P300/HDAC1 regulates the acetylation/deacetylation and autophagic activities of LC3/Atg8–PE ubiquitin-like system

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Abstract
Protein acetylation plays potential roles in regulating autophagy occurrence. However, it varies greatly between yeast and mammals, and has not been thoroughly investigated in other organisms. Here, we reported that the components of BmAtg8–PE ubiquitin-like system (BmAtg3, BmAtg4, BmAtg7, and BmAtg8) in Bombyx mori were localized in the nucleus under nutrient-rich conditions, whereas they were exported to the cytoplasm upon autophagy induction. RNAi of BmP300 and inhibition of BmP300 activity resulted in nucleo-cytoplasmic translocation of BmAtg3 and BmAtg8, as well as premature induction of autophagy in the absence of stimulus. Conversely, RNAi of BmHDAC1 and inhibition of class I/II HADCs activities led to the nuclear accumulation of BmAtg3 and BmAtg8. In addition, acetylation sites in Atg proteins of BmAtg8–PE ubiquitin-like system were identified by mass spectrometry, and acetylation-site mutations caused nucleo-cytoplasmic translocation of BmAtg3, BmAtg4, and BmAtg8 along with autophagy promotion. Similarly, the subcellular localization of human ATG4b is determined by acetylation modification. In general, BmP300-mediated acetylation sequesters the components of BmAtg8–PE ubiquitin-like system in the nucleus, thus leading to the autophagy inhibition. Oppositely, BmHDAC1-mediated deacetylation leads to the nucleo-cytoplasmic translocation of the components of BmAtg8–PE ubiquitin-like system and promotes autophagy. This process is evolutionarily conserved between insects and mammals.

Introduction
Macroautophagy/autophagy, which is implicated in neurodegenerative diseases, tumorigenesis, and pathogen invasion, is a finely regulated cellular process for bulk degradation of cellular components1. Autophagosome biogenesis requires the participation of a series of autophagy-related (Atg) proteins, and the maturation of autophagosome involves in the ubiquitin-like systems, LC3/Atg8–phosphatidylethanolamine (PE)1. LC3/Atg8–PE conjugation is mainly catalyzed by Atg4, Atg7, and Atg3 in a sequential manner1,2. Atg4 is the sole cysteine protease responsible for the cleavage of nascent LC3/Atg83. After exposure of the C-terminal, LC3/Atg8 is adenylated by the E1-like enzyme Atg7 to form a covalent thioester-linked intermediate, and then is transferred to E2-like enzyme Atg3 before conjugation with PE3,4,5. There are four ATG4 genes in mammals, one in yeast, two in Caenorhabditis elegans and Drosophila melanogaster, respectively1,6. The protease activity of different ATG4 to different LC3 homologs is notably varied1,8. Mammalian ATG4b cleaves most LC3 homologs, and is considered as a potential biomarker and therapeutic target in human diseases9,10.
Atg7 and Atg3 homologs are associated with the control of autophagosome size and number, and act as the indicators of adverse disease prognosis and anticancer targets in mammals. Several catalytic components of LC3/Atg8–PE conjugation system have been functionally identified in invertebrates; however, the molecular mechanism of regulation by acetylation remains largely unknown.

Autophagy is strictly regulated by nutrients and energy signaling mainly through the phosphorylation of ULK1/Atg1-ATG13/Atg13 protein complex2,16,17. Moreover, acetylation of lysine sites in Atg proteins represents a universal mechanism involved in the regulation of autophagy. Starvation rapidly depletes deacetylase BmRpd3/HsHDAC1, and their derivatives can induce the dephosphorylation of histone deacetylase BmRpd3/HsHDAC1, and determine their derivatives can induce the dephosphorylation of histone H4 at lysine16 (H4K16ac), thus leading to autophagy induction. In Drosophila, knockdown of AcCoA:acyl-coenzyme A synthetase in the brain enhances autophagy and lifespan, indicating a regulation of autophagy by acetylation both in insects and mammals. Whereas, it is worthy to note that yeast Atg3 is hyperacetylated during starvation-induced autophagy. Yeast acetyltransferase Esa1-dependent acetylation facilitates Atg3 to participate in autophagy, in comparison, deletion of deacetylase Rpd3 increases autophagy. In mammals, deacetylation of ATG proteins, including ATG5, ATG7, LC3, and ATG12, by sirtuin 1 (SIRT1), histone deacetylase 6 (HDAC6), or HDAC1 promotes, while acetylase EP300/P300-mediated acetylation inhibits, autophagy. Accordingly, EP300/P300 sequesters LC3 in the nucleus under nutrient-rich conditions, while deacetylation of LC3, catalyzed by the deacetylase SIRT1, results in nucleo-cytoplasmic translocation of LC3 and consequent autophagy promotion under starvation. Up to now, the variation of regulation by acetylation in autophagy through the modification of LC3/Atg8–PE ubiquitin-like system observed in mammals and yeast is not documented in other species.

In insects, 20-hydroxyecdysone (20E), the steroid hormone synthesized from dietary cholesterol, is a key regulator of multiple processes during molting and metamorphosis. During larval–pupal transition, 20E markedly induces autophagy by upregulating the expression of Atg genes and simultaneously inhibiting MTORC1 (mechanistic target of rapamycin kinase complex 1) activity in both D. melanogaster and Bombyx mori. Our previous study has shown that cholesterol and its derivatives can induce the dephosphorylation of histone deacetylase BmRpd3/HsHDAC1, and determine their nucleo-cytoplasmic translocation to promote autophagy both in B. mori and humans, suggesting an involvement of deacetylation in autophagy regulation. However, the precise regulatory mechanism of BmHDAC1 (BmRpd3) on autophagy still needs to be determined. In order to investigate the discrepancy in acetylation regulation of LC3/Atg8–PE ubiquitin-like system components in organisms other than yeast and mammals, here we investigated this regulatory mechanism in B. mori, an organism of intermediate evolutionary status. Our results demonstrate that the regulation of autophagy by acetylation, through the modification of the components of Atg8–PE ubiquitin-like system in B. mori, is evolutionarily conserved with mammals, while is opposite to that of yeast.

Results

BmAtg3 and BmAtg8 are required for autophagy occurrence during larval–pupal metamorphosis

To investigate the autophagic function of BmAtg8–PE ubiquitin-like system, BmAtg3 (NP_001135961.1), BmAtg8 (NM_001046779.1), BmAtg4 (XM_021346895), and BmAtg7 (KY608887.1) were identified in B. mori. For the transcriptional levels of BmAtg3 and BmAtg8 are fairly high during autophagy occurrence, their protein levels and subcellular localization were analyzed in the fat body from 5L2D to PP2. Western blotting and immunofluorescent staining showed that the protein level of BmAtg3 gradually reduced from late fifth instar to prepupal stage, similar to the variation of BmSqstm1, the selective autophagy receptor/adaptor. Interestingly, BmAtg3 located in the nucleus at the feeding stage, but weak BmAtg3 staining was observed in the cytoplasm at the prepupal stage (Fig. 1A–1A′ and Supplementary Fig. S1A). The protein level of BmAtg8–PE gradually increased from 5L2D to PP2. BmAtg8 located in both nucleus and cytoplasm at the feeding stage, while BmAtg8-positive puncta were clearly visible in the cytoplasm at the prepupal stage when autophagy occurred (Fig. 1B–1B′ and Supplementary Fig. S1B). We then separated nuclear proteins from cytoplasmic proteins, and western blotting showed that the cytoplasmic distribution of BmAtg3 and BmAtg8 significantly enhanced at PP2 compared to 5L4D (Fig. 1C). In summary, BmAtg3 and BmAtg8 are exported from the nucleus to cytoplasm when autophagy occurs.

The autophagic function of BmAtg3 and BmAtg8 was investigated via CRISPR/Cas9-mediated knockout experiments. The efficiency of BmAtg3 and BmAtg8 knockouts was ~30% and ~40%, respectively (Supplementary Fig. S1C, D). The BmAtg3 protein level in the fat body was significantly diminished by BmAtg3 knockout; meanwhile, BmAtg8–PE conjugation was reduced and BmSqstm1 protein level was accumulated during the autophagy induction (Fig. 1D). Transmission electron microscopy (TEM) analysis, LysoTracker staining, and immunofluorescent staining confirmed the decrease in autophagosome and autolysosome...
formation, notably, BmAtg8 was accumulated in the nucleus after BmAtg3 knockout (Fig. 1E). BmAtg8 knockout reduced both BmAtg8 and BmAtg8–PE protein, as well as autophagy monitored by TEM analysis and LysoTracker staining, moreover, BmAtg3 was accumulated in the nucleus (Fig. 1F, G). These results demonstrate that both BmAtg3 and BmAtg8 are required for autophagy, and exhibit nucleo-cytoplasmic translocation during autophagy induction.

BmAtg8 and BmAtg3 are essential for autophagy induction in B. mori fat body. A–A’ Protein levels of BmSqstm1 and BmAtg3 (A), immunofluorescent staining of BmAtg3 (A’) in B. mori fat body from 5L2D to PP2 stage. EW early wandering stage, LW later wandering stage. Scale bar: 10 µm. B–B’ Protein levels (B) and immunofluorescent staining (B’) of BmAtg8 in B. mori fat body from 5L2D to PP2 stage. Arrows: typical fat body cell. Scale bar: 10 µm. C Protein levels of BmAtg3 and BmAtg8 in the nucleus and cytoplasm at 5L4D and PP2 stages. D Protein levels of BmSqstm1, BmAtg3, and BmAtg8 after BmAtg3 knockout. E LysoTracker Red staining, TEM analysis, and immunofluorescent staining of BmAtg3 and BmAtg8 after BmAtg3 knockout. F Protein levels of BmSqstm1, BmAtg3, and BmAtg8 after BmAtg8 knockout. G LysoTracker Red staining, TEM analysis, and immunofluorescent staining of BmAtg3 and BmAtg8 after BmAtg8 knockout.

Fig. 1 BmAtg8 and BmAtg3 are essential for autophagy induction in B. mori fat body. A–A’ Protein levels of BmSqstm1 and BmAtg3 (A), immunofluorescent staining of BmAtg3 (A’) in B. mori fat body from 5L2D to PP2 stage. EW early wandering stage, LW later wandering stage. Scale bar: 10 µm. B–B’ Protein levels (B) and immunofluorescent staining (B’) of BmAtg8 in B. mori fat body from 5L2D to PP2 stage. Arrows: typical fat body cell. Scale bar: 10 µm. C Protein levels of BmAtg3 and BmAtg8 in the nucleus and cytoplasm at 5L4D and PP2 stages. D Protein levels of BmSqstm1, BmAtg3, and BmAtg8 after BmAtg3 knockout. E LysoTracker Red staining, TEM analysis, and immunofluorescent staining of BmAtg3 and BmAtg8 after BmAtg3 knockout. F Protein levels of BmSqstm1, BmAtg3, and BmAtg8 after BmAtg8 knockout. G LysoTracker Red staining, TEM analysis, and immunofluorescent staining of BmAtg3 and BmAtg8 after BmAtg8 knockout.

BmAtg3 and BmAtg8 are exported from the nucleus to cytoplasm during 20E/starvation-induced autophagy

20E and starvation are two most important inducers of autophagy in insects. After treatments with different doses of 20E (1.25, 2.5, and 5 µM) or different time intervals of starvation (2, 4, and 8 h), BmSqstm1 and BmAtg3-c-Myc protein levels gradually decreased, while FLAG-BmAtg8–PE increased in BmN cells, showing a consistent variation of BmAtg3 and BmAtg8–PE
formation in 20E- and starvation-induced autophagy (Fig. 2A, B). In addition, BmAtg3-c-Myc and FLAG-BmAtg8 were exported from the nucleus to cytoplasm in response to 20E or starvation, and puncta for both BmAtg3 and BmAtg8 were visible in the cytoplasm (Fig. 2C, D and Supplementary Fig. S1E, F). Similarly, both 20E and starvation treatments induced BmAtg8–PE conjugation, the decrease of BmAtg3 protein level, and their protein nuclear export, along with BmSqstm1 degradation in the fat body (Supplementary Fig. S2A, B). These results indicate that BmAtg3 and BmAtg8 undergo nucleo-cytoplasmic translocation during 20E- or starvation-induced autophagy.

BmP300 and BmHDAC1 oppositely regulate the acetylation/deacetylation and nuclear localization of BmAtg3 and BmAtg8

Deacetylase BmHDAC1 facilitates autophagy in B. mori34, thus, whether BmAtg3 and BmAtg8 were regulated by acetylation was investigated. Results showed that the immunoprecipitated BmAtg3-His and FLAG-BmAtg8 were both acetylated under nutrient-rich...
conditions, while deacetylated after the starvation treatment (Fig. 2E). To analyze the regulatory machinery of acetylation in the silkworm, the developmental profiles of acetyltransferases, including BmP300 (XP_021204790.1), BmTip60 (XP_004928298.1), BmChp (XP_021206531.1), and BmKat2a (XP_004922629.1), as well as deacetylases BmSirt2 (NP_001036937.1), BmHDAC3 (XP_012552478.1), BmHDAC8 (XP_021205467.1), and BmHDAC1 (XP_04931440.2) in the fat body were evaluated from 5L2D to PP2 stages. Detection showed that the mRNA level of BmP300, an acetylase displaying high homology with mammalian P300, was high during the feeding stage (5L3D) but low during the prepupal stage, showing the opposite trend to autophagy induction (Fig. 2F and Supplementary Fig. S2C). In comparison, the expression of BmHDAC1 was low during the feeding stage, but steady increased during larval–pupal transition, indicating the consistency with autophagy occurrence (Fig. 2F and Supplementary Fig. S2C).

Since the expression level of BmP300 was almost consistent with, while BmHDAC1 was opposite to, the nuclear localization of BmAtg3 and BmAtg8, they were assumed to be involved in the acetylation of BmAtg3 and BmAtg8. SiRNA for BmP300, BmTip60, BmChp, and BmKat2a were co-transfected with BmAtg3-His or FLAG-BmAtg8 in BmN cells. Results showed that siBmP300 treatment significantly decreased the acetylation levels of BmAtg3-His and FLAG-BmAtg8, and also led to their nucleo-cytoplasmic translocation (Fig. 3A–A′ and Supplementary Fig. S2D, E). Conversely, overexpression of BmHDAC1 led to the deacetylation of BmAtg3-His and FLAG-BmAtg8, as well as BmSqstm1 degradation (Fig. 3B). Summarizing, we prove that BmP300 and BmHDAC1 are the two key enzymes in B. mori to catalyze acetylation and deacetylation of BmAtg3 and BmAtg8, respectively.

**BmP300 inhibits while BmHDAC1 promotes autophagy**

Since BmP300 and BmHDAC1 regulate the acetylation status and subcellular localization of BmAtg3 and BmAtg8, their activities in regulating autophagy were further investigated. Knockdown of BmP300 and BmHDAC1 was performed in vivo using RNAi at 5L3D and 12 h before IW, respectively. BmP300 RNAi for 24 h reduced its mRNA levels to ~40% compared to egfp RNAi (Supplementary Fig. S2F). Autophagy, monitored by the degradation of BmSqstm1 and BmAtg8–PE conjugation, was markedly induced, meanwhile, the BmAtg3 and BmAtg8 nuclear localization were significantly decreased along with increased BmAtg8 puncta in the cytoplasm of fat body cells after BmP300 RNAi (Fig. 3C–C′). BmHDAC1 mRNA levels were reduced to ~50% after RNAi, consequently, the decrease of BmSqstm1 and BmAtg3, as well as BmAtg8–PE conjugation were blocked. Moreover, BmAtg3 and BmAtg8 were arrested in the nucleus along with decreased BmAtg8 puncta in the cytoplasm (Fig. 3D–D′ and Supplementary Fig. S2G).

To complement with the RNAi results, chemical compounds to modify the acetylation status in vivo were used. The effects of C646 (a selective inhibitor of mammalian P300), Trichostatin A (TSA, an effective inhibitor of deacetylase Rpd3 and its class I/II HDAC homologs in yeast and mammals), and N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (CTB, a P300/CRBP specific activator) on autophagy and acetylation of BmAtg3 and BmAtg8 were analyzed. Similar to 20E and starvation treatments, C646 administration markedly decreased the acetylation levels of immunoprecipitated BmAtg3-His and FLAG-BmAtg8. The results agreed that BmP300 inhibition resulted in the deacetylation of BmAtg proteins (Fig. 4A). Moreover, treating 5L3D feeding larvae, when autophagy was rarely observed, with different doses of C646 for 24 h revealed the decrease in BmSqstm1 and BmAtg3 protein levels with the simultaneous increase in BmAtg8–PE conjugation, indicating autophagy promotion (Fig. 4B–B′). Immunofluorescent staining showed that the nuclear localization of BmAtg3 and BmAtg8 were significantly decreased, while BmAtg8 cytoplasmic puncta were increased in C646-treated larvae (Fig. 4C). In contrast, different doses of TSA injection into the larvae at early wandering (EW) stage, when autophagy is initiated, resulted in BmSqstm1 and BmAtg3 accumulation, as well as inhibition of BmAtg8–PE conjugation (Fig. 4D–D′); moreover, BmAtg3 and BmAtg8 were mainly accumulated in the nucleus, and a decrease of BmAtg8 puncta in the cytoplasm of fat body cells was observed, suggesting the involvement of class I/II HDAC activities in autophagy occurrence and nucleo-cytoplasmic translocation of BmAtg3 and BmAtg8 (Fig. 4E). Similarly, the administration of different doses of CTB to larvae at EW stage inhibited the degradation of BmSqstm1 and BmAtg3, and decreased BmAtg8–PE formation, along with the nuclear accumulation of BmAtg3 and BmAtg8 (Fig. 4F–F′, G).

Similarly, the exposure of BmN cells to different doses of C646 resulted in a gradual degradation of BmSqstm1, along with an increase in BmAtg8–PE conjugation (Supplementary Fig. S3A, B). In addition, overexpressed BmAtg3 and BmAtg8 were translocated from the nucleus to cytoplasm, where they formed the puncta (Supplementary Fig. S3C). Immunofluorescent staining showed that TSA pretreatment partially blocked 20E-induced nuclear export of BmAtg3 and BmAtg8, as well as BmAtg8 punctation in the cytoplasm (Supplementary Fig. S3D). Finally, TSA or CTB pretreatment reduced 20E-induced autophagy as indicated by the decrease in BmAtg8–PE formation, as well as the accumulation of BmSqstm1, while C646 pretreatment intensively promoted BmSqstm1 degradation and BmAtg8–PE
conjugation (Supplementary Fig. S3E). In summary, BmP300 and BmHDAC1 oppositely regulate the nuclear localization of BmAtg3 and BmAtg8, and thus autophagy induction in B. mori.

BmAtg4 and BmAtg7 are deacetylated during autophagy induction

Atg4 and Atg7 are important mediators involved in the LC3/Atg8–PE ubiquitin-like system and autophagic flux\(^1,12\). Nevertheless, BmAtg4 and BmAtg7 have not been functionally identified in B. mori yet. To investigate their roles in the silkworm, we first performed BmAtg4 and BmAtg7 RNAi in larvae 12 h before IW. The mRNA levels of BmAtg4 and BmAtg7 were reduced to ~50% and ~30% 24 h after RNAi, respectively (Supplementary Fig. S2H, I). Decrease of BmAtg8–PE conjugation and accumulation of BmSqstm1 indicated the blockage of autophagy (Fig. 5A, B). Moreover, both of BmAtg3 and BmAtg8 increased in the nuclei (Fig. 5A’, B’). These results show that BmAtg4 and BmAtg7 are required for the nuclear export of BmAtg3.
and BmAtg8, and thus the autophagic activities of LC3/Atg8–PE ubiquitin-like system in B. mori.

We next investigated whether the subcellular localization of BmAtg4 and BmAtg7 were regulated by 20E or starvation via acetylation. Results showed that starvation-induced deacetylation of both BmAtg4-V5 and BmAtg7-HA (Fig. 5C, D). Moreover, the overexpression of BmAtg4 or BmAtg7 enhanced 20E- or starvation-induced autophagy, and the two Atg proteins were translocated from the nucleus to cytoplasm after 2.5 μM 20E and 4 h starvation treatments in BmN cells (Fig. 5E–E′, F–F′). Finally, the acetylation levels of BmAtg4-V5 and BmAtg7-HA were reduced by 20E, starvation, and 800 nM C646 treatments (Fig. 5G, H).

Fig. 4 P300 and class I/II HDAC activities oppositely regulate BmAtg3 and BmAtg8 nuclear localization and autophagy. A Acetylation levels of BmAtg3-His and FLAG-BmAtg8 after 5 μM 20E, starvation, 800 nM C646, 20 μM TSA, or 20 μM CTB treatment for 6 h in BmN cells. IP immunoprecipitation. B–B′ Protein levels of BmSqstm1, BmAtg3, and BmAtg8 in the fat body after 4.5, 9, 18, 27, or 36 μg/larva C646 treatment for 24 h, 0 μg/larva treatment is used as control (B). Quantification of BmAtg8–PE in B (B′). Significance test was performed between the control and the highest dose. C Immunofluorescent staining of BmAtg3 and BmAtg8 in the fat body after 4.5, 9, 18, 27, or 36 μg/larva C646 treatment for 24 h. Arrows typical treated cells. Scale bar: 10 μm. D–D′ Protein levels of BmSqstm1, BmAtg3, and BmAtg8 in the fat body after 3, 6, 12, or 18 μg/larva TSA treatment for 24 h (D). Quantification of BmAtg8–PE in D (D′). Significance test was performed between the control and the highest dose. E Immunofluorescent staining of BmAtg3 and BmAtg8 in the fat body after 3, 6, 12, or 18 μg/larva TSA treatment for 24 h. Scale bar: 10 μm. F–F′ Protein levels of BmAtg3, BmAtg8, and BmSqstm1 in the fat body after 5.5, 11, 22, or 33 μg/larva CTB treatment for 24 h (F). Quantification of BmAtg8–PE in F (F′). Significance test was performed between the control and the highest dose. G Immunofluorescent staining of BmAtg3 and BmAtg8 in the fat body after 5.5, 11, 22, or 33 μg/larva CTB treatment for 24 h. Scale bar: 10 μm.
Acetylation on lysine residues regulates subcellular localization and autophagic activities of LC3/Atg8–PE ubiquitin-like system

Since the autophagic functions of BmAtg3, BmAtg8, BmAtg7, and BmAtg4 were regulated by acetylation, their potential acetylation sites were further identified by mass spectrometry (MS). Two silkworm-specific acetylation sites at K94 and K195, which were not conserved with yeast Atg3, were identified in purified BmAtg3 (Supplementary Figs. S4A and S5A). In comparison, five acetylation sites at K6, K13, K20, K24, and K46 were identified in BmAtg8. An additional acetylation site, which was predicted to be conserved in LC3B, was found in BmAtg8 at K48 (Supplementary Figs. S4B and S5B–B'). Finally,
three acetylation sites at K125, K237, and K269 in BmA\(\text{t}g4\), and seven acetylation sites at K32, K37, K302, K311, K350, K415, and K515 in BmA\(\text{t}g7\) were identified (Supplementary Figs. S4C, D and S5C, D).

Subsequently, acetylation sites in BmA\(\text{t}g3\), BmA\(\text{t}g8\), and BmA\(\text{t}g4\) were verified. Results showed that single mutation partly attenuate and double mutation of acetylation sites totally abolished BmA\(\text{t}g3\) acetylation, which consequently resulted in its cytoplasmic localization and premature autophagy induction, as indicated by BmA\(\text{t}g3\) immunofluorescent staining, BmSqstm1 degradation, and BmA\(\text{t}g8\)–PE conjugation (Fig. 6A–C–C). Mutation of acetylation sites at K13, K20, K24, or K46/K48 but not K6 partly decreased, while sextuple mutation of acetylation sites abolished, the acetylation of BmA\(\text{t}g8\), which led to completely cytoplasmic localization of BmA\(\text{t}g8\) and premature autophagy induction (Fig. 6D–F–F). Interestingly, most of the double acetylation-site mutated BmA\(\text{t}g3\) and sextuple acetylation-site mutated BmA\(\text{t}g8\) were colocalized in the cytoplasm after their co-overexpression (Supplementary Fig. S6A). Since the regulatory mechanism of acetylation in Atg4 homologs has not been elucidated yet, we further analyzed the acetylation sites in BmA\(\text{t}g4\). Single mutation of acetylation sites at K237 and K269, but not K125, partially decreased acetylation of BmA\(\text{t}g4\); similar to the triple mutation, double mutation of acetylation sites at K237 and K269 totally abolished acetylation of BmA\(\text{t}g4\) (Fig. 6G–6G). Double mutation of BmA\(\text{t}g4\) acetylation sites at K237 and K269 led to its completely cytoplasmic localization and premature autophagy induction (Fig. 6H, 1–1). In addition, Bm\(\text{H}D\)AC1 overexpression led to the deacetylation of BmA\(\text{t}g4\)-c-Myc, showing the deacetylation of BmA\(\text{t}g8\)–PE ubiquitin-like system by Bm\(\text{H}D\)AC1 (Fig. 6I). Overall, deacetylation of the components in BmA\(\text{t}g8\)–PE ubiquitin-like system at lysine residues leads to their nucleo-cytoplasmic translocation.

It is known that the components of LC3/Atg8–PE ubiquitin-like system, such as ATG7 and LC3 are sequestered in the nucleus by acetylation in mammals\(^{11,12,19}\), however, no information is available for ATG4. Results showed that Homo sapiens ATG4b (HsATG4b) overexpression notably promoted starvation-induced LC3-II formation; moreover, HsATG4b overexpression directly induced SQSTM1/p62 degradation and LC3-II formation (Supplementary Fig. S7A, B). Further analysis revealed that starvation induced the deacetylation and nucleo-cytoplasmic translocation of HsATG4b-HA in human embryonic kidney (HEK293) cells (Supplementary Fig. S7C, D). Four acetylation sites were identified in HsATG4b by MS analysis, of which only one site at K137 was conserved with BmA\(\text{t}g4\) at K125 (Supplementary Figs. S6B, C and S7E). Mutation of single acetylation site partially decreased, while quadruple mutation of acetylation sites completely abolished, the acetylation of HsATG4b-HA, and consequently resulted in its completely cytoplasmic localization (Supplementary Fig. S7F, G). Moreover, quadruple mutation of HsATG4b-HA acetylation sites led to the degradation of SQSTM1/p62 and formation of LC3-II under nutrient-rich conditions, and further promoted starvation-induced autophagy (Supplementary Fig. S7H).

Finally, Hs\(\text{H}D\)AC1 overexpression led to the deacetylation of HsATG4b (Fig. S7I). Taken together, our results show that human HsATG4b is deacetylated and shuttled from the nucleus to the cytoplasm to participate in autophagic process under starvation conditions.

Collectively, our data demonstrate that P300-mediated acetylation arrests the components of LC3/Atg8–PE ubiquitin-like system in the nucleus of both silkworm and humans under nutrient-rich conditions. After deacetylation by HDAC1 (which is activated after dephosphorylation induced by 20E/cholesterol derivatives and starvation), they are translocated from the nucleus to the cytoplasm, thus facilitating autophagy induction (Fig. 7).

**Discussion**

**Regulation of autophagy by acetylation/deacetylation is evolutionarily conserved between insects and mammals**

As introduced at the beginning, acetylation/deacetylation of the Atg8–PE ubiquitin-like system displays controversial function in autophagy regulation in yeast and mammals. In yeast, Atg3 and Atg8 are acetylated under nutrient-rich conditions. The acetylase ESA1 facilitates autophagy by catalyzing hyperacetylation of Atg3 under starvation when Atg8 is deacetylated; in contrast, the deacetylase Rpd3 inhibits autophagy\(^{24}\). In mammals, the deacetylases HDAC1 (homologous to yeast Rpd3), HDAC6, and SIRT1 facilitates autophagy, while the acetylase P300 inhibits autophagy\(^{18,27,28,36}\). Importantly, P300/SIRT1-mediated acetylation/deacetylation regulates LC3 subcellular localization, and thus autophagic activities of the Atg8–PE ubiquitin-like system\(^{19,29}\).

The regulatory mechanisms of Atg proteins by acetylation are poorly understood in insects. Although knockdown of acetyl-coenzyme A synthase in Drosophila brain has been shown to enhance autophagy and lifespan, the precise mechanism is not well documented\(^{23}\). Here, we demonstrate that the components of BmA\(\text{t}g8\)–PE ubiquitin-like system are necessary for BmA\(\text{t}g8\)–PE conjugation and autophagy induction in B. mori. BmP300 acetylates the components of the BmA\(\text{t}g8\)–PE ubiquitin-like system and sequesters them in the nucleus, thus inhibits autophagy in B. mori. Conversely, once deacetylated by Bm\(\text{H}D\)AC1, these proteins are transferred from the nucleus to cytoplasm and promote autophagy. Similar to the mammalian LC3, here the BmA\(\text{t}g8\) is studied in detail, including the acetylation sites; and so does BmA\(\text{t}g3\). Notably, BmA\(\text{t}g4\), the sole cysteine protease in this ubiquitin-like system, has been identified as an
acetylated and nuclear localized protein under nutrient condition. Similar to BmAtg4, human ATG4b is subjected to deacetylation and nucleo-cytoplasmic translocation during autophagy induction. Mammalian ATG7 is acetylated by P300 under nutrient-rich conditions, but its subcellular localization and precise acetylation site are not observed. We did not study the details for too many acetylation sites of BmAtg7 were found. Taken together,
results show a consistent acetylation pattern of the Atg8–PE ubiquitin-like system components in response to nutrient status in *B. mori*, displaying a mechanism which is evolutionarily conserved between insects and humans.

Transcriptional and posttranslational regulation of autophagy in insects

In insects, autophagy is mainly regulated at both transcriptional and posttranslational levels. Nutrients and energy are universal upstream signals to elicit autophagy. In insects, the exclusively present development regulator 20E regulates autophagy by inducing Atg gene expression after binding to the EcR-USP receptor. In mammals, transcription factor forkhead box O3 (FoxO3), which is inhibited by insulin signaling, induces a set of Atg gene expressions. In *Helicoverpa armigera*, 20E upregulates the transcriptional activity of FoxO, as well as its nuclear localization, thus autophagy promotion. *dFOXO* mutation significantly reduced the transcription of *Atg1* and *Atg18* in *D. melanogaster*, indicating the involvement of FOXO in autophagy induction at transcriptional level in insects.

In addition, 20E also modulates autophagy by direct antagonizing insulin/insulin-like growth factor signaling and activating the AMP-activated protein kinase–protein phosphatase 2A axis, which both lead to the inhibition of MTOR activity and the initiation of autophagosome formation through phosphorylation modification of Atg1/Atg13 protein complex. Recently, we demonstrated that dephosphorylation of BmHDAC1 and its human homolog HsHDAC1 leads to their nucleo-cytoplasmic translocation after inhibition of MTOR signaling along with autophagy promotion. Here, the precise mechanism of autophagy regulated by acetylation is revealed as that *B. mori* acetyltransferase BmP300 and deacetylase BmHDAC1 oppositely regulate the acetylation levels and subcellular localization of Atg proteins in BmAtg8–PE ubiquitin-like system, and thus autophagy upregulation. In general, the multiple signals including nutrient, energy, and 20E serve as upstream regulators of autophagy through induction of Atg gene expression and acetylation of ATG proteins in insects.

Notably, the nuclear localization of BmAtg3 and BmAtg8 are accumulated after their mutual RNAi or knockout treatment, which are also similar to that of *BmA* or *BmA* RNAi. These results indicate that there may exist an inner operating mechanism of BmA8–PE ubiquitin-like system to maintain the autophagic flux or a feedback between the ubiquitin-like system and autophagy induction. Besides, whether and how other Atg proteins are regulated by acetylation, as well as the autophagic function of other acetylases and deacetylases in insects, deserve further investigation. Several PTMs in addition to phosphorylation and acetylation of Atg protein are reported to regulate their autophagic activities in yeast and mammals, thus whether there are unrevealed PTMs on regulating autophagy is waiting for further exploration.
Materials and methods

Animals and cell culture
Silkworms (Dazao) were reared with fresh mulberry leaves at 25 °C under 14 h light/10 h dark cycle. BmN cells derived from B. mori ovary were cultured at 28 °C in Grace’s Insect Medium (Sigma-Aldrich, MO, USA, G9771) supplied with 10% heat-inactivated fetal bovine serum (AusGeneX, Molendinar, Australia, FBS500-S); HEK293 cells (ATCC, CRL-1573™) were cultured in DMEM medium (Thermo Fisher, MA, USA, 10569044) supplemented with 5% fetal bovine serum. Escherichia coli DH5α (TIANGEN, Beijing, China, CB101-03) was used for subcloning, and E. coli BL21 (TIANGEN, Beijing, China, CB105-02) was used for prokaryotic expressions of proteins.

Plasmid construction and transfection
Full-length of BmAtg3, BmAtg4, BmAtg7, and BmAtg8 were cloned from total cDNA obtained from prepupal B. mori fat body and fused with c-Myc, His, V5, HA, or FLAG, respectively. Then they were inserted into the multiple cloning sites of pIEX4 vectors (Novagen, Beijing, China, 71235-2). The HaATG4αa, b, c, d fused with tag sequence were cloned and constructed into pcMV3 vectors (Sino Biological, Beijing, China, HG22147-UT, HG20407-CY, HG15081-CM, and HG15537-CY). BmN or HEK293 cells were transiently transfected with the respective plasmids using FuGENE® HD Transfection Reagent (Promega, WI, USA, E2311) or TransIntro™ EL Transfection Reagent (TransGen Biotech, Beijing, China, FT201-01), according to the manufacturer’s instruction.

Production of recombinant proteins

Full-length BmAtg3, BmAtg8, BmAtg4, and BmAtg7 were constructed into the ppSUMO vector fused with His and SUMO tags, respectively. BL21 (DE3) cells were transformed with these plasmids and then cultured in 600 mL Luria-Bertani medium at 37 °C until OD600 reached 0.5–0.6. Subsequently, protein expression was induced by incubating the cells with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 25 °C for 10 h. Then, the cells were harvested by centrifugation at 5000 × g for 5 min. The pellet was resuspended in phosphate-buffered saline (PBS; NaCl 8 g/L, KCl 0.2 g/L, Na2HPO4 1.42 g/L, and KH2PO4 0.27 g/L, pH 7.0) supplemented with 1 mg/mL lysozyme and 1 mM PMSF (phenyl methane sulfonyl fluoride). The cell suspension was incubated for 30 min on ice and then lysed by sonication (Vibracell VCX 750 Sonifier, Sonics Inc, Japan). The supernatant was collected by centrifugation at 12,000 × g for 30 min, and then was passed through a Nickel-charged Sepharose column (60 mL, GE Healthcare, Illinois, USA) pre-equilibrated with the binding buffer. After washing with binding buffer (20-fold column volume), the resin column was eluted using 10 mL imidazole elution buffer at different concentrations.

LC–MS/MS

The acetylation sites in BmAtg3, BmAtg4, BmAtg7, and BmAtg8, and HsATG4b were identified by liquid chromatography associated with MS. Recombinant proteins were subjected to trypsin-mediated digestion. The digested peptides were dissolved in 0.1% formic acid and 2% acetonitrile, directly loaded onto a reversed-phase analytical column (Thermo Fisher, MA, USA, 75 μm i.d. × 150 mm, packed with Acclaim PepMap RSLC C18; 2 μm, 100 Å, nanoViper), and separated by a gradient elution buffer. The peptides were analyzed by Nano-Spray Ionization (NSI) linear ion trap tandem MS (Thermo Fisher, MA, USA, Scientific Q Exactive™). Protein identification was performed with MASCOT software (Mascot Soft Web Solution Pvt. Ltd., Uttarakhand, India) and using Uniprot (https://www.uniprot.org/) search for B. mori.

Chemicals and starvation treatments

Twelve hours before initiation of wandering stage (IW), larvae were injected with different doses of TSA (MCE, NJ, USA, HY-15144; 0, 3, 6, 12, and 18 μg/larva) and CTB (Sigma-Aldrich, MO, USA, C6499; 0, 5.5, 11, 22, and 33 μg/larva). Larvae at 5L3D were injected with different doses of C646 (MCE, NJ, USA, 328968-36-1; 0, 4.5, 9, 18, 27, and 36 μg/larva) or 10 μg/larva 20E. The fat body was collected 24 h later for further analyses. BmN cells were treated with different concentrations of 20E (Sigma-Aldrich, MO, USA, H5142; 1, 2.5, and 5 μM) for 6 h, or starved for 2, 4, or 8 h. BmAtg3, BmAtg4, BmAtg7, BmAtg8, or HaATG4b overexpressed cells were treated with TSA (20 μM) for 2 h, C646 (800 nM) for 6 h, or CTB (20 μM) for 2 h, and then collected for further analyses.

RNAi knockdown

For RNA interference, the templates of BmAtg4 and BmAtg7 were amplified by PCR from total cDNA obtained from B. mori fat body. Double-stranded RNA (dsRNA) was generated using the T7 RibomAX™ Express RNAi system (Promega, WI, USA, P1700), according to the manufacturer’s instructions. dsRNA (50 μg/larva) was injected into the larvae 12 h before IW, with egfp dsRNA injection as control. The fat body was collected 24 h after injection for further analyses. All primers used in this study are listed in Table S1.

Gene editing in silkworms

Cas9 mRNA and BmAtg3 or BmAtg8 sgRNA were synthesized, using mMESSAGE mMACHINE T7 Kit (Thermo Fisher, MA, USA, AM1344) and MEGAscript™ T7 Kit (Thermo Fisher, MA, USA, AM1334), respectively.
A mixture of Cas9 mRNA (300 ng/μL) and BmAtg3 sgRNA (150 ng/μL) was injected into silkworm eggs within 6 h after oviposition using a microinjector (Eppendorf, InjectMan®4, Hamburg, Germany). Cas9 mRNA injection was used as control. The embryos were incubated at 25 °C in a humidified incubator until hatching, and then raised with fresh mulberry leaves at 25 °C under 14 h light/10 h dark cycle. The fat body was collected 24 h after IW for further analyses. The genomic segments with length near 500 bp for BmAtg3 and BmAtg8 containing the knockout site were cloned and sequenced.

**Immunoprecipitation and western blotting**

BmN cells overexpressing BmAtg3, BmAtg4, BmAtg7 or BmAtg8, and HEK293 cells overexpressing HsATG4a, b, c or d were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) supplemented with a complete protease inhibitor cocktail (Roche, Basel, Switzerland, 0469313201). The supernatant was incubated with antibodies against BmAtg antibodies at 4 °C for 4 h, and then incubated with protein A/G agarose beads (Thermo Fisher, MA, USA, 20421) overnight according to a standard immunoprecipitation procedure.

Western blotting was performed using antibodies against BmAtg3 (Abclonal Technology, Wuhan, China, 017751D; 1:3000), BmAtg8 (1:3000), BmSqstm1 (Abclonal Technology, Wuhan, China, A18679; 1:3000), c-Myc (Sigma-Aldrich, MO, USA, 11667149001; 1:1000), His (TransGen Biotech, Beijing, China, HT-501; 1:1000), FLAG (Invitrogen, CA, USA, MA1-142; 1:1000), V5 (TransGen Biotech, Beijing, China, HT-401; 1:1000), HA (Santa Cruz Biotechnology, Texas, USA, sc-7392; 1:2000), SQSTM1/p62 (Cell Signaling Technology, MA, USA, 5114; 1:1000), LC3B (Abcam, Cambridge, UK, 192890; 1:2000), and acetylated-l-lysine (Cell Signaling Technology, MA, USA, 9441; 1:2000). LMBN/Lamin B1 (Bioworld Technology, Illinois, USA, AP6001; 1:5000), TUBA1A/ tubulin alpha 1a (Beyotime Biotechnology Co., Ltd., Shanghai, China, AF0001; 1:5000), and ACTB/actin beta (ProMab Biotechnology, Hunan, China, 20270; 1:1000) were used as reference proteins. All western blotting images were acquired using confocal fluorescence microscope equipped with an Olympus digital camera (FV3000, Olympus, Tokyo, Japan). To quantify Alexa Fluor 488-labeled BmAtg3 and BmAtg8, 150–200 fat body cells or a total of 30 overexpressed BmN cells from three independent biological repeats were recorded and analyzed, using ImageJ software.

**Isolation of nuclear and cytoplasmic proteins**

Fat body from day 2 of fifth larval instar (5L2D) and day 2 of prepupa (PP2) were then harvested for nuclear and cytoplasmic extraction using a NE-PERTM Nuclear Cytoplasmic Extraction Reagent Kit (Pierce, Texas, USA, 78833), according to the manufacturer’s instruction. The separated proteins were evaluated with western blotting.

**Lysotracker red staining**

The fat body was collected, thoroughly washed with PBS (pH 7.0, 0.1 M), and then stained with Lysotracker Red dye (Invitrogen, CA, USA, L7528, 50 nM) for 5 min at 37 °C, as previously described. All images were taken using confocal fluorescence microscope (Olympus FV3000). Three independent experiments were performed.

**Transmission electron microscopy analysis**

The fat body was collected and fixed in 2.5% glutaraldehyde at least for 24 h at 4 °C, and then samples were processed, as previously described. Specimens were analyzed under a TEM to observe autolysosomes and autophagosomes, three independent biological repeats were conducted.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted using RNeasy Plus reagent (TaKaRa, Dalian, China, 9108). cDNA was obtained by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China,
RR047A). qRT-PCR was performed as previously described\(^{30,42}\). *Rpa49* was used as reference gene. PCR primers used are listed in Table S1.

**Statistics analysis**

One-way analysis of variance was performed by SPSS, *p* < 0.05, ** *p* < 0.01, and *** *p* < 0.001. The values are shown as mean ± standard deviation of three independent experiments. Ten animals were used for each repeat, and three biological replicates were conducted.

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L.T., S.L., and Y.C. designed the study; M.W. and K.L. conducted most of the experiments, S.G., J.X., S.L., and X.X. performed parts of experiments; Z.H., Y.Z., L.T., S.L., and Y.C. designed the study; M.W. and K.L. conducted most of the experiments available at https://doi.org/10.1038/s41420-021-00513-0.

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**Supplementary information**

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