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Ubiquitylation of Autophagy Receptor Optineurin by HACE1 Activates Selective Autophagy for Tumor Suppression

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Summary

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In selective autophagy, receptors are central for cargo selection and delivery. However, it remains yet unclear whether and how multiple autophagy receptors might form complex and function concertedly to control autophagy. Optineurin (OPTN), implicated genetically in glaucoma and amyotrophic lateral sclerosis, was a recently identified autophagy receptor. Here we report that tumor suppressor HACE1, a ubiquitin ligase, ubiquitylates OPTN and promotes its interaction with p62/SQSTM1 to form the autophagy receptor complex, thus accelerating autophagic flux. Interestingly, the K48-linked polyubiquitin chains that HACE1 conjugates onto OPTN might predominantly target OPTN for autophagic degradation. By demonstrating that the HACE1-OPTN axis synergistically suppresses growth and tumorigenicity of lung cancer cells, our findings may open an avenue for developing autophagy-targeted therapeutic intervention into cancer.

Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that cells use to deliver cytosolic proteins to the lysosome for degradation (Klionsky et al., 2012; Ohsumi, 2014). Although autophagy has generally been thought to be non-selective, recent work has identified mechanisms for selective uptake of cytoplasmic proteins into nascent autophagic vesicles. Selectivity in autophagy is achieved through cargo recognition by a family of autophagy receptors, the common feature of which is a functionally conserved LIR (LC3-interacting region) domain (Hurley and Schulman; Kirkin et al., 2009a; Pankiv et al., 2007). The LIR domain targets the autophagy receptor to ATG8-family proteins on the surface of the nascent autophagic vesicle. Currently known autophagy receptors include p62/Squestosome-1 (p62/SQSTM1, hereafter referred to as p62), NBR1, NDP52, Nix, Cbl, Stbd1 and OPTN (Optic neuropathy-inducing protein, Optineurin) (Wild et al., 2011). Among them, p62 and NBR1 are known to oligomerize through their N-terminal PB1 (Phox and Bem1p) domains, thus playing central role in recruiting and targeting cargoes to autophagosome. So far, multiple autophagy receptors are known to be involved in selective autophagy, in a conceptualized complex (Johansen and Lamark, 2011; Mijaljica et al., 2012). However, it remains yet unclear whether and how multiple autophagy receptors might actually form complex and operate concertedly to control selective autophagy.

There is also emerging evidence that the functionality of autophagy receptors might be regulated to modulate cellular autophagic activity in response to diverse stimuli. Optineurin (OPTN), a most recently identified autophagy receptor, has been implicated genetically in human glaucoma, Paget's Disease or amyotrophic lateral sclerosis (ALS) (Maruyama et al., 2010; Rezaie et al., 2002). Recently, TBK1-mediated phosphorylation of OPTN was shown to promote interaction between OPTN and LC3, activating selective autophagy to clear invading *Salmonella* (Wild et al., 2011). OPTN was also found to be ubiquitylated and degraded in mammalian cells (Shen et al., 2011), but the molecular basis, regulatory mechanism and impact of its ubiquitylation on the function of OPTN as an autophagy receptor remain unclear.

Meanwhile, impaired autophagy has been linked to many types of malignancies, owing to functional defects in various pathway components. Allelic loss of autophagic regulator

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Beclin1 leads to increased tumorigenesis in mice, and *Beclin1* gene was found to be mono-allelically deleted in 40-75% of sporadic human breast cancers and ovarian cancers (Liang et al., 1999). Mice lacking Atg5 or Atg7, which are essential components in autophagy pathway, are defective in autophagy and show elevated rates of spontaneous tumor formation (Komatsu and Ichimura, 2010). Impaired autophagy was also widely observed in human lung cancers, with yet unknown mechanistic basis (Moscat and Diaz-Meco, 2009). Therefore, defining the context-specific roles of autophagy in cancer and the regulatory mechanisms involved will be critical for developing autophagy-targeted therapeutics against specific types of cancer (White, 2012).

Here we set out to examine the ubiquitylation status of endogenous OPTN protein, identified and further characterized the E3 Ub ligase that could mediate the ubiquitylation of OPTN. We further investigated the impact of such ubiquitylation of OPTN on its function as an autophagy receptor and a potential tumor suppressor.

Results

Endogenous OPTN is ubiquitylated *in vivo*

Previously, OPTN (Figure 1A) was found to be ubiquitylated and to undergo degradation, but the regulatory mechanisms are unknown. As shown in Figure 1B, endogenous OPTN in human HEK293FT cells is heavily modified by ubiquitylation, as assessed by immunoprecipitation (IP) using anti-OPTN antibodies, followed by immunoblotting with anti-ubiquitin. Of note, the IP was performed in modified radioimmunoprecipitation (RIPA) buffer to remove the ubiquitylated autophagy cargos that might interact with the UBAN domain of OPTN. Moreover, since autophagy inhibitor Bafilomycin (Baf) rather than proteasome inhibitor Bortezomib (BTZ) seemed to stabilize endogenous OPTN (Figure S1A), cells were treated with Baf prior to the IP using anti-OPTN. The high molecular weight signal of modified OPTN was eliminated after incubation with Usp2cc, the catalytic core of human USP2 (ubiquitin-specific protease 2), confirming that endogenous OPTN was indeed ubiquitylated in HEK293FT cells (Figure S1B).

Tumor suppressor E3 Ub ligase HACE1 interacts with, and ubiquitylates OPTN

We next sought to identify the E3 Ub ligase responsible for ubiquitylation of OPTN, using yeast two hybrid (Y2H) screening (Fields and Song, 1989). Human OPTN was employed as the bait to screen potential OPTN-interacting proteins from an Y2H prey library containing ORFs (open-reading frames) from human cDNAs encoding over 400 putative ubiquitin ligases or their substrate binding subunits. Positive colonies were selected and found to harbor the prey vector expressing the HECT domain and ankyrin repeat containing E3 Ub ligase (HACE1). HACE1-OPTN interaction was indicated by colony formation on yeast SD-4 (SD-*Leu-Trp-His-Ura*) selection media as well as plate assays for β-galactosidase activity (Figure 1C). HACE1, a member of the HECT family of Ub ligases, consists of an N-terminal stretch of six ankyrin repeats, the C-terminal HECT ubiquitin ligase domain, and a connecting linker sequence (Figure 1A).When using HACE1 as bait, Y2H screening also revealed that OPTN interacted with wild-type HACE1 as well as $HACE1_{C876S}$, the E3 Ub ligase activity dead mutant (Figure 1D), suggesting that the Ub ligase activity is not required

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for such interaction. It was previously reported that genetic ablation of HACE1 promotes tumorigenesis in multiple tissues of mice, and down-regulation or mutation of HACE1 is associated with multiple human malignancies, which established HACE1 as a potent tumor suppressor (Zhang et al., 2007). However, the mechanism underlying the tumor-suppressive function of HACE1 remains unknown.

In vivo, both tagged and endogenous HACE1 and OPTN proteins seemed to indeed form a complex that could survive the multi-step procedure in co-immunoprecipitation (CO-IP) assay (Figure 1E, 1F). GST pull-down assays showed that OPTN directly interacted with HACE1 *in vitro*, likely through the N-terminal ankyrin repeats of HACE1 (Figure 1G, 1H). Reciprocal pull-down assay also mapped the 411-456 residues in OPTN as the major HACE1-interaction region (HIR) (Figure 1A, 1I, 1J). HACE1 and OPTN, tagged respectively with GFP (Green fluorescence protein) and RFP (Red fluorescence protein), did co-localize mostly in extra-nuclear region of the cell (Figure S1C).

To examine whether HACE1 actually catalyzes the ubiquitylation of OPTN, we assembled an *in vitro* ubiquitylation system that contained ATP, the E1 Ub-activating enzyme (Uba1), the E2 Ub conjugating enzyme (UbcH7), the substrate (OPTN) and the E3 ub ligase (HACE1). As shown in Figure 2A, wild-type HACE1 ubiquitylated OPTN *in vitro*, apparently without involving the UBAN domain of OPTN. HACE1-conjugated ubiquitin chains on OPTN were primarily in Lys 27 (K27) and Lys 48 (K48) Ub linkages *in vivo*, as Ub containing K27 or K48 as the only Lys residue was conjugated efficiently but Ub with K27R or K48R mutation was not (Figure 2B, 2C). Furthermore, knocking down endogenous HACE1 in HEK293 cells with short-hairpin RNA efficiently blocked the formation of polyubiquitin (poly-Ub) chains on OPTN (Figure S1E, S2A, S2B), suggesting that endogenous HACE1 might be the major E3 Ub ligase ubiquitylating OPTN in HEK293 cells.

HACE1-mediated K48-linked poly-Ub chains targets OPTN for autophagic degradation

Having known that endogenous OPTN was stabilized by autophagy inhibitor bafilomycin A1 (Baf) but not as much by proteasome inhibitor bortezomib (BTZ) (Figure S1A), we are particularly interested in what K48-linked poly-Ub chain would target OPTN for. As shown in Figure 2D, interestingly, K48-linked polyubiquitylation of OPTN turned out to be slightly stabilized in cells treated with BTZ, but significantly accumulated when autophagy was inhibited upon Baf treatment. The K48-linked polyubiquitylation of OPTN was also accumulated in autophagy deficient *ATG7* $^{-/-}$ cells, suggesting that OPTN with K48-linked poly-Ub chains might be predominantly targeted for autophagy-dependent degradation. Autophagy activator rapamycin destabilized endogenous OPTN protein, while genetic ablation of *ATG7* gene or treatment with autophagy inhibitor 3-Methyladenine (3-MA) had the opposite effect, suggesting that endogenous OPTN indeed undergo autophagic degradation (Figure 3A, 3B). These observations depart from the generally accepted notion that proteins with K48-linked poly-Ub chains are exclusively targeted to proteasome-dependent degradation (Chau et al., 1989; Deshaies and Joazeiro, 2009; Finley, 2009; Finley et al., 2004; Komander and Rape, 2012; Weissman et al., 2011). Notably, it has been

reported that K48-linked ubiquitin conjugates accumulate when autophagy is impaired (Riley et al., 2010).

Lys^{193} in OPTN is one of the major sites for HACE1-mediated ubiquitylation

After an *in vitro* ubiquitylation reaction, ubiquitylated OPTN was recovered (Figure S2C) and subjected to tryptic digestion, followed by mass spectrometry (MS) analysis. Attachment of ubiquitin to the side chain of a Lysine (Lys, K) residue renders it resistant to trypsin cleavage, and tryptic digestion of ubiquitin (chains) attached to the site leaves a –Gly-Gly- group, originating from the C-terminus of ubiquitin, on the side chain of the modified Lys (Kim et al., 2011). Based on these signature features, ubiquitylation sites in OPTN were identified from MS/MS spectra. Altogether, a total of 12 Lys residues were found to be ubiquitylated by HACE1 *in vitro* (Figure 2E; Figure S2D).

We then asked which Lys residues in OPTN might be the major ubiquitylation sites *in vivo*. We generated OPTN mutants bearing single or multiple Lys-to-Arg (K-to-R) substitutions in every potential ubiquitylation site. As shown in Figure 2F, K-to-R substitutions on all the potential ubiquitylation sites indeed almost completely abolished the HACE1-catalyzed ubiquitylation on OPTN *in vivo*. Remarkably, Ub conjugation to OPTN mutant with Lys-193-to-Arg (K193R) substitution, $OPTN_{K193R}$, was substantially reduced, compared with that of either wild-type OPTN or any mutant bearing single K-to-R substitutions on each of the other ubiquitylation sites (Figure 2G; Figure S2E, S2F). This result suggested that ubiquitylation on Lys^{193}, which reside close to the LIR motif in OPTN (Figure 1A), might be functionally important. Therefore we proceeded to investigate the biological consequences of HACE1-mediated ubiquitylation on OPTN, particularly that taking place on Lys^{193}.

Lys^{193} in OPTN is not involved into the interaction between OPTN and LC3 II

Since Lys^{193}, a major site for HACE1-conjugated ubiquitylation on OPTN, resides close to the LIR ($F^{178}VEI^{181}$) of human OPTN, it was natural to ask whether above K193R mutation in OPTN would have any effect on the interaction between LC3 and OPTN. GST pull-down assay was thus performed between bacterially expressed LC3 and wild-type OPTN or the $OPTN_{K193R}$ mutant. As shown in Figure S2H, compared to that of wild-type OPTN, K193R mutation in OPTN seemed to have no appreciable effect on the interaction between GST-tagged LC3 and OPTN, suggesting that $OPTN_{K193R}$ might be structurally intact, at least in terms of its affinity to LC3.

OPTN and HACE1 are down-regulated in multiple human lung cancer cells

HECT family of ubiquitin ligases have been known to play multiple roles in cancer development (Bernassola et al., 2008; Rotin and Kumar, 2009). Specifically, HACE1 has been recognized as a potent tumor suppressor, as previous work has shown that HACE1 is inactivated or down-regulated in multiple types of human malignancies, such as Wilms' tumor (Diskin et al., 2012; Zhang et al., 2007). In addition, sporadic tumors occurred frequently in various tissues of HACE1 knockout mice (Zhang et al., 2007). Impaired autophagy was also widely observed in human malignancies including lung cancers, with mechanism yet unknown (Moscat and Diaz-Meco, 2009).

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Interestingly, an analysis of a recently published dataset revealed a strong correlation between higher expression of OPTN and better relapse-free survival in 1715 lung cancer patients (Gyorffy et al.) (Figure S3F). Meanwhile, highly frequent mutations in OPTN or HACE1 gene were uncovered in thirteen different human cancer types, according to data extracted from TCGA (The Cancer Genome Atlas) datasets (Cerami et al.; Gao et al., 2013) (Figure S3D, S3E). Taken together, these results strongly underscored the potential tumor-suppressing roles of OPTN and HACE1 in human malignancies including lung cancer.

Therefore, we used lung cancer cells as a model to directly assess the potential association between lung cancer and down-regulation of OPTN and/or HACE1. We first surveyed the expression of OPTN and HACE1 in multiple lung cancer cell lines from ATCC (American Type Culture Collection). In these cells, the levels of OPTN protein appeared to be low in 3 lines. CRL-5872 cells were then selected for subsequent studies, as they expressed endogenous HACE1 and OPTN at fairly low level (Figure S3A-S3C). This would allow functional study of the exogenous wild-type HACE1, OPTN or their indicated mutants, free of potential interference from the endogenous counterparts.

HACE1-OPTN promotes the formation of LC3 puncta and lipidation of LC3II in human lung cancer cells

Previous studies established OPTN as an autophagy receptor, and phosphorylation of OPTN by TBK1 on Ser^{177} was shown to promote autophagic clearance of *Salmonella* (Wild et al., 2011). Accordingly, we asked whether HACE1-mediated ubiquitylation of OPTN might impact cellular autophagic activity, through assessing through monitoring LC3-containing puncta, lipidation of LC3 (the ratio of [LC3 II] to [LC3 I]) and cellular autophagic flux (Klionsky et al., 2012).

Consistently, only expression of wild-type HACE1, but not $HACE1_{C876S}$, resulted in increased levels of phosphatidyl-ethanolamine (PE)-modified LC3 (LC3-II-PE), with concomitant increase in OPTN ubiquitylation (See the ratio of [LC3 II] to [LC3 I], Figure 3C). As shown in Figure 3D-3E, fluorescence microscopic analysis with the reporter cell line CRL-5872 stably expressing GFP-LC3 and subsequent quantitation revealed that overexpression of human HACE1 or OPTN alone induced the formation of the GFP-LC3 puncta, similar to, but to a lesser extent than that observed in cells treated with rapamycin, a known autophagy inducer. Co-expression of exogenous OPTN and HACE1 further enhanced the formation of GFP-LC3 puncta, whereas the enzymatically inactive HACE1 mutant, $HACE1_{C876S}$, did not, suggesting the E3 Ub ligase activity of HACE1 was important for induction of puncta formation. Remarkably, this effect was almost abolished when $OPTN_{K193R}$ was co-expressed with HACE1, suggesting that HACE1-mediated ubiquitylation on Lys^{193} was critical for induction of LC3 puncta formation (Figure 3E). Consistent data were also obtained with endogenous LC3 as indicated by immunomicroscopy analysis using anti-LC3 antibodies (Figure S3J). Moreover, significantly fewer LC3 puncta were formed in the cells that overexpressed HACE1 but had endogenous OPTN knocked down with RNA interference (Figure 3JA), suggesting that HACE1-induced autophagy might be OPTN-dependent.

Therefore, HACE1-mediated ubiquitylation of OPTN appeared to activate autophagy.

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HACE1-OPTN promotes autophagic removal of p62/SQSTM1 and oxidatively damaged proteins in human lung cancer cells

Accumulation of protein carbonyl groups is a biomarker of cellular oxidative stress and compromise in cellular proteolytic capacity (Dalle-Donne et al., 2003). Cellular levels of oxidatively damaged proteins can be efficiently assessed by derivatizing the carbonyl groups with hydrazines such as DNPH (2, 4-dinitrophenylhydrazine), followed by immunoblotting with anti-DNP antibodies to visualize the carbonylated proteins. Meanwhile, the DNP group also has a characteristic absorbance at 370 nm, making possible a facile quantitative assay of the content of carbonyl groups on a pool of proteins. Normally, oxidatively damaged proteins are removed in timely manner, largely through autophagy, as their degradation could be promoted by rapamycin, but blocked by autophagy inhibitor 3-MA or Baf stabilized them (also Figure S3G). N-acetyl-cysteine (NAC) effectively scavenges cellular oxidants, while pyocyanin (PCN) is a secondary metabolite from *Pseudomonas aeruginosa* that can drastically increase the oxidative flux of cellular proteins. Therefore, cellular protein oxidation can be reliably gauged through anti-DNP blotting, using cells treated with NAC or PCN as negative or positive controls, respectively.

As shown in Figure 3F, overexpression of wild-type OPTN alone slightly decreased the total level of oxidized proteins in the lung cancer cells. Strikingly, co-expression of wild-type HACE1 and OPTN reduced the cellular level of oxidized proteins so much that signal became barely detectable in anti-DNP blotting, which could be reversed by addition of Baf or knocking down HACE1. Further experiment with the ligase activity dead $HACE1_{C876S}$ or $OPTN_{K193R}$, the OPTN mutant that harbored Lys-to-Arg substitution on Lys^{193}, indicated that HACE1-mediated ubiquitylation on Lys^{193} of OPTN is critical for removal of the carbonylated proteins during autophagy activated by HACE1-OPTN (Figure 3G, 3H). p62 along with oxidized proteins were accumulated in human lung cancer cells (CRL-5872) (Figure S3H). Similarly, when endogenous OPTN was knocked down with shRNA, overexpression of wild-type HACE1 alone did not increase removal of p62 or cellular oxidized proteins (Figure S3H). Interestingly, p62 and oxidized proteins remained high in cells overexpressing both wild-type HACE1 and $OPTN_{K193R}$, the OPTN mutant that harbored Lys-to-Arg substitution on Lys^{193} (Figure 5A, S6C). Taken together, these results suggested that HACE1-mediated ubiquitylation of OPTN, particularly that taking place on Lys^{193}, might be essential for activating autophagy, as determined by removal of oxidatively damaged proteins, the conversion of LC3 I into lipidated LC3 II, and the degradation of p62 (Figure 3C-3H; Figure S3H).

HACE1 and OPTN form a functional axis to promote autophagy in a p62-dependent manner

We next used macrophages derived from either OPTN wild-type (*OPTN* $^{+/+}$) or knockout (*OPTN* $^{-/-}$) mice (Skarnes et al., 2011) to further investigate the role of OPTN during HACE1-activated autophagy. As shown in Figure 4A, in *OPTN* $^{+/+}$ macrophages, overexpression of HACE1, but not the catalytically inactive $HACE1_{C876S}$, induced significantly more conversion of LC3 I into LC3 II in lipidated form, removal of p62 or oxidized proteins. However, in *OPTN* $^{-/-}$ macrophages, neither p62 nor the cellular oxidized proteins were eliminated to a greater extent than in control cells regardless of the expression

of HACE1 or $HACE1_{C876S}$. This strongly suggested that existence of endogenous OPTN is required for HACE1-activated autophagy (Figure 4A).

Interestingly, in *p62* $^{-/-}$ MEF cells, the HACE1-OPTN axis did not promote autophagic removal of oxidatively damaged proteins either. However, this could be reversed when wild-type human p62 was re-introduced into the cells (Figure 4B), suggesting that the HACE1-OPTN axis might accelerate cellular autophagic flux in a p62 dependent manner. Interestingly, even in the presence of p62, K193R mutation in OPTN markedly compromised the autophagy-promoting activity of the HACE1-OPTN axis, suggesting that HACE1-mediated ubiquitylation on K^{193} of OPTN might functionally interact with p62. It is intriguing to ask whether HACE1-conjugate poly-Ub chain on K^{193} of OPTN would directly interact with p62, whose UBA domain was known to bind to K48 or K63-linked poly-Ub chains (Ciani et al., 2003).

HACE1-mediated ubiquitylation of Lys^{193} in OPTN promotes the formation of p62/SQSTM1-OPTN complex

As shown in Figure 4C, in *OPTN* $^{+/+}$ MEF cells, endogenous HACE1, OPTN and p62 did physically interact to form a complex. However, knocking down HACE1 or OPTN with RNA interference abolished the interaction. Furthermore, removing poly-Ub chains on immunoprecipitated OPTN with deubiquitinating enzyme Usp2cc also disrupted OPTN-p62 interaction but not that for HACE1-OPTN, suggesting that HACE1-mediated ubiquitylation of OPTN might be critically involved into OPTN-p62 interaction (Figure 4D).

As shown in Figure 5A, in lung cancer cells that overexpress wild-type HACE1 and OPTN, OPTN was ubiquitylated and interacted with endogenous p62. However, in cells over-expressing $OPTN_{K193R}$ and wild-type HACE1, much weaker or no interaction between $OPTN_{K193R}$ and p62 was detected, despite the fact that they had higher level of endogenous p62 protein. Meanwhile, in mouse p62 knockout embryonic cells (*p62* $^{-/-}$ MEF) (Komatsu et al., 2007) expressing human HACE1, full-length p62 interacted with HACE1-ubiquitylated OPTN, which could be abolished by deletion of UBA domain in p62. Similarly, much weaker interaction was observed between $OPTN_{K193R}$ and the overexpressed p62. (Figure 5B). These data suggested that UBA domain of p62 and HACE1-conjugated Ub chains on Lys^{193} of OPTN were critically involved into OPTN and p62 interaction *in vivo*.

As shown in Figure 5C, OPTN ubiquitylated by HACE1 *in vitro* interacted with UBA domain of p62, but the unmodified OPTN did not (Figure 5C). Meanwhile, there was little or no interaction observed between p62 and $OPTN_{K193R}$, when the latter was marginally ubiquitylated by HACE1 *in vitro* (Figure 5D). As shown in Figure 5E and 5F, only K48-linked poly-Ub chain that HACE1-conjugated onto OPTN interacted with the p62 *in vitro* while K27-linked one did not. This suggested that p62 preferential bound to ubiquitylated OPTN in K48 Ub linkage.

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The HACE1-OPTN axis accelerates degradation of p62, suppresses ROS production and mitigates oxidative DNA damage in human lung cancer cells

Autophagy receptor p62 is also a known substrate for selective autophagy (Komatsu and Ichimura, 2010). Specifically, levels of endogenous p62 in mouse *OPTN* $^{-/-}$ macrophage cells were significantly higher than those in the wild-type cells, which could be reversed by re-introduction of wild-type OPTN (Figure 4A). Similar effect was also observed in CRL-5872 cells that express endogenous OPTN at low level, where level of endogenous p62 went down upon co-expression of wild-type HACE1 and OPTN (Figure 5A). However, co-expression of wild-type HACE1 and $OPTN_{K193R}$ did not reduce the level of endogenous p62, which strongly suggested that accelerated degradation of p62 was mainly dependent on HACE1-mediated ubiquitylation of K193 in OPTN. In HEK293FT cells, knocking down HACE1 or OPTN also led to appreciable increase in static level of endogenous p62 protein (Figure 4D). Therefore, the co-expression of HACE1 and OPTN appeared to promote the interaction between autophagy receptors, OPTN and p62, and accelerates autophagic degradation of p62.

Taken samples from those for Figure S3H, through IP with anti-DNP followed by quantitative mass spectra analysis, our data clearly indicated that co-expression of HACE1 and OPTN could significantly reduce the carbonylation of more than 60 cellular proteins in CRL-5872, including heat shock protein 70 KDa protein 8 (data not shown).

Previous work demonstrated that p62 is also a positive regulator of cellular ROS (reactive oxygen species) production, and that ROS at elevated level triggers translocation of Nrf2 (nuclear factor [erythroid-derived 2]-like 2) into the nucleus, thus activating Nrf2-mediated p62 transcription (Komatsu et al., 2010). These findings suggest a feedback mechanism between p62 and intracellular ROS production. During the autophagy activated by the HACE-OPTN axis, accelerated removal of p62 and the oxidatively damaged proteins would significantly suppress ROS production. Indeed, production of ROS in cells expressing both wild-type HACE1 and OPTN was less than 10 % percent of that in control cells (Figure S4A-F). Similar results were observed in DNA damage assays with cells expressing both HACE1 and OPTN (see Figure S4G).

Excess of p62 is known to associate with multiple type of human cancer, and elimination of p62 has been well accepted as one of the major mechanisms through which autophagy suppresses tumorigenesis (Komatsu et al., 2010; Mathew et al., 2009). The HACE1-OPTN axis might suppress ROS production and mitigate oxidative DNA damage, most likely through promoting autophagic removal of p62 and the other oxidatively damaged proteins (Figure S3H).

The HACE1-OPTN axis suppresses anchorage-independent growth of human lung cancer cells

We proceeded to investigate the potential tumor-suppressive effect of the HACE1-OPTN axis. Tumor colony formation assay was performed with lung cancer cells (CRL-5872) expressing wild-type HACE1, OPTN or both, or their indicated mutants (Figure 6A-B; Figure S5A). Cancer cells expressing wild-type HACE1 formed approximately 58% fewer

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colonies in soft agar than did control cells transfected with empty vectors. Cells expressing the catalytically inactive $HACE1_{C876S}$ formed as almost efficiently as the controls did. This result is consistent with the previous observation that the ubiquitin ligase activity of HACE1 is critical for its potent suppressive effect on the anchorage-independent growth of the cancer cells (Zhang et al., 2007). Overexpression of OPTN alone inhibited tumor proliferation by approximately 63%. This strongly suggested that OPTN might also function as a tumor suppressor in lung cancer cells, which was reminiscent of the previous finding that higher OPTN expression strongly correlated with better relapse-free survival in human lung cancer patients (Figure S3F).

Strikingly, cancer cells expressing both wild-type HACE1 and OPTN formed colonies at a frequency less than 10 % of that by control cells, suggesting that HACE1 and OPTN could synergistically suppress tumor growth. In contrast, cancer cells that co-expressed wild-type OPTN and the enzymatically inactive $HACE1_{C876S}$ formed 63 % more colonies than the controls. Similar results were observed with cancer cells that expressed wild-type HACE1 but had endogenous OPTN or p62 knocked down through RNA interference (Figure 6A, 6B; Figure S5A, S5E and S5F), suggesting that the tumor-suppressive function of HACE1 is dependent on the presence of endogenous OPTN, p62 and the ubiquitin ligase activity of HACE1 (Figure S5C and S5G).

Interestingly, in the cancer cells that overexpressed wild-type HACE1 and $OPTN_{K193R}$, the OPTN mutant that harbored Lys-to-Arg substitution on Lys^{193}, the inhibitory effect of HACE1 on tumor colony formation was partially abrogated (Figure 6A-B). In contrast, Lys-to-Arg substitution on every ubiquitylation site other than Lys^{193} of OPTN still synergistically suppressed tumor with HACE1. Therefore, ubiquitylation on Lys^{193} of OPTN, which was demonstrated to be critical for HACE1-activated autophagy, also significantly contributed to HACE1-mediated suppression of tumor colony formation.

The HACE1-OPTN axis suppresses tumorigenicity of human lung cancer cells in nude mice

Subsequently, a tumorigenicity model of cancer cells in nude mice was employed to further probe the tumor-suppressive effect of HACE1-mediated ubiquitylation on OPTN *in vivo.* As shown in Figure S5C, the lung cancer cells (CRL-5872) stably expressing wild-type HACE1, OPTN or mutant forms of these proteins were injected into nude mice. Efficiency for tumorigenicity of the cancer cells in each group was assessed 21 days after the injection (Figure 6C, 6D). CRL-5872 cells expressing either exogenous HACE1 or OPTN alone formed tumors of sizes significantly (38% or 25%, respectively) smaller than those in the control group. Cancer cells with endogenous OPTN knocked down formed tumors approximately 87% larger than the controls, which again suggested tumor-suppressing function for OPTN. In cancer cells stably expressing both HACE1 and OPTN, the size of tumor formation was only one fifth of that of the control, indicating a synergistic tumor-suppressing effect of the HACE1-OPTN axis. Interestingly, cancer cells that expressed HACE1 but had endogenous OPTN knocked down formed tumors in nude mice ~ 67% larger than that of the control.

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Remarkably, cancer cells expressing HACE1 with Lys-193-to-Arg substitution mutant, $OPTN_{K193R}$, formed tumor ~129 % larger than that in the control. In contrast, cancer cells expressing HACE1 and OPTN that harbored Lys-to-Arg substitutions on all potential ubiquitylation sites except Lys^{193} in OPTN, $OPTN_{K193only}$, formed tumors of ~51 percent smaller size than those of the control. This result highlighted the possibility that tumor-suppression by the HACE1-OPTN functional pair might be largely mediated by HACE1-conjugated poly-Ub chain on Lys^{193} residue of OPTN.

The HACE1-OPTN axis also critically modulates cellular autophagy in the actual tumors in dependence on ubiquitylation of Lys^{193} OPTN, as assessed by assaying the increased formation of lipidated LC3 II and removal of carbonylated proteins (Figure 6E). Altogether, these data indicated that HACE1-mediated ubiquitylation of OPTN, particularly that taking place on Lys^{193} of OPTN, plays critical role in activating autophagy to suppress tumorigenicity of the cancer cells *in vivo*.

Through immunohistochemistry microscopy analyses, we also examined the levels of endogenous HACE1 and OPTN proteins in human cancer samples, with those in adjacent normal tissues as controls. Indeed, HACE1 and OPTN appeared to be downregulated in multiple cancers, such as liver cancer (Figure 7A-D), stomach or lung cancer (Figure S6). The results further underscored the existence of a strong association between human malignancies and the deficiency of the HACE1-OPTN axis.

Discussions

Through recruiting and delivering cargo to autophagosome, receptors are known to play pivotal roles in selective autophagy. However, the distinct domain structures of different autophagy receptors actually distinguish one receptor from another. For example, p62 and NBR1 are known to self-oligomerize through their N-terminal PB1 (Phox and Bem1p) domains, while the other receptors may not (Itakura and Mizushima, 2011; Kirkin et al., 2009b). p62 and NBR1 are thus critical in organizing autophagy receptor complex and targeting autophagy cargos to autophagosome, followed by autophagic degradation. We report here that HACE1-mediated ubiquitylation of OPTN, particularly that taking place on Lys^{193}, might activate autophagy through promoting the physical interaction between autophagy receptors, OPTN and p62. Therefore, besides the previously known interaction between LC3 and the individual autophagy receptors, selective autophagy also seemed to be controlled by delicately regulated interactions between autophagy receptors. Our data not only underscore the critical role of p62 in autophagy, but also provide mechanistic insight into the functional dynamics of autophagy receptors during selective autophagy. Particularly, our findings have strongly suggested the actual existence of a large complex of multiple autophagy receptors (encompassing p62 and OPTN), in which the autophagy receptors work concertedly to recruit more cargoes and target them for efficient autophagic degradation. This model was strongly supported by the observations that endogenous OPTN, p62 and HACE1 indeed form a complex and formation of such complex is dependent on HACE1-mediated ubiquitylation on OPTN, mainly on its K^{193}. In light of the central role of p62 in forming autophagic complex, it is conceivable that such interaction *in vivo* could significantly augment recruitment of the autophagic cargos to the autophagy receptor

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complex including both p62 and OPTN, before targeting to autophagosome. This might mechanistically account for how the overall cellular autophagic flux was promoted by the HACE1-OPTN axis. With the emerging roles of Ub signaling in regulating autophagy (Kuang et al., 2013; Tasaki et al., 2013), our work has revealed a mechanism through which the Ub system delicately regulates selective autophagy through controlling the formation of autophagy receptor complex.

In this study, we report that the HACE1-OPTN axis accelerate removal of p62, carbonylated proteins as well as the total cellular autophagic flux regardless of cell types. Given wide-range of cellular functions that these proteins are involved in and the critical importance of oxidative stress in tumor (Xu et al., 2011), removal of these oxidatively damaged proteins along with p62 and reduced production of ROS (Reactive oxygen species) might thus mechanistically underlie the tumor suppression mediated by the HACE1-OPTN axis (Figure 7E).

Of note, consistent with previous observations (Komatsu and Ichimura, 2010; Mathew et al., 2009), our work have again shown that autophagy receptors such as p62 and OPTN are, on one hand, required for autophagy, but also themselves undergo autophagic degradation. These seemingly paradoxical phenomena exactly highlight the multiple roles that autophagy receptors function in autophagy: as autophagy cargo carriers, regulators as well as substrates of autophagy.

Given the fact that OPTN is critically implicated into neurological functions and host-microbe interactions (Mankouri et al., 2010; Maruyama et al., 2010; Wild et al., 2011; Ying and Yue, 2012), and the HACE1-OPTN axis activates autophagy in disregard of cell types, our findings might thus be of broad significance. Particularly, with the strong association between human malignancies and the deficiency of HACE1 or OPTN (Diskin et al., 2012; Zhang et al., 2007), targeting the HACE1-OPTN axis may thus open an avenue for therapeutic intervention in cancers.

Experimental Procedures

Reagents and plasmid construction

See Supplemental information for details.

Yeast two-hybrid screen

The yeast two hybrid (Y2H) screening was carried out to screen for E3 ub ligases that may ubiquitylate OPTN. Using OPTN or HACE1 as bait, HACE1 interacts with OPTN in yeast. OPTN and HACE1 or $HACE1_{C876S}$ co-transformed into yeast strain *Mav203* can activate the expression of β-glycosidase

In vitro and *in vivo* ubiquitylation assays

In vitro ubiquitylation assays were performed as described before (Kim et al., 2014; Li et al., 2009). Immunoprecipitation with anti-OPTN, followed by anti-Ubiquitin immunoblotting, was performed in RIPA buffer to check the ubiquitylation status of endogenous OPTN. See supplemental experimental procedures for details.

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Mass spectra and mutagenesis analyses

Mass spectra (MS) and mutagenesis analyses, were performed to determine the sites of the poly-Ub chains that HACE1 conjugates to OPTN.

Growth-independent colony formation and tumorigenicity assays

Human lung cancer cells (CRL-5872), co-transfected with HACE1, OPTN or their mutants were subjected to colony formation assay on soft agar or tumorigenicity experiments in nude mice. Soft agar colony formation assays were carried out as described before (Li et al., 2010). Tumorigenicity assay of the lung cancer cells in nude mice was performed in CRL-5872 cells. After infection with the lentivirus or retrovirus, the infected CRL-5872 cells were then selected and maintained in puromycin (2.5 μg/ml). CRL-5872 stably cell lines were trypsinized, and counted. 100 μl aliquots of Dulbecco's modified Eagle's medium containing 1×10^6 cells were mixed with Matrigel (cell suspension : Matrigel = 1 : 1) were implanted subcutaneously into 5-week-old female nude mice according to standard procedures (n = 6 mice per group). 21 days after injection, the mice were euthanized with carbon dioxide or and tumor masses were isolated and tumor weight measured.

Autophagy assay and detection of oxidatively damaged proteins

Formation of GFP-LC3 puncta, the level of lipidated LC3, level of oxidatively damaged proteins, the production of radical oxidative species (ROS), DNA damage, autophagic turnover of p62 were assayed in CRL-5872 cells. CRL-5872 cells were transfected with genes of interest, and ROS production was detected using the total ROS detection kit (ENZO ENZ-51011) as described before (Tarpey and Fridovich, 2001). Protein oxidation was determined by OxyBlot Protein Oxidation Detection Kit (Millipore), according to the manufacturer's instruction.

OPTN knockout Mice and animal handling

The *OPTN* $^{-/-}$ mice were generated in the European Conditional Mouse Mutagenesis Program (EUCOMM) (Skarnes et al., 2011) and distributed by University of Oulu, Finland. All animals were handled following the protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Clinical Specimens

The liver and stomach cancer tissues were collected from patients in Zhongshan Hospital, Medical College of Fudan University (Shanghai). The lung cancer tissue microarrays (Number: HLug-NSCLC150PT-01), which contained samples from 75 cases of human non-small cell lung cancer (NSCLC) in Taizhou Hospital (Zhejiang), were provided by Shanghai Outdo Biotech Co.. Informed consents were obtained from all subjects in accordance with the protocol approved by the individual institutional Ethic Committees.

Isolation of murine peritoneal macrophages

Murine peritoneal macrophages isolated as described before (Zhang et al., 2008) with slight modifications.

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Statistical Methods

Data were analyzed by using Prism 5 (GraphPad Software, Inc). Unpaired T-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. Results are presented as means ± S.E.M.

Full methods and any associated references are available in the online supplemental information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Receptors are central for cargo selection and delivery during selective autophagy, but how autophagy receptors are organized *in vivo* is yet unknown. In this article, we report that tumor suppressor E3 Ub ligase HACE1 ubiquitylates autophagy receptors Optineurin (OPTN), which promotes the formation of autophagy receptor complex involving OPTN and p62/SQSTM1 and activates autophagy. This suggests an underappreciated mode of regulation for autophagy by Ub signaling. As HACE1 is frequently downregulated or mutated in human tumors, which would cause deficiency in autophagy that is known to affect tumorigenesis and tumor drug responses, targeting autophagy in these cases might represent a promising strategy for cancer therapy.

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Highlights

1. HACE1 mediates ubiquitylation of autophagy receptor OPTN *in vitro* and *in vivo;*
2. HACE1-ubiquitylated OPTN interacts with p62 to form autophagy receptor complex;
3. K48-linked polyubiquitin chains on OPTN target it for autophagic degradation;
4. HACE1 and OPTN constitute an axis to activate autophagy and suppress tumor.

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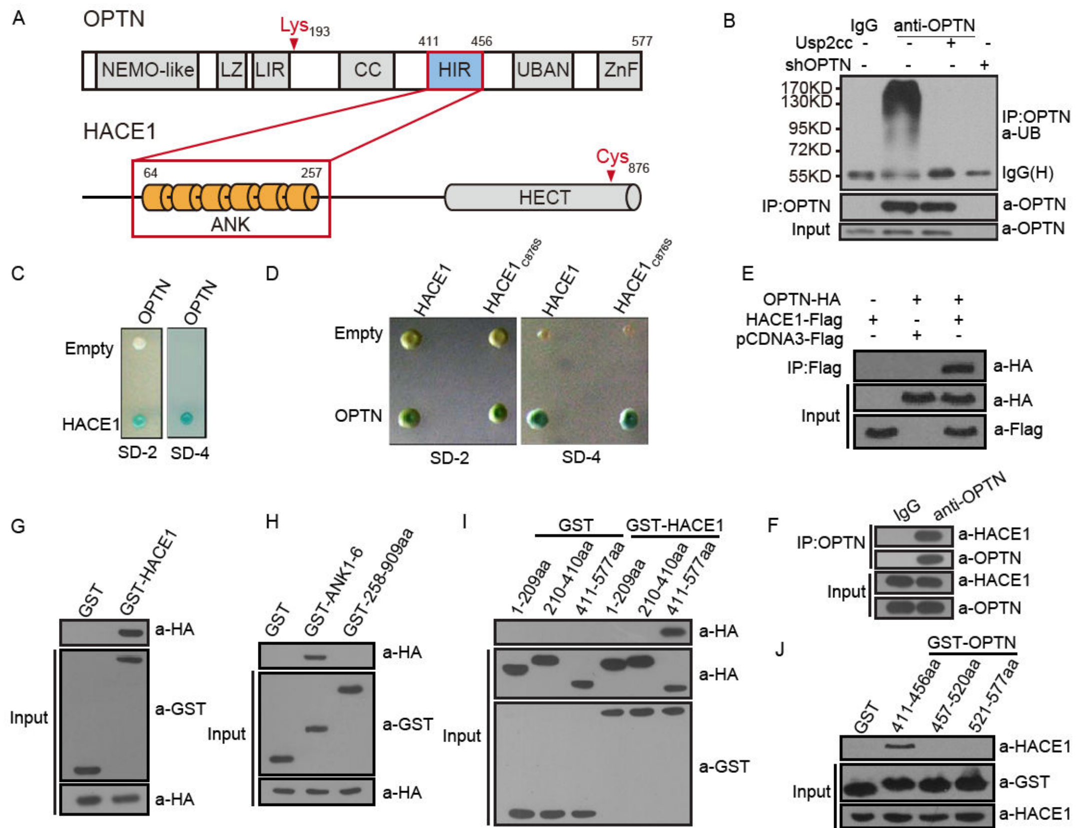

Figure 1. Human OPTN interacts with HACE1 *in vitro* and *in vivo*

(**A**) Schematic representation of the domain architecture of OPTN. LIR, LC3-interacting region; UBAN, ubiquitin binding in ABIN and NEMO domain; ZnF, zinc Finger; Ank, ankyrin repeats; HECT, homologous to the E6-AP Carboxyl Terminus; HIR, HACE1-interacting region.

(**B**) Endogenous OPTN was ubiquitylated in HEK293FTcells. OPTN was immunoprecipitated from HEK293FT cells using anti-OPTN in modified RIPA buffer, followed by immunoblotting with anti-Ub (See Figure S1C for information on shRNA targeting OPTN).

(C-D) Human OPTN interacted with tumor suppressor HACE1 in yeast two hybrid system. Using OPTN (C) or HACE1 (D) as bait, HACE1 interacts with OPTN in yeast. In (D), $HACE1_{C876S}$ was also used as bait. OPTN and HACE1 or $HACE1_{C876S}$ co-transformed into yeast strain Mav 203 activated expression of β-glycosidase.SD-2: deficient in Leu, Trp; SD-4: deficient in Leu, Trp, His and Ura; C876S, the HACE1 active site Cys-876-to-Ser mutant.

(E-J) OPTN interacted with HACE1. Co-immunoprecipitation assay shows that tagged (E) or endogenous (F) OPTN and HACE1 formed a complex in HEK 293FT cells. GST pull-down assays indicates that recombinant GST-tagged HACE1 but not GST only interacts with HA-tagged OPTN (G); the N-terminal ankyrin repeats of HACE1 (H) and the fragment spanning residues 411-456 of OPTN are implicated in such interaction (I, J).

See also Figure S1.

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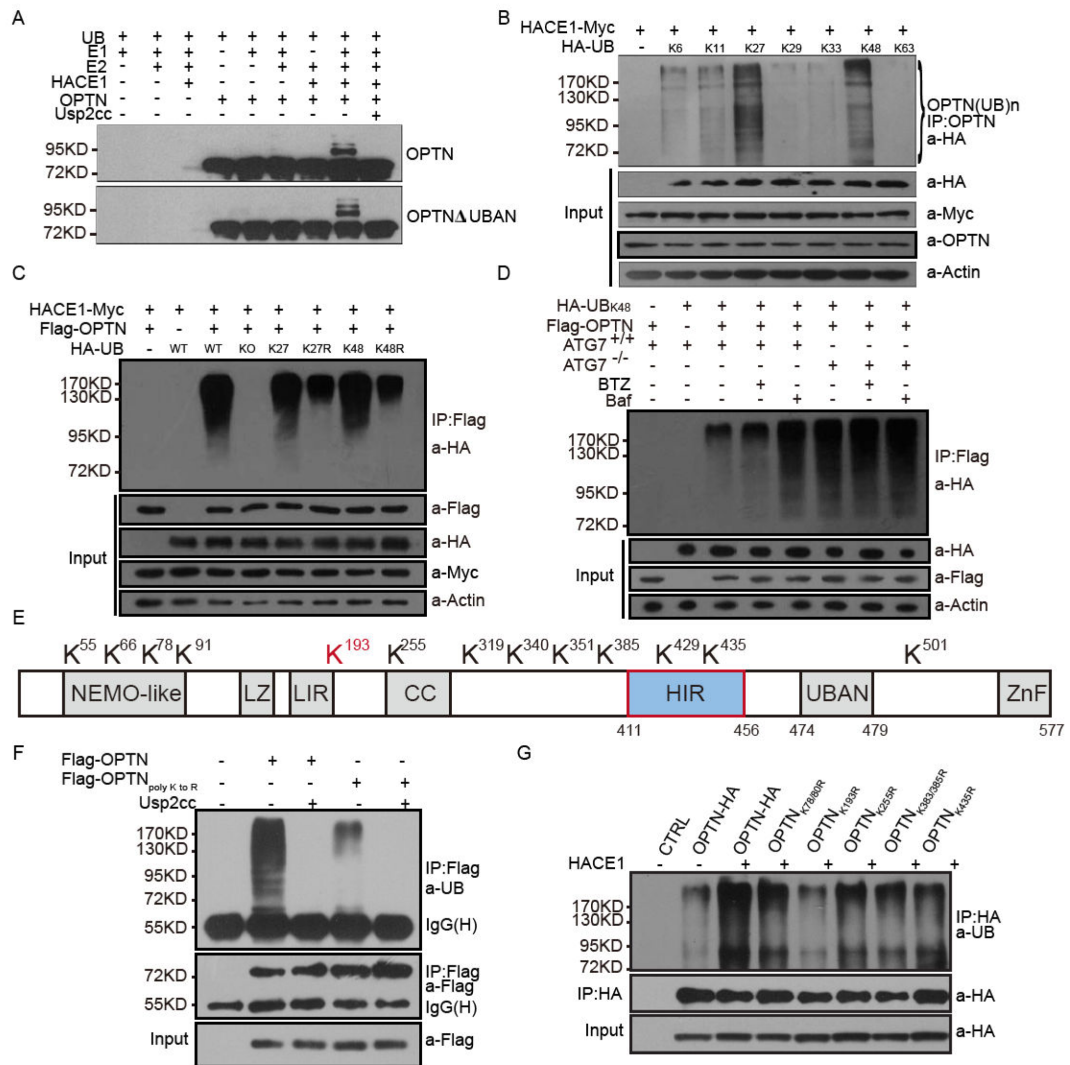

Figure 2. Tumor suppressor HACE1 ubiquitylates OPTN with K27 and K48 ubiquitin linkages, and on multiple lysine (K) residues of OPTN, including K193

(A) Human HACE1 ubiquitylated OPTN *in vitro*. *In vitro* ubiquitylation assay was carried out with the recombinant proteins: OPTN-HA, E1, UbcH7 as E2, and HACE1 together with indicated components.

(B-C) HACE1 ubiquitylated OPTN with K27 and K48 ubiquitin linkages. HEK 293FT cells were expressing HACE1-Myc, and HA-Ub (K6-, K11-, K27-, K29-, K33-, K48-, or K63-only) as indicated (B); HEK 293FTcells stably overexpressing HACE1-Myc were transfected with HA-Ub (WT, KO, K27, K27R, K48 or K48R) as indicated **(C)**.

(D) OPTN with K48-linked poly-Ub chains is stabilized upon autophagy inhibition. *ATG7* $^{+/+}$ or *ATG7* $^{-/-}$ MEF cells were co-transfected with HA-Ub (K48) and Flag-OPTN as indicated, OPTN was immunoprecipitated with anti-Flag M2 beads, and immunoblotted with anti-HA antibodies to detect ubiquitylated OPTN. Bafilomycin A1 (Baf) is an autophagy inhibitor.

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(E) Schematic distribution of the 13 lysine (K) residues in OPTN ubiquitylated by HACE1 *in vitro*. Mass spectra analyses were performed on OPTN recovered from an *in vitro* ubiquitylation assay, K^{501} ubiquitylation was reported elsewhere (Kim et al., 2011).

(F) Lys-to-Arg substitution at all 13 ubiquitylation sites in OPTN almost abolished HACE1-mediated ubiquitylation of OPTN. HEK 293FTcells were transfected with Flag-tagged wild-type OPTN or its poly K-to-R mutants. Note that conjugated ubiquitin in this experiment was endogenous.

(G) Lys^{193} is one of the major sites for HACE1-mediated ubiquitylation on OPTN. HEK 293FTcells were transfected with HA-tagged wild-type OPTN or its mutants bearing single K-to-R substitutions at all potential ubiquitylation sites (Also see Figure S2E, S2F).

See also Figure S2.

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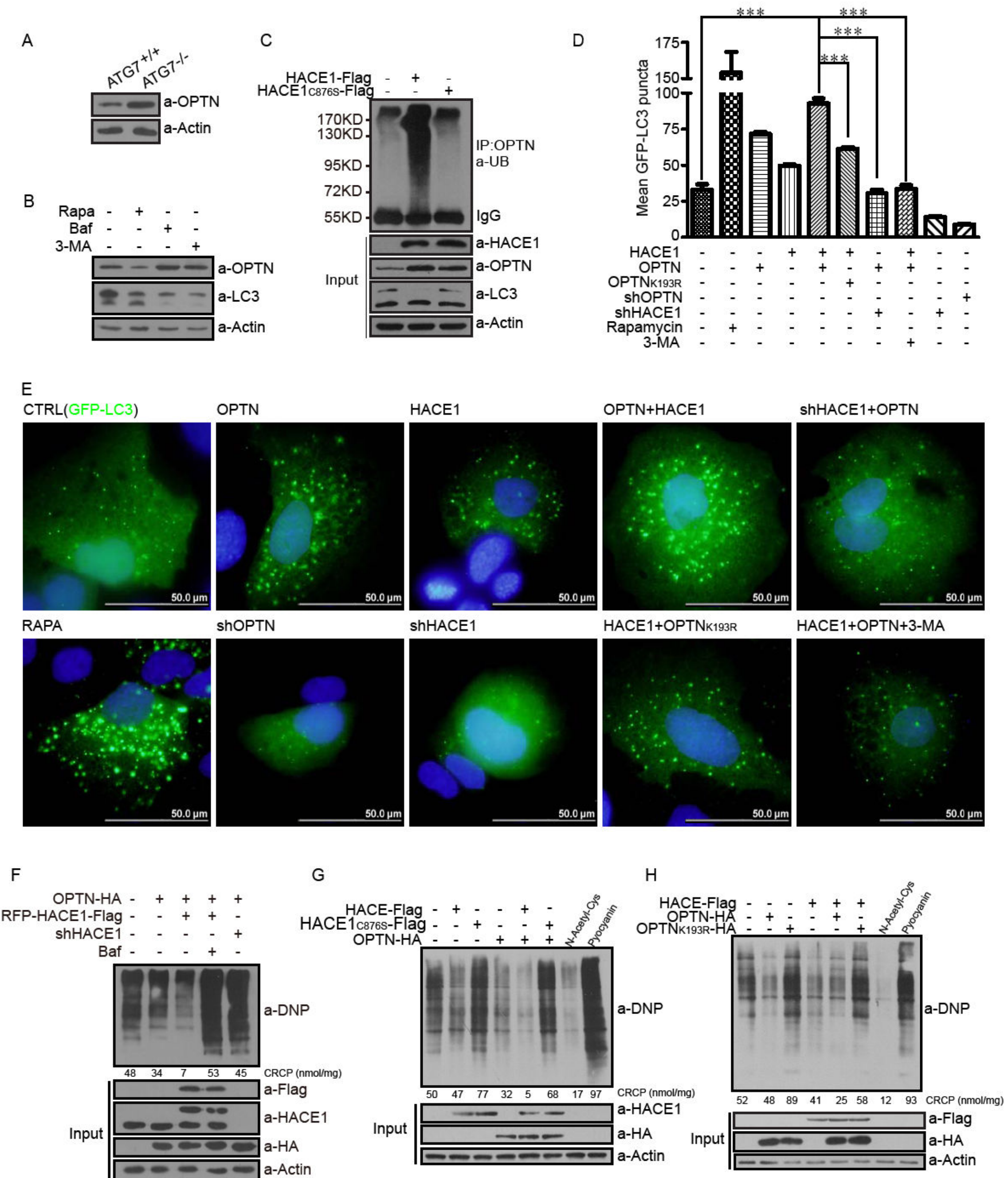

Figure 3. HACE1 and OPTN activate autophagy

(A, B) Endogenous OPTN is an autophagy substrate. Compared with that in wild-type MEF cells, OPTN was accumulated in autophagy deficient *ATG7* $^{-/-}$ mouse MEF cells (A). Treatment with rapamycin (Rapa, 10 μg/ml) induced degradation of endogenous OPTN, while the Baf (20nM) or 3-MA (10 mM) blocked the degradation of endogenous OPTN in HEK 293FT cells (B).

(C-E) HACE1-mediated ubiquitylation activated autophagy in human lung cancer cells. Overexpression of Flag-tagged HACE1 but not its catalytically inactive mutant,

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HACE1$_{C876S}$, increased the ratio of [LC3II]/[LC3 I] (C). Fluorescence microscopy shows formation of GFP-LC3 puncta in CRL-5872 cells transfected with indicated plasmids. Autophagy inducer, Rapamycin (Rapa, 10 μg/ml), was used as a positive control, 3-Methyladenine (3-MA, 10 mM) is an autophagy inhibitor. Statistics of puncta formation by GFP-LC3 in indicated samples, data are presented in mean ± S.E.M. ***, n = 10, $p < 0.001$ (D). Representative images were shown in (E). Cell nuclears were stained with DAPI.

(F) Co-expression of HACE1 and OPTN activated autophagy and removes carbonylated proteins in the human lung cancer cells, CRL-5872. Baflinomycin (Baf, 20 nM) is an autophagy inhibitor.

(G, H) HACE1-mediated ubiquitylation promoted removal of carbonylated proteins in human lung cancer cells (CRL-5872), in dependence of E3 Ub ligase activity of HACE1 (G) and the ubiquitylation of K^{193} in OPTN (H). Pyocyanin (PCN) or N-Acetyl-L-Cysteine (NAC) was used as positive or negative controls, respectively. Expression of the genes of interest was probed with indicated antibodies. Actin was also probed for loading control. Carbonylated proteins were visualized through derivatization with DNPH, followed by immunoblotting with anti-DNP. The contents of residual carbonylated proteins (CRCP) (nmol/mg total proteins) were determined using absorbance at 370 nm for each group (See Experimental Procedures for details).

See also Figure S3.

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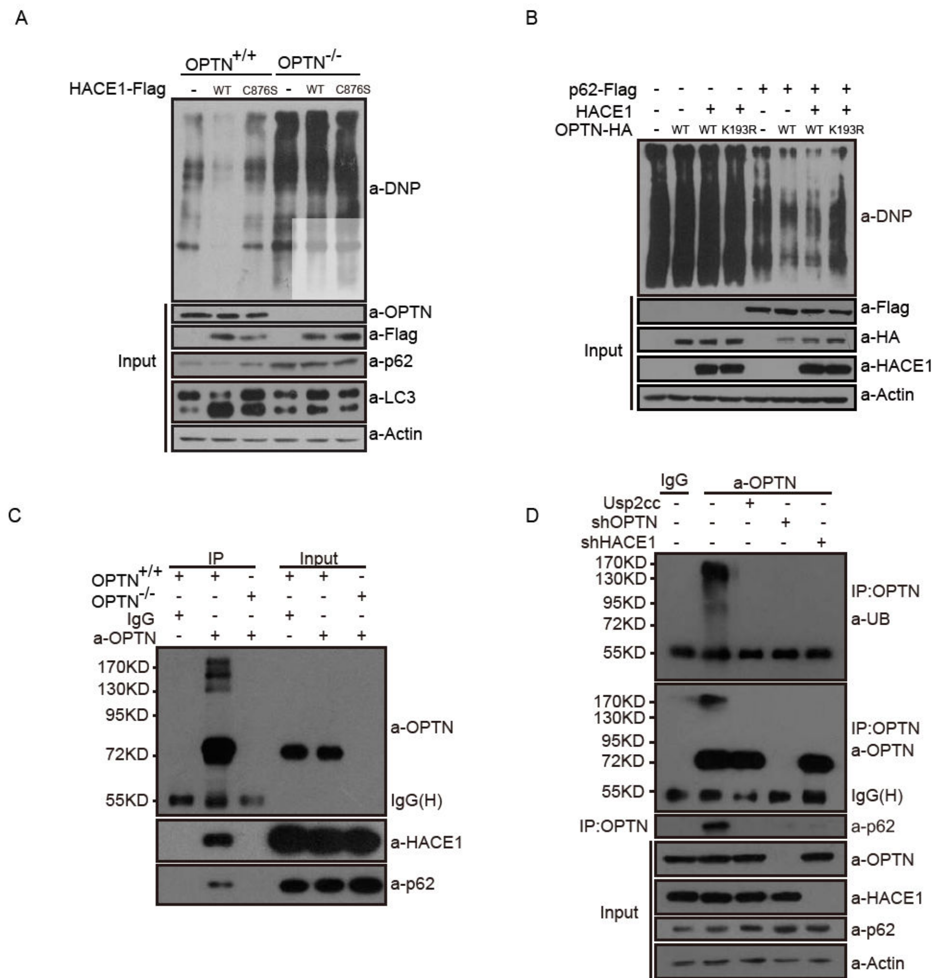

Figure 4. HACE1 and OPTN form a functional axis to activate autophagy in dependence of p62/ SQSTM1

(A) HACE1-mediated ubiquitylation accelerated the removal of carbonylated proteins in OPTN-dependent manner. Macrophage cells were isolated from *OPTN* $^{+/+}$ and *OPTN* $^{-/-}$ mice, cultured, and transfected with plasmids encoding HACE1 or HACE1$_{C876S}$.

(B) HACE1-mediated ubiquitylation of Lys^{193} in OPTN promoted degradation of oxidized proteins in*p62/SQSTM1* $^{-/-}$MEF cells overexpressing p62-Flag.

(C) Endogenous OPTN, HACE1 and p62 form a complex in *OPTN* $^{+/+}$ cells.

(D) Endogenous HACE1 ubiquitylates OPTN in HEK293FT cells and promotes its interaction with p62. OPTN was immunoprecipitated from extracts of *OPTN* $^{+/+}$ or HEK293FT cells, using anti-OPTN, followed by immunoblotting with anti-OPTN, anti-HACE1 or anti-p62 antibodies. IgG served as a negative control.

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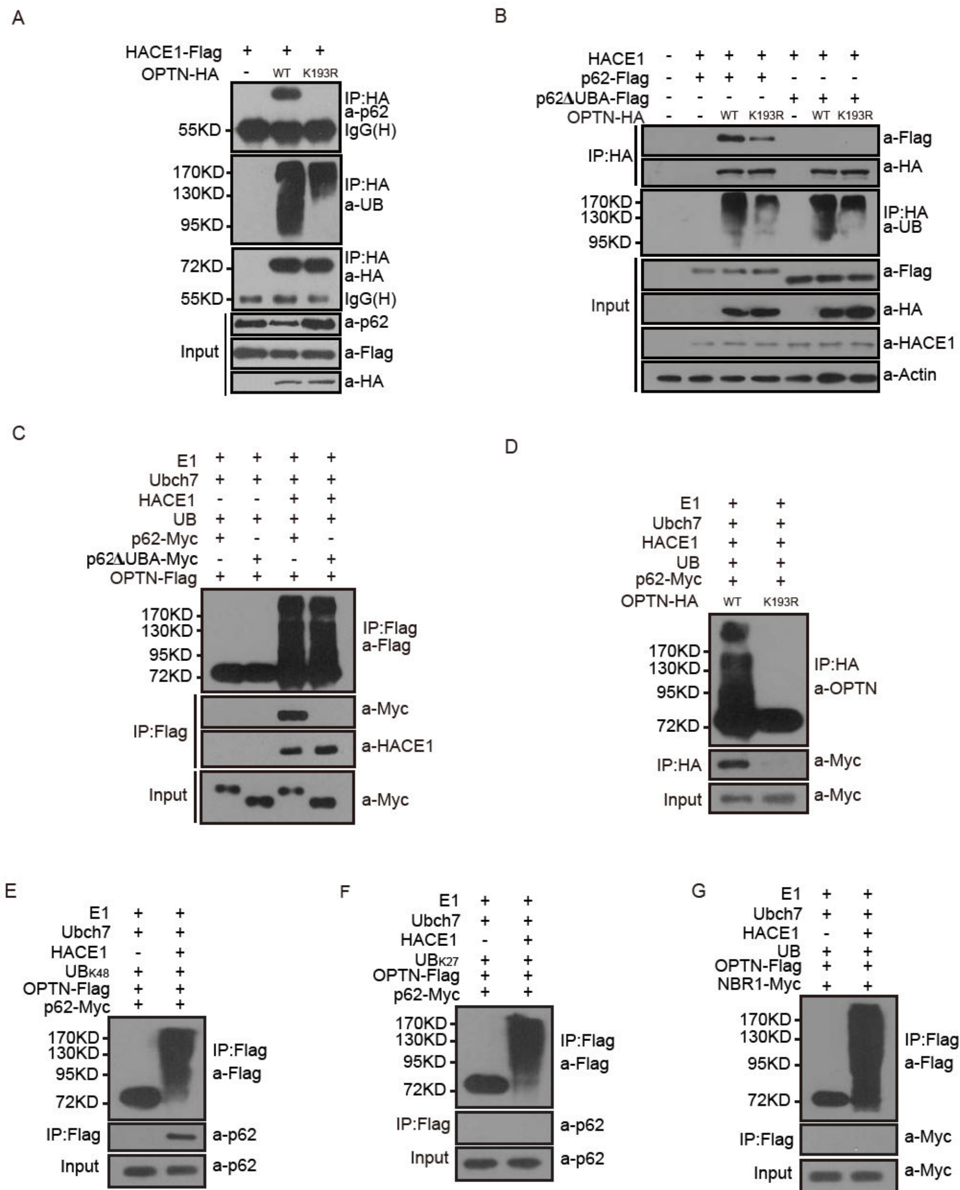

Figure 5. HACE1-mediated ubiquitylation of Lys^{193} in OPTN promotes the interaction between OPTN and p62/SQSTM1 to activate autophagy

(A) HACE1-mediated ubiquitylation of Lys^{193} in OPTN promoted the interaction between OPTN and endogenous p62/SQSTM1 in CRL-5872 cells.

(B, C) UBA domain in p62 interacted with HACE1-ubiquitylated OPTN. (B) *p62* $^{-/-}$ MEF cells were infected with lenti-virus for expression of HACE1, HA-tagged wild-type OPTN or $OPTN_{K193R}$, the full-length p62 or its UBA domain deletion mutant, p62 $_{UBA}$, in indicated combinations. OPTN or $OPTN_{K193R}$ was IP-ed using anti-HA. (C) Bacterially expressed OPTN was ubiquitylated by HACE1 *in vitro*, and interacted with p62 but not p62 $_{UBA}$.

(D) Bacterially expressed p62/SQSTM1 interacted with OPTN through poly-Ub chains that HACE1 conjugated onto Lys^{193} in OPTN *in vitro*.

(E, F) Bacterially expressed p62 interacted K48- but not K27-linked poly-Ub chains that HACE1-conjugated onto OPTN.

(G) Bacterially expressed NBR1 did not interact with HACE1-ubiquitylated OPTN. The presence of indicated proteins was confirmed with immunoblotting using respective antibodies.

See also Figure S4.

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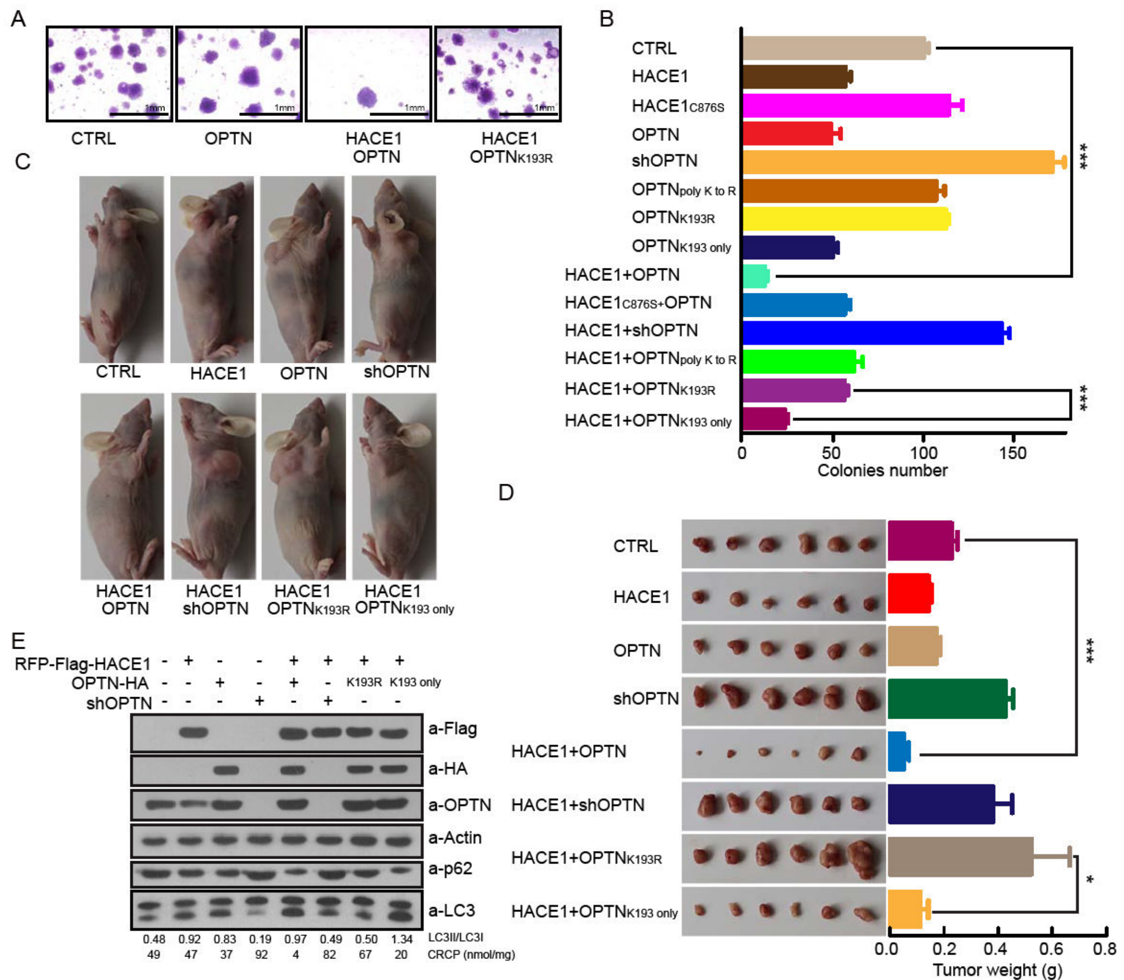

Figure 6. HACE1-mediated ubiquitylation of OPTN induces autophagy, suppressing anchorage-independent growth and tumorigenicity of human lung cancer cells in nude mice

(A-B) HACE1-mediated ubiquitylation of OPTN inhibits anchorage-independent proliferation of human lung cancer cells, CRL-5872. The representative images show typical colony formation by cells expressing indicated genes, 14 days after cell plating (A). Colony formation efficiency of each group was assessed in percentages of that for the control (B). Data are presented in mean ± S.E.M. ***, n = 3, $p < 0.001$.

(C-E) HACE1-mediated ubiquitylation of OPTN, particularly which on Lys^{193} of OPTN, activates autophagy and suppresses tumorigenicity of the lung cancer cells in nude mice. Images showed tumor formation in nude mice (C) or the dissected tumors 21 days after injection (D). Tumor masses for each group were shown in mean ± S.E.M. ***, n = 6,p = 0.001; *, n = 6,p = 0.0177. Expression of the genes of interest in the xenografts were examined with immunoblotting (E). The ratios of static level of lipidated LC3 II to that of LC3 I, [LC3 II] / [LC3 I], were assayed for autophagic activities in the tumors. The contents of residual carbonylated proteins (CRCP) (nmol/mg total proteins) were assessed for each group of the tumor masses. Data were shown in mean ± S.E.M (n = 3). ***, $p < 0.001$.

See also Figure S5.

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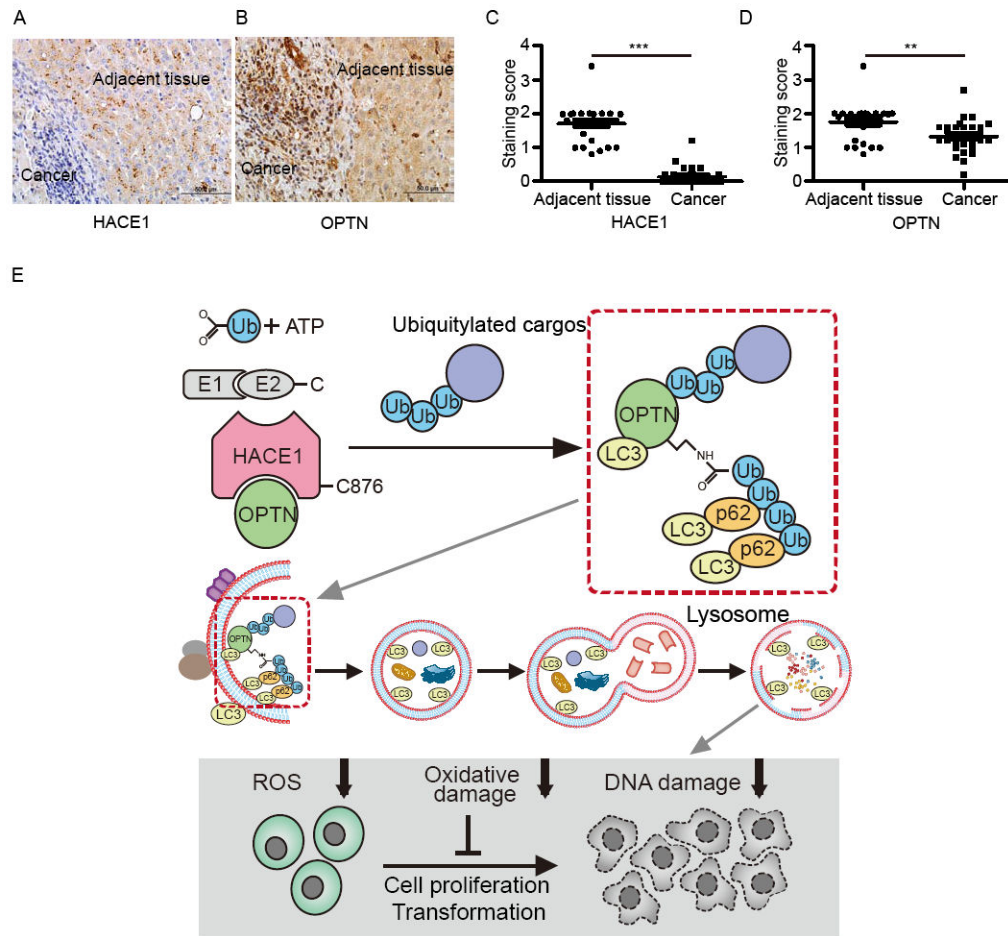

Figure 7. The HACE1-OPTN axis suppresses tumor through promoting formation of the complex of autophagy receptors

(A-D) HACE1 (A) or OPTN (B) is downregulated in human liver cancer. Shown here are representative immunohistochemical staining images and Statistics score of the staining of HACE1 (A, C) or OPTN (B, D) in human liver cancer or adjacent tissues, respectively. Data are presented in mean ± S.E.M. ***, n = 30. For HACE1, $p < 0.001$; For OPTN, $p = 0.0027$. (See Supplemental experimental procedures for more details)

(E) A model depicting how the HACE1-OPTN axis might suppress tumor through promoting autophagy *in vivo*.

See also Figure S6.