Biochemical and Biophysical Research Communications 441 (2013) 970–975

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

LC3B-II deacetylation by histone deacetylase 6 is involved in serum-starvation-induced autophagic degradation

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ARTICLE INFO

Article history:
Received 30 October 2013
Available online 9 November 2013

Keywords:
Autophagic degradation
Histone deacetylase 6
Microtubule-associated protein 1 light chain 3
Deacetylation

ABSTRACT

Autophagy is a conserved mechanism for controlling the degradation of misfolded proteins and damaged organelles in eukaryotes and can be induced by nutrient withdrawal, including serum starvation. Although differential acetylation of autophagy-related proteins has been reported to be involved in autophagic flux, the regulation of acetylated microtubule-associated protein 1 light chain 3 (LC3) is incompletely understood. In this study, we found that the acetylation levels of phosphotidylethanolamine (PE)-conjugated LC3B (LC3B-II), which is a critical component of double-membrane autophagosome, were profoundly decreased in HeLa cells upon autophagy induction by serum starvation. Pretreatment with lysosomal inhibitor chloroquine did not attenuate such deacetylation. Under normal culture medium, we observed increased levels of acetylated LC3B-II in cells treated with tubacin, a specific inhibitor of histone deacetylase 6 (HDAC6). However, tubacin only partially suppressed serum-starvation-induced LC3B-II deacetylation, suggesting that HDAC6 is not the only deacetylase acting on LC3B-II during serum-starvation-induced autophagy. Interestingly, tubacin-induced increase in LC3B-II acetylation was associated with p62/SQSTM1 accumulation upon serum starvation. HDAC6 knockdown did not influence autophagosome formation but resulted in impaired degradation of p62/SQSTM1 during serum starvation. Collectively, our data indicated that LC3B-II deacetylation, which was partly mediated by HDAC6, is involved in autophagic degradation during serum starvation.

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that plays an important role in the turnover of misfolded or aggregated proteins and damaged organelles in eukaryotic cells [1]. Such self-degradation not only provides nutrients for the maintenance of cellular functions, but also serves as an adaptive mechanism for protecting the organism against various pathologies, such as neurodegeneration, infection, cancer, aging, and heart diseases [2]. Thus, autophagy acts as a pro-survival mechanism for coping with various metabolic stresses, including energy deficiency, nutrient starvation, and growth factor withdrawal [3].

Numerous studies have reported that acetylation plays a significant role in autophagy regulation. The clearance of mutant huntingtin through autophagic degradation can be regulated by the acetylation at its Lys444 residue [4]. In cells deprived of growth factors, glycogen synthase kinase-3 (GSK3) activates acetyltransferase TIP60 through phosphorylating TIP60-Ser86. The activated TIP60 directly acetylates and thereby stimulates the protein kinase ULK1, which is required for autophagy induction [5]. Genetic analysis of *Saccharomyces cerevisiae* also identified Esa1 as a histone acetyltransferase required for autophagy [6]. Thus, the acetylation of autophagy-related proteins plays an important role in regulating autophagic flux. It has reported that the acetyltransferase p300 can acetylate Atg5, Atg7, Atg8 (the yeast homolog of the mammalian LC3 gene product) and Atg12 proteins; acetylation by p300 inhibits autophagy, whereas silencing of p300 increases autophagic flux [7]. Deacetylation of Atg8 is regulated by Sirt1, a well-known histone deacetylase [8]. LC3 is a core component of autophagosome and functions as an adaptor for delivering the cargoes to autophagosomes. Newly synthesized LC3 precursor was processed to form a soluble LC3 (LC3-I) with a C-terminal glycine residue [9]. Upon autophagy induction, LC3-I is conjugated with phosphatidyzlethanolamine (PE) through its C-terminus to form PE-conjugated LC3 (also known as LC3-II) that is tightly associated with the

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http://dx.doi.org/10.1016/j.bbrc.2013.11.007

autophagosomal membrane [10]. LC3-PE mediates the degradation of autophagosome contents by their fusion with lysosomes. However, such a process requires cleavage and removal of LC3-PE by Atg4 prior to the fusion, at least in yeast. Inability to carry out the second cleavage confines LC3-PE at the outer surface of the autophagosome, which prevents the release of LC3 from PE and the fusion of autophagosomes with lysosomes thus limiting autophagic degradation of their contents [9,11]. Thus, protein modification of LC3 may regulate the final completion of autophagic degradation.

HDAC6 is a member of histone deacetylases (HDACs), which are potential regulators for autophagy. There are four classes of HDACs: class I (HDACs 1–3 and 8), class II (HDACs 4–7, 9 and 10), class III (the Zn-independent, NAD-dependent deacetylases Sirts 1–7), and class IV (HDAC11) [12]. By deacetylating α-tubulin, HDAC6 modulates retrograde transport of aggregate-containing inclusion bodies to be degraded via autophagy [13]. Parkin-mediated clearance of damaged mitochondria also requires the participation of HDAC6 [14]. It is also reported that HDAC6 recruits and deacetylates cortactin, thereby promoting F-actin remodeling important for autophagosome–lysosome fusion and protein aggregate clearance [15]. In the present study, we observed that LC3B-II, which is essential for autophagy, was markedly deacetylated during serum-starvation-induced autophagy in HeLa cells. High levels of LC3B-II acetylation, upon inhibiting the deacetylase activity of HDAC6 by tubacin or siRNA knockdown, were correlated with impaired degradation of p62/SQSTM1 during serum starvation, suggesting a linkage between LC3B-II deacetylation and autophagic degradation.

2. Materials and methods

2.1. Reagents

Chloroquine (CQ), rapamycin, tubacin and Hoechst33342 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rapamycin was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) and kept at −20 °C. The final concentration of DMSO never exceeded 0.2%, which had no cytotoxicity on cells. Dulbecco's modified Eagle's medium (DMEM), antibiotics, fetal bovine serum (FBS), and Lipofectamine RNAi MAX were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membranes (Hybond-P) and Protein A-Sepharose were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Enhanced chemiluminescence (ECL) kit was obtained from Beyotime (Haimen, China). Antibodies against acetyl-lysine, acetyl-α-tubulin, α-tubulin, LC3B, p62/SQSTM1, HDAC6, and HRP-conjugated sheep anti-rabbit IgG were all obtained from Cell Signaling Technology (Danvers, MA, USA). DyLight 488-conjugated LC3B polycolonal antibody was purchased from Pierce (Rockford, IL, USA). HDAC6 siRNA was purchased from Abgent (San Diego, CA, USA).

2.2. Cell culture and transfection

HeLa cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and maintained in DMEM supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. HeLa cells were seeded in 6-well plates for 24 h. Then cells were treated with HDAC6 siRNA in DMEM with Lipofectamine RNAi MAX for transfection. After 72 h, cells were used for experiments.

2.3. Immunofluorescence microscopy

Immunofluorescence was performed essentially as previously reported [16]. In brief, cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. The permeabilized cells were incubated with appropriate primary antibodies at 4 °C overnight. After PBS wash, cells were incubated with CF488-conjugated goat-anti-mouse IgG or CF568-conjugated goat-anti-rabbit IgG (Biotium, Hayward, CA, USA) at room temperature for 1 h. Nuclei were revealed by Hoechst33342 staining. Fluorescence images were collected under a Leica DMIRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany) armed with a Spinning Disk Confocal Microscopy system (UltraView cooled CCD; Perkin Elmer, Waltham, MA, USA).

2.4. Protein extraction

Cells were washed thoroughly with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime) for assaying degradation of proteins and immunoprecipitation [17]. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples were subjected to Western blotting or immunoprecipitation.

2.5. Immunoprecipitation and Western blotting

Protein lysates (200 μg) were prepared from HeLa cells and mixed with the indicated antibodies (4 °C overnight) followed by incubation with 30 μl of Protein A-Sepharose (Cell Signaling Technology) at 4 °C for 2 h. Immune complexes were washed five times with lysis buffer (Beyotime). Then samples were boiled in 2× loading buffer and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes. After incubation in blocking buffer (50 mM Tris-buffered saline (pH7.4) containing 5% non-fat milk and 0.1% Tween-20), the membranes were probed with indicated antibodies, followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Bands were revealed by ECL and recorded on X-ray films (Kodak, Xiamen, China). Images were acquired by using FluorChem 8000 (AlphaInnotech, San Leandro, CA, USA).

3. Results

3.1. Serum starvation induced autophagy in HeLa cells

As serum-deprivation is an effective stimulus to induce autophagy in HeLa cells [18], we used this cellular model to study serum-starvation-induced autophagy. To monitor autophagy induction, we assayed the accumulation of LC3B-II and the formation of LC3B puncta in HeLa cells cultured under serum-deprived medium. Western blot analysis revealed that serum starvation time-dependently increased the level of LC3B-II compared to control (0 h), and culminated in its peak level at 8 h (Fig. 1A). Moreover, serum deprivation in the presence of chloroquine (CQ), a lysosomal inhibitor that blocks autophagic degradation, induced a higher level of LC3B-II compared to serum deprivation alone (Fig. 1B). These results confirmed an increased autophagic flux of LC3B-II during serum starvation. In support of this, we observed a robust formation of LC3B puncta in serum-deprived cells compared to control, and more LC3 puncta in the presence of CQ (Fig. 1C). These data indicated that 8 h serum starvation induced a marked autophagy in HeLa cells, thus this time point was adopted for the following experiments.

3.2. Acetylation levels of LC3B-II were decreased during serum-starvation-induced autophagy

To evaluate the involvement of LC3 acetylation during autophagy, we next tested whether the acetylation levels of LC3B are regulated upon autophagy induction by serum starvation. The

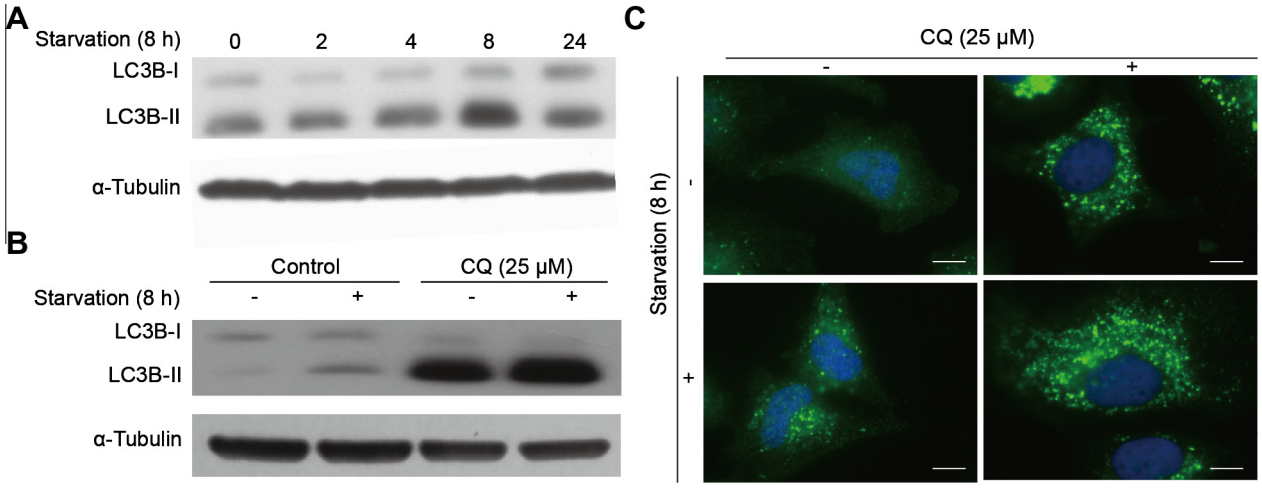

Fig. 1. Induction of autophagy by serum withdrawal in HeLa cells. (A) Western blot analysis showing the time course of LC3B-II levels upon autophagy induction by serum deprivation. α-Tubulin was used as a loading control. (B) Western blotting showing enhanced accumulation of LC3B-II when cells were deprived of serum for 8 h in the presence of chloroquine (CQ). (C) Fluorescence microscopy analysis showing LC3B puncta formation by serum starvation for 8 h with or without CQ. Scale bars, 10 μm.

acetylation levels of LC3B were detected by Western blotting using antibody against acetyl-lysine residues after immunoprecipitation of LC3B with its specific antibody. As shown in Fig. 2A and B, both LC3B-I and LC3B-II were highly acetylated under normal culture condition. In contrast, the acetylation levels of LC3B-II were markedly reduced upon serum starvation or rapamycin treatment, indicating that the acetylation of LC3B-II was associated with autophagy induction. Moreover, the acetylation levels of LC3B-II were also reduced by CQ treatment, and were further reduced when treated with CQ in combination with serum deprivation (Fig. 2C). These results indicated that LC3B-II was deacetylated upon serum-starvation-induced autophagy.

3.3. High levels of LC3B-II acetylation upon inhibition of HDAC6 by tubacin correlated with impaired autophagic degradation

As both HDAC6 and the autophagosome decorated with LC3-II are mainly distributed in the cytoplasm, we sought to explore whether HDAC6 is involved in the regulation of LC3B-II acetylation. To address this, we used a HDAC6-specific inhibitor tubacin, which can specifically bind to the catalytic domain of HDAC6 and thus inhibits its deacetylase activity [19]. In view of the evidence that HDAC6 inhibitors can induce hyperacetylation of α-tubulin in cells [20], we thus monitored the HDAC6 activity upon culture with or without tubacin by assaying the acetylation levels of α-tubulin. Treatment with tubacin culminated in a time- and dose-dependent increase in the acetylation levels of α-tubulin along with increased accumulation of LC3B-II as compared with control (Fig. 3A). Although LC3B-II levels were increased and autophagosomes were induced by tubacin treatment (Fig. 3B), CQ co-treatment did not further increased LC3-II accumulation (Fig. 3C) and serum-deprivation-induced p62/SQSTM1 degradation was attenuated in the presence of tubacin (Fig. 3D), indicating a blockage of autophagic degradation [21]. In addition, the acetylation levels of LC3B-II were markedly increased by tubacin treatment compared with control cells, whereas tubacin-induced increase in LC3B-II acetylation was partly counteracted by serum starvation (Fig. 3E), suggesting that other deacetylases than HDAC6 may also regulate LC3B-II acetylation upon serum-starvation-induced autophagy. Together, these results demonstrated that LC3B-II acetylation was involved in autophagic degradation during serum starvation and that HDAC6 was at least partly responsible for LC3B-II deacetylation.

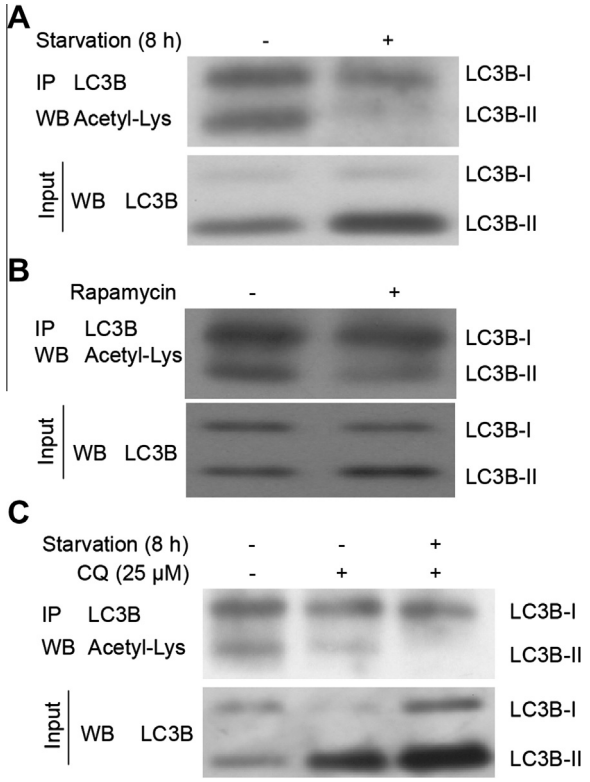

Fig. 2. Decline of LC3B-II acetylation levels upon autophagy induction. (A) Analysis of acetylation levels of endogenous LC3B-II after serum starvation in HeLa cells. LC3B proteins were immunoprecipitated (IP) with a LC3B-specific antibody and their acetylation levels were detected by Western blotting (WB) with an antibody against acetyl-lysine residues. (B) Acetylation levels of LC3B-II in cells treated with or without rapamycin (200 ng/ml) were determined in HeLa cells as in (A). (C) Acetylation levels of LC3B-II in HeLa cells treated with chloroquine (CQ) in the presence or absence of serum-starvation were determined in HeLa cells as in (A).

3.4. HDAC6 knockdown attenuated LC3B-II deacetylation and autophagic degradation

To further confirm the involvement of HDAC6 in LC3B-II deacetylation, we knocked down the expression of HDAC6 in HeLa cells by small interfering RNA (siRNA). The efficiency of HDAC6

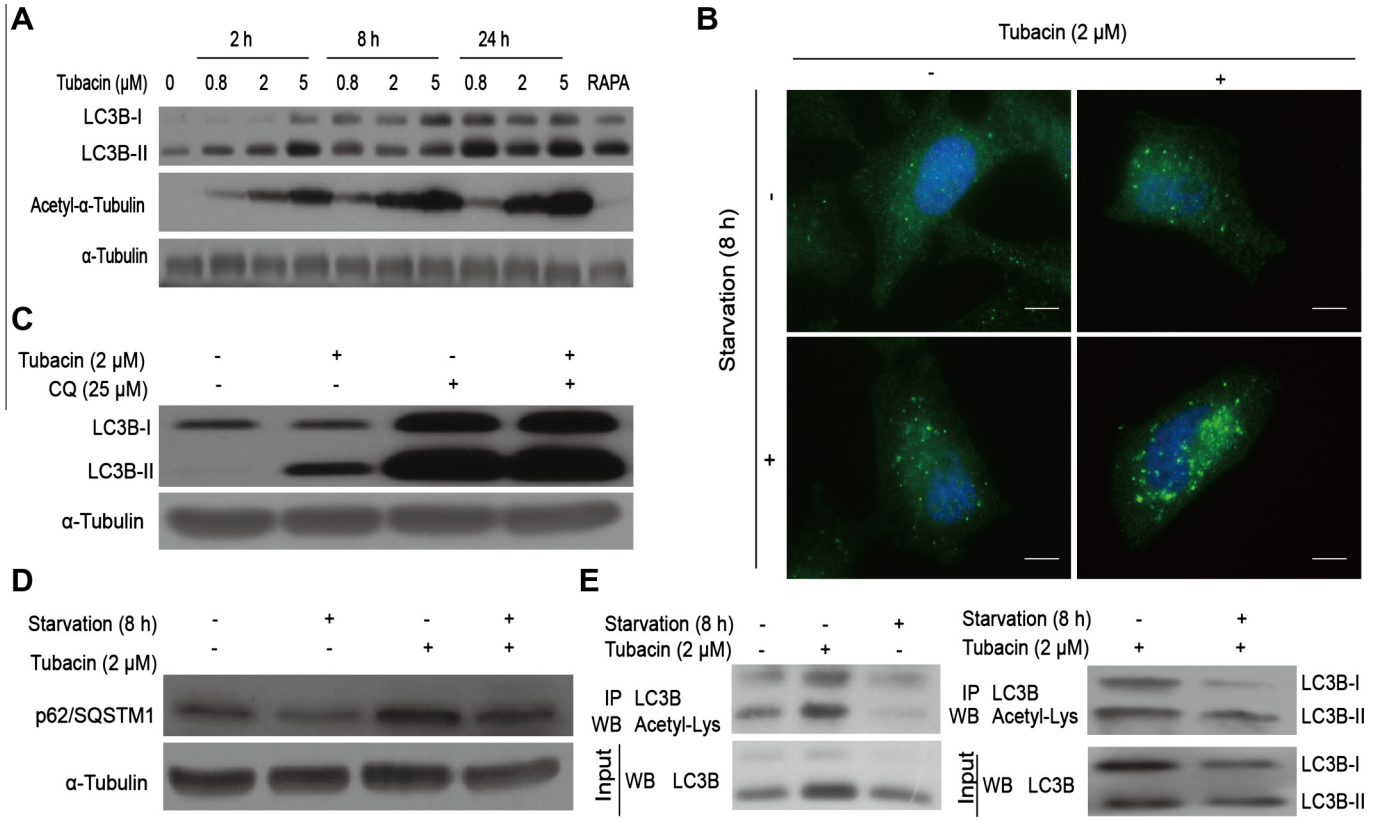

Fig. 3. Involvement of HDAC6 in the regulation of LC3B-II acetylation upon serum starvation. (A) Western blot analysis of HDAC6 activity by the level of acetylated α-tubulin and LC3B accumulation in HeLa cells treated with or without tubacin. (B) Fluorescence microscopy analysis showing LC3B puncta formation by serum starvation for 8 h with or without tubacin. Scale bars, 10 μm. (C) Western blot analysis of LC3B levels in HeLa cells treated with or without tubacin in the presence or absence of CQ. (D) Western blot analysis of p62/SQSTM1 accumulation in HeLa cells cultured in serum withdrawal condition with or without tubacin. (E) Determination of the acetylation levels of endogenous LC3B-II after tubacin treatment with or without serum starvation. LC3B proteins were immunoprecipitated (IP) and their acetylation levels were detected by Western blotting (WB) with an antibody against acetyl-lysine residues.

knockdown was validated by Western blot analysis (Fig. 4A). After HDAC6 knockdown, LC3B-II conversion and LC3B puncta formation were slightly increased compared to cells treated with control siRNA, but their p62/SQSTM1 levels showed no difference (Fig. 4A and B). Consistent with the result of tubacin treatment, HDAC6 knockdown also suppressed serum-starvation-induced degradation of p62/SQSTM1 concomitant with increased LC3B-II acetylation levels compared with control siRNA (Fig. 4A). In support of the tubacin assay, HDAC6 knockdown only partly blocked LC3B-II deacetylation. These results confirmed that HDAC6 was partly responsible for LC3B-II deacetylation during serum starvation, which was required for autophagic degradation in this setting.

We finally sought to explore whether HDAC6, is co-localized with LC3B puncta. Immunofluorescence revealed that a substantial proportion of diffuse HDAC6 appeared to co-localize with LC3B in cells cultured under normal condition (Fig. 4C). Interestingly, LC3B puncta, indicative of autophagosome formation, did not co-localized with HDAC6 in cells either upon serum starvation or CQ treatment. This result suggested that LC3B-II deacetylation by HDAC6 might occur before or during the autophagosome–lysosome fusion process.

4. Discussion

Acetylation/deacetylation has an important role in regulating autophagy. In this study, we demonstrated that the levels of LC3B-II acetylation were dramatically declined upon serum-starvation-induced autophagy in HeLa cells. This may be at least partly mediated by HDAC6 as inhibition of its activity by tubacin or siRNA-mediated knockdown of its expression prevented the deacetylation of LC3B-II. In view of the observations that chloroquine-treated cells exhibited a decreased level of LC3B-II acetylation and that HDAC6 did not co-localize with LC3B puncta (autophagosomes), it is probable that such deacetylation occurs before or during the autophagosome–lysosome fusion process. Notably, decreased LC3B-II acetylation was concomitant with increased degradation of p62/SQSTM1, indicative of autophagic degradation. Thus, our findings provide a new insight into the role of HDAC6-mediated deacetylation of LC3B-II during autophagy degradation upon serum deprivation.

Several possible mechanisms have been proposed to explain the role of HDAC6 in regulating autophagic degradation. One possibility is that the enzymatic activity of HDAC6 may be involved in the regulation of HSP90, which is a known substrate of this deacetylase [22]; suppression of this activity led to increased acetylation of HSP90, thereby inhibiting the binding of proteins to HSP90 and enhancing their chaperone-mediated autophagic degradation [23,24]. Another possibility relates to the role of HDAC6 in the formation of aggresome. HDAC6 acts as an adaptor to control the pathway shuttling polyubiquitinated substrates to a location which is beneficial to the engulfment by the autophagosome [25]. A third possibility is that HDAC6 may be involved in the transport of lysosomes to the sites of autophagy [26], which is supported by the observation that HDAC6 knockdown resulted in dispersal of lysosomes away from the microtubule organizing centre [13]. Whereas much is known about the adaptor role of HDAC6 in the protein turnover via autophagy, less is understood about this

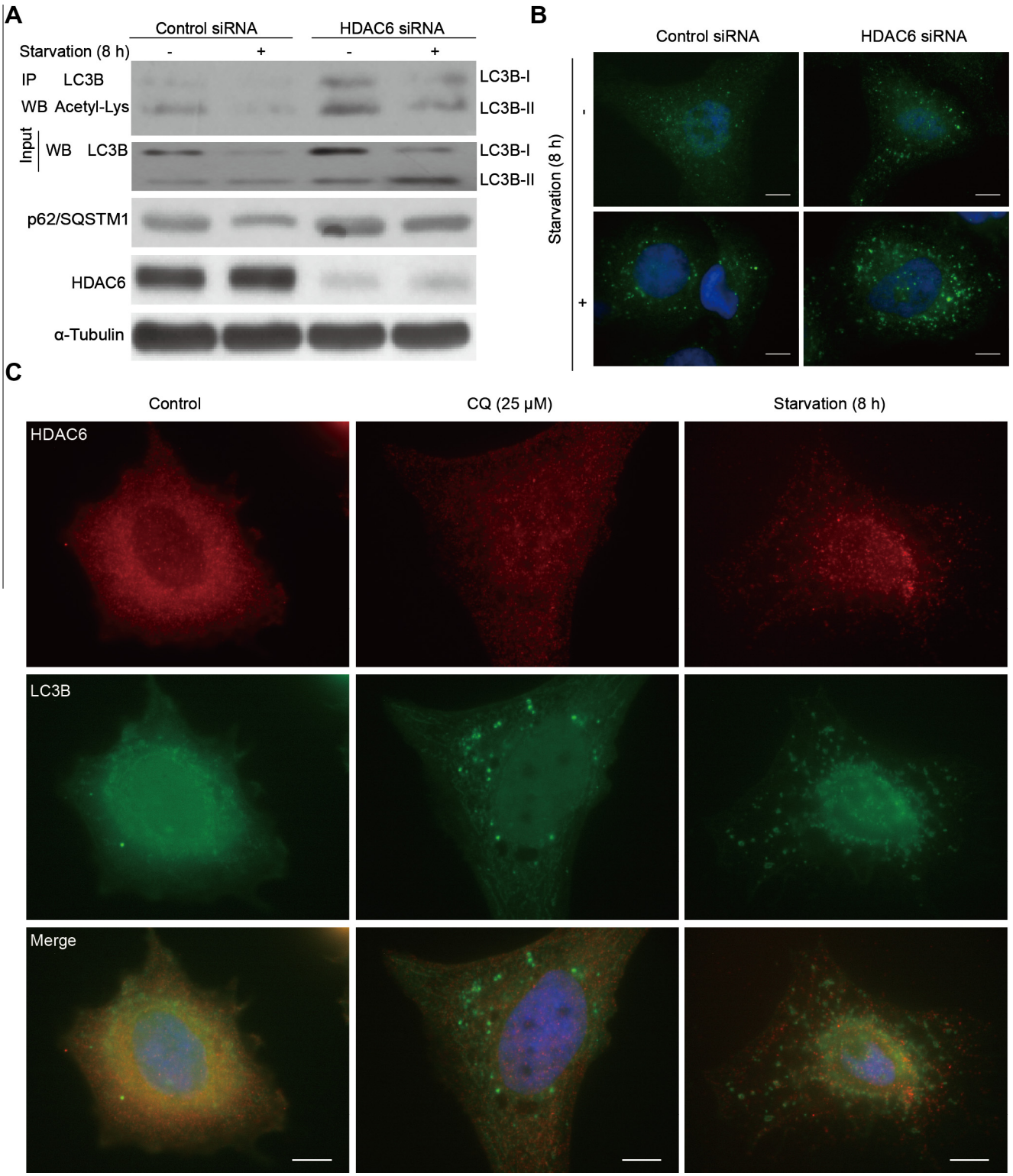

Fig. 4. LC3B-II acetylation and autophagic degradation in HeLa cells after HDAC6 knockdown (A) Levels of LC3B-II acetylation and p62/SQSTM1 upon serum-starvation-induced autophagy in HeLa cells treated with or without HDAC6 siRNA. (B) Fluorescence microscopy analysis showing LC3B puncta formation, which was assessed in the presence or absence of serum for 8 h in HeLa cells with or without HDAC6 siRNA transfection. (C) Distribution of HDAC6 and LC3B in HeLa cells. After cultured under indicated conditions for 8 h, the subcellular distribution of endogenous LC3B (green), HDAC6 (red), and their overlap (yellow) were observed by immunofluorescence microscopy. Nuclei were revealed by Hoechst33342 staining. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HDAC acting as a deacetylase in the regulation of autophagic flux. In this study, we found that LC3B-II deacetylation was reduced concomitant with increased p62/SQSTM1 degradation upon serum withdrawal in HeLa cells whereas HDAC6 inhibition by tubacin or by siRNA-mediated knockdown partially blocked LC3B-II deacetylation as well as p62/SQSTM1 degradation. It is reported that

HDAC6 recruits and deacetylates cortactin, thereby promoting F-actin remodeling important for autophagosome–lysosome fusion and protein aggregate clearance [15], suggesting that blockage of autophagosome–lysosome fusion by removing HDAC6 is attributed to suppressed deacetylation of cortactin. However, according to this same report, HDAC6 and F-actin assembly were dispensable for starvation-induced autophagy, indicating that HDAC6-mediated cortactin deacetylation is not involved in this process [15]. Thus, under the setting of serum starvation in this study, HDAC6-mediated deacetylation of LC3B-II, instead of cortactin, plays a role in regulating autophagic degradation. Taken together, in addition to cortactin, LC3B-II deacetylation mediated by HDAC6 is also involved in autophagic degradation.

As our current results showed that HDAC6 only partially deacetylated LC3B-II during serum starvation, we proposed that other deacetylases are likely involved in this process. To support this hypothesis, it has been reported that, as a deacetylase of Atg8 (homologue of LC3), Sirt1 knockout in mouse embryonic fibroblasts (MEFs) led to hyperacetylation of Atg8 and impaired degradation of p62/SQSTM1 [8]. Thus, both HDAC6 and Sirt1 may mediate the deacetylation of LC3B upon serum deprivation, which probably act together to promote efficiently the autophagic degradation even though further investigations are warranted to uncover the precise action mechanism for this process.

Although our data suggested that LC3B-II deacetylation might occur before or during the autophagosome–lysosome fusion process, it is unclear how CQ alone induced such deacetylation. One possible explanation is that CQ treatment blocks autophagic degradation, thereby activating potential pathway(s) leading to LC3B-II deacetylation. The pathway(s) underlying this process remains to be defined.

In summary, we demonstrated that HDAC6 was partly responsible for LC3B-II deacetylation upon serum starvation, which is involved in autophagic degradation. HDAC6-mediated regulation of the acetylation status of autophagy-related proteins may be an important way to modulate autophagic self-digestion and provides a potential strategy for the therapeutic intervention of various diseases.

Acknowledgments

This study was supported by Grants from the Specialized Research Program of "Twelfth Five-Year Plan" of China (No. 2011ZX09307-303-03), the Major State Basic Research Development Program of China (973 Program) (No. 2010CB833603) and the Science and Technology Planning Project of GuangdongProvince of China (No.2011B031600001).

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