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# CCCP-Induced LC3 lipidation depends on Atg9 whereas FIP200/Atg13 and Beclin 1/Atg14 are dispensable

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ABSTRACT

Treatment of cells with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial proton gradient uncoupler, can result in mitochondrial damage and autophagy activation, which in turn eliminates the injured mitochondria in a Parkin-dependent way. How CCCP mobilizes the autophagy machinery is not fully understood. By analyzing a key autophagy step, LC3 lipidation, we examined the roles of two kinase complexes typically involved in the initiation and nucleation phases of autophagy, namely the ULK kinase complex (UKC) and the Beclin 1/Atg14 complex. We found that CCCP-induced LC3 lipidation could be independent of Beclin 1 and Atg14. In addition, deletion or knockdown of the UKC component FIP200 or Atg13 only led to a partial reduction in LC3 lipidation, indicating that UKC could be also dispensable for this step during CCCP treatment. In contrast, Atg9, which is important for transporting vesicles to early autophagosomal structure, was required for CCCP-induced LC3 lipidation. Taken together, these data suggest that CCCP-induced autophagy and mitophagy depends more critically on Atg9 vesicles than on UKC and Beclin 1/Atg14 complex.



## 1. Introduction

Macroautophagy (hereafter referred to as autophagy) represents an evolutionarily conserved self-degradation process, in which cellular constituents are sequestered into double-membraned autophagosomes and delivered to the lysosome for hydrolytic digestion [1,2]. The autophagy machinery is controlled by autophagy-associated (Atg) proteins. More than 30 Atg genes have so far been characterized in yeast [1–3]. The mammalian homologues of most Atg proteins have been identified. Among them, the mammalian ULK kinase complex (UKC) is composed of ULK1/ULK2, Atg13, focal adhesion kinase family interactional protein of 200 kD (FIP200) and Atg101, which can be regulated by metabolic signals through the mammalian target of rapamycin (mTOR) [4,5]. The autophagy-specific class III phosphatidylinositol 3-kinase complex (PI-3KCIII), or the Beclin 1/Atg14 complex, has been identified as a Beclin 1-Atg14-Vps34-Vps15 complex [6,7]. The hierarchical relationship of Atg proteins has been well established in canonical autophagy, such as that stimulated by nutrient deprivation [8], in which the above two kinase complexes are required for the initiation and nucleation of autophagosomes. Beclin 1 complex plays an essential role in bridging the upstream UKC to the downstream Atg12-Atg5-Atg16 complex [7,9], and the Atg8/microtubule-associated protein light chain 3 (LC3) conjugation system [10]. Finally, vesicle transportation by Atg9-mediated process is critical in the assembly of pre-autophagosomal structure [11].

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is known as an uncoupling agent, increasing the proton permeability across the mitochondrial inner membranes and thus depolarizing the mitochondria. CCCP has been used extensively in recent years to study mitochondrial damage and to induce autophagic degradation of damaged mitochondria (i.e. mitophagy) [12]. Previous studies demonstrated that CCCP can induce autophagy via a ROS-mediated mechanism [13], and anti-oxidants, such as N-acetyl cysteine, can inhibit autophagy induced by CCCP. CCCP-induced autophagy and LC3 lipidation required the Atg7 and Atg5-mediated conjugation system [13]. In addition, the depolarized

*Abbreviations:* CCCP, carbonyl cyanide m-chlorophenylhydrazone; CQ, chloroquine; EBSS, earle's balanced salt solution; LC3, microtube-associated protein light chain 3; MEFs, mouse embryonic fibroblasts; UKC, ULK kinase complex.

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mitochondria were targeted by structures containing ULK1, Atg14 or Atg9 [14]. However, the contribution of these canonical Atg proteins to CCCP-induced autophagy remained unknown. In this study we used genetically manipulated cells to define the role of UKC, Beclin 1/Atg14 complex and Atg9 using LC3 lipidation as the parameter. We found that the importance of these molecules varies in CCCP-induced autophagy.

## 2. Materials and methods

### 2.1. Reagents and antibodies

The following primary antibodies were used: anti-FIP200 (Gene Tex, Irvine, CA); anti-Atg13 and anti-β-actin (Sigma–Aldrich, St. Louis MO); anti-LC3 (MBL International, Woburn, MA); anti-Beclin 1 (Santa Cruz Biotechnology, Dallas, TX); anti-Atg14 (Cell Signaling Technology, Danvers, MA); anti-Atg9 (Abgent, San Diego, CA); and peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The Atg13 and Atg14 shRNA were purchased from Santa Cruz Biotechnology (catalogue # sc-97013) and Cell Signaling Technology (catalogue #6286), respectively.

### 2.2. Cell culture and fluorescence microscopy

All cells were maintained in DMEM with 10% FBS and the standard supplements. Mouse embryonic fibroblasts (MEF), and human cervical cancer cell line HeLa stably expressing GFP-LC3 were constructed as previously described [13,15]. FIP200-deficient and Atg9A-deficient MEFs, and the glioblastoma cell line U251 with constitutive knock-down of Beclin 1 had been described [16–18]. Some experiments were conducted with a prior infection of the cells with an adenoviral vector encoding GFP-LC3. For gene knock-down experiments, Atg13 or Atg14 siRNA (100 nM) was transfected into $1 \times 10^6$ cells using Lipofectamine2000 (Invitrogen) for 48 h. To induce autophagy, cells were treated with CCCP (20 μM) for 6 h, or cultured in Earle's balanced salt solution (EBSS) in the presence or absence of chloroquine (CQ, 10 μM) (Sigma) for 4 h. Images were acquired using an inverted epifluorescence microscope (Nikon Eclipse TE 2000). GFP-LC3 punctation were quantified and calculated as the average number of puncta per cell.

### 2.3. Immunoblotting Assay

Cells ($5 \times 10^5$ per well) were seeded into 6-well plates for indicated treatments, washed in PBS and lysed in RIPA buffer with protease/phosphatase, inhibitors (Sigma). Thirty micrograms of protein were separated by SDS–PAGE and then transferred to PVDF membranes. The membranes were probed with the indicated antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Images were taken using a Kodak Image 4000MM with the companion Software (Carestream Health).

### 2.4. Statistical analysis

Each experiment was performed with replicates for at least three times. Images were acquired from multiple randomly selected fields for each group. At least 100 or more cells per condition were analyzed for quantification. Data were presented as mean ± S.D, which were subjected to One-Way ANOVA with post hoc analysis using the GraphPad software. $P < 0.05$ was considered significant.

## 3. Results and discussion

*CCCP is able to initiate autophagy in FIP200 or Atg13-deficient cells.* A well-defined example of autophagy is the one induced by starvation, in which mTOR is inhibited, which in turn allows the activation of UKC to initiate autophagy [19]. We had previously shown that CCCP can inhibit mTOR via reactive oxygen species [13]. We thus investigated whether CCCP-induced autophagy would depend on UKC.

We assessed the autophagy process by measuring LC3 lipidation, a well-defined parameter for autophagy, using both fluorescence microscopy and immunoblot assay. MEFs deficient in FIP200, a key component of UKC were unable to mount autophagy response to starvation [16]. Indeed, starvation of cells by incubating them in EBSS induced an upregulation of GFP-LC3 puncta (Fig. 1A–B) and LC3-II (Fig. 1C) in wild type MEFs but not in FIP200-deficient MEF. Addition of a lysosome inhibitor, such as chloroquine (CQ), blocked the degradation of GFP-LC3, thus further elevating the number of GFL-LC3 dots in starved wild type cells, but again not in starved FIP200-deficient cells. The data confirmed the dependence of starvation-induced autophagy on FIP200. As previously shown [13] CCCP induced both GFP-LC3 puncta and LC3-II in wild type MEFs, which was dependent on Atg5 and Atg7. The level of LC3 lipidation was more significant than that induced by starvation. Strikingly, LC3 lipidation remained prominent, although reduced, in FIP200-deficient cells, suggesting that CCCP, unlike starvation, could still promote LC3 lipidation in the absence of FIP200.

We then explored the importance of another key component of UKC, Atg13, in CCCP-induced autophagy. Immunoblotting analysis confirmed the reduction of Atg13 protein level following the transfection of a specific siRNA into HeLa cells (Fig. 1D). However, the level of LC3-II was only slightly decreased in the knockdown cells after CCCP treatment (Fig. 1D). Atg13 knockdown also resulted in no significant change in GFP-LC3 dots upon CCCP challenge (data not shown). These observations suggested that CCCP could employ a FIP200/Atg13-independent pathway to activate autophagy in addition to the FIP200/Atg13-dependent mechanism.

*CCCP can induce LC3 lipidation in the absence of functional Beclin 1/Atg14 complex.* In starvation-induced autophagy the Beclin 1/Atg14 complex acts downstream of UKC and plays important roles in the nucleation of autophagosomal membrane [8]. To determine the role of Beclin 1/Atg14 complex in CCCP-induced autophagy, we first analyzed a glioblastoma cell line (U251), which stably expressed a Beclin 1-specific shRNA construct [18]. Immunoblotting assay confirmed Beclin 1 knock-down, which was accompanied by a decrease of Atg14 expression (Fig. 2A). The Beclin 1 complex components stabilize each other so that a reduction in one component can result in disintegration of the whole complex and the reduction in other components [6,7]. As anticipated starvation-triggered elevation in GFP-LC3 punctation and LC3-II were observed in wild type, but not in Beclin 1 knockdown U251 cells (Fig. 2A–B).

Following the treatment with CCCP there was no difference in GFP-LC3 punctation between Beclin 1-wild type and -knockdown cells (Fig. 2A–B). Both types of cells had significantly increased GFP-LC3 punctation following CCCP treatment. Consistently, the level of lipidated LC3-II also remained high in the absence of Beclin 1(Fig. 2A). These findings suggested that there were Beclin 1-independent mechanisms for CCCP-induced LC3 lipidation.

To confirm the finding with the Beclin 1 knockdown cells we inhibited the expression of Atg14 in HeLa cells by transient transfection of a specific siRNA. Immunoblotting analysis indicated that the siRNA successfully diminished Atg14 expression and promoted a concomitant degradation of Beclin 1 (Fig. 2C). We observed that

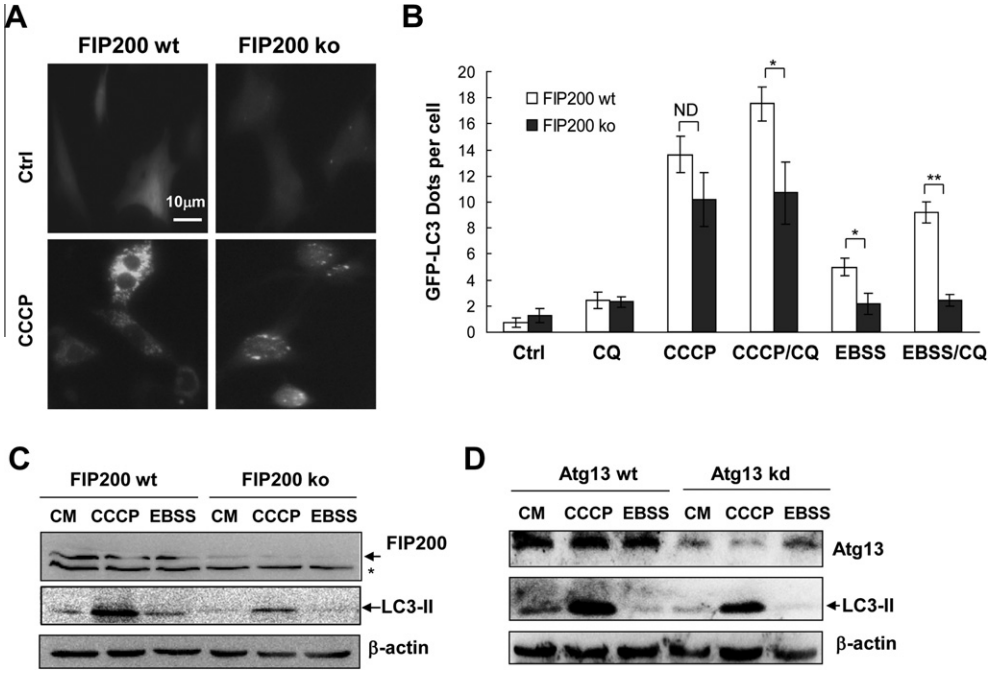


**Fig. 1.** CCCP induces LC3 lipidation in cells with deficient FIP200 or Atg13 expression. (A–C) Wild type (wt) and FIP200-deficient (ko) MEFs stably expressing GFP-LC3 were treated with CCCP or EBSS in the presence or absence of CQ. Fluorescent images were taken (A) for quantification of GFP-LC3 punctation (B). Cell lysates were analyzed by immunoblotting assay with indicated antibodies (C). The asterisk indicates a nonspecific band. (D) HeLa cells with or without Atg13 knocked down by a specific siRNA were treated with CCCP with or without CQ. Cell lysates were prepared for immunoblotting assay with indicated antibodies (A). $^{*}P < 0.05$; $^{**}P < 0.01$; ND: no statistical difference.

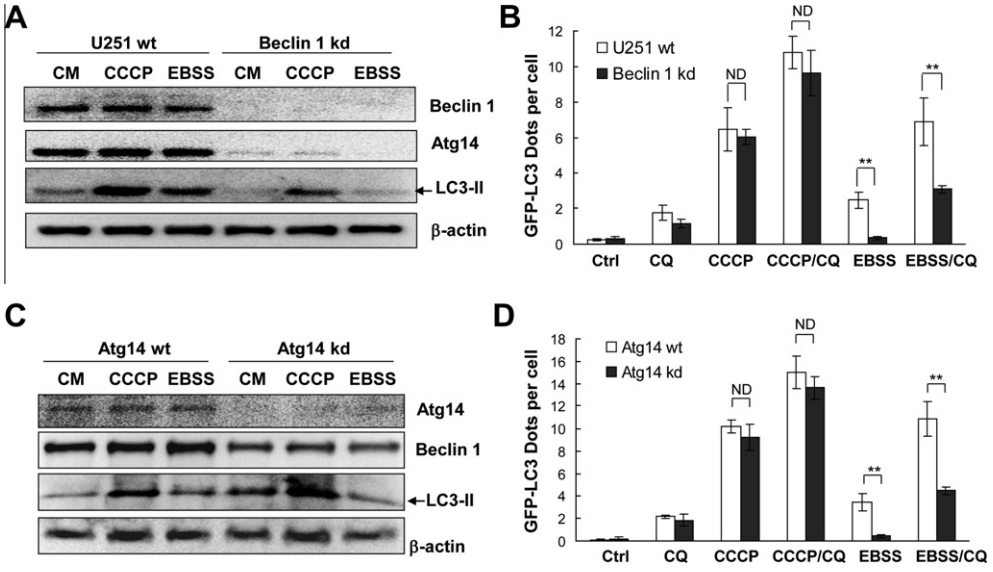


**Fig. 2.** CCCP induces LC3 lipidation in cells with compromised Beclin 1/Atg14 complex. (A–B) Wild type (wt) U251 cells and U251 cells with constitutive Beclin 1 knock-down (kd) were infected with Ad-GFP-LC3 overnight and then treated with CCCP or EBSS in the presence or absence of CQ. Cell lysates were analyzed by immunoblotting assays with indicated antibodies (A). Fluorescent images were taken for quantification of GFP-LC3 punctation (B). (C–D) HeLa cells expressing GFP-LC3 were transfected with Atg14-specific siRNAs for 48 h and then treated with CCCP or EBSS in the presence or absence of CQ. Cell lysates were analyzed by immunoblotting assay with indicated antibodies (C). Fluorescent images were taken for quantification of GFP-LC3 punctation (D). $^{*}P < 0.05$; $^{**}P < 0.01$; ND: no statistical difference.

Atg14 knockdown significantly suppressed starvation-induced GFP-LC3 punctation and LC3 lipidation (Fig. 2C–D). In contrast, Atg14 knockdown resulted in no changes in GFP-LC3 punctation or LC3-II level after CCCP treatment (Fig. 2C–D). Consistent with the above studies, we had also found that 3-MA, a class III PI-3 K inhibitor, did not affect CCCP-induced LC3 lipidation (data not shown). Taken together, these experiments suggested that the Beclin 1/Atg14 complex was indispensable for starvation-induced autophagy, but dispensable for CCCP-initiated autophagy.

The Beclin 1-independent autophagy had been frequently seen in cases where ROS and/or mitochondrial damage are involved [20–23], which is also the case in CCCP-induced autophagy. Indeed, CCCP-induced autophagy can be effectively suppressed by antioxidants [13]. ROS-triggered Beclin 1 independent autophagy had

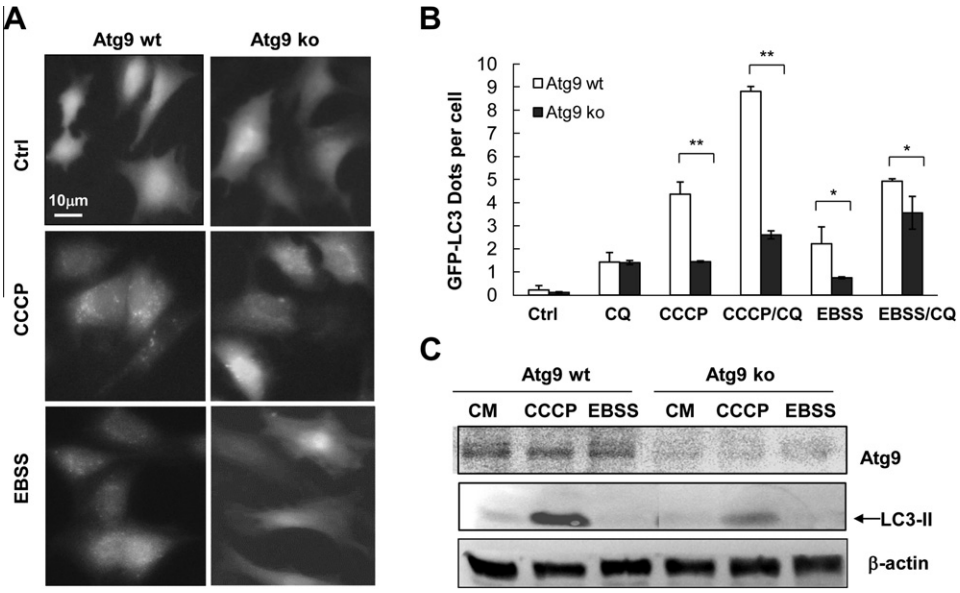


**Fig. 3.** CCCP-induced LC3 lipidation is largely abrogated in Atg9-deficient cells. Wild type (wt) and Atg9-deficient (ko) MEFs expressing GFP-LC3 were stimulated with CCCP or cultured in EBSS in the presence or absence of CQ. Fluorescent images were taken (A) for GFP-LC3 puncta quantification (B). Cell lysates were prepared for immunoblotting assay with the indicated antibodies (C). $^{*}P < 0.05$; $^{**}P < 0.01$.

been associated with ERK and JNK [21,24]. We found that ERK and JNK could be activated by CCCP and suppression of these kinases partially inhibited CCCP-induced autophagy (data not shown), suggesting that MAP kinases could play an important role in the signaling of CCCP-induced autophagy.

*Atg9 contributes to CCCP-induced autophagy and mitophagy*. Atg9 is the only membrane protein involved in autophagy and it shuttles between different compartments especially between the Golgi apparatus and endosomes [11,25]. It is speculated to be a membrane carrier that contributes to autophagosome expansion. We thus would like to determine whether CCCP-induced autophagy would dependent on Atg9 by comparing the wild type and Atg9A-deficient MEFs [17].

We found that consistent with previous reports [17,26] loss of Atg9 significantly decreased EBSS-induced GFP-LC3 punctation (Fig. 3A–B). Notably, Atg9 deletion also resulted in significantly reduced levels of GFP-LC3 puncta and LC3-II level following CCCP treatment (Fig. 3A–C), indicating that Atg9 was important for autophagosome biogenesis induced by CCCP.

Although Atg9 trafficking can be regulated by ULK1, Atg13 and PI-3 kinase activity [25,27,28] a recent study found that following CCCP treatment Atg9 and ULK1/Atg14 were independently recruited to depolarized mitochondria [14]. In addition, LC3 is also found to colocalize with the damaged mitochondria [12,13]. Consistently we found that deletion of Atg9 reduced the colocalization of LC3 with the fragmented mitochondria following CCCP treatment (data not shown). It is thus possible that LC3 lipidation during CCCP-triggered mitophagy could be independently regulated by Atg9 and UKC/Beclin 1/Atg14, and that Atg9 may mediate a more crucial process since UKC could be dispensable (Fig. 1).

To summarize, in the work presented here we demonstrate that CCCP-induced LC3 lipidation is more dependent on Atg9 than on UKC and Beclin 1 complex, suggesting that CCCP can utilize initiating mechanisms other than that of ULK and Beclin 1 complexes. Autophagy activation in the absence of ULK1 and/or ULK2 had been reported [29,30]. Notable, a kinase-inactivated ULK1 could inhibit starvation-induced autophagy via an Atg13-indpenent mechanism [28], supporting the notion that other autophagy proteins could be involved in parallel to the classical UKC to activate autophagy.

Though not well defined a major role of UKC in autophagy would have to be related to the activation of the Beclin 1/Atg14-directed class III PI3-kinase. Thus it may not be surprising that Beclin 1 and Atg14 could be also dispensable for CCCP-induced LC3 lipidation when the UKC was dispensable. Interestingly, Beclin 1-independent autophagy has been well reported in several other studies [20,31,32] and has been considered as a form of non-canonical autophagy [33].

How does the Beclin 1-indepenednet pathway leading to the engagement of the conjugation system is still an open question and could vary in different scenarios. There are potentially many factors that could affect the Atg7-Atg5-LC3conjugation system, such as those affecting the level of phosphatidylinositol-3 phosphate, the extent of enzymatic reactions, the selection of the membrane source, and the processing/deconjugation of LC3 by Atg4. It is therefore important to sort through these different regulatory steps to determine whether disturbance at one of them could offer the compensation to the loss of Beclin 1 complex components. It is also possible that LC3 lipidation initiated by the mechanisms independent of UKC and/or Beclin 1/Atg14 may not be ultimately associated with productive autophagosomes and/or efficient autophagy process. Future works would have to focus more on the physiological significance of the UKC and Beclin 1/Atg14-independent LC3 lipidation and autophagy. In addition, careful dissections of the non-canonical pathway will likely lead to the revelation of new molecular information for autophagy regulation under different contexts.

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