Auto-ubiquitination of NEDD4-1 Recruits USP13 to Facilitate Autophagy through Deubiquitinating VPS34

Highlights

- NEDD4-1 promotes autophagy through inhibiting K48-linked ubiquitination of VPS34
- NEDD4-1 recruits USP13 to deubiquitinate VPS34
- Auto-ubiquitination of NEDD4-1 is required for its interaction with USP13 and VPS34

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In Brief

Xie et al. demonstrate that HECT ubiquitin E3 ligase NEDD4-1 undergoes lysine 29 (K29)-linked auto-ubiquitination and serves as a scaffold to recruit the ubiquitin-specific protease 13 (USP13) to form a deubiquitination complex, which subsequently stabilizes VPS34 to promote autophagy through the removal of K48-linked poly-ubiquitin chains on VPS34.

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Auto-ubiquitination of NEDD4-1 Recruits USP13 to Facilitate Autophagy through Deubiquitinating VPS34

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SUMMARY

The class III phosphoinositide 3-kinase vacuolar protein sorting 34 (VPS34) is a core protein of autophagy initiation, yet the regulatory mechanisms responsible for its stringent control remain poorly understood. Here, we report that the E3 ubiquitin ligase NEDD4-1 promotes the autophagy flux by targeting VPS34. NEDD4-1 undergoes lysine 29 (K29)-linked auto-ubiquitination at K1279 and serves as a scaffold for recruiting the ubiquitin-specific protease 13 (USP13) to form an NEDD4-1-USP13 deubiquitination complex, which subsequently stabilizes VPS34 to promote autophagy through removing the K48-linked poly-ubiquitin chains from VPS34 at K419. Knockout of either NEDD4-1 or USP13 increased K48-linked ubiquitination and degradation of VPS34, thus attenuating the formation of the autophagosome. Our results identify an essential role for NEDD4-1 in regulating autophagy, which provides molecular insights into the mechanisms by which ubiquitination regulates autophagy flux.

INTRODUCTION

Macroautophagy, hereafter called autophagy, is an evolutionarily conserved eukaryotic bioprocess that plays a critical role in cellular homeostasis by facilitating the capture and clearance of intracellular macromolecules, organelles, and invading pathogens (Galluzzi et al., 2017; Mizushima, 2018). Autophagy initiation is inducible and occurs in response to different forms of stress, including nutrient starvation, energy deprivation, infection, and hypoxia (Galluzzi et al., 2017; Liu et al., 2016). The rapid induction of autophagy functions in maintaining cell homeostasis and survival, thus playing an essential role in a variety of biological processes ranging from development to aging (Dikic and Elazar, 2018; Hansen et al., 2018). Autophagy deficiency has been implicated in various disease states, such as cancers, neurodegenerative diseases, infectious diseases, and myopathy (Deretic and Levine, 2018; Mizushima, 2018).

Autophagy is orchestrated by a number of autophagy-related proteins (ATGs) (Hansen et al., 2018; Jin et al., 2016). Vacular protein-sorting 34 (VPS34), the only PI3KC3 in mammals, is the catalytic subunit of the class III phosphatidylinositol 3-kinase (PI3KC3) complex (Dikic and Elazar, 2018; Hurley and Young, 2017; Kemp, 2017). It is activated at phagophore and endosomal membranes, where key proteins harboring phosphatidylinositol 3-phosphate (PI3P)-binding domains are recruited for membrane remodeling, and is important for autophagy and endosomal transport (Hurley and Young, 2017; Mizushima, 2018). Under autophagy activation conditions, VPS34 interacts with Beclin-1, VPS15, and ATG14L at the phagophore to form functional complexes (Dikic and Elazar, 2018; Mizushima, 2018). It has been well demonstrated that posttranslational modification (PTM), especially the phosphorylation of VPS34, plays a critical role in the strict control of autophagy initiation (Hurley and Young, 2017). ULK1 can phosphorylate VPS34 on serine (S) 249 (Egan et al., 2015). 5′ AMP-activated protein kinase (AMPK) differentially controls the endocytic and autophagic pools of VPS34. AMPK inhibits endosomal VPS34 activity by mediating the phosphorylation of threonine (T) 163 and S165 on VPS34, thereby shutting down this process when external energy sources become deficient. On the contrary, with the existence of ATG14L, an autophagy-essential protein present only in the pro-autophagy VPS34 complex, AMPK loses its ability to phosphorylate VPS34 (Kim et al., 2013). DAPK (death-associated protein kinase), a stress kinase responsible for sensing oxidative and endoplasmic reticulum (ER) stress, is capable of activating VPS34 indirectly through phosphorylating protein kinase D, which subsequently binds to and phosphorylates VPS34 (Eisenberg-Lerner and Kimchi, 2012). Furthermore, CDK1 phosphorylates VPS34 at T159 to negatively regulate its interaction with Beclin-1 during the mitotic arrest induced by DNA damage agents (Furuya et al., 2010).

In addition to phosphorylation, another PTM type, ubiquitination, as well as its reversal process, deubiquitination, play pivotal roles in the regulation of the autophagy process. Recently, the ubiquitination modification of VPS34 has received broad attention. Cullin1 is responsible for suppressing autophagy by
Figure 1. NEDD4-1 Positively Regulates Autophagy by Targeting VPS34

(A and B) Representative images (A) and quantification of GFP-LC3B puncta (B) of HeLa-GFP-LC3B cells transfected with scrambled (Scr) siRNA or NEDD4-1-specific siRNAs in normal medium (DMEM) or Earle’s balanced salt solution (EBSS) medium for 2 h. Arrows denote representative autophagosomes. The images in (A) were taken using Leica DMI3000 B microscopy with a 100× numerical aperture (NA) oil-immersion objective. Scale bar, 20 μm. The bars in (B) represent means ± SEMs of triplicate samples (80 cells per sample). ***p < 0.001 (2-tailed Student’s t test).

(C) 293T cells were transfected with plasmid encoding HA-NEDD4-1 and treated with rapamycin (250 nM) for 12 h. Protein extracts were harvested after bafilomycin A1 (Baf A1) (0.2 μM) treatment for 6 h.

(D and E) Protein extracts of THP-1 cells (D) or human peripheral blood mononuclear cells (PBMCs) (E) treated with EBSS for various time points (above lanes) were subjected to immunoprecipitation with anti-NEDD4-1 and immunoblot analysis with indicated antibodies.

(F and G) HeLa cells were incubated in EBSS medium for 1 h after co-transfection with EGFP-NEDD4-1 and mCherry-VPS34 vectors for 48 h. Cells were imaged for EGFP and mCherry and images were taken using Zeiss LSM 710 microscopy with a 100× NA oil-immersion objective (F). Scale bar, 40 μm. Quantitative data are means ± SEMs of triplicate samples (80 cells per sample) (G). ***p < 0.001 (2-tailed Student’s t test).

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targeting VPS34 for ubiquitination and proteasomal degradation (Xiao et al., 2015). The Cul3-KLHL20 ubiquitin ligase controls autophagy-induced degradation of VPS34 complex members, including VPS34 and Beclin-1 as direct substrates (Liu et al., 2016). In Caenorhabditis elegans, UBC-13 functions with UEV-1, a noncatalytic E2 variant, and CHN-1, a U-box-containing E3 ubiquitin ligase, to catalyze K63-linked ubiquitin chains on VPS34, hence leading to the stabilization of VPS34 protein abundance (Liu et al., 2018a). Despite these findings, the function and mechanism underlying the regulation of VPS34 deubiquitination remain unclear.

Neural precursor cell expressed developmentally downregulated protein 4-1 (NEDD4-1) belongs to the HECT ubiquitin E3 ligase family (Sun et al., 2014; Wang et al., 2007). It has been reported to ubiquitinate the epithelial sodium channel (ENaC) and accelerate its proteasomal degradation (Tofaris et al., 2011; Xu et al., 2016). Both in yeast and mammalian cells, NEDD4 regulates the cargo sorting, intracellular trafficking, and degradation of a large number of proteins in multiple cellular compartments by the ubiquitination of its specific substrates (Fajner et al., 2017; Lin et al., 2017; Sun et al., 2017; Xu et al., 2016; Yang and Kumar, 2010). Moreover, NEDD4-1 functions in autophagy by mediating the ubiquitination of Beclin-1, thus leading to its stabilization (Pei et al., 2017). In addition, NEDD4-1 ubiquitinates p62/SQSTM1 with the help of autophagy marker light chain 3 (LC3) (Lin et al., 2017; Sun et al., 2017). However, whether NEDD4-1 affects the stabilization of other key proteins in autophagy is incompletely understood. In this study, we have identified a pivotal role for NEDD4-1-mediated VPS34 deubiquitination in autophagy. NEDD4-1 undergoes K29-linked auto-ubiquitination at lysine (K) 1279 and serves as a platform to bridge ubiquitin-specific protease 13 (USP13) to VPS34, leading to the decreased K48-linked ubiquitination of VPS34 at K419. Our findings provide insight into the mechanisms underpinning VPS34 deubiquitination mediated by the NEDD4-1-USP13 axis, thus promoting the stabilization of VPS34 and contributing to autophagosome formation.

RESULTS

NEDD4-1 Positively Regulates Autophagy by Targeting VPS34

Previous studies have revealed that NEDD4-1 affects autophagy at multiple levels by targeting Beclin-1 and p62 (Lin et al., 2017; Pei et al., 2017; Sun et al., 2017). To further study the intricate role of NEDD4-1 in autophagy, we synthesized specific small interfering RNAs (siRNAs) targeting three different regions of NEDD4-1 to knock down the expression of NEDD4-1. All of these siRNAs efficiently inhibited the expression of transfected and endogenous NEDD4-1 in human embryonic kidney (HEK) 293T cells and HeLa cells (Figure S1A). We next assessed the effects of NEDD4-1 knockdown on the formation of autophagosomes under both basal conditions and autophagy-induced conditions treated with Earle’s balanced salt solution (EBSS) and found that NEDD4-1 depletion resulted in considerably fewer numbers of GFP-LC3B puncta (Figures 1A and 1B). We also examined the autophagic flux by detecting LC3 and the autophagy substrate p62 with immunoblot analysis, and observed that the overexpression of NEDD4-1 significantly enhanced the accumulation of LC3 II and consumed more p62 (Figure 1C). To further demonstrate the role of NEDD4-1 in autophagy, we generated an FLAG-tagged NEDD4-1 doxycycline (Dox)-inducible A549 cell line (Figure S1B) and found that the ectopic expression of NEDD4-1 resulted in higher levels of LC3 II and more consumption of p62 (Figure S1C). These results confirmed that NEDD4-1 is a positive regulator of autophagy flux, as previously reported (Lin et al., 2017; Pei et al., 2017; Sun et al., 2017).

To determine how NEDD4-1 promotes autophagy besides associating with Beclin-1, ATG8, and p62, we co-transfected 293T cells with plasmids encoding autophagy-related (ATG) proteins together with NEDD4-1 vector and found that NEDD4-1 interacted with VPS34, Beclin-1, Atg14L, and UVRAG, while among them, the interaction between NEDD4-1 and VPS34 was the strongest (Figure S1D). As VPS34 is an indispensable member of the VPS34 complex and affects the assembly of other subunits to form a functional complex, we next determined whether knockdown of VPS34 would affect the interaction between NEDD4-1 and other members of the VPS34 complex. Our analysis showed that VPS34 depletion remarkably diminished the interaction between NEDD4-1 and other members of the VPS34 complex, indicating that VPS34 is essential for the association between NEDD4-1 and other VPS34 complex members (Figures S1E and S1F). Meanwhile, we did not observe any apparent interaction between NEDD4-1 and the ULK1 complex members, including ULK1, ATG13, ATG101, and FIP200 (Figure S1G). The association between VPS34 and other VPS34 complex subunits was not altered by NEDD4-1, indicating that NEDD4-1 does not affect the assembly of VPS34 complex (Figure S1H). We next treated THP-1 cells and human peripheral blood mononuclear cells (PBMCs) with EBSS medium and found that the interaction between NEDD4-1 and VPS34 was significantly increased at 0.5–1 h upon starvation treatment (Figures 1D and 1E). Further confocal microscopy analysis revealed that VPS34 colocalized with NEDD4-1 under EBSS incubation (Figures 1F and 1G). These results suggest that VPS34 is an unrecognized target of NEDD4-1 during autophagy.

To determine which domain of NEDD4-1 is responsible for the interaction with VPS34, we generated three truncations of NEDD4-1 and found that VPS34 interacted with the NEDD4-1 mutant containing the HECT domain (amino acids [aa] 955–1,319), but not the mutants containing its N-terminal (aa 1–581) or the WW domain (aa 582–954) (Figure 1H). Next, we examined which domain of VPS34 was needed for its interaction with NEDD4-1. We observed that the VPS34 mutant containing the C2 domain (aa 1–233) but not other domains interacted with

(H) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with vectors for NEDD4-1 and its deletions along with vector encoding FLAG-VPS34.

(i) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with vectors for VPS34 and its deletions along with vector encoding HA-NEDD4-1. All of the experiments are representative of 3 independent biological experiments with similar results.
NEDD4-1 (Figure 1). These results suggest that the HECT domain of NEDD4-1 binds to the C2 domain of VPS34.

**NEDD4-1 Stabilizes VPS34**

To discover the molecular mechanism by which NEDD4-1 regulates autophagy via VPS34, we overexpressed NEDD4-1 in FLAG-NEDD4-1-inducible A549 cells with increasing doses of Dox and found that the protein abundance of VPS34, as well as other VPS34 complex members, including Beclin-1, VPS15, ATG14L, and UVRAG, positively correlated with increasing amounts of NEDD4-1 (Figure 2A). To exclude the possibility that the upregulation of VPS34 protein was caused by transcription, we examined the mRNA levels of VPS34 and found that the amount of VPS34 transcripts remained unchanged (Figure 2A). As VPS34 depletion remarkably diminished the interaction between NEDD4-1 and other members of the VPS34 complex, we further assessed the effects of VPS34 in NEDD4-1-regulated autophagy and found that the knockdown of VPS34 resulted in the impaired function of NEDD4-1 to stabilize Beclin-1, VPS15, ATG14L, and UVRAG (Figure 2B). To test whether the role of NEDD4-1 in VPS34 stabilization is dependent on Beclin-1, we examined the protein level of VPS34 in BECN1-deficient cells and found that NEDD4-1 could still stabilize VPS34 in the absence of Beclin-1 (Figure S2A). These results suggest that NEDD4-1 promotes autophagy by stabilizing VPS34 in a Beclin-1-independent manner. Likewise, we found that the overexpression of NEDD4-1 stabilized endogenous VPS34 under both basal and autophagy-induced conditions (Figure 2C). To discover the molecular mechanism by which NEDD4-1 regulates autophagy, we examined the mRNA levels of VPS34 and found that the upregulation of VPS34 protein was caused by transcription.

Figure 2. NEDD4-1 Stabilizes VPS34

(A) FLAG-tagged NEDD4-1-inducible A549 cells were treated with increasing doses of doxycycline (Dox) for 12 h, and the protein was harvested for immunoblot analysis. Below, RT-PCR analysis of VPS34 mRNA; RPL13 mRNA serves as a loading control.

(B) 293T cells were transfected with Scr siRNA or VPS34-specific siRNAs (#4), together with empty vector (EV) or vector for HA-NEDD4-1. Protein extracts were immunoblotted with indicated antibodies.

(C) Immunoblot analysis of protein extracts of 293T cells transfected with EV or vector for HA-NEDD4-1, and treated with EBSS for the indicated time points.

(D) A549 cells were transfected with Scr siRNA or NEDD4-1-specific siRNAs. The lysates were analyzed with the indicated antibodies.

(E) PBMCs were transfected with Scr siRNA or NEDD4-1-specific siRNAs, followed by treatment with rapamycin (250 nM) for 12 h. Protein extracts were analyzed with the indicated antibodies.

(F) Wild-type (WT) and NEDD4-1 knockout (KO) A549 cells were cultured in EBSS for the indicated time points, and the protein expression levels of VPS34 were detected by immunoblot. The cell lysates were analyzed by immunoblot.

(G) All of the experiments are representative of 3 independent biological experiments with similar results.
Figure 3. NEDD4-1 Inhibits K48-Linked Ubiquitination of VPS34 on K419

(A) Coimmunoprecipitation and immunoblot analysis of extracts of 293T cells transfected with Scr siRNA or NEDD4-1 siRNAs, together with the expression vector of FLAG-VPS34, and treated with MG132 (10 \( \mu \)M) for 3 h.

(B) Coimmunoprecipitation and immunoblot analysis of extracts of HeLa cells transfected with Scr siRNA or NEDD4-1 siRNAs and treated with MG132 (10 \( \mu \)M) for 3 h using anti-VPS34 antibody and immunoblotted with anti-K48-ubiquitin (Ub) antibody.

(C) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with vectors expressing FLAG-VPS34 or its mutants, together with HA-K48-Ub, and treated with MG132 (10 \( \mu \)M) for 3 h.

(D) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with vectors expressing WT FLAG-VPS34 or its K419R mutant, together with EV or vector for Myc-NEDD4-1, and treated with MG132 (10 \( \mu \)M) for 3 h.

(E) Immunoblot analysis of protein extracts of 293T cells transfected with EV or vector for HA-NEDD4-1, together with plasmids expressing WT FLAG-VPS34 or its indicated mutants. Different amounts of plasmids for various mutants of FLAG-VPS34 were transfected into cells for equal expression without NEDD4-1 overexpression.

(F) Immunoblot analysis of protein extracts of 293T cell transfected with WT FLAG-VPS34 or its K419R mutant, and treated with CHX (100 \( \mu \)g/mL) for the indicated time points.

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VPS34, we generated NEDD4-1 knockout (KO) A549 cells and found that NEDD4-1 deficiency led to the degradation of VPS34 under both basal and autophagy-induced conditions (Figure 2F). In addition, we found that NEDD4-1 was degraded after 6–9 h of autophagy induction and that this degradation could be rescued by bafilomycin A1 (Baf A1) treatment (Figures S2B and S2C). NEDD4-1-directed autophagy can further mediate the autophagic degradation of NEDD4-1, thus preventing the overactivation of autophagy. Further investigation showed that VPS34 could still be stabilized by NEDD4-1 in the presence of autophagy inhibitor 3-methyladenine (3MA) and autolysosome inhibitor Baf A1, but not proteasome inhibitor MG132 (Figure 2G), indicating that NEDD4-1 mediates VPS34 stabilization via a ubiquitin-proteasome pathway. These results demonstrate that NEDD4-1 stabilizes VPS34 through the ubiquitin-proteasome pathway.

NEDD4-1 Inhibits K48-Linked Ubiquitination of VPS34 on K419

To determine how NEDD4-1 regulates the stability of VPS34 protein, we analyzed the ubiquitination of VPS34 and found that NEDD4-1 depletion remarkably increased the poly-ubiquitination of VPS34 (Figure 3A). We further demonstrated that NEDD4-1 specifically inhibited K48-linked ubiquitination of VPS34 under autophagy-induced conditions (Figure S3A). In addition, we observed that knockdown of NEDD4-1 enhanced the K48-linked ubiquitination of VPS34 in HeLa cells (Figure 3B). Likewise, we found that NEDD4-1 could still inhibit the K48-linked ubiquitination of VPS34 in BECN1 KO cells (Figure S3B).

To gain insight into the mechanism of NEDD4-1 in cleaving the K48-linked ubiquitin chains on VPS34, we next identified five conserved lysine sites on VPS34, and generated the VPS34 mutants bearing single lysine (K)-to-arginine (R) substitution in every potential ubiquitin site for analysis. The ubiquitination assay showed that the VPS34 K419R mutant displayed reduced K48-linked ubiquitination (Figure 3C). We further found that NEDD4-1 failed to cleave the K48-linked ubiquitin chains of the VPS34 K419R mutant (Figure 3D), indicating that K419 of VPS34 is an essential residue for its K48-linked ubiquitination. Further experiments showed that unlike wild type (WT) or other mutants of VPS34, VPS34 K419R mutant could no longer be stabilized by NEDD4-1 (Figures 3E and S3C). We next determined whether K48-linked ubiquitination on K419 functions as a degradation signal for VPS34 by a cycloheximide (CHX)-chase assay and found that the degradation rate of VPS34 K419R mutant was slower, compared with WT VPS34 (Figures 3F and 3G). In addition, we observed the dramatically enhanced formation of GFP-LC3B puncta in VPS34 K419R reconstituted GFP-LC3B HeLa cells when compared to WT VPS34 reconstituted cells (Figures 3H and 3I). Consistently, the VPS34 K419R mutant resulted in an increased accumulation of LC3 II and consumed more p62 (Figure S3D). Collectively, these results suggest that NEDD4-1 specifically removes K48-linked poly-ubiquitin chains from VPS34 on K419 to promote autophagy.

NEDD4-1 Recruits USP13 to Deubiquitinate VPS34

Since NEDD4-1 is a well-studied E3 ubiquitin ligase that mainly catalyzes the ubiquitination of certain substrates (Lin et al., 2017; Pei et al., 2017; Sun et al., 2014; Wang et al., 2007; Yang and Kumar, 2010), we speculated that certain DUBs may be involved in removing K48-linked ubiquitin chains on VPS34 mediated by NEDD4-1. USP10 and USP13 have been reported to regulate the stability of the VPS34 complex (Liu et al., 2011). We next examined whether NEDD4-1 recruits USP10 or USP13 for VPS34 stabilization. We found that NEDD4-1 markedly enhanced the association between VPS34 and USP13, but not USP10 (Figures 4A and S4A). Since previous reports indicated that the knockdown of USP10 led to a reduction in the level of USP13 (Liu et al., 2011), we further examined the interaction between USP13 and VPS34, as well as the stabilization of VPS34 by USP13 in USP10 KO cells. We found that USP13 could still interact with VPS34 and stabilize VPS34 in USP10 KO cells, indicating that USP10 is not required for USP13 to interact and stabilize VPS34 (Figures S4B and S4C). Consistently, knockdown of NEDD4-1 resulted in impaired interaction between VPS34 and USP13 (Figure S4D). Moreover, we observed that the diminished K48-linked ubiquitination of VPS34 mediated by NEDD4-1 was almost abrogated in USP13 KO cells (Figure 4B). We further performed an in vitro deubiquitination assay to show that USP13 removed the K48-linked ubiquitin chain on VPS34 in the presence of NEDD4-1 (Figure 4C). These results suggest that USP13 is indispensable for the NEDD4-1-regulated removal of K48-linked poly-ubiquitin chains on VPS34.

Since knockdown of VPS34 did not affect the association between NEDD4-1 and USP13 (Figure 4D), we hypothesized that NEDD4-1 may first bind to USP13 to form an NEDD4-1–USP13 complex. In addition, KO of BECN1 could not affect the interaction of NEDD4-1 with USP13 or VPS34, indicating that the NEDD4-1–USP13 complex binds to VPS34 in a Beclin-1-independent manner (Figure S4E). As expected, KO of NEDD4-1 completely abrogated the interaction between USP13 and VPS34 (Figure 4E). To confirm whether USP13 could directly bind to VPS34, we performed in vitro His-pull-down assays and found that purified NEDD4-1 could directly promote the association between USP13 and VPS34 (Figure 4F). To further determine the sequential events of the interaction of NEDD4-1 with USP13 or VPS34 under autophagy-induced conditions, we collected cells incubated with EBSS at various time points via microscopy with a 100× NA oil-immersion objective. Scale bar, 20 μm. The bars (I) represent means ± SEMs of triplicate samples (80 cells per sample). ***p < 0.001 (2-tailed Student’s t test).

The samples in (A)–(D) were incubated for 5 min with 1% SDS before immunoprecipitation. All of the experiments are representative of 3 independent biological experiments with similar results.

(Q) Quantification of the expression levels of WT and K419R VPS34 shown in (F). Bars represent means ± SEMs of triplicate samples. ***p < 0.001 (2-tailed Student’s t test).

(H and I) Representative images (H) and quantification of GFP-LC3B puncta (I) of HeLa-GFP-LC3B cells transfected with VPS34-specific siRNA (#4), together with plasmids expressing WT FLAG-VPS34 or its K419R mutant treated with DMSO or rapamycin (250 μM) for 12 h. Images (H) were taken using Leica DMi3000 B microscopy with a 100× NA oil-immersion objective. Scale bar, 20 μm. The bars (I) represent means ± SEMs of triplicate samples (80 cells per sample). ***p < 0.001 (2-tailed Student’s t test).
and immunoprecipitated complexes from lysates with NEDD4-1, USP13, and VPS34 antibodies, respectively. Immunoblot analysis showed that NEDD4-1 interacted with USP13 at 1 h post-EBSS incubation, while the apparent interaction between NEDD4-1-USP13 and VPS34 could be detected at a later time point (at 2 h) (Figure 4G), which revealed the dynamic assembly of the NEDD4-1-USP13-VPS34 complex. We next found that USP13 lost its ability to stabilize VPS34 in NEDD4-1 KO cells (Figure 4H). Meanwhile, NEDD4-1-mediated VPS34 stabilization was almost abolished in USP13 KO cells (Figure 4I). These results suggest that the formation of NEDD4-1-USP13 complex is an essential prerequisite for USP34 deubiquitination.

To determine whether USP13 stabilizes VPS34 in a catalytic activity-dependent manner, we overexpressed NEDD4-1 with the WT USP13 or the USP13 CA (C345A) mutant, the catalytically inactive form of USP13, in USP13 KO cells. We found that NEDD4-1 failed to stabilize VPS34 when restored with the USP13 CA mutant in USP13 KO cells (Figure 4J). Unlike WT USP13, the USP13 CA mutant failed to cleave the K48-linked ubiquitination of VPS34 and promote the stabilization of WT VPS34 (Figure 4K), indicating that the protease activity of USP13 is required for NEDD4-1-mediated VPS34 stabilization. USP13 could not affect the ubiquitination and protein stability of the VPS34 K419R mutant (Figure 4K), further confirming that the NEDD4-1-USP13 complex mainly cleaves K48-linked polyubiquitin chains from VPS34 on K419 to mediate its stability.

NEDD4-1 Bridges USP13 to VPS34 through Its E3 Ligase Activity

To investigate whether the ubiquitin E3 ligase activity of NEDD4-1 is required to promote autophagy flux, we generated the construct encoding catalytically inactive mutant of NEDD4-1, NEDD4-1 HA/CA (H1284A/C1286A), and restored the WT or HA/CA mutant of NEDD4-1 in NEDD4-1 deficient HeLa-GFP-LC3B cells. Fluorescence microscopy analysis showed that cells expressing the NEDD4-1 HA/CA mutant displayed a reduced accumulation of GFP-LC3B puncta compared with the cells expressing WT NEDD4-1 (Figures 5A and 5B). Next, we transfected NEDD4-1 KO 293T cells with the WT or HA/CA mutant of NEDD4-1 and found that the NEDD4-1 HA/CA mutant failed to stabilize VPS34 (Figure 5C). Moreover, the NEDD4-1 HA/CA mutant could not interact with VPS34 (Figure 5D). Meanwhile, we found that the NEDD4-1 HA/CA mutant failed to interact with endogenous USP13 (Figure 5E). These results suggest that the catalytic activity of NEDD4-1 is responsible for the association with VPS34 and USP13, thus promoting the formation of autophagosomes.

Auto-regulated K29-Linked Ubiquitination of NEDD4-1 Is Required for Its Interaction with USP13 and VPS34

Next, we investigated how NEDD4-1 mediates the deubiquitination and stabilization of VPS34 via its E3 ligase activity. We noticed that NEDD4-1 did not affect the ubiquitination of USP13, suggesting that USP13 is not a direct substrate of NEDD4-1 (Figure S5A). We observed that the poly-ubiquitination of NEDD4-1 HA/CA mutant was much weaker compared with WT NEDD4-1 and that the ubiquitination of NEDD4-1 HA/CA mutant could be restored by WT NEDD4-1 (Figure 6A). These results indicated that NEDD4-1 acts as its own substrate for ubiquitination, and the catalytic activity of NEDD4-1 is responsible for its auto-ubiquitination. A further ubiquitination assay revealed that the K29-linked ubiquitination was NEDD4-1 responsive (Figures 6B and S5B). To further confirm the K29-linked ubiquitination of NEDD4-1, we performed ubiquitin KR mutant analysis and found that NEDD4-1 could undergo K29-linked auto-ubiquitination (Figure S5C). Moreover, the auto-ubiquitination of NEDD4-1 increased with autophagy induction (Figures S5D and S5E). To further investigate the auto-interaction of NEDD4-1, we performed the binding assay and found that NEDD4-1 underwent auto-interaction and that this association remarkably increased with EBSS treatment, suggesting that NEDD4-1 auto-ubiquitination can form oligomers (Figure S5F).

Figure 4. NEDD4-1 Recruits USP13 to Deubiquitinate VPS34

(A) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with vectors expressing FLAG-VPS34, Myc-USP13, and HA-NEDD4-1.

(B) Coimmunoprecipitation and immunoblot analysis of protein lysates of WT and USP13 KO 293T cells transfected with vectors expressing FLAG-VPS34, together with EV or vector for Myc-NEDD4-1, followed by MG132 (10 μM) treatment for 3 h.

(C) Purified ubiquitinated VPS34 was incubated with immunopurified USP13 and NEDD4-1 in vitro in deubiquitinating buffer. The immunoblot was probed with anti-K48-Ub antibody.

(D) 293T cells were transfected with Scr siRNA or VPS34-specific siRNAs, together with plasmids encoding FLAG-USP13 and EV or vector for Myc-NEDD4-1, followed by treatment with MG132 (10 μM) for 3 h and Baf A1 (0.2 μM) for 6 h. Protein lysates were immunoprecipitated using anti-Myc beads and immunoblotted with anti-FLAG antibody.

(E) Protein lysates of WT and NEDD4-1 KO 293T cells treated with MG132 (10 μM) for 3 h were immunoprecipitated using anti-USP13 antibody and immunoblotted with anti-VPS34 antibody.

(F) Glutathione S-transferase (GST)-VPS34 and His-USP13 were purified from bacteria. FLAG-NEDD4-1 was expressed in 293T cells, purified using FLAG affinity column, and eluted with FLAG peptide. Purified GST-VPS34 was incubated with immunopurified His-USP13 and FLAG-NEDD4-1 in reaction buffer in vitro. After His pull-down, the bound material was analyzed by western blot using the indicated antibodies.

(G) HeLa cells were treated with EBSS for various time points. Protein lysates were immunoprecipitated and immunoblotted with indicated antibody.

(H) Protein lysates of WT and NEDD4-1 KO 293T cells transfected with Myc-USP13 (wedge) were immunoblotted with indicated antibodies.

(I) Protein lysates of WT and USP13 KO 293T cells transfected with HA-NEDD4-1 (wedge) were immunoblotted with indicated antibodies.

(J) Protein lysates of WT and USP13 KO 293T cells transfected with EV or HA-NEDD4-1, together with WT USP13 or its CA mutant, were immunoblotted with indicated antibodies.

(K) 293T cells were transfected with vectors expressing WT FLAG-VPS34 or its K419R mutant, together with WT USP13 or its CA mutant. Protein lysates were immunoprecipitated with anti-FLAG and immunoblotted with indicated antibodies.

Samples in (B) and (K) were incubated for 5 min with 1% SDS before immunoprecipitation. All of the experiments are representatives of three independent biological experiments with similar results.
We next found that like the full-length NEDD-1, the NEDD4-1 HECT domain alone could undergo auto-ubiquitination (Figure S5G), indicating that the HECT domain is needed for NEDD4-1 to trigger auto-ubiquitination. To further identify which K site of the HECT domain is associated with the auto-ubiquitination of NEDD4-1, we identified two conserved K sites by amino acid sequence analysis and generated mutants of NEDD4-1 with the substitution of R for K. We found that the NEDD4-1 K1279R mutant displayed reduced K29-linked ubiquitination (Figure 6C). In addition, we found that the NEDD4-1 K1279R mutant failed to stabilize the VPS34 (Figure 6D). Consistently, the NEDD4-1 K1279R mutant failed to interact with VPS34 and USP13 (Figure 6G), indicating that K1279 of NEDD4-1 is essential for its binding ability with USP13 and VPS34. Neither the NEDD4-1 HA/CA nor the NEDD4-1 K1279R mutant could inhibit the K48-linked ubiquitination of VPS34 (Figure 6H). Further immunoprecipitation analysis revealed that USP13 failed to interact with VPS34 in the presence of K1279R or HA/CA mutants of NEDD4-1 (Figure S5I). These results suggest that the auto-ubiquitination of NEDD4-1 at K1279 is indispensable for NEDD4-USP13 complex formation, thus mediating the deubiquitination and stabilization of VPS34.

**DISCUSSION**

Ubiquitination is one of the most important posttranslational modifications of proteins, as it functions for the vast majority of cellular proteins during their lifetime through diverse mechanisms (Swatek and Komander, 2016; Yau and Rape, 2016). To edit the ubiquitin code, eukaryotic cells have evolved a plethora of E3 ubiquitin ligases and DUBs to deal with the vast complexity of the ubiquitin system (Rape, 2018; Zhou et al., 2017). The
Figure 6. Auto-regulated K29-Linked Ubiquitination of NEDD4-1 Is Required for Its Interaction with USP13 and VPS34

(A) 293T cells transfected with HA-Ub, together with WT FLAG-NEDD4-1 or FLAG-NEDD4-1 HA/CA mutant or WT Myc-NEDD4-1, were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-HA antibody.

(B) Lysates of 293T cells transfected with plasmids expressing FLAG-NEDD4-1 HA/CA and HA-tagged Ub and its indicated mutants, together with the EV or expression vector of Myc-NEDD4-1 WT were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-HA.

(C) Lysates of 293T cells transfected with plasmids expressing HA-K29-Ub, together with Myc-NEDD4-1 or its indicated mutants, were immunoprecipitated with anti-Myc beads and immunoblotted with anti-HA.

(D) Protein lysates of 293T cells transfected with plasmids encoding WT Myc-NEDD4-1 or its mutants were immunoblotted with the indicated antibody.

(E and F) Representative images (E) and quantification of GFP-LC3B puncta (F) of HeLa-GFP-LC3B cells transfected with NEDD4-1-specific siRNA (#3), together with plasmids expressing the WT or K1279R mutant of Myc-NEDD4-1 treated with DMSO or rapamycin (250 nM) for 12 h. Images (E) were taken using Leica DMI3000 B microscopy with a 100x oil-immersion objective. Scale bar, 20 μm. The bars (F) represent means ± SEMs of triplicate samples (80 cells per sample). ***p < 0.001 (2-tailed Student’s t test).

(G) Protein lysates of 293T cells transfected with plasmids expressing WT Myc-NEDD4-1 or its indicated mutants were immunoprecipitated with anti-Myc beads and immunoblotted with indicated antibodies.

(legend continued on next page)
ligation of ubiquitin to certain substrates is tightly regulated by an E1-E2-E3 ubiquitin system, in which a great number of E3 ubiquitin ligases determine the specificity of the substrates and the ubiquitin linkages, while hundreds of DUBs reverse this process through cleaving poly-ubiquitin chains from the substrates (Kwon and Ciechanover, 2017; Rape, 2018). Recently, we found that E3 ligases and DUBs could physically and functionally interact with one another to synergistically edit the ubiquitination of substrates (Liu et al., 2018b). Here, we demonstrated that the HECT ubiquitin E3 ligase NEDD4-1 positively regulates autophagy by editing VPS34.

Ever since NEDD4-1 was identified as a factor of autophagy, several key proteins in the autophagy process, including ATG8 family proteins p62, ULK1, and Beclin-1, have been reported to interact with NEDD4-1. In particular, NEDD4-1 can catalyze the ubiquitin chains on p62 and Beclin-1 (Lin et al., 2017; Pei et al., 2017; Sun et al., 2017). VPS34, the class III PI3K, produces PI3P on phagosomes, which plays a pivotal role in both phagophore closure and maturation (Morel et al., 2017; Su et al., 2017). VPS34 is assembled into tetrameric complexes with Beclin-1, VPS15, and Atg14L to regulate autophagy or with Beclin-1, VPS15, and UVRAG to control vesicular trafficking (Liu et al., 2011; Morel et al., 2017). The presence of VPS34 appears to be essential for the stabilization of Beclin-1, VPS15, ATG14L, and UVRAG, suggesting that VPS34 is responsible for the stability of other complex subunits (Antonioli et al., 2017; Liu et al., 2011). Thus, it is critical to decode the regulatory mechanism of VPS34 stability.

Although much attention has been focused on the regulation of VPS34 activity, the molecular mechanism of VPS34 ubiquitination has not been clearly addressed. In this report, we have demonstrated that NEDD4-1 promotes autophagy by stabilizing VPS34 in an enzyme activity-dependent manner. NEDD4-1 maintains VPS34 stability by removing K48-linked ubiquitin chains from VPS34. The K48-linked ubiquitination of VPS34 was reduced by the mutation of the K419R. NEDD4-1 was likely to remove K48-linked ubiquitin chains from VPS34 on K419. Furthermore, we have discovered that NEDD4-1 undergoes K29-linked auto-polyubiquitination at K1279 and functions as a platform to recruit USP13 to form an NEDD4-1-USP13 deubiquitination complex. The formation of the NEDD4-1-USP13 complex plays a critical role in cleaving the K48-linked ubiquitin chains of VPS34 and then stabilizing VPS34, thus promoting the formation of autophagosomes. Our results suggested that K48-linked ubiquitination is a signal for VPS34 degradation and that NEDD4-1-USP13 complex reverses this process.

Previous studies indicated that several ubiquitin ligases and deubiquitinates target the VPS34 complex; however, the ubiquitination sites and the linkage types of ubiquitin chains that attach to VPS34 remain unexplored. Our study reveals the mechanism underlying the stabilization of VPS34 mediated by deubiquitination. NEDD4-1-USP13 axis promoted autophagy flux at the basal level, and the enhancement was largely increased at the early time of autophagy-induced conditions (0.5–4 h). In addition, we discovered that NEDD4-1 was degraded after 6–9 h of autophagy induction (Figures 2C and 2F). The precise regulation of NEDD4-1 protein abundance through autophagic degradation prevents the persistent activation of autophagic flux in a negative feedback loop, hence conferring the tight control of autophagy. Thus, NEDD4-1 functions as a guardian for VPS34 stabilization and autophagosome turnover. Our results identify an essential unidentified role for NEDD4-1 to ensure the optimal activation of autophagy flux by targeting VPS34 for direct ubiquitination.

DUBs may collaborate with the HECT enzyme to edit the ubiquitination state of substrates, as has been shown in the yeast ortholog of the NEDD4 family, Rsp5, which can mediate the degradation of cytosolic protein under stress conditions. Upon heat stress, the deubiquitinases Ubp2 and Ubp3 associate with Rsp5 to prevent the assembly of K63-linked ubiquitin chains on Rsp5 substrates (Fajner et al., 2017; Fang et al., 2014, 2016). DUBs can also physically and functionally interact with one another to modify the ubiquitination of the target protein. In vitro, USP18 does not deubiquitinate stimulator of interferon (IFN) genes (STING), but it does facilitate USP20 to catalyze the deubiquitination of STING in an enzymatic activity-independent manner, which in turn mediates the stabilization of STING and the activation of type I IFN signaling (Zhang et al., 2016). In this study, we have demonstrated that NEDD4-1 catalyzes the K29-linked ubiquitin chains on itself, thus forming a deubiquitination complex with USP13 to remove the K48-linked ubiquitination of VPS34. The various combinations among members of E3 ubiquitin ligases and DUBs constitute the diversity of regulation modes for ubiquitination modification. Our findings enrich the theory of ubiquitination regulation generated by E3 ubiquitin ligases and deubiquitinases.

We identified the function of NEDD4-1 in autophagy by targeting VPS34 for deubiquitination via the recruitment of USP13. Based on our findings, we proposed a working model to illustrate how NEDD4-1 plays an important role in autophagy. NEDD4-1 interacts with itself and promotes the K29-linked ubiquitination at K1279 as a platform for recruiting deubiquitinating enzyme USP13 to cleave the K48-linked ubiquitination of VPS34 at K419. The NEDD4-1-USP13 axis suppresses the proteasomal degradation of VPS34 and promotes autophagic flux. Our findings provide evidence to support the function of NEDD4-1 in autophagy flux by targeting VPS34, and understanding such underlying biology is required for autophagy-related clinical therapy.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**

(H) Protein lysates of 293T cells transfected with plasmids expressing WT Myc-NEDD4-1 or its indicated mutants, together with FLAG-VPS34, were harvested with treatment of MG132 (10 μM) for 3 h, immunoprecipitated with anti-FLAG beads, and immunoblotted with the indicated antibodies. The samples in (A)–(C) and (H) were incubated for 5 min with 1% SDS before immunoprecipitation. All of the experiments are representative of 3 independent biological experiments with similar results.

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METHOD DETAILS
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- In vitro deubiquitination assay
- In vitro His-pulldown binding assay
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QUANTIFICATION AND STATISTICAL ANALYSIS

DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.088.

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AUTHOR CONTRIBUTIONS

W.X. and S.J. performed the experiments and analyzed the results. Y.W., H.X., S.T., Z.G., and D.-A.L. provided technical assistance. J.C. initiated and directed the research. W.X., S.J., and J.C. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| NEDD4-1 Antibody (H-135) | Santa Cruz Biotechnology | Cat# sc-25508; RRID: AB_2149316 |
| goat anti-rabbit IgG-HRP | Santa Cruz Biotechnology | Cat# sc-2004; RRID: AB_631746 |
| goat anti-mouse IgG-HRP | Santa Cruz Biotechnology | Cat# sc-2005; RRID: AB_631736 |
| Mouse monoclonal anti-ubiquitin | Santa Cruz Biotechnology | Cat# sc-8017; RRID: AB_628423 |
| USP13 Antibody (D-11) | Santa Cruz Biotechnology | Cat# sc-390316 |
| LC3B (D11) XP® Rabbit mAb | Cell Signaling Technology | Cat# 3868S; RRID: AB_2137707 |
| mouse anti-rabbit IgG-HRP | Cell Signaling Technology | Cat# 5127; RRID: AB_10892860 |
| anti-Beclin-1 | Cell Signaling Technology | Cat# 3793; RRID: AB_490837 |
| anti-UVRAG (D2Q12) | Cell Signaling Technology | Cat# 13115; RRID: AB_2687988 |
| anti-Pik3r4 (VPS15) | Cell Signaling Technology | Cat# 14580 |
| K48-Ub-HRP | Cell Signaling Technology | Cat# 12805 |
| Alexa Fluor 488 conjugated anti-mouse-IgG secondary antibody | Cell Signaling Technology | Cat# 4408S; RRID: AB_10694704 |
| Anti-Pi3 Kinase Class 3 antibody | Abcam | Cat# ab5451; RRID: AB_304898 |
| Rabbit polyclonal antibody anti-USP10 | Abcam | Cat# ab72486; RRID: AB_1271412 |
| Anti-Atg14 pAb | MBL | Cat# PD026; RRID: AB_1953054 |
| Anti-Ubiquitin Antibody, Lys48-Specific, clone Apu2, rabbit monoclonal | Millipore | Cat# 05-1307; RRID: AB_1587578 |
| Rabbit polyclonal anti-p62/SQSTM1 | Proteintech Group | Cat# 18420-1-AP; RRID: AB_10694431 |
| Mouse monoclonal anti-Flag (M2) peroxidase (HRP) | Sigma-Aldrich | Cat# A8592; RRID: AB_439702 |
| Mouse monoclonal anti-β-actin | Sigma-Aldrich | Cat# A1978; RRID: AB_476692 |
| Hemagglutinin (HA)-HRP antibody | Roche Applied Science | Cat# 3F10; RRID: AB_2314622 |
| Mouse monoclonal unlabeled anti-c-Myc | Roche Applied Science | Cat# 11667203001; RRID: AB_390911 |
| CF350 donkey anti-rabbit-IgG secondary antibody | Biotium | Cat# 20389 |
| **Bacterial and Virus Strains** |        |            |
| E. coli BL21 | TIANGEN | Cat# CB105 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Lipofectamine 2000 | Invitrogen | Cat# 11668019 |
| Lipofectamine RNAiMAX | Invitrogen | Cat# 13778100 |
| Puromycin | Sigma-Aldrich | Cat# P9620 |
| MG132 | Sigma-Aldrich | Cat# C2211 |
| Doxycycline | Sigma-Aldrich | Cat# D9891 |
| Rapamycin | Sigma-Aldrich | Cat# 37094 |
| Baflomycin A1 | Selleck | Cat# S1413 |
| 3-methyladenine (3-MA) | Sigma-Aldrich | Cat# M9281 |
| **Critical Commercial Assays** |        |            |
| HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) | Vazyme | Cat# R223-01 |
| **Experimental Models: Cell Lines** |        |            |
| HEK293T/17 | ATCC | CRL-11268 |
| HeLa | ATCC | CCL-2 |
| THP-1 | ATCC | TIB-202 |
| A549 | ATCC | CRM-CCL-185 |
| NEDD4-1 KO HEK293T | This paper | N/A |
| NEDD4-1 KO A549 | This paper | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jun Cui (cuij5@mail.sysu.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HEK293T, HeLa and A549 cells were maintained in DMEM medium (GIBCO) with 10% (vol/vol) fetal bovine serum (GIBCO) and 1% L-glutamine (GIBCO). Human PBMCs and THP-1 cells were maintained in RPMI-1640 medium (GIBCO) 10% fetal bovine serum. To induce starvation, cells were washed with phosphate-buffered saline (PBS) and incubated in EBSS (GIBCO). All cells were incubated at 37°C/4°C incubator with 5% CO2.

METHOD DETAILS

Plasmids and transfection
Plasmids for HA-, Flag-, or Myc-tagged NEDD4-1, VPS34 and USP13 and their fragment were cloned into the pcDNA3.1 vector for transient expression and into the FG-EH-DEST (provided by Xiaofeng Qin laboratory) for retroviral expression. HEK293T transfection...
was performed using Lipofectamine 2000 (Invitrogen) according to procedures recommended by the manufacturer. Chemically synthesized 21-nucleotide siRNA duplexes were obtained from TranSheepBio and transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. RNA oligonucleotides used in this study can be found in Key Resources Table.

**Generation of NEDD4-1-inducible and knockout cell lines**

The retroviral vectors (15 g) were co-transfected with 5 μg of an expression plasmid for the vesicular stomatitis virus G protein into the HEK293T cells. The medium was changed the following day and the viral containing supernatant was collected 48 hr after transfection, filtered through a 0.45 mm filter and subsequently used to infect cells with Polybrene (8 μg/mL). For NEDD4-1 ectopic expression, lentiviral particles were produced by transfecting HEK293T cells with FG-EH-DEST-NEDD4-1, VSVG and Δ8.9. A549 cells were infected by incubation with retrovirus-containing supernatant for 48 hr. To generate NEDD4-1 KO, BECN1 KO, USP13 KO and USP10 KO cells, target sequences were cloned into pLentiCRISPRv2 by cutted with BsmBI. Infected cells were purified by puromycin selection. The sequences of target sgRNAs can be found in Key Resources Table.

**Immunoprecipitation and immunoblot analysis**

For immunoprecipitation, whole-cell extracts were prepared after transfection or stimulation with appropriate ligands, followed by incubation overnight with the appropriate antibodies plus Protein A/G beads (Pierce), anti-Flag or anti-HA agarose beads (Sigma). Beads were then washed five times with low-salt lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1.5 mM MgCl₂, and 1% Triton X-100), and immunoprecipitates were eluted with 2 × SDS Loading Buffer and resolved by SDS-PAGE. For deubiquitination assays in cultured cells, the cells were lysed with low-salt lysis buffer and the supernatants were denatured at 95°C for 5 min in the presence of 1% SDS. The denatured lysates were diluted with lysis buffer to reduce the concentration of SDS below 0.1% followed by immunoprecipitation (denature-IP) with the indicated antibodies. Proteins were transferred to PVDF membranes (Bio-Rad) and further incubated with the appropriate antibodies. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used for protein detection.

**In vitro deubiquitination assay**

Ubiquitinated VPS34 was isolated from 293T cells transfected with expression vector for Flag-VPS34. Ubiquitinated VPS34 was purified from the cell extracts with anti-Flag affinity column in Flag-lysis buffer (50 mM Tris-HCl (pH 7.8), 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarcosyl, 1 mM DTT, 10% glycerol, and fresh proteinase inhibitors). After extensive washing with the Flag-lysis buffer, the proteins were eluted with Flag peptides (Sigma). The recombinant Flag-USP13 and Flag-NEDD4-1 were respectively expressed in 293T cells and purified using Flag affinity column and eluted with Flag peptide. For in vitro deubiquitination assay, ubiquitinated VPS34 protein was incubated with recombinant USP13 or NEDD4-1 in the deubiquitination buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) for 2 hr at 37°C.

**In vitro His-pulldown binding assay**

GST-VPS34 was cloned into the vectors pGEX-5 x, and His-USP13 was cloned into pET-28a (+), for bacterial expression. E. coli BL21 were transformed with pGEX-5 x -GST-VPS34 and pET-28a (+)-His-USP13 vectors. GST-VPS34 and His-USP13 protein expression were induced with IPTG and purified using Glutathione Agarose (a kind gift from Dr. Xiya Zhang) and Ni-NTA Agarose beads (Qiagen, #30210) according to standard protocols. The recombinant Flag-NEDD4-1 was expressed in 293T cells and purified using Flag affinity column and eluted with Flag peptide. Purified GST-VPS34, His-USP13 and Flag-NEDD4-1 were incubated in reaction buffer at 4°C for 1 hr and then with His-beads overnight at 4°C. Afterward, the beads were washed four times with low-salt buffer, and bound proteins were eluted and subjected to immunoblot analysis.

**Fluorescence microscopy**

Cells were cultured on Glass Bottom culture dishes (Nest Scientific) and directly observed as previously described (Chen et al., 2016). For examination by immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde for 10 min at 20°C. After washing with PBS for 3 times, cells were permeabilized in 0.1% Triton X-100 and incubated with primary antibodies diluted in 1% bovine serum albumin overnight. The cells were washed, and then incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody (Alexa Fluor 488 conjugated anti-mouse-IgG and CF350 donkey anti-rabbit-IgG). Confocal images were examined using a Leica DMi3000 B microscopy or Zeiss LSM 710 confocal microscope (LSM710; Carl Zeiss) equipped with a ×100 NA oil-immersion objective.

**Quantitative RT-PCR**

Total RNA was extracted from cells using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. For RT-PCR analysis, cDNA was generated with HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, R223-01) and was analyzed by PCR using the 2 × Taq PCR StarMix (GenStar). The sequences of primers can be found in Key Resources Table.
QUANTIFICATION AND STATISTICAL ANALYSIS

Data are represented as mean ± SEM unless otherwise indicated, and Student’s t test was used for all statistical analyses with the GraphPad Prism 5 software. Differences between two groups were considered significant when P value was less than 0.05.

DATA AND CODE AVAILABILITY

Original data for figures in the paper is available by contacting the Lead Author.