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Involvement of acetylation of ATG4B in controlling autophagy induction

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ABSTRACT

ATG4B, a cysteine protease promoting autophagosome formation by reversibly modifying Atg8-family proteins, plays a vital role in controlling macroautophagy/autophagy initiation in response to stress. However, the molecular mechanism underlying the regulation of ATG4B activity is far from well elucidated. In the current study, we firstly revealed that the acetylation level of ATG4B at lysine residue 39 (K39) is strongly involved in regulating its activity and autophagy. Specifically, SIRT2 deacetylates ATG4B K39, enhancing ATG4B activity and autophagic flux, which can be antagonized by EP300/p300. Starvation treatment contributes to EP300 suppression and SIRT2 activation, promoting the deacetylation of ATG4B K39, which leads to the elevation of ATG4B activity and finally autophagy initiation. Mechanistic investigation showed that starvation reduces CCNE (cyclin E), resulting in the downregulation of the CCNE-CDK2 protein complex, decreasing the phosphorylation of SIRT2 Ser331 and finally activating SIRT2. In addition, we confirmed that SIRT2 promotes autophagy via suppressing acetylation of ATG4B at K39 using *sirt2* gene knockout (*sirt2*$^{-/-}$) mice. Collectively, our results have revealed the acetylation-mediated regulation of ATG4B cysteine protease activity in autophagy initiation in response to nutritional deficiency.

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(Macro)autophagy, a lysosome-dependent cellular degradation process, is a tightly regulated catabolic process whereby cells degrade their constituents to dispose of unwanted cytoplasmic elements and recycle nutrients for cellular remodeling when facing unfavorable environmental changes. Intracellular materials targeted for autophagic destruction are sequestered into newly synthesized double-membrane compartments termed phagophores, that mature into autophagosomes, which are subsequently delivered to lysosomes for degradation. The biogenesis of the autophagosome involves two sequential ubiquitin-like conjugation steps: one involving ATG12–ATG5 and the other involving Atg8-family protein (MAP1LC3/LC3 and GABARAP subfamilies) conjugation to phosphatidylethanolamine (PE). With respect to the latter, ATG4 cysteine proteases cleave the LC3 precursors (pro-LC3) to reveal a glycine residue for conjugation with the amino group of PE in the cell membrane. ATG4 proteases also deconjugate LC3 homologs from the autophagosome outer membrane. The membrane-conjugated form of LC3 (LC3-II) plays a vital role in engulfing autophagic cargoes by the phagophore. There are four ATG4 isoforms have been described in mammals (ATG4A to ATG4D), among which ATG4B displays a highly selective preference toward its substrate LC3. Recently, the impaired activity or level of ATG4B has been demonstrated to be involved in various physiological and pathological process such as development, inflammation and cancer. Therefore, there is no doubt that unveiling the mechanism of ATG4B activity regulation will aid us in exploring novel therapeutic strategies for ATG4B-related diseases.

Acetylation, here meaning acetylation of the ε-amino group of lysine residues, is a key post-translation modification/PTM in regulating protein functions. Importantly, a plethora of regulators and autophagic machinery key components such as ULK1, ATG5, ATG7, Atg8-family proteins, ATG12, SQSTM1 and LC3 are regulated by acetylation. However, whether ATG4B, a key "scissor" for processing pro-LC3 and lipidated LC3 to drive the autophagy progress under starvation conditions can be regulated by acetylation remains unclear. To this end, we recently observed that acetylated ATG4B dramatically decreases during starvation and characterized the corresponding mechanism *in vitro* and *in vivo* [1]. We first detected whether acetylated ATG4B can be influenced after starvation. Interestingly, we found that nutrient deprivation significantly decreases the acetylation of ATG4B. We next identified the acetylation sites on ATG4B using mass spectrometry analysis. Although several potential acetylation sites (including K39, K137, K153, K154, K244 and K259) on ATG4B have been detected, only the K39 site of ATG4B influences its activity and biogenesis of autophagosomes. As is known to us, there are seven mammalian homologs of yeast Atg8: MAP1LC3A/LC3A (microtubule associated protein 1 light chain 3 alpha), LC3B, LC3B2, LC3C, GABARAP (GABA type A receptor-associated protein), GABARAPL1

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and GABARAPL2. Therefore, we examined whether acetylated ATG4B K39 correlates with lipidation of GABARAPL2. Interestingly, we found that ATG4B K39 acetylation has a subtle influence on the lipidation of GABARAPL2, suggesting that deacetylated ATG4B K39 preferably processes LC3 rather than GABARAPL2. Subsequently, we made a polyclonal antibody against acetylated ATG4B K39. This antibody is applicable to immunoblotting, immunofluorescence and immunohistochemical approaches. Using this antibody, we observed that starvation significantly reduces acetylated ATG4B K39 *in vitro* and *in vivo*. From the above results, we concluded that starvation downregulates ATG4B K39 acetylation, enhancing ATG4B cysteine protease activity, which finally promotes autophagy.

We further explored the acetyltransferase (KAT)-mediated ATG4B K39 acetylation. Previous studies report that five acetyltransferases, including EP300 (E1A binding protein p300), CREBBP/CBP (CREB binding protein), KAT2B/PCAF (lysine acetyltransferase 2B), KAT8/MOF/MYST1 and KAT2A/GCN5 are closely related to autophagy in mammals. Therefore, we transfected five siRNAs targeting the above acetyltransferases individually into HepG2 cells. The results showed that EP300 knockdown decreases acetylated ATG4B K39 and upregulates ATG4B activity. Moreover, we identified the deacetylase that mediates the deacetylation of ATG4B using four siRNAs targeting *SIRT1*, *SIRT2*, *SIRT3* or *SIRT6*. We found that knockdown of SIRT2 markedly increases acetylated ATG4B K39. Collectively, these results indicate that EP300 acetylates KTG4B K39, whereas SIRT2 is the deacetylase of ATG4B at K39. However, it is still unclear why acetylated ATG4B K39 suppress autophagy whereas deacetylated ATG4B K39 promotes autophagy. To this end, the expression plasmids for K39 R mutation of ATG4B (mimicking the loss of acetylation of ATG4B) and K39Q mutation of ATG4B (mimicking an acetylated state of ATG4B) were constructed. Interestingly, we found that $ATG4B^{K39\,R}$ mainly interacts with pro-LC3 while $ATG4B^{K39Q}$ almost has no interaction with pro-LC3, indicating that deacetylated ATG4B K39 has higher affinity for its substrate pro-LC3. We also found that the level of acetylated ATG4B is negatively related to the level of LC3-II in cells. Combined with the above data, we speculate that deacetylated ATG4B K39 leads to the activation of ATG4B, enhancing its binding with pro-LC3 and promoting the generation of LC3-I by cleaving pro-LC3; then, LC3-I is further turned into LC3-II rapidly, which finally promotes autophagy induction. Additionally, we explored whether SIRT2 is involved in the regulation of starvation-induced autophagy via deacetylating ATG4B. We found that SIRT2 inhibition weakens starvation-induced autophagy and the deacetylation of ATG4B, indicating that starvation promotes ATG4B deacetylation and autophagy via activating SIRT2. Last, we explored the mechanism by

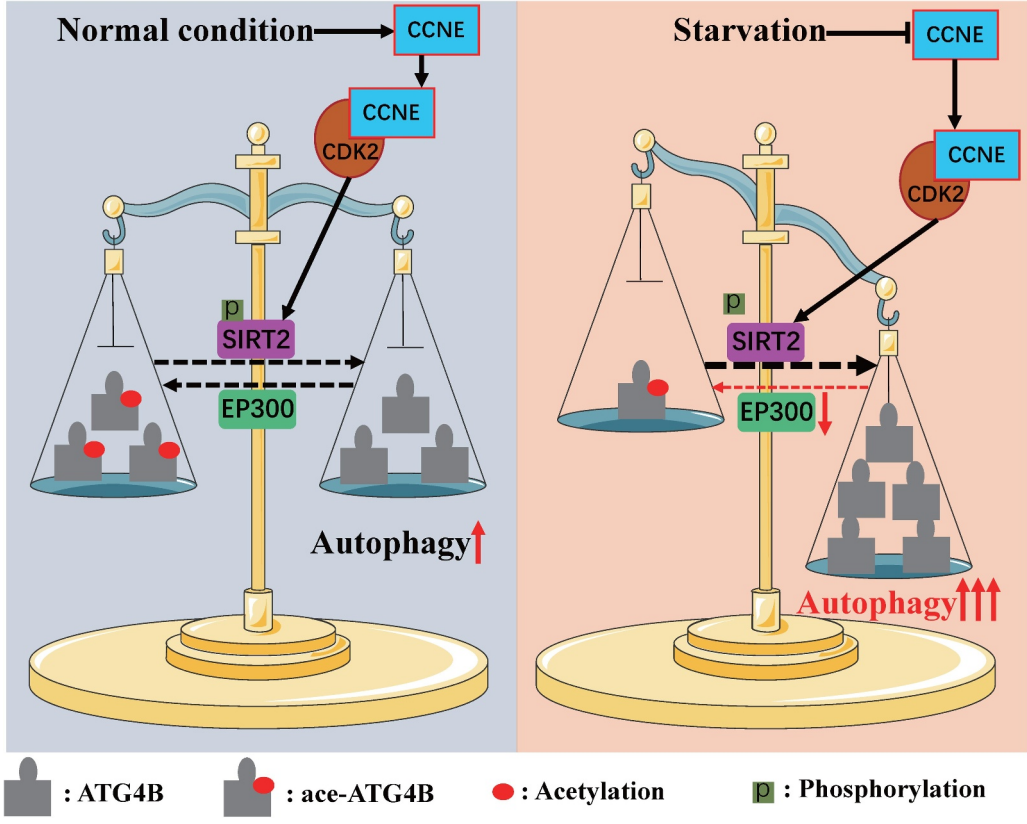

Figure 1. A proposed model illustrating the molecular mechanism of ATG4B acetylation/deacetylation and its role in controlling autophagy. Under basal conditions, the acetylation and deacetylation of ATG4B is sustained in a dynamic balance by EP300 and SIRT2, respectively to maintain the basal level of autophagy. Specifically, CCNE forms a protein complex with CDK2, phosphorylating SIRT2 at the Ser331 site and suppressing its activity to deacetylate ATG4B, which limits ATG4B cysteine protease activity and subsequent autophagy induction. Upon nutrition deprivation, the balance between acetylated and deacetylated ATG4B is broken, which immediately triggers autophagy. Briefly, nutrient deprivation on one side downregulates EP300, and on the other side activates SIRT2 by suppressing the CCNE-CDK2 protein complex-induced phosphorylation of SIRT2 Ser331, promoting the deacetylation of ATG4B K39, which leads to the elevation of ATG4B activity and autophagy induction.

which starvation activates SIRT2 and found that SIRT2 is activated by the downregulation of the CCNE-CDK2 complex under starvation conditions, which leads to the deacetylation of ATG4B K39.

In summary, our study presents three novel findings: (1) ATG4B K39 acetylation regulates its activity and autophagy initiation; (2) EP300 is the KAT, whereas SIRT2 is the deacetylase that mediates the acetylation of ATG4B K39; (3) starvation treatment contributes to the EP300 suppression and SIRT2 activation, deacetylating ATG4B K39, which enhances ATG4B activity and finally autophagy (Figure 1). Mechanistic investigation revealed that starvation downregulates CCNE, leading to the reduction of the CCNE-CDK2 protein complex, decreasing the phosphorylation of SIRT2 S331 and subsequently activating SIRT2. In this study, we for the first time identify an acetylation-dependent regulatory mechanism governing ATG4B activity and function in autophagy, which may supply a clinical opportunity for treating ATG4B- and autophagy-related diseases.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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