

USP14 regulates autophagy by suppressing K63 ubiquitination of Beclin 1

Daichao Xu,¹ Bing Shan,¹ Huawang Sun,¹ Juan Xiao,^{1,3} Kezhou Zhu,¹ Xingxing Xie,¹ Xingyan Li,¹ Wei Liang,¹ Xiaojuan Lu,¹ Lihui Qian,¹ and Junying Yuan^{1,2}

¹Interdisciplinary Research Center on Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 201210, China; ²Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

The ubiquitin–proteasome system (UPS) and autophagy are two major intracellular degradative mechanisms that mediate the turnover of complementary repertoires of intracellular proteomes. Simultaneously activating both UPS and autophagy might provide a powerful strategy for the clearance of misfolded proteins. However, it is not clear whether UPS and autophagy can be controlled by a common regulatory mechanism. K48 deubiquitination by USP14 is known to inhibit UPS. Here we show that USP14 regulates autophagy by negatively controlling K63 ubiquitination of Beclin 1. Furthermore, we show that activation of USP14 by Akt-mediated phosphorylation provides a mechanism for Akt to negatively regulate autophagy by promoting K63 deubiquitination. Our study suggests that Akt-regulated USP14 activity modulates both proteasomal degradation and autophagy through controlling K48 and K63 ubiquitination, respectively. Therefore, regulation of USP14 provides a mechanism for Akt to control both proteasomal and autophagic degradation. We propose that inhibition of USP14 may provide a strategy to promote both UPS and autophagy for developing novel therapeutics targeting neurodegenerative diseases.

[*Keywords:* USP14; autophagy; Beclin 1; Akt]

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Autophagy and the ubiquitin–proteasome system (UPS) are two major intracellular degradative mechanisms that function in a complementary manner. UPS mediates the degradation of short-lived proteins conjugated with K48 ubiquitin chains (Komander and Rape 2012). On the other hand, autophagy mediates the turnover of long-lived proteins and intracellular organelles encapsulated in autophagosomes that eventually fuse with lysosomes to allow degradation by lysosomal proteases. Ubiquitination is also involved as a signaling mechanism in targeting both protein substrates and organelles such as depolarized mitochondria for degradation by autophagy (Sarraf et al. 2013; Ordureau et al. 2014). Ubiquitination of protein substrates is a reversible process, as ubiquitin chains can be removed by deubiquitinating enzymes (DUBs). Deubiquitination is an important negative regulatory mechanism for reducing the levels of protein ubiquitination. Ubiquitin-specific protease-14 (USP14), a DUB reversibly associated with the proteasome, has been shown to negatively regulate the activity of proteasomes by trimming K48 ubiquitin chains on proteasome-bound substrates (Borodovsky et al. 2001; Koulich et al. 2008; Lee et al. 2010). USP14 can also be activated by Akt-mediated phosphory-

lation, which promotes its deubiquitinating activity for both K48 and K63 ubiquitin linkages (Xu et al. 2015a). The activity of USP14 in deubiquitinating K63 ubiquitin linkages is likely to be physiologically relevant, as inhibition of USP14 in vivo leads to increases in the levels of K63-linked ubiquitin conjugates in both spinal cords and neurons (Vaden et al. 2015). However, the mechanism by which USP14 regulates K63 ubiquitination in control of cellular processes and its functional significance are not well characterized.

Both UPS and autophagy are implicated in the removal and degradation of misfolded proteins that play critical roles in the pathogenesis of neurodegenerative diseases (Ciechanover and Kwon 2015). Since UPS and autophagy mediate the turnover of complementary repertoires of intracellular proteomes, simultaneously activating both UPS and autophagy might provide a powerful strategy for the clearance of misfolded proteins. However, UPS and autophagy have not been demonstrated to share a common regulatory mechanism. Here, we explore the mechanism and function of the K63 deubiquitinating activity of USP14. We show that USP14 can regulate

³Present address: Youjiang Medical University for Nationalities, Guangxi 533000, China.

Corresponding author: jyuan@hms.harvard.edu

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autophagy by controlling K63 ubiquitination of Beclin 1. Furthermore, we show that activation of USP14 by Akt-mediated phosphorylation provides a mechanism for Akt to negatively regulate autophagy by removing K63 ubiquitin chains from Beclin 1. Our study suggests that USP14 regulates both proteasomal degradation and autophagy through controlling K48 and K63 ubiquitination, respectively. Our results suggest that USP14 regulates both proteasomal and autophagic degradation. Therefore, inhibition of USP14 might provide a mechanism to promote both UPS and autophagy.

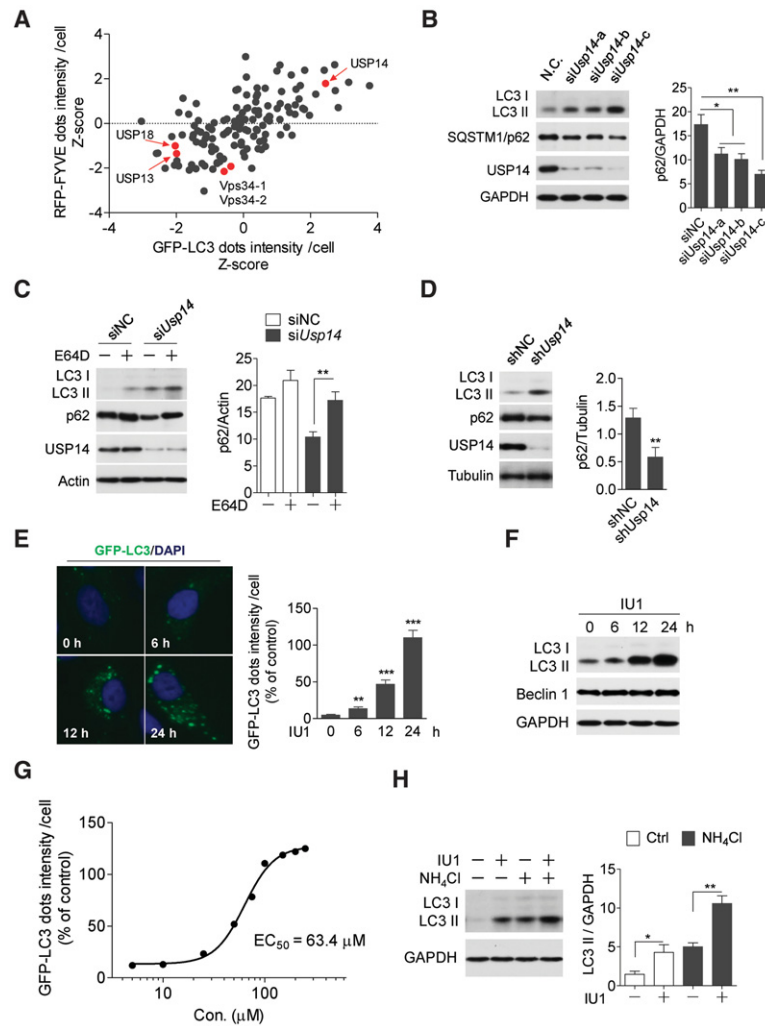
Results

USP14 negatively regulates autophagy

USP14 was identified as a gene that, when knocked down, led to up-regulation of autophagy in a siRNA screen targeting 127 DUBs (Fig. 1A). To confirm this finding, we

transiently transfected H4 cells with siRNAs targeting USP14 and found that reduction of USP14 expression led to increased autophagy as detected by up-regulation of LC3-II protein levels and down-regulation of SQSTM1/p62 protein levels (Fig. 1B). The reduction of p62 protein levels could be restored by treatment with E64D (Mizushima et al. 2010), a lysosomal inhibitor that blocks autophagic flux, which is indicated by further increases in the levels of LC3-II (Fig. 1C). In contrast, treatment with MG132, an inhibitor of proteasomal degradation, had no effect on p62 degradation when USP14 was knocked down (Supplemental Fig. S1A). Thus, these results suggest that inhibition of USP14 promotes autophagic flux.

To further verify the role of USP14 in autophagy, we established a stable USP14 knockdown H4 cell line. Consistent with induction of autophagy, the levels of LC3-II were higher and the levels of p62 were lower in this USP14 knockdown cell line compared with that of the



ed with different concentrations of IU1 for 24 h. The GFP-LC3⁺ puncta were quantified as in E. (H) IU1 induces autophagic flux. H4 cells were treated with 50 μM IU1 for 6 h in the presence or absence of 10 mM NH_4Cl and harvested for Western blot analysis. Ratios of LC3-II/GAPDH were calculated and are shown at the right.

control under basal conditions (Fig. 1D). Furthermore, treatment with IU1 (Lee et al. 2010), an inhibitor of USP14, induced the accumulation of LC3-II in a time- and dose-dependent manner as determined by both GFP-LC3 assay and Western blotting (Fig. 1E,F; Supplemental Fig. S1B). The EC₅₀ of IU1 in inducing autophagy is 63.4 μM as determined by GFP-LC3 assay (Fig. 1G), which is comparable with an EC₅₀ of 62 μM in inducing UPS activity (Lee et al. 2010) as determined by GFPu assay (Supplemental Fig. S1C; Bence et al. 2005; Xu et al. 2015a). Similarly, treatment with IU1 also induced autophagy in other cell lines tested, including Huh7, HeLa, HCT116, and mouse embryonic fibroblasts (MEFs) (Supplemental Fig. S1D–G). Consistent with its role as a negative regulator of autophagic flux, inhibition of USP14 by IU1 also induced the accumulation of LC3-II in the presence of NH₄Cl, a lysosomal inhibitor that blocks autophagic flux (Fig. 1H). Notably, the ability of IU1 to induce autophagy was blocked after USP14 knock-out in H4 cells, suggesting that the activity of IU1 to induce autophagy requires USP14 (Supplemental Fig. S1H). Taken together, we conclude that USP14 is a negative regulator of autophagy, and, furthermore, inhibition of USP14 can simultaneously activate both autophagy and UPS.

USP14 is important for Akt to inhibit autophagy

Since USP14 is activated by Akt-mediated phosphorylation (Xu et al. 2015a), we next examined the possible role of USP14 as a downstream mediator of Akt in the control of autophagy. The expression of activated Akt (myristoylation signal [MGSSKSKPK]-attached Akt [Myr-Akt]) could reduce autophagy even in the presence of rapamycin, an inhibitor of mTOR, suggesting that Akt can inhibit autophagy in mTOR-independent mechanisms as report-

ed (Supplemental Fig. S2; Wang et al. 2012). However, the ability of activated Akt to negatively regulate autophagy was significantly reduced when USP14 was knocked down (Fig. 2A), suggesting that USP14 is an important downstream mediator for Akt to regulate autophagy. Consistently, when the activity of Akt was inhibited by MK2206, the effect of USP14 knockdown in stimulating autophagy was reduced (Fig. 2B), further supporting the role of USP14 as a downstream mediator of Akt in suppressing autophagy. In addition, we tested the role of growth factors such as insulin-like growth factor 1 (IGF1) or epidermal growth factor (EGF), both of which are known to promote activation of Akt (Burgering and Coffey 1995). We found that treatment with IGF1 or EGF reduced the levels of autophagy in serum-starved wild-type H4-GFP-LC3 cells, which was restored when the cells were treated with MK2206 (Fig. 2C). However, treatment with IGF1 or EGF failed to inhibit autophagy in H4-GFP-LC3 cells with USP14 stable knockdown (Fig. 2C). Taken together, these results suggest that USP14 plays a critical role as a downstream mediator for Akt to inhibit autophagy.

Akt-mediated phosphorylation of USP14 is required for USP14 to inhibit autophagy

We next tested the role of USP14 phosphorylation in Akt regulation of autophagy. To this end, expression vectors of wild-type USP14 or phosphorylation mutants of USP14 were stably transfected into *Usp14* knockdown cells. As shown in Figure 3A, the expression of wild-type USP14 or a phosphorylation mimic mutant of USP14, USP14-DD (S143D/S432D), but not the USP-AA (S143A/S432A) mutant, was able to reduce autophagy in USP14 stable knockdown cells. This result was further validated by using a GFP-LC3 assay in H4-GFP-LC3 cells with *Usp14*

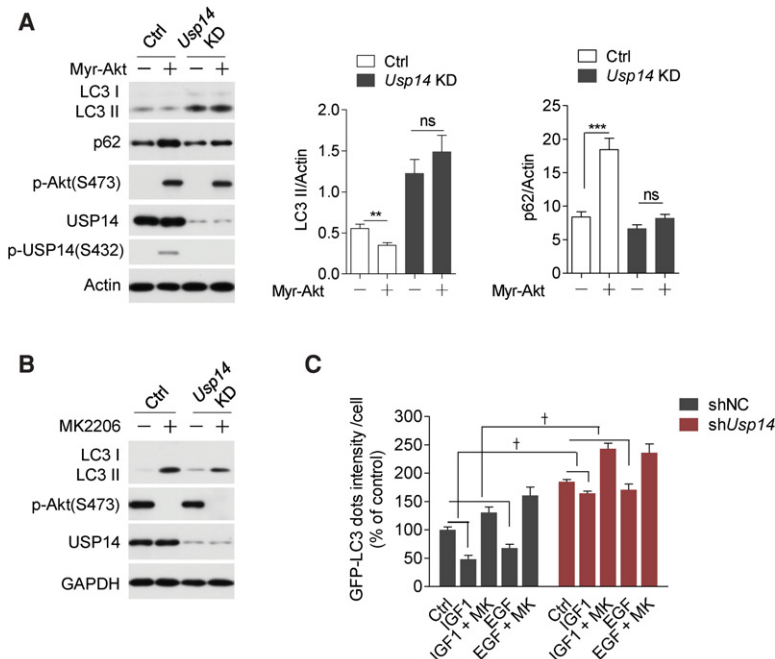


Figure 2. USP14 is important for Akt to inhibit autophagy. (A) Either wild-type or *Usp14* stable knockdown (*Usp14* KD) H4 cells were infected with a lentiviral vector expressing Myr-Akt for 24 h. The cells were then harvested and subjected to Western blot analysis using the indicated antibodies. The ratios of LC3-II/Actin and p62/Actin were calculated and are shown at the right. Results are shown as means ± SD of three independent sets of experiments. (ns) Not significant. (B) Wild-type and *Usp14* stable knockdown H4 cells were treated with 1 μM MK2206 for 4 h and then harvested and analyzed by Western blotting using the indicated antibodies. (C) Wild-type and *Usp14* stable knockdown H4-GFP-LC3 cells were serum-starved overnight and pretreated with 1 μM MK2206 for 30 min before stimulation with 100 ng/mL IGF1 or 100 ng/mL EGF for 1 h. Images of the cells were collected using an Array-Scan HCS 4.0 reader. The average spot intensity in 1000 cells from each indicated sample was determined. Data are displayed as means ± SD of the spot intensity per cell. (+) *P* < 0.01 (ANOVA).

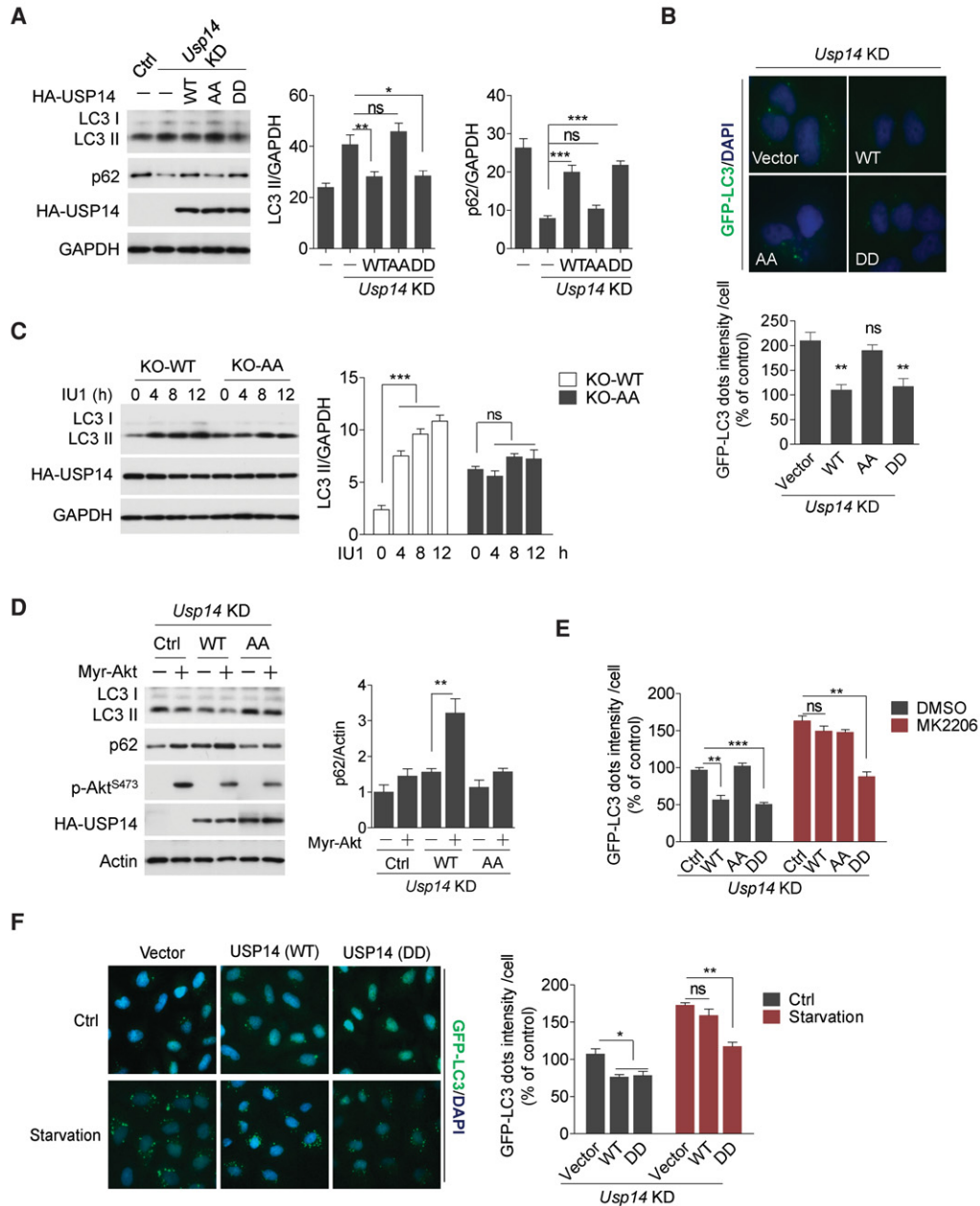


Figure 3. Akt-mediated phosphorylation of USP14 is required for USP14 to inhibit autophagy. (A) *Usp14* stable knockdown H4 cells were infected with lentiviral vectors expressing wild-type USP14, the USP14-AA (S143A/S432A) mutant, or the USP14-DD (S143D/S432D) mutant as indicated for 24 h. The cells were then harvested and subjected to Western blot analysis using the indicated antibodies. The ratios of LC3-II/GAPDH and p62/GAPDH were calculated and are shown at the right. Results are shown as means \pm SD of three independent sets of experiments. (B) *Usp14* stable knockdown H4-GFP-LC3 cells were transfected as in A and then imaged using an ArrayScan HCS 4.0 reader. The average spot intensity in 1000 cells from each indicated sample was determined. Bars represent mean \pm SEM of triplicate samples. (C) *Usp14*^{-/-} H4 cells expressing wild-type USP14 or the USP14-AA mutant were treated with 50 μ M IU1 for indicated periods of times. Cells were then harvested for Western blot analysis. The ratios of p62/Actin were calculated and are shown at the right. (D) *Usp14* stable knockdown H4 cells were infected with lentiviral vectors expressing either wild-type USP14 or the USP14-AA mutant in the presence or absence of Myr-Akt as indicated for 24 h. The cells were then harvested and subjected to Western blot analysis using the indicated antibodies. The ratios of p62/Actin were calculated and are shown at the right. Results are shown as means \pm SD of three independent sets of experiments. (ns) Not significant. (E) *Usp14* stable knockdown H4-GFP-LC3 cells were infected with lentiviral vectors expressing wild-type USP14, the USP14-AA mutant, or the USP14-DD mutant as indicated for 20 h and then treated with or without 1 μ M MK2206 for another 4 h. The cells were imaged and quantified as in B. (F) *Usp14* stable knockdown H4-GFP-LC3 cells were infected with lentiviral-expressing vectors of wild-type USP14, the USP14-AA mutant, or the USP14-DD mutant as indicated for 12 h and then serum-starved for another 12 h. The cells were imaged and quantified as in B.

knockdown (Fig. 3B). Furthermore, inhibition of USP14 by IU1 in *Usp14*^{-/-} cells stably expressing wild-type USP14, but not the USP14-AA mutant, induced autophagy in a time-dependent manner (Fig. 3C), suggesting that phosphorylation of USP14, which promotes its activation (Xu et al. 2015a), is important for USP14 to inhibit autophagy.

To further validate the role of USP14 phosphorylation by Akt in the regulation of autophagy, we tested the effect of activated Akt in *Usp14* knockdown cells expressing wild-type USP14 or the USP14-AA mutant. As shown in Figure 3D, the expression of activated Akt was able to inhibit autophagy in cells expressing wild-type USP14 but not in USP14-AA mutant cells. Consistently, when Akt was inhibited by MK2206 (Fig. 3E) or serum starvation (Fig. 3F; Franke et al. 1995), the expression of wild-type USP14 failed to inhibit autophagy, while that of the USP14-DD mutant could still inhibit autophagy, suggesting the importance of phosphorylation by Akt for USP14 to inhibit autophagy.

USP14 suppresses Vps34 activity by interacting with Beclin 1

We next characterized the mechanism by which USP14 regulated autophagy. PtdIns3P (phosphatidylinositol 3-phosphate) is a key lipid signaling molecule involved in the nucleation of autophagosomes and is especially important for regulation of autophagy under normal nutritional conditions (Fig. 1A; Lipinski et al. 2010). The recruitment of FYVE-RFP to endosomal localized PtdIns3P has been used as an assay for measuring the cellular levels of PtdIns3P (Zhang et al. 2007). We found that knockdown of USP14 induced the formation of FYVE-RFP dots (Figs. 1A, 4A), suggesting that USP14 may negatively regulate Vps34 activity critical for the production of PtdIns3P under basal condition.

Since inhibition of USP14 promoted the degradation of a set of proteins through UPS (Xu et al. 2015a), we first considered the possibility that inhibition of USP14 promoted the degradation of an inhibitor of class III PI3 kinase

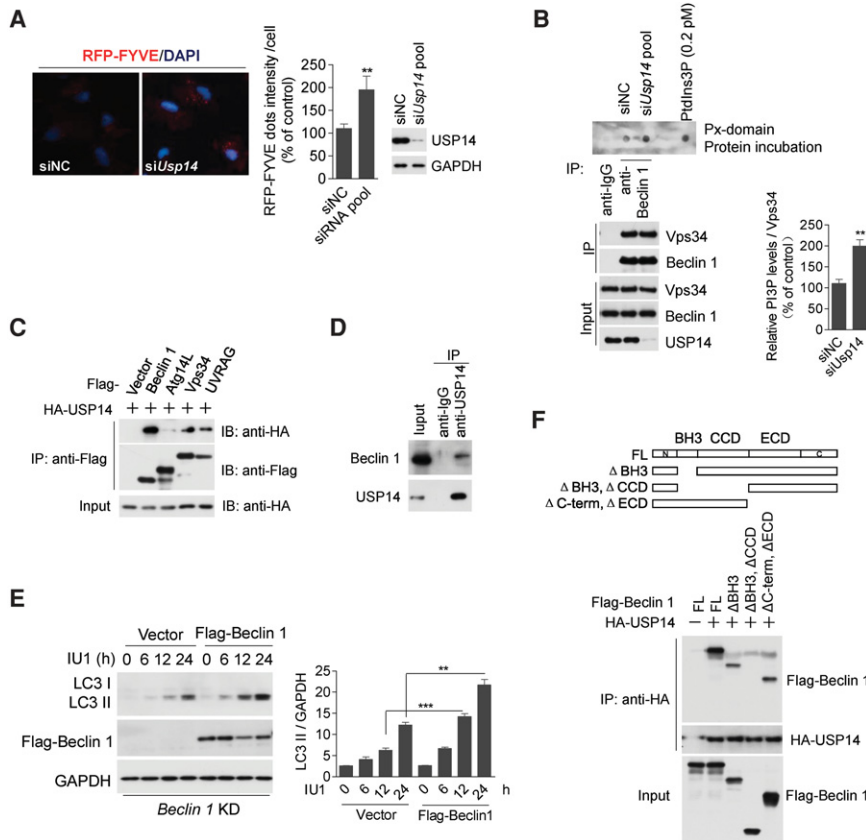


Figure 4. USP14 suppresses Vps34 activity by interacting with Beclin 1. (A) USP14 deficiency enhances Vps34 activity. H4-RFP-FYVE cells were transfected with control siRNA or siRNA pools targeting *Usp14*. Seventy-two hours after transfection, the cells were imaged using an Array-Scan HCS 4.0 reader. Representative cells are shown. The average RFP-FYVE spot intensity in 1000 cells from each indicated sample was determined. The data are displayed as the means ± SEM of the spot intensity per cell. USP14 knockdown efficiency is shown at the right. (B) The Beclin 1-associated Vps34 lipid kinase assay. H4 cells (2 × 10⁷ cells) were lysed after being transfected with either a control siRNA or siRNA pools targeting *Usp14* for 72 h, and the cell lysates were incubated with anti-Beclin 1 antibody to precipitate the Beclin 1/Vps34 complex. The immunoprecipitates were separated into two parts: one for input detection and the other for kinase assays. The production of PtdIns3P (PI3P) was detected by protein–lipid blot assay. Lipids were extracted and applied onto a Hybond C-extra membrane. The commercial PtdIns3P was spotted as indicated for control. The levels of PtdIns3P were detected using the GST-PX-p40 domain protein, which binds to PtdIns3P and anti-GST antibody. Ratios of PtdIns3P levels/Vps34 were calculated and are shown.

PI3P (0.2 pM) was used as a positive control. (C) USP14 associates with Vps34 complexes. The expression vectors of HA-tagged USP14 and Flag-tagged Beclin 1, Vps34, Atg14L, or UVRAG were cotransfected into HEK293T cells, respectively, and immunoprecipitations (IP) were performed at 24 h after transfection. (D) H4 cells (2 × 10⁷ cells) were lysed, the lysates were analyzed by immunoprecipitation with anti-IgG control or anti-USP14, and the immunocomplexes were analyzed by Western blot analysis. (E) H4 cells with *Beclin 1* stable knockdown were virally infected with expression vectors for either control or Flag-tagged Beclin 1 for 12 h and then treated with 50 μM IU1 for the indicated periods of times. The cells were harvested for Western blot analysis. The ratios of LC3-II/GAPDH were calculated and are shown at the right. (F) Mapping the interacting regions of Beclin 1 with USP14. The expression vectors of Flag-tagged Beclin 1 truncations encoding the indicated regions were cotransfected with that of full-length USP14 into HEK293T cells and analyzed by immunoprecipitation and Western blotting.

activity. However, no obvious candidate was identified in the list of proteins whose levels were reduced after USP14 knockout. We further experimentally tested whether inhibition of USP14 might promote the degradation of Rubicon, a well-established inhibitor of the Vps34 complex (Zhong et al. 2009). As shown in Supplemental Figure S3A, treatment with IU1 had no effect on the levels of Rubicon. To directly test the effect of USP14 knockdown and inhibition by IU1 on the activity of the Vps34/Beclin 1 complex in converting PtdIns to PtdIns3P, we conducted an *in vitro* kinase assay using the isolated Vps34/Beclin 1 complex and lipid blots (Fig. 4B; Supplemental Fig. S3B). These results showed that inhibition of USP14 by pharmacological means or siRNA-mediated knockdown led to activation of class III PI3 kinase activity; however, it was unlikely that this was accomplished by promoting the degradation of the inhibitory activity of Vps34 complexes.

To explore the mechanism by which USP14 regulates the production of PtdIns3P, we screened for the components of the Vps34 complex that can interact with USP14. We found that overexpressed USP14 showed a strong interaction with Beclin 1 (Fig. 4C; Supplemental Fig. S3C). Furthermore, we detected the interaction of endogenous Beclin 1 and USP14, both of which were predominantly present in the cytoplasm (Fig. 4D; Supplemental Fig. S3D). To test the possible role of Beclin 1 in USP14-regulated autophagy, a stable Beclin 1 knockdown H4 cell line was generated (Supplemental Fig. S3E; Xu et al. 2015b). As shown in Figure 4E, the effect of IU1 on the levels of LC3-II was smaller in this Beclin 1 knockdown cell line compared with that of the knockdown-reconstituted Beclin 1 cell line, suggesting that USP14 regulates autophagy in a Beclin 1-dependent manner.

To further characterize the interaction of USP14 with Beclin 1, we determined the domains of Beclin 1 that interact with USP14. Expression vectors of truncated Beclin 1 mutants were coexpressed with that of USP14 in HEK293T cells, and the interaction of USP14 with Beclin 1 mutants was analyzed by coimmunoprecipitation. The binding of USP14 with Beclin 1 was significantly reduced upon deletion of its CC domain (Fig. 4F), suggesting that the CC domain of Beclin 1 is important for the interaction with USP14.

USP14 regulates Beclin 1 K63-linked ubiquitination

Since knockdown or inhibition of USP14 had no effect on the levels of key components of Vps34 complexes (Supplemental Fig. S4), we hypothesized that USP14 might regulate autophagy through a nondegradative mechanism independently of K48 ubiquitination. Since USP14 can deubiquitinate K63 ubiquitin linkage (Jung et al. 2013; Xu et al. 2015a; Lee et al. 2016), we considered the possibility that USP14 may regulate autophagy by deubiquitinating K63 ubiquitinated proteins. To directly test this possibility, we performed a quantitative mass spectrometry analysis to compare the changes in K63 ubiquitinated proteins with H4 cells expressing USP14 wild type and those expressing vector only or the USP14-AA mutant (Fig. 5A). We established H4 cells sta-

bly expressing Flag and His-double-tagged K63-only ubiquitin with vector only, USP14 wild type, or USP14-AA. K63 ubiquitinated proteins were first immunoprecipitated using anti-Flag from the cell lysis. The anti-Flag immunocomplexes were then denatured in 8 M urea, and individual K63 ubiquitinated proteins were isolated by Ni²⁺ beads, which bind to the His tag. Using an isobaric tandem mass tag (TMT) labeling approach, our mass spectrometry analysis identified 19,503 peptides with high confidence ($q < 0.01$), corresponding to 2651 proteins with at least one unique peptide in each protein. One-thousand-six-hundred-six proteins were quantified in at least two of three replicates. Among those 1606 proteins, we found 1117 proteins that were significantly decreased ($P < 0.05$) from immunoprecipitation of K63 ubiquitinated proteins in H4 cells overexpressing wild-type USP14 compared with that in H4 cells expressing control vector. In this set of 1117 proteins, we found that the levels of 74 proteins were decreased at least 1.3-fold, suggesting that these proteins may be substrates of K63 deubiquitination by USP14 (Fig. 5B, Lane 1).

In addition, we compared the mass spectrometry analysis of K63 ubiquitinated proteins from cells expressing wild-type USP14 with those expressing the USP14-AA mutant. Importantly, we identified a set of 59 proteins in common with the set of 74 ubiquitinated proteins (80%) identified above from the comparison of wild-type USP14/vector. This set of 59 proteins was significantly increased ($P < 0.05$) from immunoprecipitation of K63 ubiquitinated proteins in cells overexpressing the USP14-AA mutant, an inactive form of USP14 (Xu et al. 2015a), compared with that of USP14 wild type (Fig. 5B, lanes 2,3). Interestingly, Beclin 1 was identified as a common top hit, with a twofold decrease on both lists of K63 ubiquitinated proteins that were altered in response to changes in the expression of wild type when compared with vector alone or the USP14-AA mutant. Since Beclin 1 is an essential component of Vps34 complexes, this result suggests that USP14 may regulate K63 ubiquitination of Beclin 1.

Next, we directly characterized the effect of USP14 on K63 ubiquitination of Beclin 1. We found that inhibition of USP14 by either siRNA knockdown or IU1 led to increases in the levels of K63 ubiquitination on endogenous Beclin 1 (Fig. 5C). Conversely, we found that overexpression of USP14 significantly reduced the K63, but not K48, ubiquitination levels of Beclin 1 (Fig. 5D). To demonstrate that USP14 can directly deubiquitinate Beclin 1 K63 ubiquitination, we performed *in vitro* deubiquitination assays. Incubation of purified activated USP14-S432E—but not USP14 wild type, which is known to be inactive *in vitro* in the absence of proteasomes (Hu et al. 2005; Lee et al. 2010; Xu et al. 2015a)—with K63 ubiquitinated Beclin 1 *in vitro* led to its deubiquitination (Fig. 5E).

To characterize the mechanism underlying Beclin 1 K63 ubiquitination-mediated promotion of autophagy, we performed a mass spectrometry analysis to determine the interactome changes of Beclin 1 with or without K63 ubiquitination. Interestingly, we found that K63 ubiquitination of Beclin 1 increased its interaction with two well-characterized Beclin 1-binding partners—

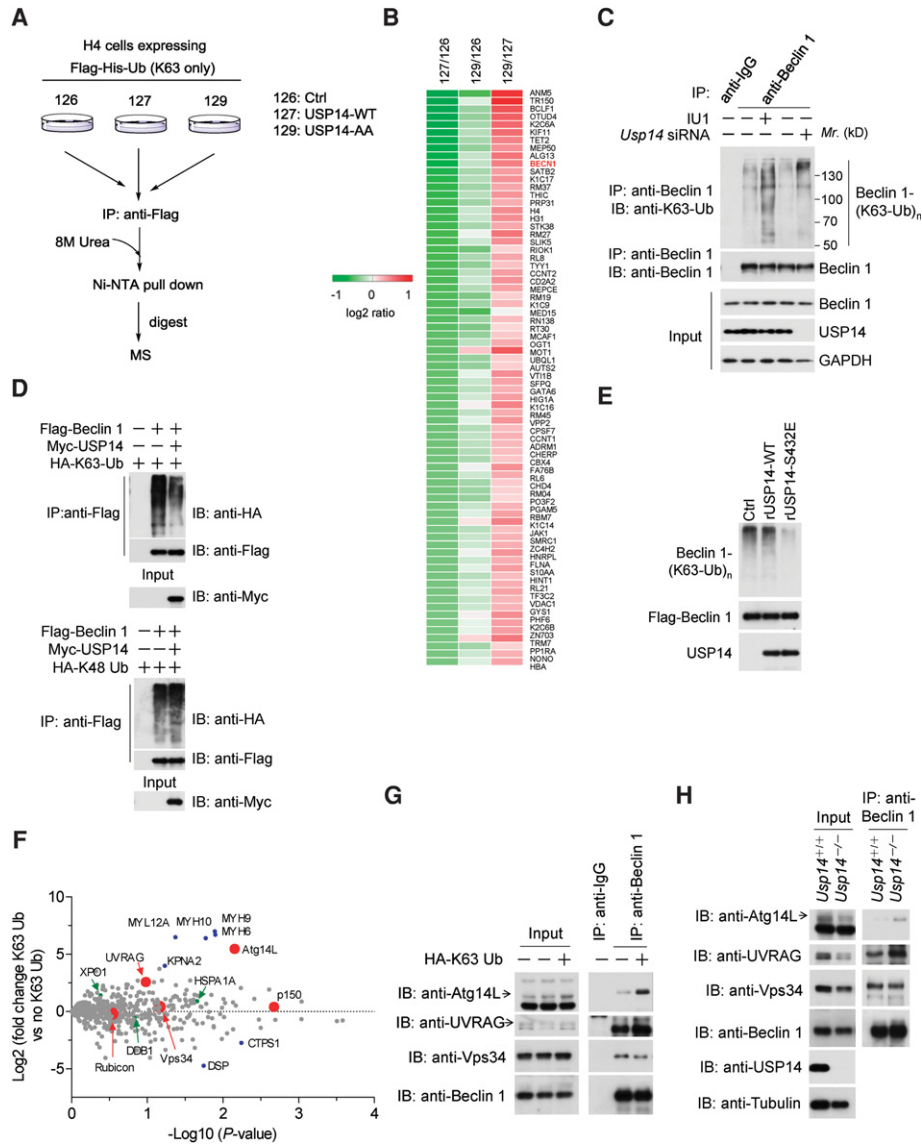


Figure 5. USP14 regulates Beclin 1 K63-linked ubiquitination. (A) Schematic representation of mass spectrometry assay to determine the changes in K63 ubiquitinated proteins in response to overexpression of USP14. (B) The quantitative analysis of ubiquitinated proteome changes in control cells or cells overexpressing wild-type USP14 or the USP14-AA mutant expressing Flag-His-K63-only ubiquitin were performed by TMT isobaric labeling followed by shotgun analysis. The heat map was plotted based on the set of 74 proteins that were significantly decreased ($P < 0.05$) at least 1.3-fold from K63 ubiquitination immunoprecipitation in the presence of wild-type USP14 compared with control cells. The log base 2 of average ratios was plotted as indicated. (C) H4 cells (2×10^7 cells) were transfected with siRNA pools targeting *Usp14* for 72 h or treated with 50 μ M IU1 for 24 h and then lysed, and the lysates were incubated with anti-Becn1 1 antibody followed by Western blot analysis to determine the K63 ubiquitination on Beclin 1. (D) USP14 reduced the K63, but not K48, ubiquitination levels of Beclin 1. HEK293T cells were transfected with expression vectors of HA-tagged K63-only ubiquitin or K48-only ubiquitin and Flag-tagged Beclin 1 as indicated with or without Myc-tagged USP14 and cultured for 24 h. The cell lysates were immunoprecipitated with anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting with anti-HA antibody. (E) In vitro K63-linked deubiquitination of Beclin 1 by USP14. K63 ubiquitinated Beclin 1 was incubated with purified recombinant USP14 or USP14-S432E in vitro and then blotted with anti-HA antibody. (F) A scatter plot depicting interactome changes of Beclin 1 identified and quantified in a quantitative proteomics experiment. Proteins were plotted as a function of fold change with K63 ubiquitin relative to the control. Some of the known Beclin 1 interactors (from BioGRID, <http://thebiogrid.org>) are highlighted in red and green. Some of the unknown binding targets with significant fold changes are highlighted in blue. The targets in Beclin 1-containing Vps34 complexes (Vps34, p150, Atg14L, UVRAG, and Rubicon) are highlighted by larger red dots. (G) K63 ubiquitination promotes the association of Atg14L and UVRAG with Beclin 1. Control H4 cells or K63 ubiquitin stably expressing H4 cells were harvested, the cell lysates were incubated with anti-Becn1 1 antibody to precipitate the Beclin 1/Vps34 complex, and the immunocomplexes were analyzed by Western blotting with the indicated antibodies. (H) Knockout of USP14 promotes the association of Atg14L and UVRAG with Beclin 1. *Usp14*^{+/+} H4 cells or *Usp14*^{-/-} H4 cells were harvested, the cell lysates were incubated with anti-Becn1 1 antibody to precipitate the Beclin 1/Vps34 complex, and the immunocomplexes were analyzed by Western blotting with the indicated antibodies.

Atg14L and UVRAG—that are important regulators of Vps34 complex activity (Fig. 5F; Itakura et al. 2008; Matsunaga et al. 2009; Zhong et al. 2009; Russell et al. 2013). On the other hand, the association of Vps34, p150, and Rubicon with Beclin 1 did not change significantly (Fig. 5F). These results suggest that K63 ubiquitination of Beclin 1 may promote autophagy through the increased association of Atg14L and UVRAG with Beclin 1.

To verify these results, we then performed immunoprecipitation–immunoblot assays to determine the interaction of Atg14L and UVRAG with Beclin 1 in the presence of K63 ubiquitin. Consistent with the results from mass spectrometry analysis, we found that K63 ubiquitination promoted the interaction of Beclin 1 with Atg14L and UVRAG but not with Vps34 (Fig. 5G). Furthermore, we found that the interaction of Beclin 1 with Atg14L and UVRAG in *Usp14*^{-/-} H4 cells was significantly increased compared with that of wild-type H4 cells (Fig. 5H). Since Atg14L and UVRAG promote Vps34 complex activity in mediating autophagy induction and maturation, respectively, these results suggest that, in addition to regulating autophagy initiation through Atg14L complexes, K63 ubiquitination of Beclin 1 may also play a role in promoting autophagosome maturation through regulating the UVRAG-containing Vps34 complex.

Akt-mediated phosphorylation regulates USP14 DUB activity toward Beclin 1

Since Akt-mediated USP14 phosphorylation activates USP14 DUB activity (Xu et al. 2015a) and since we found that USP14 could regulate K63 ubiquitination of Beclin 1, we next examined whether USP14 might regulate K63 ubiquitination of Beclin 1 in an Akt-dependent manner. We first characterized the levels of K63 ubiquitination

on Beclin 1 under starvation conditions, which lead to inactivation of Akt. We found that the K63 ubiquitination levels of endogenous Beclin 1 were increased under starvation conditions (Fig. 6A). A similar increase in K63 ubiquitination levels on exogenous transfected Beclin 1 in *Beclin 1* knockdown cells was also found (Supplemental Fig. S5). Moreover, starvation-induced K63 ubiquitination of Beclin 1 was inhibited upon the expression of activated Akt (Fig. 6B).

To test whether phosphorylation of USP14 is required for its DUB activity toward K63 ubiquitination of Beclin 1, we stably expressed wild-type USP14 or mutant USP14 in *Usp14* knockout cells and examined the effect of Beclin 1 ubiquitination. As shown in Figure 6C, the expression of wild-type USP14 or the USP14-DD mutant, but not the USP14-AA mutant, was able to inhibit the K63 ubiquitination of Beclin 1. To further validate that USP14-regulated K63 ubiquitination of Beclin 1 is dependent on Akt-mediated phosphorylation, we expressed activated Akt in *Usp14* knockout cells complemented with wild-type USP14 or the USP14-AA mutant. The expression of wild-type USP14 was able to inhibit ubiquitination of Beclin 1 in both the presence and absence of Myr-Akt expression, while that of the USP14-AA mutant failed to inhibit ubiquitination of Beclin 1 even in the presence of activated Akt (Fig. 6D), suggesting that the USP14-regulated deubiquitination of Beclin 1 is dependent on Akt-mediated phosphorylation. Moreover, the USP14-DD mutant was still able to reduce ubiquitination levels of Beclin 1 even in the presence of the Akt inhibitor MK2206, while wild-type USP14 lost its deubiquitination activity toward Beclin 1 upon inhibition of Akt (Fig. 6E). Taken together, these results suggest that Akt-mediated phosphorylation of USP14 regulates its K63 deubiquitinating activity toward Beclin 1.

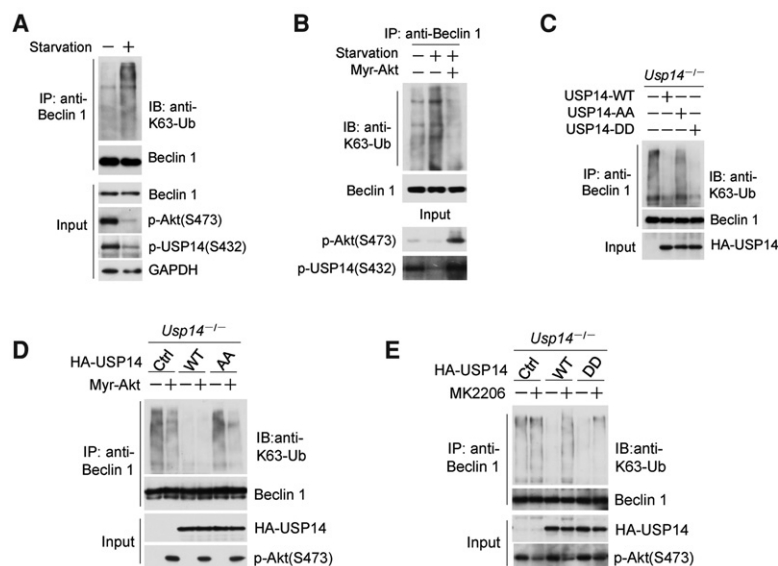


Figure 6. Akt-mediated phosphorylation regulates USP14 DUB activity toward Beclin 1. (A) H4 cells were serum-starved overnight, the lysates were analyzed by immunoprecipitation with anti-Beclin 1, and the immunocomplexes were analyzed by Western blot analysis using an anti-ubiquitin K63-specific antibody. (B) H4 cells were virally infected with a lentiviral vector for Myr-Akt for 12 h and then serum-starved for another 12 h, the lysates were analyzed by immunoprecipitation with anti-Beclin 1, and the immunocomplexes were analyzed as in A. (C) *Usp14*^{-/-} H4 cells were virally transfected with wild-type USP14 or the mutant as indicated for 24 h, the lysates were analyzed by immunoprecipitation with anti-Beclin 1, and the immunocomplexes were analyzed by Western blotting. (D) *Usp14*^{-/-} H4 cells were virally transfected with wild-type USP14 or the USP14-AA mutant as indicated with or without Myr-Akt for 24 h, the lysates were analyzed by immunoprecipitation with anti-Beclin 1, and the immunocomplexes were analyzed as in C. (E) *Usp14*^{-/-} H4 cells were virally transfected with wild-type USP14 or the USP14-DD mutant as indicated for 18 h and

then treated with or without 1 μ M MK2206 for another 6 h, the lysates were analyzed by immunoprecipitation with anti-Beclin 1, and the immunocomplexes were analyzed as in D.

USP14 regulates autophagy by modulating K63-linked ubiquitination of Beclin 1

Since both USP14-mediated Beclin 1 deubiquitination and autophagy inhibition are dependent on Akt activation, we reasoned that Akt-activated USP14 might regulate autophagy through deubiquinating K63-linked ubiquiti-

nation of Beclin 1. To test this hypothesis, we first characterized the role of K63 ubiquitination of Beclin 1 in the regulation of autophagy by Akt. We transfected an expression vector encoding K63-only ubiquitin into H4 cells in the presence or absence of an Akt inhibitor. As shown in Figure 7A, overexpression of K63-only ubiquitin induced autophagy as indicated by the increase in the levels of

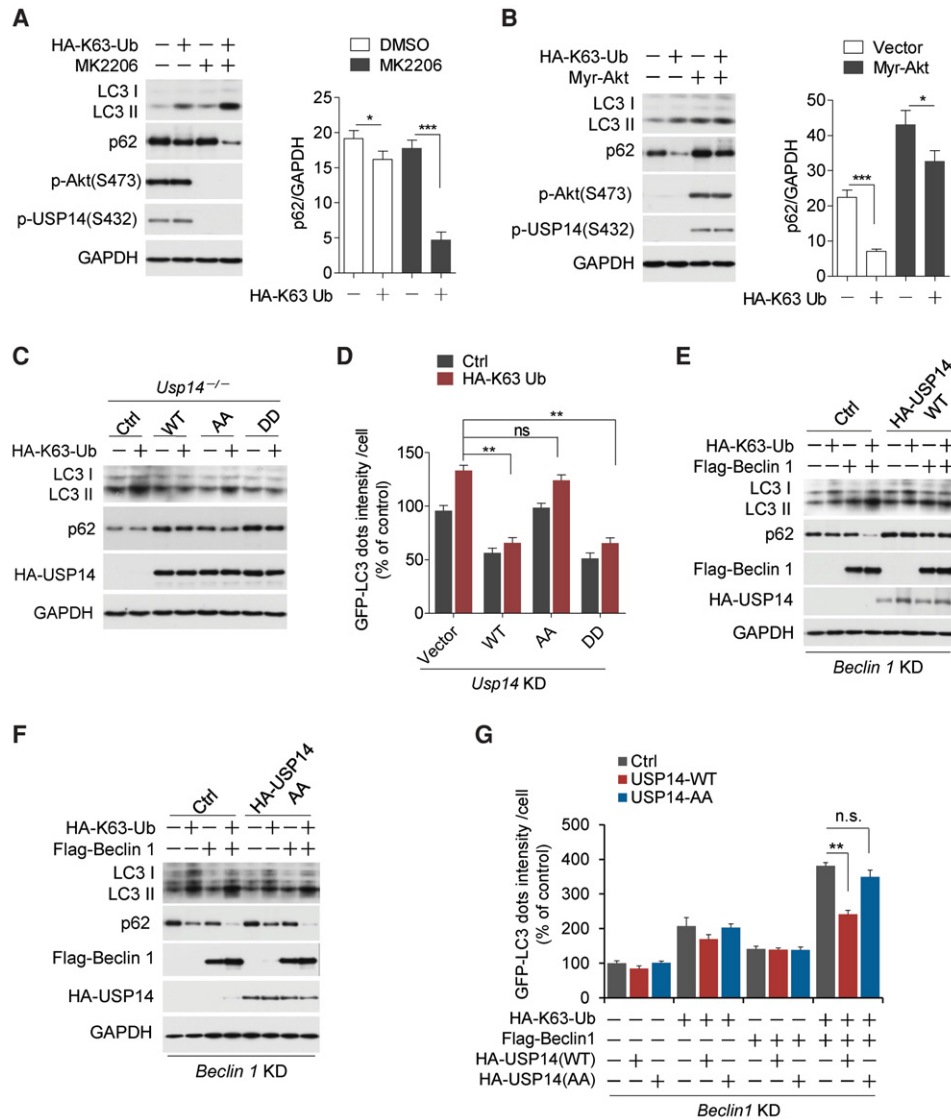


Figure 7. USP14 regulates autophagy by modulating Beclin 1 K63-linked ubiquitination. (A) H4 cells were virally infected with an expression vector for HA-tagged K63-only ubiquitin for 18 h and then treated with or without 1 μ M MK2206 for another 6 h, and the lysates were analyzed by Western blotting. The ratios of p62/GAPDH were calculated and are shown at the right. Results are shown as means \pm SD of three independent sets of experiments. (B) H4 cells were virally infected with an expression vector for HA-tagged K63-only ubiquitin with or without that of Myr-Akt for 24 h, and the lysates were analyzed by Western blotting. The ratios of p62/GAPDH were calculated and are shown at the right. Results are shown as means \pm SD of three independent sets of experiments. (C) *Usp14*^{-/-} H4 cells were virally infected with expression vectors for wild-type USP14 or the mutant as indicated with or without HA-tagged K63-only ubiquitin for 24 h, and the lysates were analyzed by Western blotting. (D) H4-GFP-LC3 cells with *Usp14* stable knockdown were virally infected with expression vectors for wild-type USP14 or the mutant as indicated with or without HA-tagged K63-only ubiquitin for 24 h and imaged using an ArrayScan HCS 4.0 reader. The average spot intensity in 1000 cells from each indicated sample was determined. (E,F) H4 cells with *Beclin 1* stable knockdown were virally infected with expression vectors for Flag-tagged Beclin 1 and HA-tagged K63-only ubiquitin as indicated in the presence or absence of wild-type USP14 (E) or the USP14-AA mutant (F) for 24 h, and the lysates were analyzed by Western blotting. (G) H4-GFP-LC3 cells with *Beclin 1* stable knockdown were virally infected as in E and F and then were imaged and quantified as in D.

LC3-II and the decrease in the levels of p62, and this effect was further enhanced when the cells were treated with MK2206. Conversely, when coexpressed with activated Akt, the expression of K63-only ubiquitin was not able to further increase autophagy, as determined by p62 protein levels (Fig. 7B). These results suggest that K63 ubiquitination-promoted autophagy is negatively regulated by Akt.

To demonstrate that Akt-mediated USP14 phosphorylation inhibits autophagy through Beclin 1 K63-linked deubiquitination, we overexpressed wild-type USP14 or mutants together with K63-only ubiquitin in *Usp14* knockout and knockdown H4 cells (Fig. 7C,D). Strikingly, both wild-type USP14 and phosphomimic mutant USP14 (USP14-DD), but not USP14-AA, were able to inhibit K63 ubiquitination-induced autophagy as determined by both Western blotting and GFP-LC3 assay (Fig. 7C,D). To further verify the role of USP14-mediated Beclin 1 K63-linked deubiquitination in autophagy regulation, we transfected expression vectors for K63-only ubiquitin and Beclin 1 in *Beclin 1* stable knockdown H4 cells (Xu et al. 2015b) in the presence or absence of USP14. As shown in Figure 7E, coexpression of K63-only ubiquitin and Beclin 1 activates autophagy activity, as determined by the reduction in p62 protein levels and increase in LC3-II protein levels, while expressing K63-only ubiquitin or Beclin 1 alone only slightly increased LC3-II levels. However, the activation of autophagy activity by coexpression of K63-only ubiquitin and Beclin 1 was totally blocked when coexpressing USP14. Moreover, the AA mutant of USP14, which cannot be phosphorylated by Akt, failed to block Beclin 1 K63 ubiquitination-induced autophagy (Fig. 7F). In addition, we further verified these results using a GFP-LC3 assay on *Beclin 1* stable knockdown H4-GFP-LC3 cells (Fig. 7G; Xu et al. 2015b). From these results, we conclude that Akt-mediated phosphorylation of USP14 regulates autophagy by controlling K63-linked ubiquitination of Beclin 1 under normal nutritional conditions (Supplemental Fig. S6).

Discussion

Substrate selection of both UPS and autophagy involves protein ubiquitination, although the nature of the linkage and/or topology of the ubiquitination might be different. UPS targets primarily K48 ubiquitinated proteins, whereas K63 ubiquitination may provide one of the mechanisms to target proteins for degradation through autophagy (Olzmann et al. 2007; Tan et al. 2008). Since ubiquitination of different linkages such as K48 or K63 is accomplished by distinct E3 ubiquitin ligases through their respective E2 enzymes under tightly regulated conditions, ubiquitination processes are unlikely to provide common mechanisms to simultaneously control both UPS and autophagy. On the other hand, DUBs can often target more than one type of ubiquitin linkage and thus are often less selective in their targeting substrates than E3 ubiquitin ligases. In this regard, since USP14 is a DUB that can trim K48 ubiquitin chains on different substrates that bind with proteasomes, it is conceivable that USP14

might be able to regulate K63 ubiquitination on different substrates. Using mass spectrometry analysis, we demonstrated the ability of USP14 to regulate K63 ubiquitination of Beclin 1, a key component of Vps34 complexes. Since USP14 can modulate K63 ubiquitination of multiple targets, our study does not rule out that other targets of USP14 can potentially also regulate autophagy. Our study demonstrates that regulation of protein deubiquitination may provide a mechanism to control the activity of both UPS and autophagy. As a key regulator that controls both UPS and autophagy, USP14 might act as a key signaling molecule in coordinating these two major intracellular proteolytic pathways.

As a critical mediator of multiple intracellular signaling pathways, Akt regulates both anabolic and catabolic mechanisms. Akt is known to negatively regulate autophagy in both mTOR-dependent (Levine and Klionsky 2004; Levine and Kroemer 2008) and mTOR-independent (Lipinski et al. 2010; Wang et al. 2012) mechanisms. Previous studies have shown that Akt can control UPS and autophagy through distinct mechanisms. Our results suggest that Akt-mediated phosphorylation of USP14 negatively regulates both UPS and autophagy. These results suggest that activated Akt in *Pten*-negative cancer cells might be able to stabilize a significant portion of the proteome by inhibiting both UPS and autophagy through regulating USP14 to promote tumorigenesis. Consistent with this possibility, increased expression of USP14 has been found in a variety of cancers, including multiple myeloma (Tian et al. 2014), colorectal cancer (Shinji et al. 2006), lung cancer (Wu et al. 2013), epithelial ovarian cancer (Wang et al. 2015), and endometrial cancer (Vogel et al. 2016). Future studies will be needed to elucidate the functional role of elevated USP14 expression in tumorigenesis. Finally, our results suggest the possibility of developing inhibitors of USP14 for modulating both UPS and autophagy as a strategy to promote the degradation of misfolded proteins for the treatment of neurodegenerative diseases.

Materials and methods

Cell culture

HEK293T cells were cultured in DMEM (Gibco) with 10% (v/v) FBS (Gibco) and 1% penicillin/streptomycin. H4, H4-GFP-LC3, H4-RFP-FYVE, and H4-*Usp14*^{-/-} cells were maintained in DMEM supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin, and 1×sodium pyruvate (Invitrogen).

Antibodies and reagents

The commercial antibodies used for Western blot analysis include the following: Anti-phospho-Akt (Ser473) (1:1000 dilution; no. 3787), anti-LC3B (1:1000 dilution; no. 2775), anti-USP14 (rabbit, 1:1000 dilution; no. 11931), anti-Atg14L (1:1000 dilution; no. 5504), and anti-Flag (1:1000 dilution; no. 2368) were from Cell Signaling Technology. Anti-Beclin 1 (1:1000 dilution for Western blot [sc-11427]; 1:200 dilution for immunoprecipitation [sc-48341]), anti-Akt (1:1000 dilution; sc-8312), and anti-USP14 (mouse, 1:100 dilution for immunoprecipitation; sc-393872) were from Santa Cruz Biotechnology. Anti-Myc (1:1000 dilution;

16286-1-AP), anti-HA (1:1000 dilution; 51064-2-AP) anti-Rubion (1:1000 dilution; 21444-1-AP), and anti-GAPDH (1:10,000 dilution; 60004-1-Ig) were from Proteintech. Anti-Tubulin (1:10,000 dilution; PM054), anti-UVRAG (1:1000 dilution; PD027), and anti-p62 (1:5000; PM045) were from MBL. Anti-ubiquitin Lys63-specific antibody (1:2000 dilution; no. 2210353) was from Millipore. Mouse monoclonal anti-Flag (1:500 for immunoprecipitation; F1804), anti-Myc, and anti-HA affinity gel (E6779) were from Sigma-Aldrich. IU1 (S7134), MK2206 (S1078), E64D (S7379), rapamycin (S1039), and MG132 (S2619) were from Selleckchem.

Plasmids and siRNA transfection

cDNAs for USP14 and a constitutively active form of Akt (Myr-Akt) were cloned into pcDNA3.1 using Phanta Max Super-Fidelity DNA polymerase (Vazyme Biotech Co., Ltd.) and ClonExpress II cloning kit (Vazyme Biotech Co., Ltd.). Mutagenesis was performed using the MutExpress II mutagenesis kit (Vazyme Biotech Co., Ltd.). Cells were transfected with plasmid DNA using PolyJet DNA in vitro transfection reagent (Signagen Laboratories) according to the manufacturer's instructions. The sequences of siRNAs used in this study were as follows: si*Usp14-a* (5'-GGA GAAUUUGAAGGUGUA-3'), si*Usp14-b* (5'-GCAGCCCUUA GAGAUUUGU-3'), and si*Usp14-c* (5'-GCCUCGAGAGUUG AAAUA-3'). siRNA transient transfections were performed using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions.

Generation of knockdown and reconstitution lines

H4 and H4-GFP-LC3 cells were stably infected with shRNA against *Usp14* 3' untranslated region (UTR) (CTTTAGAGGAA GACACATA), *Beclin 1* 3' UTR (CTCTGTGTTAGAGATATGA) (Xu et al. 2015b), or scramble control in the pLVX lentiviral background. Lentiviral particles were made according to the manufacturer's instructions (Clontech). Viral supernatants were collected 48 h after transfection. Cleared supernatant was filtered through a 0.45- μ m filter. Polybrene (8 μ g/mL) was supplemented with viral supernatants. Twenty-four hours after infection, cells stably expressing shRNA were obtained by selection with 10 μ g/mL puromycin (Invivogen). *Usp14* 3' UTR shRNA-expressing H4 or H4-GFP-LC3 cells and *Usp14*^{-/-} H4 cells (Xu et al. 2015a) were infected with lentiviral particles expressing HA-USP14 (wild-type or mutant). Polyclonal populations were screened until wild-type and mutant lines were generated that had near-endogenous USP14 reconstitution levels.

Vps34 lipid kinase assay and protein-lipid blot assay

Vps34/Beclin 1 complexes were immunoprecipitated by antibody against Beclin 1. The immunoprecipitated beads were washed three times and added with 0.1 mg/mL PL3PS and 25 μ M ATP in 50 μ L of reaction buffer (50 mM HEPES at pH 7.5, 50 mM NaCl, 3 mM MgCl₂, 0.025 mg/mL BSA) for 1 h at 25°C. The production of PtdIns3P was detected by protein-lipid blot assay as reported (Liu et al. 2011; Xu et al. 2015b). Briefly, lipid extracted from the reaction was spotted onto a Hybond C-extra membrane (Amersham) using a Bio-DOTTM apparatus (Bio-Rad). The membrane was allowed to dry overnight in the dark. Next, the membrane was incubated with blocking buffer (5% BSA in TBST) for 1 h, washed once in TBST for 30 min, and incubated with protein buffer (1 mg of GST-tagged PX-p40 domain protein, which binds to PtdIns3P, per 1 mL of TBST with 5% BSA) overnight at 4°C. The membrane was then washed in TBST three times for

30 min each, incubated with anti-GST (Sigma) in 5% BSA buffer for 4 h, washed in TBST for three changes for 5 min each, incubated with secondary antibody for 1 h, and washed in TBST for three changes for 5 min each. All incubations were at room temperature unless noted otherwise. The signals were visualized with ECL.

Mass spectrometry and data analysis

The quantitative analysis of ubiquitinated proteome change in the absence or presence of USP14 in H4 cells expressing Flag-His-K63-only ubiquitin was performed by TMT isobaric labeling followed by shotgun analysis. The immunoprecipitation for Flag followed by His pull-down was performed to obtain ubiquitinated proteome. The proteins pulled down from control cells, or cells overexpressing wild-type USP14 or the USP14-AA mutant were trypsin-digested and labeled with 126-TMT, 127-TMT, and 129-TMT labeling reagent (Thermo Scientific), respectively, as the instructions described. The same amount of peptides was mixed with each TMT tag, and the resulting mixture of peptides was analyzed on a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer. Three replicates were performed. The protein identification and quantification were done by Thermo Proteome discoverer (version 1.4). The peptide false positive rate was controlled to be <1%. The peak integration tolerance was set as 10 ppm. Only unique peptides were used for protein quantitation. The isobaric tag purity correction was performed. The labeling efficiency was measured and was >99%. The average ratios of each protein from three replicates were used for analysis.

The binding partners of endogenous Beclin 1 with overexpression of K63 ubiquitin were identified by mass spectrometry. The proteins obtained by immunoprecipitation against Beclin 1 in cells with or without K63 ubiquitin overexpression were trypsin-digested on beads. The resulting peptides were analyzed on a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer. Three replicates were performed. The protein identification and quantification were done by MaxQuant (Cox and Mann 2008). The tandem mass spectra were searched against the UniProt human protein database and a set of commonly observed contaminants. The precursor mass tolerance was set as 20 ppm, and the fragment mass tolerance was set as 0.5 Da. The cysteine carbamidomethylation was set as a static modification, and the methionine oxidation was set as a variable modification. The false discovery rate at the peptide spectrum match level and protein level was controlled to be <1%. The unique peptides plus razor peptides were included for quantification. The summed peptide intensities were used for protein quantification.

In vitro deubiquitination assay

For preparation of ubiquitinated Beclin 1 as the substrate for the in vitro deubiquitination assay, HEK293T cells were transfected with expression vectors of HA-K63-ubiquitin and Flag-Beclin 1 for 24 h, and ubiquitinated Beclin 1 was purified from the cell extracts with anti-Flag affinity column in lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 1% NP-40, protease inhibitor cocktail, 100 μ g/mL PMSF, 1 mM Na₃VO₄, 50 mM NaF). After extensive washing with the lysis buffer, the proteins were eluted with Flag peptides (Sigma). The recombinant USP14 and USP14-S432E were obtained as previously reported (Xu et al. 2015a). Ubiquitinated Beclin 1 protein was incubated with recombinant USP14 in a deubiquitination buffer (50 mM Tris-HCl at pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) for 2 h at 37°C.

Cell imaging and statistical analysis

Cells were fixed with 4% paraformaldehyde (Sigma) and stained with 3 $\mu\text{g}/\text{mL}$ DAPI (Sigma). Image data were collected with an ArrayScan HCS 4.0 reader with a 203 objective (Cellomics ArrayScan VTI) for DAPI-labeled nuclei and GFP/RFP-tagged intracellular proteins. Error bars for microscopy are presented as the standard deviation of triplicate samples. Error bars for Western blot analysis represent the standard deviation between densitometry data from three unique experiments. Student's *t*-test was used as statistical analysis by using GraphPad Prism.

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