The transcription factor p8 regulates autophagy during diapause embryo formation in *Artemia parthenogenetica*

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Abstract Autophagy is an essential homeostatic process by which cytoplasmic components, including macromolecules and organelles, are degraded by lysosome. Increasing evidence suggests that phosphorylated AMP-activated protein kinase (p-AMPK) and target of rapamycin (TOR) play key roles in the regulation of autophagy. However, the regulation of autophagy in quiescent cells remains unclear, despite the fact that autophagy is known to be critical for normal development, regeneration, and degenerative diseases. Here, crustacean *Artemia parthenogenetica* was used as a model system because they produced and released encysted embryos that enter a state of obligate dormancy in cell quiescence to withstand various environmental threats. We observed that autophagy was increased before diapause stage but dropped to extremely low level in diapause cysts in *Artemia*. Western blot analyses indicated that the regulation of autophagy was AMPK/TOR independent during diapause embryo formation. Importantly, the level of p8 (Ar-p8), a stress-inducible transcription cofactor, was elevated at the stage just before diapause and was absent in encysted embryos, indicating that Ar-p8 may regulate autophagy. The results of *Ar-p8* knockdown revealed that Ar-p8 regulated autophagy during diapause formation in *Artemia*. Moreover, we observed that activating transcription factors 4 and 6 (ATF4 and ATF6) responded to

Ar-p8-regulated autophagy, indicating that autophagy targeted endoplasmic reticulum (ER) during diapause formation in *Artemia*. Additionally, AMPK/TOR-independent autophagy was validated in human gastric cancer MKN45 cells overexpressing Ar-p8. The findings presented here may provide insights into the role of p8 in regulating autophagy in quiescent cells.

Keywords p8 · Autophagy · Diapause · *Artemia* · AMPK/TOR

Introduction

Autophagy is an essential catabolic and evolutionarily conserved process that targets cytoplasmic components, including macromolecules and organelles, to lysosomes for bulk degradation in all eukaryotic cells in response to prolonged starvation periods, nutritional fluctuations in the environment, developmental tissue remodeling, and organelle quality control (Klionsky et al. 2012; Levine and Kroemer 2008; Levine et al. 2011; Mizushima 2007; Mizushima and Levine 2010). The autophagy process involves more than 30 genes and proteins and can be divided into three major stages: initiation, elongation, and maturation. The autophagy process is related to the plasma membrane and the membranes of the endoplasmic reticulum (ER), mitochondria, and Golgi (He and Klionsky 2009; Levine and Klionsky 2004; Yorimitsu and Klionsky 2005). As a key energy sensor, AMP-activated protein kinase (AMPK) regulates cellular metabolism to maintain energy homeostasis and promotes autophagy by activating UNC-51-like kinase 1 (ULK1) through phosphorylation of its Ser317 and Ser777 residues. Conversely, the central cell growth regulator target of rapamycin (TOR) inhibits autophagy by phosphorylating Ser757 of ULK1 and disrupting its interaction with AMPK (Kim et al. 2011; Mizushima 2010).

Cheng Lin and Sheng-Nan Jia contributed equally to this work.

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This coordinated pattern of phosphorylation is important for the proper control of autophagy.

Previous studies have demonstrated the existence of ER-related autophagy (reticulophagy), a selective autophagic pathway that targets ER in fibroblasts and myoblasts (Oh and Lim 2009; Younce and Kolattukudy 2010). The regulation of reticulophagy triggered by ER stress is TOR independent but requires protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1) (Cebollero et al. 2012; Deegan et al. 2013). p8, also called nuclear protein transcriptional regulator 1 (NUPR1), is a member of the high mobility group of transcriptional regulators. p8 can respond to divergent stresses, such as lipopolysaccharide, serum withdrawal, and mitotic cancer drugs (Jiang et al. 1999; Valacco et al. 2006; Zinke et al. 2002). p8 was first identified as a gene strongly induced in pancreatitis in rats in 1997 (Mallo et al. 1997). Recently, p8 has been found to be overexpressed in many human cancers, including pituitary tumors and pancreatic tumors (Mohammad et al. 2004; Su et al. 2001), and pathological conditions, such as the diabetic kidney (Goruppi et al. 2002; Goruppi and Iovanna 2010). p8 is also involved in autophagy induced by lipopolysaccharide treatment of vascular endothelial cells (Meng et al. 2012).

Under certain conditions, cells enter a quiescent state for extended periods that can last for years or throughout their lifetime while retaining their ability to proliferate or differentiate (Rossi et al. 2012). Furthermore, cell quiescence and its reversible transition into proliferation are often triggered in response to signals from tissue homeostasis or repairing environmental stresses (Coller et al. 2006; Yao 2014). The cardinal features of this quiescence are its differences from cycling cells with respect to metabolism, autophagy, cell size, and terminal differentiation. In some systems, cell quiescence is associated with reduced autophagy and senescence (Blagosklonny 2011; Valcourt et al. 2012). However, the regulation of prohibited autophagy in quiescent cells is unknown.

The crustacean *Artemia parthenogenetica* resides in hypersaline environments, such as salt lakes, that are among the most hostile environments on the earth; in fact, only a small number of animals can survive in the extreme conditions of high salinity, low oxygen levels, and large fluctuations in temperature. As a survival strategy, *Artemia* possesses two independent reproductive pathways that allow for adaptation to widely fluctuating environments. Under unfavorable conditions, mature females produce and release encysted embryos (cysts) that enter diapause, a state of obligate dormancy (oviparous pathway), whereas swimming nauplius larvae are released under favorable conditions (ovoviviparous pathway) (Chen et al. 2011). The cells in diapause embryos do not divide, exhibit a low level of metabolism for prolonged periods, and are considered to be in a quiescent state (Dai et al. 2008). However, under certain conditions, diapause is

terminated, and the activated cysts progress to the post-diapause stage in which development and metabolism resume, but the cell cycle is arrested (Duan et al. 2014). Under suitable conditions, activated cysts resume development and eventually hatch as nauplius larvae, at which point, metabolism and cell division resume (Li et al. 2012).

Here, we used *Artemia* as a model system to study the regulation of autophagy in encysted embryos during diapause formation, in which the embryonic cells enter quiescence from a cycling state. The results presented here demonstrate that the ER stress-related autophagy is AMPK/TOR independent but regulated by Ar-p8 during diapause formation in the oviparous reproductive pathway of *Artemia*. However, autophagy is controlled via the AMPK/TOR signaling pathway during embryonic development in the ovoviviparous reproductive pathway. Moreover, AMPK/TOR-independent autophagy was validated in human gastric cancer MKN45 cells overexpressing Ar-p8. The findings presented here may provide insights into the regulation of autophagy in quiescent cells.

Materials and methods

Animals

The crustacean *A. parthenogenetica* cysts from Gahai Lake were a kind gift from Prof. Feng-Qi Liu (College of Life Sciences, Nankai University, Tianjin, China) (Dai et al. 2008) who cultured *Artemia* during 1990s when *Artemia* cysts were collected freely for scientific research and Gahai Lake was not a state-level nature reserves. *Artemia* cysts can store for decades and hatch. Animal experiments were approved and performed in accordance with the institutional guidelines for animal care of animal ethics committee of Zhejiang University. The animals were raised in artificial seawater (blue starfish, Hangzhou, China) at 25 °C and were fed once every 2 days with chlorella powder. Ovoviviparous *Artemia* adults, releasing nauplius larvae, were raised in 40‰ artificial seawater under a regime of 16 h light and 8 h dark. Oviparous adults, releasing diapause cysts, were raised in 80‰ artificial seawater under a regime of 4 h light and 20 h dark. In both reproductive pathways, early oocytes are formed in the paired ovaries and mature in the oviducts to form late oocytes, which pass into the ovisac (uterus), where they become early embryos. The late embryo stage gives rise to either nauplius larvae or diapause cysts, both of which are released into the environment.

Cell lines and transfection

Human gastric cancer cells (MKN45) (Bioteleaf, Shanghai, China) were maintained separately in RPMI 1640 medium (Corning, New York, USA) containing 10 % fetal bovine

serum (GIBCO, USA) and 1 % penicillin/streptomycin (Sigma, Dorset, UK). The cells were cultured at 37 °C in a 5 % CO₂ atmosphere. The pEGFP-C1 vectors were used for the construction of the pEGFP-Ar-p8 plasmids. MKN45 cells were transfected with these recombinant expression plasmids and pEGFP-C1 vectors as control using Attractene transfection reagent (Qiagen, Germany), according to the manufacturer's instructions.

Reagents and antibodies

Chloroquine (CQ) was purchased from Sigma-Aldrich. The primary antibodies used in this study were as follows: polyclonal anti-Ar-ATG5, polyclonal anti-Ar-ATG8, polyclonal anti-Ar-p8, and polyclonal anti-Ar-AMPK (HuaAn, Hangzhou, China); polyclonal anti-p-AMPK (Epitomics, Burlingame, USA); polyclonal anti-p70s6k, polyclonal anti-p-p70s6k, polyclonal anti-AMPK, and polyclonal anti-Histone H3 (Cell Signaling Technology, Leiden, Netherlands); polyclonal anti- α -Tubulin and polyclonal anti-LC3B (Sigma); and polyclonal anti-ATF4 and polyclonal anti-ATF6 (Abcam, Cambridge, UK).

Western blot

Proteins extracted from the tissues of *Artemia* at different developmental stages were prepared using TRIzol reagent (Invitrogen Life Technologies, Burlington, ON) according to the manufacturer's instructions and were quantified using the Bradford method. Each total protein sample (4 μ g) was separated on SDS-PAGE gel and then transferred to PVDF membranes (Bio-Rad, CA, USA). After incubation of the membranes with the primary antibodies at 4 °C overnight, specific proteins were detected using the BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany).

Cell fractionation

Cell fractionation was performed as described previously. Briefly, specimens were homogenized on ice in HEPES buffer (10 mM HEPES, 4 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) using a Dounce homogenizer. To obtain the cytoplasmic fraction, the homogenates were centrifuged at 3300 $\times$ g for 15 min at 4 °C, and the supernatant was retained. To obtain the nuclear fraction, the pellet was re-suspended and re-homogenized in fractionation buffer (10 mM Tris, 10 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, and 4 mM MgCl₂, pH 7.4), loaded onto a 45 % (w/v) sucrose buffer drop by drop, and centrifuged at 13,000 $\times$ g for 15 min at 4 °C. The pellets were washed once and then dissolved in EBC buffer (50 mM Tris, 1 mM EDTA, 120 mM MgCl₂, 0.5 % NP-40, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). The proteins were quantified using the Bradford

method. Equal amounts of each protein were used for immunoblotting using anti- α -Tubulin (Sigma) and anti-Histone H3 (Cell Signaling Technology) antibodies as cytoplasmic and nuclear markers, respectively.

Real-time PCR

Specimens from different developmental stages of *Artemia* were snap-frozen in liquid nitrogen, and total RNA was prepared from homogenized specimens using TRIzol reagent (Invitrogen Life Technologies). The extracted RNA was quantified by measuring the absorbance at 260 nm with a UV/visible spectrophotometer (Eppendorf). Total RNA (1 μ g) was reverse transcribed in a 10 μ l reaction containing oligo (dT) primers and M-MLV Reverse Transcriptase (TaKaRa, Shiga, Japan). All real-time PCR analyses were performed using gene-specific primers and the Bio-Rad MiniOpticonTM Real-Time PCR System (iQ5) using SYBR Premix Ex TaqTM (TaKaRa). The relative amounts of each messenger RNA (mRNA) were analyzed using the comparative CT method. All data are expressed as the mean $\pm$ SE of three independent repeats. All statistical analyses were performed using a two-tailed, paired Student's *t* test, and *P* < 0.05 was considered significant.

RNA interference

For preparation of the double-strand RNA (dsRNA), PET-T7 plasmid that contained two inverted T7 polymerase sites flanking the cloning region was used as the expression vector. A 550 bp fragment including the 5' UTR and parts of the 5' coding region of the *AMPK* gene, as well as a 500 bp fragment of the *Ar-p8* gene, were amplified by PCR. These amplified fragments were digested using *Xba*I and *Nco*I (TaKaRa) and subcloned into PET-T7. The plasmid expressing the dsRNA targeting *GFP* was constructed as described previously and used as a negative control. Approximately 1 μ g of *AMPK*-, *Ar-p8*-, or *GFP*-specific dsRNA was injected into the body cavity of *Artemia* using an Ultra-MicroPump II equipped with a Micro4TM MicroSyringe pump controller (World Precision Instruments, Micro4).

Immunohistochemistry by transmission electron microscopy analysis

Cysts were soaked in ice-cold 2 M sucrose for 20 min. The cyst walls were then nicked, and the cysts were fixed in 4 % paraformaldehyde and 0.2 % glutaraldehyde overnight at 4 °C. To obtain ultrathin sections, the cysts were embedded in 1 $\times$ PBS containing 12 % (w/v) gelatin at 37 °C for 30 min and then incubated at 4 °C until they solidified. The cysts in gelatin were cut into sections of approximately 1 mm³; these sections were sequentially immersed in 30, 50, and 70 % (w/v)

sucrose solutions while sedimenting. Sections of 70 nm were cut using a microtome (Leica EM UC6), incubated with the anti-Ar-ATG8 antibody (HuaAn), and coated with 15 nm of gold nanoparticle-conjugated secondary antibody (Sigma). The cysts were viewed by transmission electron microscopy (TEM; JEM-1230) and photographed at a voltage of 80 kV.

Results

Characterization of autophagy during diapause embryo formation of *Artemia*

To identify the level of autophagy in *Artemia*, we cloned *ATG5* and *ATG8* from *Artemia* (*Ar-ATG5* and *Ar-ATG8*, respectively) using degenerate primer PCR and rapid amplification of complementary DNA (cDNA) ends (RACE) (Table 1). Sequence analyses revealed that the *Ar-ATG5* and *Ar-ATG8* cDNA each contains an open reading frame that encodes a protein of 267 or 117 amino acids, with a predicted mass of 31.4 or 14 kDa, respectively (Gene Bank ID KP31725 and KP31726, Supplementary Fig. S1a and S2a). The deduced amino acid sequences of these proteins are similar to those of the ATG5 and ATG8 proteins from other species (43.5–51.5 % and 29.7–89 % identities, respectively; Supplementary Fig. S1b and S2b).

As shown in Fig. 1a, *Artemia* possesses two independent reproductive pathways, including ovoviviparous pathway (Fig. 1a, 1–4) and oviparous pathway (Fig. 1a, 1'–5'), that allow adaptation to widely fluctuating environments. The levels of autophagy in each developmental stages of *Artemia* in the two pathways were examined by Western blots using polyclonal antibodies specific to Ar-ATG5 and Ar-ATG8. In the nauplius-destined ovoviviparous pathway, the autophagy-related protein Ar-ATG5 was relatively constant, and Ar-ATG8 decreased (Fig. 1b, lanes 1–4). However, in the

diapause-destined oviparous pathway, the level of Ar-ATG8 increased throughout development and reached to a peak in pre-diapause stage (Fig. 1b, lanes 1'–4') and then disappeared in diapause cysts (Fig. 1b, lane 5'), suggesting that autophagy was enhanced before diapause stage and then prohibited during the long period of dormancy. To further confirm the observation above, TEM analysis of Ar-ATG8 using gold nanoparticles was performed on pre-diapause and diapause embryos. As shown in Supplementary Fig. S3, Ar-ATG8 was observed in the pre-diapause stage (Supplementary Fig. S3, 4') but was very rare in diapause embryos (Supplementary Fig. S3, 5'). Taken together, these results confirmed that in oviparous pathway, autophagy increased progressively before diapause stage but disappeared in diapause embryos.

The regulation of autophagy is AMPK/TOR independent during the diapause embryo formation

As mentioned above, autophagy is activated by AMPK and inhibited by TOR-dependent signaling (Mizushima 2010). To investigate the regulation of autophagy in the two independent reproductive pathways of *Artemia*, the levels of AMPK, phosphorylated AMPK (p-AMPK), and p-p70s6k (a direct downstream target of TOR) were assessed by Western blots. In the ovoviviparous pathway, similar to the level of autophagy (Fig. 1b, lanes 1–4), p-AMPK (AMPK) and p-p70s6k were relatively constant (Fig. 2a, lanes 1–4). In the diapause-destined oviparous pathway, the levels of p-AMPK (AMPK) and p-p70s6k stayed the same before diapause stage (Fig. 2a, lanes 1'–4'), whereas the level of autophagy was increased (Fig. 1b, lanes 1'–4'). In contrast, the level of p-AMPK was high, and p-p70s6k was extremely low (Fig. 2a, lane 5'), whereas the level of autophagy was extremely low in diapause embryos (Fig. 1b, lane 5'). These results suggested that the regulation of autophagy was AMPK/TOR independent during diapause embryo formation in the oviparous pathway.

Table 1 Nucleotide sequences of primers used in polymerase chain reactions (PCRs)

Primer	Length (bp)	Position	Direction	Sequence (5'–3')
AMPKi-F	31	413–435	F	gctctagaTATGTTTCTGGTGGTGAACCTCTT
AMPKi-R	30	839–861	R	ggaattcAGGTTACAGTGCTTTTATTCAA
Ar-p8i-F	33	1–25	F	ggtctagaCATTTGAAAGGAAGAGTTTCAGGTA
Ar-p8i-R	28	453–472	R	cgggatccCAGTGTTGCCAGCAAAAAGAA
Ar-p8 RT-F	18	276–293	F	GGACTTCCGATCTCGTTT
Ar-p8 RT-R	18	379–396	R	GTCACCAAGAGCCCTACA
AMPK RT-F	19	1336–1354	F	GCACTTGGGTATCCGTTCC
AMPK RT-R	24	1451–1474	R	TCCCGTTACAGAGTTTGTCTTCCT
Ar-Tubulin-F	20	446–465	F	GCAGTGGTCTACAAGGTTTC
Ar-Tubulin-R	22	774–795	R	ATCAAAACGAAGGCTGGCGGTG
Ar-GFPi-F	28		F	gctctagaGCCATTCTTTGGTTTGTCTC
Ar-GFPi-R	30		R	ggaattcAACTTACCCTTAATTTTATTTGC

Fig. 1 Characterization of autophagy during the development of *Artemia*. **a** Morphology of the two reproductive pathways of *Artemia*: the diapause-destined oviparous pathway (top) and the nauplius-destined ovoviparous pathway (bottom). Lanes 1–4: 1–4 days after the oocytes entered the uterus in ovoviparous pathway. Lanes 1'–4': pre-diapause, 1–4 days after the oocytes entered to the ovisac in oviparous pathway; and

5': released diapause cysts. Scale bars, 1.2 mm. **b** Representative Western blots and quantitative data using ImageJ software showed the extent of Ar-ATG5 and Ar-ATG8 at various stages during the development of *Artemia*. The numbers correspond to those described in **a**. Mean $\pm$ SD, $n=3$, $*P<0.05$

Furthermore, RNA interference (RNAi)-mediated knock-down of *AMPK* in both reproductive pathways was performed. Approximately 1 μ g of an *AMPK*-specific dsRNA was injected into the body cavity of *Artemia*, and the interference efficiency was measured by real-time PCR. After injection, the mRNA level of *AMPK* was reduced by

approximately 80 % compared with control (Fig. 2b), and the protein level of AMPK was barely detectable in *Artemia* in both reproductive pathways by Western blot (Fig. 2c). In the ovoviparous pathway, the protein levels of Ar-ATG5 and Ar-ATG8 were significantly decreased, indicating that autophagy was decreased after *AMPK* RNAi (Fig. 2c, 4). However,

Fig. 2 The regulation of autophagy is AMPK/TOR independent during the diapause embryo formation. **a** Representative Western blots and quantitative data using ImageJ software showed the extent of p-AMPK and p-p70s6k at various stages during the development of *Artemia*. The numbers correspond to those described in Fig. 1a. **b** Real-time PCR analysis of the relative *AMPK* transcript levels in *Artemia* treated with a dsRNA targeting *AMPK* or *GFP* as a negative control. *AMPK* and *GFP* RNAi in ovoviparous pathway (4 *AMPKi* and 4 *GFPi*) and *AMPK* and *GFP* RNAi in oviparous pathway (4' *AMPKi* and 4' *GFPi*). **c** Representative Western blots and quantitative data using ImageJ software showed the extent of Ar-ATG5 and Ar-ATG8 in *Artemia* after *AMPK* or *GFP* RNAi in the two reproductive pathways. Mean $\pm$ SD, $n=3$, $*P<0.05$

Ar-ATG5 and Ar-ATG8 were unchanged in response to the RNAi in the oviparous pathway (Fig. 2c, lane 4'). The results indicated that the regulation of autophagy was AMPK/TOR dependent during the embryonic development of the ovoviparous pathway and AMPK/TOR independent during diapause embryo formation in the oviparous pathway.

Ar-p8 regulates autophagy during diapause embryo formation

p8, a stress-inducible transcription cofactor, is reported to be involved in autophagy (Meng et al. 2012). We found that level of Ar-p8 was increased before diapause stage of oviparous pathway (Fig. 3a, lanes 1'–4'), while the level of autophagy was increased (Fig. 1b, lanes 1'–4'). Then, Ar-p8 decreased until barely detectable in diapause embryo (Fig. 3a, lane 5') in which autophagy was suppressed (Fig. 1b, lane 5'). To examine the subcellular location of Ar-p8, cell fractionation was performed as described in the Materials and methods section. Similar to the report by Qiu and MacRae (2007), Ar-p8 accumulated in the nuclei of cells (Fig. 3b). These results suggested that Ar-p8, a nuclei located protein, may be related to the regulation of autophagy during diapause embryo formation. However, Ar-p8 was not detected during embryonic development in the ovoviparous pathway (Fig. 3a, lanes 1–4) in which autophagy remains at a basal level (Fig. 1b, lanes 1–4).

To elucidate the function of Ar-p8 in the regulation of autophagy during diapause embryo formation, *Ar-p8* gene was

knocked down via RNAi. The result of real-time PCR showed that the mRNA level of *Ar-p8* was reduced by approximately 85 % after injecting with 1 μ g *Ar-p8*-specific dsRNA compared with the control group (Fig. 3c), and the protein level of Ar-p8 was decreased significantly (Fig. 3d). Meanwhile, the expression of Ar-ATG5 and Ar-ATG8 was significantly reduced after *Ar-p8* interference (Fig. 3d). These results indicated that Ar-p8 regulated autophagy during diapause embryo formation.

Ar-p8 regulates ATF4/ATF6-mediated ER stress-related autophagy

As mentioned, p8 plays a key role in response to ER stress (Carracedo et al. 2006). The ER stress-related autophagy-related proteins ATF4 and ATF6 were assessed by Western blot. The levels of ATF4 and ATF6 increased before diapause stage (Fig. 4a, lanes 1'–4'), in which stages, the level of Ar-p8 increased (Fig. 3a, lanes 1'–4'). In contrast, the levels of ATF4 and ATF6 were relatively constant in the ovoviparous pathway (Fig. 4a, lanes 1–4), in which Ar-p8 was hardly detected (Fig. 3a, lanes 1–4). These results suggested that ATF4- and ATF6-related ER stress was increased before diapause stage. However, the high levels of ATF4 and ATF6 were also detected in released diapause (Fig. 4a, lane 5'), in which they may be involved in resistance of diapause embryo to extreme high salinity, low oxygen, and large fluctuations in temperature. As another case, ATF4 and ATF6 act as activating

Fig. 3 Ar-p8 regulated autophagy during diapause embryo formation in *Artemia*. **a** Representative Western blot and quantitative data using ImageJ software showed the extent of Ar-p8 at various stages during the development of *Artemia*. The numbers correspond to those described in Fig. 1a. **b** Western blot analysis of the nucleic (N) and cytoplasmic (C) locations of Ar-p8 at pre-diapause stage (4') and diapause stage (5'). Histone H3 and Tubulin were used as loading

controls for the nucleus and cytoplasm, respectively. **c** Real-time PCR analysis of the relative *Ar-p8* transcript levels in *Artemia* treated with a dsRNA targeting *Ar-p8* or GFP as a negative control in pre-diapause stage. **d** Representative Western blots and quantitative data using ImageJ software showed the extent of Ar-ATG5 and Ar-ATG8 in *Artemia* after *Ar-p8* or GFP RNAi in pre-diapause stage. Mean $\pm$ SD, $n=3$, * $P<0.05$

Fig. 4 Ar-p8 regulated ATF4/ATF6-mediated ER stress-related autophagy. **a** Western blot showed the extent of ATF4 and ATF6 at various stages during the development of *Artemia*. The numbers correspond to those described in Fig. 1a. **b** Western blot analyses of Ar-p8

p8, ATF4, and ATF6 in *Artemia* after p8 or GFP RNAi. **c** Western blot analyses of AMPK, ATF4, and ATF6 in *Artemia* after AMPK or GFP RNAi. Quantitative data of optical band densitometry are shown. Mean $\pm$ SD, $n = 3$, $*P < 0.05$

transcriptional factors regulating the adaptation of cells to stress factors such as anoxic insult, endoplasmic reticulum stress, and oxidative stress (Rzymiski et al. 2008; Deegan et al. 2013). Furthermore, upon RNAi-mediated knockdown of *Ar-p8* in the oviparous pathway, the levels of ATF4 and ATF6 assessed by Western blots were significantly reduced (Fig. 4b). In contrast, ATF4 and ATF6 remained constant in both reproductive pathways after *AMPK* RNAi (Fig. 4c, lanes 4 and 4'). Thus, Ar-p8 regulated ATF4- and ATF6-mediated ER stress-related autophagy during diapause embryo formation.

Ar-p8 induces autophagy in human gastric cancer MKN45 cells

To explore the function of Ar-p8 in the regulation of ER stress-related autophagy in a model system other than *Artemia*, plasmid expressing enhanced green fluorescent protein (EGFP)-fused Ar-p8 was constructed and then transfected into the human gastric cancer MKN45 cell line for 24 h; a

plasmid expressing EGFP served as a control (Fig. 5a). The level of autophagy-related protein LC3B (homologous to ATG8) was detected by Western blot. As shown in Fig. 5b, the level of LC3B was increased significantly when the cells were treated with chloroquine (CQ) to promote autophagosome accumulation. Moreover, the protein levels of p-AMPK (AMPK) and p-p70s6k (p70s6k) remained constant (Fig. 5c). Furthermore, the similar results were observed in other cell lines including embryonic kidney (HEK293T) cells, fibrosarcoma (HT1080) cells, and breast cancer (MCF7) cells (data not shown). These results enforced the observation that Ar-p8 induced autophagy and that the induction was AMPK/TOR independent.

AMPK/TOR regulates autophagy in response to environmental stress in *Artemia* adults

In order to study the regulation of autophagy in response to environmental stress, adult *Artemia* neither ovoviviparous nor oviparous pathway were treated with nutrient starvation or

Fig. 5 Ar-p8 induced autophagy in human gastric MKN45 cell line. **a** Western blot analysis of EGFP in the MKN45 cells transfected with EGFP-fused Ar-p8 (Ar-p8) or EGFP alone for 24 h alone. **b** Western blot analysis of LC3B in MKN45 cells transfected with EGFP-Ar-p8 or EGFP alone for 24 h and treated with or without 10 μ M chloroquine (CQ) for 4 h to promote the accumulation of autophagosomes. **c** Western blot analyses of p-AMPK (AMPK) and p-p70s6k (p70s6k) in MKN45 cells overexpressing Ar-p8. Quantitative data of optical band densitometry are shown. Mean $\pm$ SD, $n = 3$, $*P < 0.05$

UV irradiation. The increased levels of Ar-ATG5 and Ar-ATG8 were observed after the treatments. The results indicated that autophagy was upregulated in response to the environmental stresses. Furthermore, we found that the level of p-AMPK was significantly increased and p-p70s6k was strongly decreased after the treatments compared to the control group (Fig. 6a, b). However, Ar-p8 was not in response to the stresses that could not be detected after the treatments (data not shown). Based on the results, we concluded that the regulation of autophagy was AMPK/TOR dependent in response to the environmental stresses, but the autophagy during diapause formation was regulated by Ar-p8, an AMPK/TOR-independent pathway.

Discussion

Accumulating studies have demonstrated that the regulation of autophagy is essential for cell homeostasis. Cell quiescence and its reversible transition into proliferation are often due to the responses to signals involved in tissue homeostasis or repairing of environmental stresses. The quiescent cells in *Artemia* diapause embryos have reversibly shut down DNA synthesis, cell division, and metabolism (Abatzopoulos et al. 2002). The results presented here demonstrated that the level of autophagy was high before diapause stage and decreased dramatically in diapause cysts, and the autophagy was

regulated by Ar-p8 but not by the AMPK/TOR signaling pathway during diapause embryo formation. Moreover, the levels of the ER stress-related proteins ATF4 and ATF6 decreased significantly after *Ar-p8* RNAi. These results indicated the essential role of Ar-p8 in regulating ER stress-related autophagy during diapause embryo formation.

Artemia diapause embryos exhibit remarkable resistance to severe desiccation, extremes of temperature and pressure, anoxia extending over periods of years (while fully hydrated at physiological temperatures), and various forms of radiation (Clegg 1984, 1997). To understand the induction and maintenance of *Artemia* diapause embryos, previous studies focused on molecules associated with extreme stress tolerance. For example, p26, a small heat shock/ α -crystallin protein that acts as a molecular chaperone, is expressed in diapause embryos at extremely high levels and is partially translocated into the nuclei during periods of environmental stress (Clegg et al. 1995; Liang and MacRae 1999). Furthermore, the cyst-specific small heat shock protein artemin, which is also present at high levels in diapause embryos, has been implicated as a cytoplasmic RNA chaperone (Chen et al. 2007; Warner et al. 2004). Another molecule that protects against environmental stresses is the non-reducing disaccharide trehalose, which accumulates in large amounts in encysted embryos, where it is thought to be involved in desiccation tolerance (Clegg 1965; Clegg and Jackson 1992). *Artemia* diapause embryos enter quiescence, which involves the reversible shutdown of DNA synthesis, cell division, and metabolism. In some systems, cell quiescence is associated with reduced autophagy and senescence (Blagosklonny 2011; Valcourt et al. 2012). We suggest that the autophagy during diapause embryo formation which still remains unknown may be essential for the *Artemia* entering the state of obligate dormancy in cell quiescence to withstand various environmental threats. Our research indicates that the level of autophagy increased before diapause stage, suggesting that organelles may undergo a special autophagy process that promotes remodeling and/or dysfunction to enable the maintenance of extremely low metabolism during long periods of dormancy. Previous work on *Artemia* demonstrated that in diapause embryos, the level of H3 lysine 56 acetylation (H3K56ac) on chromatin was high (Zhou et al. 2013). The levels of H3K56ac are controlled by histone deacetylase 1 and 2 (HDAC1 and HDAC2), and HDACs have been reported to be important during autophagy flux (Miller et al. 2010). HDAC inhibitors attenuate cardiac hypertrophy by suppressing autophagy (Cao et al. 2011). In our research, autophagy was barely detected in diapause embryos, and the very low autophagy could be the result of high level of H3K56ac and is essential during long period of dormancy.

The degree of activation of the AMPK/TOR pathway is thought to be one of the major factors governing autophagy. However, the AMPK/TOR pathway is not involved in autophagy during diapause embryo formation, and recent studies

Fig. 6 AMPK/TOR regulated autophagy in response to nutrient starvation or UV irradiation in *Artemia* adults. **a** Western blot analyses of AMPK, p-AMPK, p-p70s6k, Ar-ATG5, and Ar-ATG8 in response to nutrition starvation in *Artemia*. *Artemia* adults were treated with PBS for 4 days (st1 and st2); *Con.* control group with no stress treatment. **b** Western blot analyses of AMPK, p-AMPK, p-p70s6k, Ar-ATG5, and Ar-ATG8 in response to UV irradiation. *Artemia* adults were exposed directly to a UV lamp (310 nm) at 0.15 J/cm² for 0–30 min. Quantitative data of optical band densitometry are shown. Mean $\pm$ SD, $n = 3$, * $P < 0.05$

have suggested that autophagy regulation is a very complex process involving the integration of signals from many diverse signaling pathways (He and Klionsky 2009). Although substantial attentions have recently been focused on the direct phosphorylation of ATG1/ULK1 by AMPK or TOR, there are many reports of other kinases capable of controlling autophagy by a variety of TOR-independent mechanisms (Sakar et al. 2005; Stephan et al. 2009; Tan et al. 2012). Our research demonstrates that Ar-p8 but not AMPK/TOR regulates autophagy to control cell quiescence entry. The AMPK/TOR signaling pathway is reported to be activated when cells experience stress or starvation. Before diapause stage, the level of p-AMPK is relatively constant, indicating that embryos are not under energy stress. However, autophagy is increased before diapause stage. Thus, the autophagy during diapause embryo formation is an AMPK/TOR-independent regulation pathway and is distinct from the stress-induced AMPK/TOR-dependent passive regulation.

p8 belongs to the family of HMG-I/Y transcription factors and may play a cell-specific role in autophagy. Previous studies have demonstrated that autophagy is positively regulated by p8 in human glioma cells and human umbilical vein endothelial cells, but deficiency of p8 results in increased

autophagy in H9C2 and U2OS cells (Kong et al. 2010; Meng et al. 2012; Salazar et al. 2009). Previous work on Ar-p8 in *Artemia* found that *Ar-p8* mRNA was specifically expressed before diapause embryo formation (Qiu and MacRae 2007). In the present study, we found that Ar-p8 upregulated autophagy and knockdown of *Ar-p8* suppressed the autophagy before diapause stage. These results indicated that the autophagy occurred during diapause embryo formation in *Artemia* was regulated by Ar-p8. Furthermore, overexpression of Ar-p8 increased the level of autophagy in MKN45 cells. However, Ar-p8 was not involved in the induction of autophagy by nutrient starvation or UV exposure, both of which activated the AMPK/TOR signaling pathway. This Ar-p8-dependent autophagy could be essential for the formation of *Artemia* diapause cysts.

Autophagy is a selective process that degrades various organelles in response to specific stimuli. For example, autophagy induced by ER stress may play an important role in maintaining ER homeostasis by segregating and/or degrading a part of this structure in response to various ER stresses (Cebollero et al. 2012). Kraft et al. (2008) have demonstrated the existence of ribophagy, a selective autophagic pathway that target ribosomes, in *Saccharomyces cerevisiae*.

Ribophagy is thought to adjust the number and quality of ribosomes in response to new environments or stresses and results in a decrease or complete loss of translation when it targets mature ribosomes (Beau et al. 2008). Previous studies have reported evidences of altered numbers, structures, and functions of organelles such as the mitochondria, ER, and ribosomes in diapause *Artemia* embryonic cells (Robert et al. 1988; Vallejo et al. 1996). The regulation of autophagy may be important for the alteration of organelles during the long period of dormancy.

Overall, our findings indicate that Ar-p8 regulates ER stress-related autophagy through an AMPK/TOR-independent pathway to assist cells in entering a reversible quiescence state during diapause embryo formation. This proactive regulation of autophagy is important for maintaining the propagation and stability of *Artemia* populations in extreme environments. Furthermore, the regulation of AMPK/TOR-independent autophagy by Ar-p8 offers a new mechanistic understanding of quiescence formation, which is very important for tissue homeostasis and resistance to environmental stresses.

AMPK AMP-activated protein kinase, **ATF4** Activating transcription factor 4, **ATF6** Activating transcription factor 6, **ATG5** Autophagy-related protein 5, **ATG8** Autophagy-related protein 8, **CQ** Chloroquine, **EGFP** Enhanced green fluorescent protein, **ER** Endoplasmic reticulum, **GFP** Green fluorescent protein, **H3K56ac** H3 lysine 56 acetylation, **HDAC** Histone deacetylase, **IRE1** Inositol requiring enzyme 1, **PERK** Protein kinase-like endoplasmic reticulum kinase, **RNAi** RNA interference, **TEM** transmission electron microscopy, **TOR** target of rapamycin, **ULK1** UNC-51-like kinase 1

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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