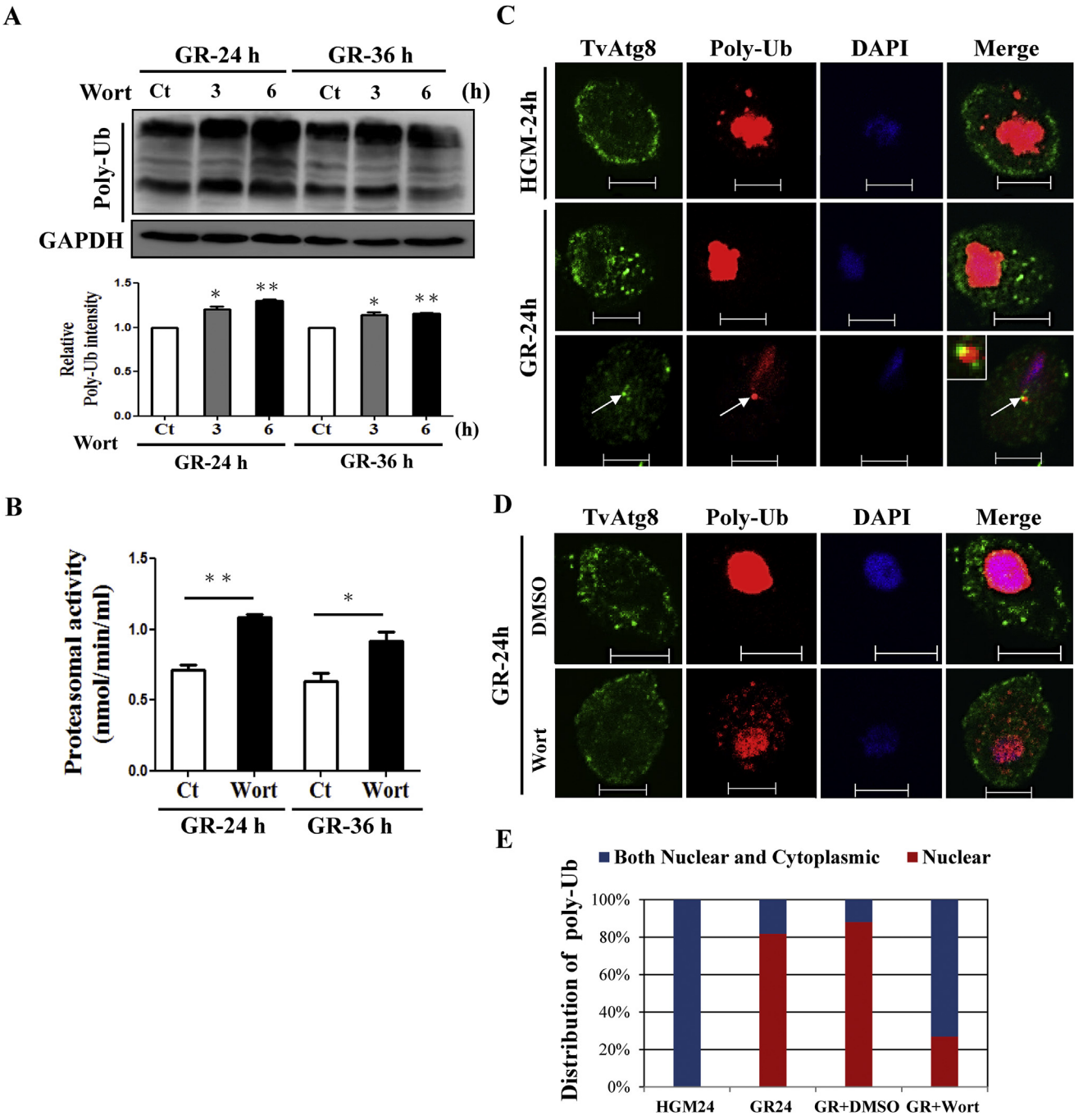

Figure 5. GR-induced autophagy is crucial for the removal of polyubiquitinated proteins in *T. vaginalis*. (A) GR-cultured cells (24 and 36 h) were treated with 100 μM Wort and the accumulation of poly-Ub-tagged proteins was detected. (B) The proteasomal activities were monitored in GR-cultured cells following 6 h of Wort treatment (100 μM). (C) The distribution of Ub-positive proteins and TvAtg8 in HGM- and GR-cultivated cells in the presence or absence of Wort (100 μM for 6 h) was monitored. Arrows indicate the colocalization of TvAtg8 with polyubiquitinated proteins in GR-cultured cells. Scale bar = 5 μm. The localization of ubiquitinated proteins in cells (n = 10) was classified into two groups: both the nuclear and cytoplasmic compartments and the nucleus only. *$p < 0.05$; **$p < 0.01$.

poly-Ub-tagged proteins in MG132-treated cells (Fig. 4B), whereas the colocalization was not observed in the untreated control, supporting that PI-induced autophagy exerts the function to remove poly-Ub-tagged proteins.

GR-induced autophagy is a major proteolytic system in *T. vaginalis*

Given that the stationary-phase GR-cultured cells were able to efficiently remove the ubiquitinated proteins irrespective of PI (Fig. 4A), we hypothesized that GR-induced autophagy is vital for the elimination of ubiquitinated proteins. Indeed, the amount of ubiquitinated proteins in GR-cultivated cells (GR-24 h and 36 h) treated with Wort markedly increased in a time-dependent manner ($P < 0.05$) compared with those in the DMSO-treated control (Fig. 5A). The massive accumulation of polyubiquitinated proteins following autophagy suppression rather than PI suggests that autophagy is a key proteolytic system in the stationary phase of GR culture. Autophagy inhibition has recently been proven to enhance the proteasomal activity for protein degradation in serum-deprived colon cancer cells.[26] Similarly, we found that the proteasomal activity was elevated in GR-cultured cells after autophagy inhibition by Wort ($P < 0.05$) (Fig. 5B). However, the upregulation of proteasomal activity appeared not to rescue the impaired autophagic protein degradation. Unexpectedly, the Ub-positive proteins were distributed predominantly in the nucleus of GR-cultured cells (Fig. 5C—E). In rare cases, the poly-Ub-tagged proteins found in the cytoplasm were partially colocalized with the TvAtg8 puncta in GR-cultured cells (Fig. 5C, arrow). These observations suggest that the cytoplasmic ubiquitinated proteins are removed very soon via autophagy in GR cultivation. This data was validated in GR-cultured cells treated with Wort, resulting in the translocation of ubiquitinated proteins from the nucleus to both the nucleus and cytoplasm (Fig. 5D, E). Together, these Results demonstrate that GR-induced autophagy is essential for the removal of polyubiquitinated proteins in *T. vaginalis*.

Discussion

There are variations in expression, localization, and function of Atg8 among the protists. For example, Atg8-PE increases during the log phase and encystation stage of *E. invadens*, suggesting that autophagy is crucial for proliferation and differentiation.[12] Interestingly, Atg8 localizes to the apicoplast and appears not to be associated with autophagosomes in *Plasmodium* liver- and blood-stage parasites, providing an unexpected role of Atg8 in apicoplast biology.[16,27] We demonstrate that TvAtg8 is associated with the autophagosomes-like vacuoles upon autophagy induction by GR, suggesting that TvAtg8 is a potential marker for autophagy detection. However, we do not exclude the possibility that autophagy may occur at a basal level under high-glucose condition and that TvAtg8 may have another function beyond autophagy, as suggested by previous reports.[28] The TvAtg8 puncta also partially colocalizes with the polyubiquitinated proteins upon PI and GR, suggesting that autophagy is involved in proteolysis. Further characterization of TvAtg8-associated complex will not only establish parasite-specific autophagy networks but unravel novel role(s) of TvAtg8 in trichomonads.

Accumulating evidence indicates that the UPS and autophagy are functionally coupled.[26] Autophagy dysfunction leads to the accumulation of ubiquitinated protein aggregates in the social amoeba *D. discoideum*.[29] Recent studies have investigated the effects of proteasome inhibitors (lactacystin and gliotoxin) on the morphology of the bovine trichomonad *Tritrichomonas foetus*, indicating that PI provokes the formation of concentric membrane whorls containing remnants of organelles, which resemble autophagy vacuoles.[30] Our Results uncover the intriguing crosstalk between the UPS and autophagy in trichomonads. PI-induced autophagy functions as a compensatory protein degradation system in *T. vaginalis* under glucose-rich condition. Additionally, although suppression of GR-induced autophagy enhances the proteasomal activity, this effect seems not to alter the massive accumulation of polyubiquitinated proteins. The molecular mechanism of autophagy triggered by GR or PI in *T. vaginalis* needs further investigations. It is possible that PI and GR can trigger different signaling pathways for autophagy induction. A previous study indicated that proteasome inhibitors elicit noncanonical autophagy, which is not suppressed by inhibitors of class III phosphatidylinositol 3-kinase (PtdIns3K),[31] supporting our data that Wort has no effect on PI-induced autophagy. Accordingly, we propose that the UPS is a major proteolytic system and autophagy induced by the compromised UPS serves as a backup in the stationary phase of HGM culture. Besides, the UPS and autophagy are predominant machinery for protein degradation in the log- and stationary phase of GR cultures, respectively.

Collectively, we have elucidated the role of autophagy in the ancient eukaryote *T. vaginalis*. Autophagy is elicited to compensate for proteolysis in HGM-cultured cells upon PI. Besides, GR-induced autophagy is a major proteolytic system. These findings highlight the importance of autophagy in proteastasis in the ancient trichomonads.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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