1 **ATG5 is instrumental in the transition from autophagy to apoptosis**
2 **during the degeneration of tick salivary glands**

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15

16 **Abbreviations**

17 RhATG5: *R. haemaphysaloides* ATG5; 20E: 20-hydroxyecdysone; TBP: tick-borne
18 pathogen; ATG: autophagy-related; TEM: Transmission electron microscopy; TUNEL:
19 Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling; PI: Isoelectric Point;
20 pcAb: polyclonal antibodies; KLH: Keyhole limpet hemocyanin; IFA: indirect
21 immunofluorescence antibody; TBS: Tris-buffered saline; PBS: phosphate-buffered
22 saline; ER: endoplasmic reticulum; qPCRs: Quantitative real-time polymerase chain
23 reactions; PE: phosphatidylethanolamine; ORF: open reading frames; DAPI: 4', 6'-
24 diamidino-2-phenylindole; Hoechst 33342: 2,5'-Bi-1H-benzimidazole, 2'-(4-
25 ethoxyphenyl)-5-(4-methyl-1-piperazinyl)- 23491-52-3; IPTG: isopropyl thio-β-D-
26 galactoside; DMSO: Dimethyl sulfoxide; dsRNA: double-strand RNA

27

28 Abstract

29 Female tick salivary glands undergo rapid degeneration several days post engorgement.
 30 This degeneration may be caused by the increased concentration of ecdysone in the
 31 hemolymph during the fast feeding period and both autophagy and apoptosis occur. In
 32 this work, we first proved ATG and Caspase gene expression peaks during degeneration
 33 of the tick salivary glands. We explored the regulatory role of *Rhipicephalus*
 34 *haemaphysaloides* autophagy related 5 (RhATG5) in the degeneration of tick salivary
 35 glands. During the fast feeding phase, RhATG5 was cleaved and both calcium
 36 concentration and the transcription of RhCalpains increased in the salivary glands.
 37 Recombinant RhATG5 was cleaved by μ -Calpain only in the presence of calcium; the
 38 mutant RhATG5^{191-199Δ} was not cleaved. Treatment with 20-hydroxyecdysone (20E) led
 39 to programmed cell death in the salivary glands of unfed ticks *in vitro*, RhATG8-
 40 phosphatidylethanolamine (PE) was upregulated in ticks treated with low concentration
 41 of 20E. Conversely, RhATG8-PE decreased and RhCaspase-7 increased in ticks treated
 42 with a high concentration of 20E and transformed autophagy to apoptosis. High
 43 concentrations of 20E led to the cleavage of RhATG5. Calcium concentration and
 44 expression of RhCalpains were also upregulated in the tick salivary glands. RNA
 45 interference (RNAi) of RhATG5 *in vitro* inhibited both autophagy and apoptosis of the
 46 tick salivary glands. RNAi of RhATG5 *in vivo* significantly inhibited the normal
 47 feeding process. These results demonstrated that high concentrations of 20E led to the
 48 cleavage of RhATG5 by increasing the concentration of calcium and stimulated the
 49 transition from autophagy to apoptosis.

50 Key words: tick; salivary gland degeneration; ATG5; apoptosis; autophagy

51 Introduction

52 Ticks, which are well-known pathogen transmission vectors, are long-lived,
 53 reproductively prolific, and can utilize a variety of hosts (1-3). Ticks also may remain
 54 attached to the host, feeding on host blood, for long periods (4). Most tick-borne
 55 pathogens (TBPs) are transmitted to hosts through tick bites assisted by saliva (3, 5-8).
 56 During the blood-sucking process, tick salivary glands secrete a variety of biologically-

57 active molecules, which reduce host pain and itching, inhibit blood coagulation, disturb
58 the innate and adaptive immune responses, and dilate the blood vessels (7-14). These
59 molecules contribute to the spread, survival, and reproduction of TBPs (15).

60 Tick salivary glands rapidly degenerate and disappear within 4 days post
61 engorgement (7, 16). During the degeneration process, many autophagic vacuoles
62 appear in salivary gland cells and tick secretion ability decreases up to 90% (17).
63 Salivary gland degeneration begins during the initial period of fast feeding, and may
64 primarily be caused by the increase in ecdysone in the hemolymph (18, 19). To verify
65 the association between ecdysone and salivary gland degradation, 20E was added to an
66 *in vitro* culture of salivary glands that had been extracted post-feeding, the wet weight
67 and secretion abilities of the salivary glands decreased significantly after incubation
68 with 20E (17). However, the mechanisms underlying these effects remain unclear.

69 Several studies have attempted to investigate these mechanisms. For example,
70 L'Amoreaux used Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
71 (TUNEL) staining to show that the rate of DNA fragmentation was significantly higher
72 in degenerated salivary glands (20). In addition, it is reported that caspase3 activity
73 levels increased significantly in tick salivary glands 72 h post-engorgement (21).
74 Similarly, metabolic activity levels and morphological characters differed substantially
75 among the salivary glands of fast-feeding, engorged ticks, and 3 d post-engorged female
76 brown dog ticks (*Rhipicephalus sanguineus*); metabolic activity levels in the salivary
77 glands of the fast feeding ticks were high, and nuclear and plasma membranes were
78 intact. In engorged ticks, the membranes of the type I and type II acini remained intact,
79 but the nuclei had changed, ATPase activity (proportional to membrane integrity) had
80 decreased, and acid phosphatase activity (inversely proportional to ATPase activity) had

81 increased. At 3 d post-engorgement, salivary gland tissue was severely degraded, the
82 membranes of most cells (some type I acini, most type II acini, and all type III acini)
83 had disintegrated, ATPase activity had decreased, acid phosphatase activity had
84 increased, and apoptotic bodies had appeared (22). Thus, during salivary gland
85 degeneration, nuclear changes precede cytoplasmic changes and are accompanied by the
86 formation of apoptotic bodies. Proteomic sequencing indicated that energy-related
87 proteins were downregulated during salivary gland degeneration, but some proteins
88 associated with the degradation of DNA or proteins were up regulated; certain proteins
89 involved in apoptosis or autophagy were also differentially expressed (23). Thus,
90 similar to some insects, the degradation of tick salivary glands represents programmed
91 cell death mediated by ecdysone (24-28). There is also a relationship between
92 autophagy and apoptosis during the degeneration of tick salivary glands (29). In
93 summary, both autophagy and apoptosis are involved in the degeneration of tick
94 salivary glands but their relationship is unclear.

95 Most organisms, from mammals to insects, switch between autophagy and apoptosis
96 (30). In mammals, autophagy related 5 (ATG5) plays a regulatory role during the
97 transition from autophagy to apoptosis, and the molecular regulation mechanisms
98 involving this protein have been characterized (31). ATG5 may promote the expansion
99 of autophagosomes by combining ATG12 and ATG16 to form ATG12-ATG5-ATG16L
100 complexes (32). Alternatively, ATG5 may be cleaved to produce NtATG5 (24 kDa,
101 amino-terminal truncated), which binds to the apoptosis inhibitor protein BCL-XL
102 located on the mitochondrial membrane; this binding leads to the activation of the pro-
103 apoptotic protein BAX to form a BAX-BAX homodimer, thereby promoting apoptosis
104 (31, 33). It was recently shown that, in most insects, the transcription levels of ATG5
105 and associated genes were the highest during metamorphosis (34, 35). In addition,

106 similar to ATG5 in mammals, insect ATG5 is cleaved by calpains, inducing apoptosis
107 after autophagy (25, 34-36). However, the mechanisms underlying the transition
108 between apoptosis and autophagy in ticks, particularly with respect to salivary gland
109 degeneration, remain unclear.

110 To address this knowledge gap, we investigated the relationship between autophagy
111 and apoptosis in the salivary glands of the tick *R. haemaphysaloides*. We first reported
112 the concentration-dependent ecdysone-mediated programmed cell death in ticks.
113 RhATG5 was cleaved to produce Nt-RhATG5 during the fast-feeding period. Nt-
114 RhATG5 mediated the transition to apoptosis following 20E-induced autophagy. High
115 20E concentrations led to high levels of intracellular Ca^{2+} , which activated the calpains
116 and mediated the cleavage of RhATG5.

117 **Materials and methods**

118 **Ethical statement**

119 The care of rabbits and mice was approved by the Institutional Animal Care and Use
120 Committee of the Shanghai Veterinary Research Institute (IACUC approve number:
121 SHVRI-mo-20180306-008; SHVRI-ra-20180415-04; shvri-ZD-2019-027), and
122 authorized by the Animal Ethical Committee of Shanghai Veterinary Research Institute.

123 **Ticks and animals**

124 Adult *R. haemaphysaloides* were attached to the ears of New Zealand White rabbits;
125 rabbits were provided by the Shanghai Laboratory Animals Center (Shanghai Institutes
126 for Biological Science, Chinese Academy of Sciences, Shanghai, China) (37).

127 **TUNEL staining**

128 The paraformaldehyde-fixed salivary glands were subjected to paraffin-sectioning and

129 antigen retrieval. Sections were then permeabilized with 0.1% Triton X-100 and
130 incubated for 1 h with 1:9 TdT mixed with fluorescent-labeled dUTP at 37°C, following
131 the instructions of the Roche In Situ Cell Death Detection Kit, POD (Roche,
132 11684817910). To stain the nuclei, sections were washed three times with phosphate-
133 buffered saline (PBS; 0.14 M NaCl, 0.0027 M KCl, 0.01 M phosphate buffer; pH
134 7.4)/0.5% Tween-20, and then incubated with 1 µg/mL 4', 6'-diamidino-2-phenylindole
135 (DAPI, Invitrogen, Carlsbad, CA, USA) in dd H₂O for 20 min. After washing, the
136 sections were mounted using fluorescent mounting medium under glass coverslips, then
137 viewed and photographed on a Zeiss LSM880 Laser Scanning Confocal Microscope.

138 **Transmission electron microscopy (TEM) analysis**

139 The glutaraldehyde-fixed sections were ultrathin sectioned and treated using standard
140 TEM procedures (38). Sections were then viewed and photographed using a Tecnai G2
141 Spirit BIOTWIN TEM (FEI, Hillsboro, Oregon, USA).

142 **Quantitative real-time polymerase chain reactions (qPCRs)**

143 All related genes were identified using a BLAST analysis (39)
144 (<http://www.ncbi.nlm.nih.gov/BLAST/>). The salivary glands of the *R.*
145 *haemaphysaloides* allowed to feed for different periods were collected for RNA
146 extraction after microdissection. RNA was converted into the first-strand cDNA using a
147 HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme Biotech, Nanjing,
148 China), following the manufacturer's protocols. The double-stranded cDNAs of each
149 gene were used as templates for qPCR, along with specific primers (Table S1), which
150 were designed using Primer Premier 5. QPCRs were performed using ChamQ Universal
151 SYBR qPCR Master Mix (Vazyme Biotech) green and gene-specific primers with a
152 QuantStudio 5 System PCR System (Applied Biosystems, Austin, TX, USA). QPCR

153 cycling parameters were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C
154 for 30 s. All samples were analyzed three times.

155 The data were normalized to the gene expression of elongation factor-1 (ELF1A,
156 Genbank accession no. AB836665) (40) using the $2^{-\Delta\Delta Ct}$ method (41, 42); ΔCt was
157 calculated by subtracting the average ELF1A Ct value from the average Ct value of the
158 target gene.

159 **Sequencing and cloning of RhATG5**

160 RhATG5-specific primers (Table S2) were designed based on a comparison of the
161 salivary glands transcriptomes of fasted and engorged *R. haemaphysaloides* (43). A
162 BLAST analysis of the translation products deduced from the open reading frames
163 (ORFs) was performed. We used ExPASy (https://web.expasy.org/compute_pi/) to
164 predict the molecular weight and Isoelectric Point (PI) of RhATG5. We aligned
165 RhATG5 with the ATG5 protein sequences of various organisms using Genetyx ver. 6
166 (GENETYX, Tokyo, Japan). For phylogenetic analysis, the alignment of the sequences
167 was performed using the MUSCLE algorithm (Edgar, 2004) and inferred using the
168 Maximum likelihood method with the default settings in MEGA X software (Kumar et
169 al., 2018). Bootstrap support values were estimated using 500 bootstrap replicates. The
170 sequences were obtained from GenBank or UniProt.

171 **RhATG5 cleavage assay**

172 The ORF sequence of *RhATG5* was subcloned into the pET-30a prokaryotic expression
173 vector, using In-Fusion HD Cloning Kits (Takara Clontech, Mountain View, CA,
174 USA). We constructed the mutant and the mutation primers for RhATG5 using the
175 Quick Mutation Gene Site-Directed Mutagenesis Kit (Beyotime, Shanghai, China),
176 following the manufacturer's instructions. The specific mutation primers that we

designed are listed (Table S2). The recombinant plasmids pET-30a-RhATG5 and pET-30a-RhATG5^{191-199Δ} were constructed and transformed into BL21 (DE3) competent *Escherichia coli* cells (Tiangen, Beijing, China). To induce the expression of the recombinant proteins RhATG5 and RhATG5^{191-199Δ}, BL21 cells were incubated in a final concentration of 1 mM isopropyl thio-β-D-galactoside (IPTG) at 25°C for 12 h. Then, recombinant RhATG5 and RhATG5^{191-199Δ} were affinity-purified using Ni-NTA His•Bind Resin (Merck-Millipore, Darmstadt, Germany). Recombinant RhATG5 and RhATG5^{191-199Δ} were then incubated with 10 μg μ-calpain (Merck) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, PH 8.0) at 25°C for 3 hours. The reaction was stopped by adding 2 × SDS loading buffer. The mixtures were heated at 100°C for 10 min and then separated on GenScript ExpressPlus PAGE Gels 10 × 8, 12%, 10 wells (Genscript, Nanjing, China). Gels were stained with Coomassie brilliant blue using eStain™ L1 (Genscript).

***In vitro* culture of tick salivary glands and 20E treatment**

Unfed or fed ticks were sterilized in 70% ethanol for 30 s and then dried with filter paper. The salivary glands of each tick were removed using microdissection, immediately transferred to *L15* medium supplemented with 1% penicillin-streptomycin at 27°C, and cultured *in vitro* as previously described (44). Cultured salivary glands were then treated with 1 μM, 5 μM, 10 μM, or 20 μM 20E (Sigma-Aldrich, St. Louis, MO, USA). Incubation was stopped and samples were collected at 24 or 48 h. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as the control. Each treatment group included the salivary glands from 10 ticks. After incubation, a pair of the salivary glands in each group was fixed in 4% paraformaldehyde for TUNEL staining or fixed in 2.5% glutaraldehyde for at least 24 hours at 4°C for TEM. The remaining salivary glands were used for western blots.

202 **Antibody generation, western blotting and indirect immunofluorescence** 203 **antibody (IFA)**

204 Recombinant his-tag RhATG5 was expressed and purified according to standard
205 protocols (45). Polyclonal antibodies (pcAb) against recombinant RhATG5 were
206 developed in mice. We tested the specific recognition of the recombinant protein by the
207 antibodies using western blots. To distinguish between autophagy and apoptosis, we
208 chose the molecular markers RhATG8 (autophagy, MN395580) and RhCaspase7
209 (apoptosis, MN395579) for antibody production. We predicted the epitopes of RhATG8
210 and RhCaspase7 online (<http://www.iedb.org/>), and synthesized Keyhole limpet
211 hemocyanin (KLH) (46)-coupled polypeptides based on the epitope amino acid
212 sequences (Table S3). Polypeptides were dissolved in PBS and Freund's adjuvant
213 (complete and incomplete; Sigma-Aldrich). Equal volumes of these solutions were then
214 emulsified together and injected into 6–8 week-old BALC/c mice or New Zealand
215 White rabbits provided by the Shanghai Laboratory Animals Center (Shanghai Institutes
216 for Biological Science, Chinese Academy of Sciences, Shanghai, China. Injections were
217 repeated three times at two-week intervals. Serum samples were collected seven days
218 after the third injection.

219 For western blots assays, total proteins from the salivary glands of 10 ticks were
220 extracted using Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.5; 150 mM NaCl
221 with 1 mM phenylmethanesulfonyl fluoride) to eliminate individual differences. Protein
222 concentrations were quantified using the Bradford Protein Assay Kit (Beyotime),
223 following the manufacturer's instructions. We used 20 µg of protein from each sample
224 for SDS-PAGE. Western blotting and IFA were then performed as previously described
225 (45), and images were captured by ChemiDoc Touch (Bio-rad, Hercules, CA, USA) or
226 Odyssey (*LI-COR*, Nebraska, USA) Imaging System. The intensity of target protein

bands was measured by Image-Pro Plus software (Media Cybernetics). The anti-Alpha-tubulin primary antibody was purchased from Proteintech (Rosemont, IL, USA). The Goat anti-Mouse IgG (H+L) Secondary Antibody conjugated with HRP, Goat anti-Rabbit IgG (H+L) Secondary Antibody conjugated with HRP, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 594, Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 were all purchased from Invitrogen.

Measurement of cellular Ca^{2+} in salivary glands

All salivary glands were washed three times with PBS, and then incubated with 3 μM AM ester Calcium Crimson TM dye (Invitrogen) for 30 min at 27°C. After incubation, the salivary glands were washed three times with PBS, then incubated with 1 mg/mL 2,5'-Bi-1H-benzimidazole, 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-23491-52-3 (Hoechst 33342, trihydrochloride, trihydrate, Life Technologies, H3570) in water for 20 min. After washing three times with PBS, each whole salivary gland was placed on a glass slide, sealed with Lab Vision PermaFluor (Thermo Scientific, Carlsbad, CA, USA), and observed under a Zeiss LSM880 Laser Scanning Confocal Microscope (Oberkochen, Germany).

RNAi *in vivo* and *in vitro*

Specific interference primers were designed based on the known sequences (Table S2), adding the T7 polymerase promoter sequence to the 5' end of each primer. After PCR amplification, the amplicons were purified to obtain templates for double-strand RNA (dsRNA) synthesis. The dsRNAs of RhATG5 and Luciferase were generated using the T7 RiboMAX Express RNAi system (Promega, Madison, WI, USA). The dsRNA of each gene was either microinjected into unfed female ticks (1 μg /tick).

Our preliminary results indicated that the dissected salivary glands of female *R. haemaphysaloides* were viable in culture (see above). This indicated that salivary gland cultures were suitable for a detailed study of RhATG5 RNAi *in vitro*. We used salivary glands extracted from ticks during the fast-feeding period (5 and 7 days) for *in vitro* RNAi experiments. Salivary gland cultures were treated with RhATG5 dsRNA (1 µg/mL) for 48 h. After 48 h, ticks and salivary glands were analyzed.

Data analysis

GraphPad PRISM 6.0 software (La Jolla, CA, USA) was used for all data analyses. Mean ± standard error (SEM) values were calculated for three independent experiments, and two-tailed Student's *t* tests were used to identify significant differences between groups (**p* < 0.05; ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

Results

Both autophagy and apoptosis occurred in the degeneration of salivary glands

To determine the exact time of tick salivary gland degeneration, we observed the morphologies of the salivary glands of ticks fed for different periods. The length, width and bubble diameter of the salivary glands increased continuously from the first feeding day until engorgement and rapidly degenerated and atrophied after engorgement (Fig. 1A, B). TEM observations showed that mitochondria large amounts of bilayer membrane autophagosomes and autophagic vacuoles during the late feeding and post engorged periods (Fig. 1C). Compared to the early feeding period, DNA fragmentation increased from the fast feeding period to post-engorgement, along with the emergence of autophagosomes (Fig. 1D). The TUNEL positivity of cytosol may relate to the presence of a large number of mitochondria. Autophagy and apoptosis occur during the

275 degeneration of tick salivary glands, which is consistent with previous observations (4,
276 16, 22, 47, 48).

277 **Relative expression levels of autophagy and apoptosis genes in tick salivary** 278 **glands of ticks fed for different periods**

279 The cDNA of tick salivary glands at different feeding times were subjected to qPCR to
280 evaluate the expression profiles of autophagy and apoptosis related genes. To
281 investigate the expression patterns of ATG homologs associated with autophagy, A total
282 of 13 putative RhATG genes, including RhATG3, RhATG4B, RhATG4D, RhATG5,
283 RhATG6 (BECN1), RhATG7, RhATG8, RhATG9, RhATG10, RhATG12, RhATG13,
284 RhATG14 and RhATG16, occur in the salivary gland transcriptome of *R.*
285 *haemaphysaloides* (43), all of the RhATG proteins contained classic ATG domains.
286 Most autophagy-related genes were upregulated between the 1st and 3rd day of feeding,
287 and then down-regulated during the fast-feeding period (days 4–7; Fig. 2A). Post-
288 engorgement, most of autophagy-related genes was sharply upregulated except
289 RhATG3, but the reason remains to be explored (Fig. 2A).

290 Using the same strategies, we identified 4 predicted RhCaspases, including
291 RhCaspase1, RhCaspase7, RhCaspase8, and RhCaspase9. All 4 RhCaspases contained
292 the conserved peptide sequence of QACR(I)G (29). All apoptosis-associated genes were
293 upregulated in fast feeding period and post-engorged period compared to slow feeding
294 period (Fig. 2B). RhCasapase8 was also sharply upregulated on day 5 of feeding (Fig.
295 2B).

296 **Cloning and recombinant expression of RhATG5**

297 Based the results above, RhATG5 was cloned to study the interaction between
298 autophagy and apoptosis. The *R. haemaphysaloides* ATG5 homolog was designated

299 RhATG5 (GenBank accession no. MK841509). This is the first reported ATG5
300 homolog in ticks. The full-length open reading frame (ORF) of RhATG5 was 810 base
301 pairs long and encoded a 270 amino acid peptide. The predicted molecular weight of the
302 RhATG5 protein was 31.3 kDa, with a theoretical pI of 6.19. Homology analyses
303 indicated that RhATG5 is a member of the ATG5 family. The APG5 domain extends
304 from amino acids 81 to 270 (marked with a black line), and has 54.58%, 53.85%,
305 59.85%, and 50.19% identity with the ATG5 proteins from (*Homo sapiens*;
306 NP_004840.1), mice (*Mus musculus*; NP_444299.1), fruit flies (*Drosophila*
307 *melanogaster*; NM_132162.4), and the cotton bollworm (*Helicoverpa armigera*;
308 AMQ76404.1), respectively (Supplement Fig. S1A). The lysine (K) 130 of Homo-
309 ATG5 is necessary for covalent bond formation in the ATG12-ATG5 complex (49).
310 This critical lysine residue of ATG5 is present at position 132 in RhATG5 (Supplement
311 Fig. S1A, marked with black frame). Amino acids His80 and Ser127 of homo-ATG5
312 (His82 and Ser129 in RhATG5) (Supplement Fig. S1A, marked with black frame) play
313 important roles in the formation of the ATG12-ATG5-ATG16 complex (49). Although
314 ticks are evolutionarily distant from other animals, our phylogeny of ATG5 homologs
315 from a variety of species suggests a close relationship among ATG5 homologs
316 (Supplement Fig. S1B). This indicates that autophagy-associated genes, such as the
317 ATG5 homologs, are ancient and have been conserved over long periods of
318 evolutionary time.

319 **RhATG5 was cleaved and intracellular calcium increased in tick salivary** 320 **glands during the fast feeding phase**

321 RhATG5, RhATG8 and RhCaspase7 were tested to show the occurrence of Autophagy
322 and Apoptosis. RhATG5, with a molecular weight of 33kDa, kept increasing during all
323 the feeding times until engorged. RhATG5 was cleaved into NtATG5 (represented a

324 fragmented form of RhATG5 with a molecular weight of 25 kDa) from the fast-feeding
325 to the engorgement phases (Fig. 3A, B). RhATG5 and NtATG5 decreased rapidly post
326 engorgement. The expression level of RhATG8-PE (16 kDa) appeared at the early
327 feeding time point and low expressed during the fast feeding periods, and then peaked
328 post-engorgement, consistent with our qPCR results (Fig. 3A, B). Pro-RhCaspase7 (34
329 kDa) increased during fast feeding periods and kept the increase post engorged.
330 Cleaved-RhCaspase7 (17 kDa) emerged followed the decrease of RhATG8-PE and kept
331 increasing until post engorgement (Fig. 3A, B).

332 The Ca^{2+} concentrations of tick salivary glands during different feeding periods were
333 studied to display the concentration changes of Ca^{2+} in the degeneration of tick salivary
334 glands. According to the results, Ca^{2+} concentration (displayed as red fluorescence) in
335 the tick salivary glands increased between the fast-feeding and the engorgement phases
336 along with the degeneration of tick salivary glands (Fig. 3C); the transcription levels of
337 two RhCalpains also increased during this period (Fig. 3D), which is consistent with the
338 appearance time of Nt-RhATG5. Our results indicated that the cleavage of RhATG5
339 may be caused by an increase in intracellular calcium concentration.

340 **RhATG5 was cleaved by μ -calpains *in vitro*, depending on calcium** 341 **concentration**

342 The position of the 6-amino-acids (QTTTER) is crucial for the calpain cleavage site
343 (marked with red frame) of Homo-ATG5 (31), which occurs at positions 192 to 197 in
344 RhATG5. One calpain cleavage site was predicted in our analyses of RhATG5
345 homology (Fig. 4A). The coding sequences of RhATG5 and RhATG5¹⁹¹⁻¹⁹⁹ were cloned
346 into prokaryotic expression vectors (pET-30a) to produce recombinant RhCaspases 7, 8,
347 and 9. All of the recombinant proteins were expressed in *E. coli*. After purification, His-
348 RhATG5 (39 kDa) was incubated with μ -Calpain at different concentrations, Ca^{2+}

349 treated RhATG5 was cleaved into one active fragment (NtRhATG5, 28 kDa) (Fig. 4A).
 350 With the same Ca^{2+} concentration and the same reaction time, along with the increased
 351 concentration of μ -calpain, the bands of NtRhATG5 were more obvious, and under the
 352 same concentrations of μ -Calpain, changing the Ca^{2+} concentration did not affect the
 353 cleavage of RhATG5 (Fig. 4A). After the cleavage site was deleted (Fig. 4B), the
 354 recombinant protein RhATG5^{191-199Δ} was not cleaved by μ -Calpain under identical
 355 experimental conditions (Fig. 4C).

356 **Induction of autophagy and apoptosis in tick salivary glands by culturing** 357 **with 20E**

358 The similarity of results between the 20E titer and the expression profile of autophagy
 359 and apoptosis genes in tick salivary glands at different feeding times indicated that 20E
 360 might induce degeneration of the salivary glands. Salivary glands of unfed ticks were
 361 cultured with different concentrations of 20E *in vitro*. The unfed tick salivary glands
 362 exhibited positive TUNEL staining after treatment with 10 μM 20E at 24 h and 48 h and
 363 apoptosis increased with time (Fig. 5A). After incubated with 5 μM 20E, ATG8 (green
 364 fluorescence) increased at 24h, along with the concentration of 20E,
 365 immunofluorescence analysis showed that RhATG8 and RhCaspase7 (red fluorescence)
 366 were co-localized after treatment with high concentrations (10 μM and 20 μM) of 20E
 367 (Fig. 5B; displayed as yellow fluorescence).

368 The qPCRs demonstrated that the expression profiles of ATGs were upregulated
 369 ($p < 0.0001$) 24 h after treatment with 10 μM 20E. These results were consistent with
 370 western blot results. All ATGs increased during the first 48 h of feeding but
 371 significantly decreased at 72 h (Fig. 6A). The apoptosis-related genes, RhCaspase7 and
 372 RhCaspase8 were upregulated at 24 h, similar to the ATGs (at 24 h); however, the

373 expression levels of these genes decreased between 24 and 48 h. All the RhCaspases
374 were upregulated at 72 h (Fig. 6B).

375 **Treatment with high concentrations of 20E increased intracellular Ca^{2+} and** 376 **mediated the cleavage of RhATG5 in tick salivary glands**

377 As mentioned above, unfed tick salivary glands were treated with different
378 concentrations of 20E. The results of western blots showed that RhATG8 and RhATG8-
379 PE was upregulated after treatment with a low concentration (5 μM) of 20E. With the
380 increase in the concentration of 20E, RhATG8 and RhATG8-PE decreased and both
381 cleaved-RhCaspase7 and NtRhATG5 increased after treatment with high concentrations
382 of 20E (10 μM and 20 μM ; Fig. 7A).

383 Ca^{2+} was tested in the 20E treated tick salivary glands to demonstrate the increased
384 Ca^{2+} during the ecdysone mediated degeneration of tick salivary glands (Fig. 7B).
385 Similarly, RhCalpainI peaked at 24 h, while RhCalpainII expression peaked at 72 h (Fig.
386 7C). The data shows that the cleavage of ATG5 and caspase-3 depends on the increase
387 of the calcium concentration after 20E induction.

388 **Inhibition of RhATG5 delayed apoptosis in the tick salivary glands**

389 RhATG5 was knocked down in tick salivary glands *in vitro*, and the results showed that
390 RhATG8-PE was downregulated relative to the control at 1 d post-engorgement and
391 cleaved-RhCaspase7 was downregulated relative to the control at day 5 of fast feeding
392 (Fig. 8A). RhATG5 knockdown result in the inhibition of apoptosis, and the degree of
393 nuclear and cytoplasmic separation was reduced (Fig. 8B). In addition, TEM analysis
394 showed that, compared with the control groups, the degree of vacuolization were
395 noticeably reduced after knockdown (Fig. 8C). These results revealed that RhATG5 is
396 crucial for both autophagy and apoptosis.

397 **RNAi knockdown of RhATG5 in vivo inhibited tick blood-feeding behavior**

398 RNAi of RhATG5 *in vivo* caused inhibition on the blood-feeding behavior of *R.*
399 *haemaphysaloides*. When RhATG5 was knocked down (Fig. 9B), *R. haemaphysaloides*
400 blood-feeding behavior was inhibited (Fig. 9A). In addition, RhATG5-knockdown ticks
401 had a decreased engorgement rate and a higher mortality rate than wild-type ticks
402 (Table 1). However, there was no difference in attachment rate at 48 h between the
403 wild-type and the RhATG5-knockdown groups (Table 1). These results suggest that
404 RhATG5 plays an important role during tick feeding.

405 **Discussion**

406 Ticks are well-known pathogen vectors which transmitted virus, bacterial and protozoan
407 and they are evolutionarily distant from mammals and insects. Understanding the tick
408 physiological characteristics is the key step for investigating the mechanism of pathogen
409 transmission in ticks. However, there are knowledge gap remained in tick physiology.
410 ATG5 is required for autophagy and apoptosis in most species. However, this was
411 previously not recognized in ticks. The 20E steroid hormone promotes programmed cell
412 death in insects but the mechanism in ticks is unclear. We first proved that 20E
413 promotes both autophagy and apoptosis and that ATG5 is necessary for autophagy and
414 apoptosis in ticks.

415 Most of the apoptosis and autophagy associated genes were sharply upregulated post
416 engorgement. This indicated that both autophagy and apoptosis occur during tick
417 salivary gland degeneration. ATG5 and ATG12, which are generally thought to form a
418 covalently-bound complex during autophagy (50, 51), had similar transcriptional
419 profiles in *R. haemaphysaloides* during feeding (Fig 2A). ATG16, other major members
420 of the ATG5-ATG12-ATG16 complex (52, 53), ATG10, the E2 ubiquitination protein,
421 as the catalyst of complex formation (50, 54), were also upregulated after engorgement.

422 Interestingly, the expression levels of most autophagy-related genes in the tick salivary
423 glands peaked during the slow-feeding phase (Fig 2A), as did the ecdysone titer changed.
424 Thus, the upregulation of autophagy-related genes during the slow-feeding period (days
425 1–4) might be associated with the elevated concentrations of ecdysone in salivary
426 glands, but this supposition requires further experimental confirmation. Our results
427 clearly showed that, during the fast-feeding phase (days 5–7), autophagy-related genes
428 were downregulated, but apoptosis-related genes were upregulated. In addition, the two
429 initial RhCaspases (RhCaspase8 and RhCaspase9) were upregulated before the two
430 effector RhCaspases (RhCaspase1 and RhCaspase7) molecules (Fig 2B). Thus, our
431 results indicated that both autophagy and apoptosis are involved in the degeneration of
432 tick salivary glands.

433 It has been suggested that the peak in hemolymph ecdysteroid concentration during
434 the fast-feeding phase contributes to the initiation of salivary gland degeneration (19).
435 This hypothesis was supported by the results of the TUNEL and TEM assays of the
436 salivary glands from ticks allowed to feed for different periods (Fig 1A, B and C). As
437 shown above, the protein NtATG5, which was a maker for the transition from
438 autophagy to apoptosis, was initially expressed during the fast-feeding phase (days 5–7)
439 and was significantly downregulated post engorgement. After a transient peak in
440 expression on day 3 of feeding, the transcriptional levels of most autophagy-related
441 genes were reduced. In contrast, the expression levels of the RhCaspases increased
442 during the fast-feeding period (Fig 2A, B). In addition, intracellular Ca^{2+} concentration
443 and RhCalpain expression peaked simultaneously with the appearance of NtATG5 (Fig
444 4C, D). The knockdown of RhATG5 *in vitro* delayed both autophagy and apoptosis,
445 demonstrating that RhATG5 is closely associated with both autophagy and apoptosis in
446 the tick salivary glands.

447 In insects, programmed cell death is usually induced by ecdysone (25, 26, 55). In
 448 addition, peak ecdysone titer peaks simultaneously with the autophagy-related gene
 449 transcription in most insects (24, 26, 55). After insects enter metamorphosis, ecdysone
 450 concentration in the hemolymph increases sharply, and is converted into highly-active
 451 20E; 20E then induces the degeneration of obligate tissues (26, 56). It has been reported
 452 that the ecdysone titer in tick salivary glands increases throughout the feeding period,
 453 and remains elevated for up to six days post engorgement (19). However, it is unclear
 454 whether these events are associated in ticks. Our results showed that, similar to insects,
 455 20E induced both autophagy and apoptosis in tick salivary glands. Interestingly, in
 456 insects, low concentrations of 20E induce autophagy, while high concentrations of 20E
 457 stimulate the transition from autophagy to apoptosis (35, 57). However, the situation
 458 appears to differ in ticks. Ticks switched from autophagy to apoptosis during the rapid
 459 blood-sucking period due to the increase of ecdysone in the hemolymph, but this
 460 transition was reversed post engorgement. Thus, our results indicated that the rates of
 461 both autophagy and apoptosis were high in tick salivary glands post engorgement. We
 462 hypothesize that this phenomenon might be due to the long feeding period of ticks, and
 463 might be caused by host-specific factors, but further studies are necessary to investigate
 464 these factors.

465 During the slow blood-feeding period, ecdysone concentration was elevated in the
 466 tick salivary glands, resulting in a general increase in the transcription of autophagy-
 467 related genes. However, during the rapid feeding phase, ecdysone concentration
 468 fluctuated in the hemolymph, intracellular Ca^{2+} concentration increased, and
 469 NtRhATG5 appeared (Fig 10). These factors reflected a transition from autophagy to
 470 apoptosis. Our findings provide a basis for advanced studies on tick salivary glands
 471 degeneration.

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476 Author Contributions

477 Yanan Wang, Houshuang Zhang and Jinlin Zhou designed research; Yanan Wang,
478 Houshuang Zhang and Jinlin Zhou analyzed data; Yanan Wang, Houshuang Zhang, Li
479 Luo and Jinlin Zhou performed research; Yanan Wang, Jinlin Zhou wrote the paper;
480 Yanan Wang, Houshuang Zhang, Yongzhi Zhou, Jie Cao, Xuenan Xuan, Hiroshi
481 Suzuki and Jinlin Zhou contributed new reagents or analytic tools.

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- 664
- 665

666 **Figure legends**

667 **Figure 1.** Autophagy and apoptosis profile of tick salivary glands at different feeding
668 times. *A)* Morphological observation *in vitro* (scale bar: 1000 μ m) of salivary glands at
669 different feeding times. *B)* Analysis of *A)*, Bars represent mean $\pm$ SD for three replicates.
670 *C)* TEM observation (scale bar: 1 μ m) of salivary glands at different feeding times.
671 Autophagosomes are marked with white arrows. *D)* TUNEL staining (green, scale bar:
672 50 μ m) of salivary glands during different feeding periods.

673
674 **Figure 2.** Transcription profiles of autophagy and apoptosis related genes. *A)* autophagy
675 and *B)* apoptosis, as quantified using qPCR. Expression levels of all genes were
676 measured in the salivary glands of female ticks at various stages during the feeding
677 process: unfed to engorged to 1–3 d post engorgement, F: fed, E: engorged. Each group
678 included 10 ticks and each experiment was repeated three times. Bars represent mean $\pm$
679 SD for three replicates.

680
681 **Figure 3.** RhATG5 cleaved and intracellular calcium increased during the fast feeding
682 phase. *A)* Relative protein expression of RhATG5 and other proteins associated with
683 autophagy and apoptosis related genes. The salivary gland samples were harvested at
684 feeding days 3, 5, 7 and post engorgement days 1, 3, and 5, following by
685 immunoblotting with the anti-RhATG5 (pcAb), anti-RhATG8 (pcAb), anti-RhCaspase7
686 (pcAb) and anti- α -tubulin (mAb). *B)* Statistical analysis of *A)*. *C)* Ca^{2+} concentration of
687 tick salivary glands in different feeding periods was measured using Calcium Crimson
688 dye (Invitrogen), and is represented by red fluorescence, scale bars: 50 μ m. *D)* QPCR
689 quantification of RhCalpains expression in the salivary glands of female ticks at various

690 stages during the feeding process: unfed to engorged to 1–3 d post engorgement. All
691 experiments were performed in triplicate; bars represent the mean $\pm$ S. D.

692

693 **Figure 4.** *In vitro* RhATG5 cleavage assays. A) SDS-PAGE showing that recombinant
694 RhATG5-His is cleaved by μ -calpain and is not cleaved by calpain in the absence of
695 Ca^{2+} . B) The RhATG5 cleavage site. C) The mutant recombinant RhATG5^{191-199 Δ} -His is
696 not cleaved by μ -calpain.

697

698 **Figure 5.** Programed cell death caused by 20E *in vitro*. A) TUNEL staining showing
699 green fluorescence, Scale bars: 50 μm . B) RhCaspase7 (red fluorescence; pcAb from
700 rabbits) and RhATG8 (green fluorescence; pcAb from mice) were co-localized after
701 treatment with high concentrations of 20E (10–20 μM ; displayed as yellow
702 fluorescence), scale bar: 20 μm .

703 **Figure 6.** QPCR quantification of the expression levels of A) autophagy-related genes
704 and B) apoptosis-related genes after treatment with 20E. Bars represent mean $\pm$ SD for
705 three replicates. Significance of differences determined by Student's *t*-test: **p* < 0.05;
706 ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

707

708 **Figure 7.** Treatment with 20E increased cellular Ca^{2+} concentration and mediated the
709 cleavage of RhATG5 *in vitro*. A) Western blots showing the expression levels of
710 proteins associated with autophagy and apoptosis after treatment with different
711 concentrations of 20E. B) Statistical analysis of A). C) Cellular concentrations of Ca^{2+} ,
712 using Calcium Crimson dye (red fluorescence, scale bars: 50 μm). D) QPCR
713 quantification of calpains after treatment with 20E. Bars represent mean $\pm$ SD for three

714 replicates. Significance of differences determined by Student's *t*-test: **p* < 0.05; ***p* <
715 0.01, ****p* < 0.001, *****p* < 0.0001.

716

717 **Fig 8.** RNAi of RhATG5 *in vitro*. A) Knockdown of RhATG5 inhibited the protein
718 expression of RhATG8-PE and cleaved-Rhcaspase7. B) Positive TUNEL staining
719 decreased after RNAi of RhATG5, scale bars: 50 μm. C) Knockdown of RhATG5
720 reduced the degree of vacuolization in the tick salivary glands, scale bars: 1 μm.

721

722 **Fig 9.** RNAi of *RhATG5* *in vivo*. A) Confirmation of RhATG5 silencing using
723 quantitative real-time polymerase chain reactions (qPCRs). Total RNA was extracted on
724 feeding day 5 from female ticks injected with dsRNA. Bars represent mean relative
725 expression of RhATG5 across three replicates; error bars represent standard error. ***,
726 *P* < 0.001, based on two-tailed Student's *t* tests. B) Representative images comparing
727 ticks injected with dsRhATG5 with control groups on day 6 of feeding. DsRhATG5
728 injection inhibited blood-feeding behavior; injected ticks were smaller than control ticks.

729

730 **Fig 10.** Schematic showing how 20E induces autophagy and apoptosis in the tick
731 salivary glands. At low concentrations (during the slow-feeding phase, days 1–4 of
732 feeding) 20E induces autophagy. As the concentration of 20E increases (during the fast-
733 feeding phase, days 5–7 of feeding), cellular Ca²⁺ increases, mediating the cleavage of
734 RhATG5, and thus the transition from autophagy to apoptosis.

735 **Supporting Information**

736 **Supplement Fig. 1** The RhATG5 protein. A) Alignment of the RhATG5 amino acid
737 sequences with ATG5 protein sequences from *H. sapiens* (NP_004840.1), *M. musculus*
738 (NP_444299.1), *H. armigera* (AMQ76404.1), and *D. melanogaster* (NM_132162.4).

739 Conserved APG5 domain is underlined with a black line, and the critical conserved
 740 amino acid residue for Atg12-Atg5 interactions and complex formation are marked with
 741 a blue or black frame. Putative calpain cleavage sites of RhATG5 are marked with a red
 742 frame. The (*) indicates the position with single fully conserved residue, and (.)
 743 indicates conservation between groups with weakly similar properties. *B*) Maximum
 744 likelihood phylogeny showing the relationship among RhATG5 (marked by a red dot)
 745 and the ATG5 homologs of other species. The evolutionary history was inferred using
 746 the Neighbor-Joining method (58). The evolutionary distances were computed using the
 747 Poisson correction method (59) and are displayed in units of the number of amino acid
 748 substitutions per site.

749

750 **Supplement Table. 1** Primers used for qPCR.

751 S, forward primer; A, reverse primer

752 **Supplement Table. 2** Primers for apoptosis and autophagy related gene cloning and
 753 vector construction.

754 S, forward primer; A, reverse primer; Sm, forward primer for deletion mutation; Am,
 755 reverse primer for deletion mutation; the sequence in bold underlined indicates the
 756 sequence of the T7 promoter.

757 **Supplement Table. 3** Predicted epitope peptide sequences

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764 TABLE 1. RNAi of RhATG5 *in vivo*

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Test groups	ds RhATG5	dsLuciferase
Attachment rate at 48h(%)	80.22±0.906	87.70±0.690
Engorgement rate (%)	0	75.79±3.255
Engorged tick weight (mg)	—	363.01±30.01

A

B

C

D

A

B

A

B

C

D

A

CalpainI	10	-	20	10	5	20	10	20
RhATG5	-	+	+	+	+	+	+	+
Ca ²⁺ (mM)	0	0	5	5	5	10	10	0

KDa

116

66

45

35

B

GGECFKHIPFRL-----RLVTPLTPS
 GGECFKHIPFRLYMPDGS LIQRLVTPLTPS

C

calpain1	10	-	10	10	10	10
RhATG5 ^{Δ191-199}	-	+	+	+	+	+
Ca ²⁺ (mM)	0	0	5	10	0	0

KDa

116

66

45

35

A

B

A

B

A

B

C

A

dsATG5

dsLuciferase

B