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Nedd4-dependent lysine-11-linked polyubiquitination of the tumour suppressor Beclin 1

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Beclin 1, a subunit of the class III phosphatidylinositol 3-kinase complex, is a tumour suppressor with a central role in endocytic trafficking, cytokinesis and the cross-regulation between autophagy and apoptosis. Interestingly, not only reduced expression but also overexpression of Beclin 1 is correlated with cancer development and metastasis. Thus it seems necessary for the cell to balance the protein levels of Beclin 1. In the present study we describe a regulatory link between Beclin 1 and the ubiquitin ligase Nedd4 (neural-precursor-cell-expressed developmentally down-regulated 4). We establish Nedd4 as a novel binding partner of Beclin 1 and demonstrate that Nedd4 polyubiquitinates Beclin 1 with Lys^{11}- and Lys^{63}-linked chains. Importantly, Nedd4 expression controls the stability of Beclin 1, and depletion of the Beclin 1-interacting protein VPS34 causes Nedd4-mediated proteasomal degradation of Beclin 1 via Lys^{11}-linked polyubiquitin chains. Beclin 1 is thus the first tumour suppressor reported to be controlled by Lys^{11}-linked polyubiquitination.

Key words: cancer, homologous with E6-associated protein C-terminus (HECT), neural-precursor-cell-expressed developmentally down-regulated 4 (Nedd4), phosphoinositide, ubiquitination, VPS34.

INTRODUCTION

Phosphoinositides, phosphorylated derivatives of PtdIns, control cellular functions through recruitment of cytosolic proteins to specific membranes. The PI3K-III (class III PI 3-kinase) complex, which catalyses the conversion of PtdIns into PtdIns3*P*, is involved in at least three topologically related membrane involution processes: autophagy [1] and cytokinesis [2], as well as endocytic trafficking and signalling [3]. The dysfunction of PtdIns3*P* generation is directly linked to the occurrence of several diseases, including different types of cancer [4]. The assembly of the PI3K-III complex is suggested to occur in a sequential manner. Initially, the regulatory subunit VPS15/PIK3R4/p150 associates with specific membranes and recruits the catalytic subunit VPS34/PIK3C3. Together with VPS15 and VPS34, the coiled-coil protein Beclin 1 is thought to form the core of the PI3K-III complex, and accumulating evidence suggests that Beclin 1 serves as a platform for the recruitment of transiently associated regulatory proteins [5,6]. The importance of Beclin 1 is underscored by the finding that low cellular concentrations of Beclin 1 are often associated with the occurrence of cancer [7,8]. However, it is important to also note that overexpression of Beclin 1 is correlated with prolonged survival of a subset of tumour cells, most likely by promoting autophagy and thereby preventing apoptosis [9,10]. In general, the question of how and when Beclin 1-mediated autophagy has a positive or negative effect on tumorigenesis is not fully resolved [11].

Beclin 1 has a modular architecture which facilitates differential interaction with various binding partners. The interaction with the PI3K-III catalytic subunit VPS34 is mediated both by the ECD (evolutionarily conserved domain) [12] and by regions of the CCD (coiled-coil domain). The latter also serves as a binding site for UVRAG (UV radiation resistance-associated gene), a positive regulator of PI3K-III complex activity [13]. The BH3 (Bcl-2 homology 3) domain is required for the association with Bcl-2 family members [14], which act as inhibitors of Beclin 1's function in autophagy. The list of Beclin 1 effectors is still growing [6] and gives rise to the notion that the stoichiometry of the Beclin 1-associated proteins targets the VPS34 complex to its different functions in autophagy, endocytic trafficking and cytokinesis [5]. Thus the availability of the active pool of Beclin 1 to associate with VPS34 and in turn to promote PtdIns3*P* signalling is tightly governed by a subset of regulatory factors. First, Beclin 1 can be regulated at the level of protein expression, which is usually increased during autophagy [15]. In this context, it has been described that the expression of the *BCN1* gene is induced via 14-3-3τ and E2F1 [16]. Furthermore, the activity of the Beclin 1 protein is titrated by its interaction with Bcl-2 family members [14]. At the post-translational modification level Beclin 1 can be phosphorylated by DAP (death-associated protein) kinase within its BH3 domain in order to reduce its interaction with Bcl-2 [17]. Recently, it has also been demonstrated that TRAF6 (tumour-necrosis-factor-receptor-associated factor 6)-dependent ubiquitination, which is supposed to function non-proteolytically, activates Beclin 1 during early autophagy, as this modification occurs within the BH3 domain and inhibits the interaction with Bcl-2 [18].

Even though several regulators of Beclin 1 have been identified, it is not known how the stability of this tumour suppressor is controlled. In the present study we provide evidence for a novel type of regulatory mechanism of human Beclin 1. We demonstrate that Beclin 1 is polyubiquitinated by the HECT (homologous with E6-associated protein C-terminus) ligase Nedd4 (neural-precursor-cell-expressed developmentally down-regulated 4) with Lys^{11}- and Lys^{63}-linked Ub (ubiquitin) chains and that the former post-translational modification regulates the stability of Beclin 1.

Abbreviations used: AIP4, atrophin-interacting protein 4; BH3, Bcl-2 homology 3; CCD, coiled-coil domain; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECD, evolutionarily conserved domain; GST, glutathione transferase; HA, haemagglutinin; HECT, homologous with E6-associated protein C-terminus; HRP, horseradish peroxidase; Nedd4, neural-precursor-cell-expressed developmentally down-regulated 4; PI3K-III, class III PI 3-kinase; siRNA, small interfering RNA; TRAF6, tumour-necrosis-factor-receptor-associated factor 6; Ub, ubiquitin.

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EXPERIMENTAL

Plasmids

The pCXN2-Nedd4 construct (from Professor Sharad Kumar, Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, SA, Australia) was cut with EcoRI and Nedd4 was ligated into pET21d, resulting in pET21d-His-Nedd4. The WW-fragment, containing the WW-motifs 1–3, was generated by using Nedd4 as a PCR template. The PCR fragment was restricted with BamHI/XhoI and cloned into the BamHI/XhoI sites of pGAD-GH2 or pcDNA3, from where it was cut with EcoRI/XbaI in order to be ligated into pET21d to generate His–Nedd4–(WW). The Myc–Nedd4 fusion and the Myc–Nedd4-2 construct (from Professor Jon Huibregtse, Cell and Molecular Biology, University of Texas at Austin, TX, U.S.A.) as well as the Myc–AIP4 (atrophin-interacting protein 4) (from Dr Tony Pawson, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) construct were expressed from pcDNA3. The fusion proteins Myc–Beclin 1, GST (glutathione transferase)–Beclin 1 and pGAD-Beclin 1 as well as pLexA-VPS34 were from Dr Anne Simonsen (Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Oslo, Norway). The Y352A point mutation was introduced in Beclin 1 using overlap extension PCR. The different HA (haemagglutinin)-tagged Ub species were expressed from pcDNA3 and comprised HA–Ub, HA–Ub-K0, HA–Ub-Lys^{6}, HA–Ub-Lys^{11}, HA–Ub-Lys^{29}, HA–Ub-Lys^{48} and HA–Ub-Lys^{63} (from Dr Vishva Dixit, Department of Physiological Chemistry, Genentech, South San Francisco, CA, U.S.A.). The sequences of the PCR oligonucleotides used are available upon request.

Cell culture and transfections

Media and reagents for cell culture were purchased from Gibco. HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10 % FBS (fetal bovine serum), 5 units/ml penicillin and 50 μg/ml streptomycin. Cells were transfected with the above expression plasmids using the FuGENE™ 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions.

siRNA (small interfering RNA) treatment

ON-Target Plus siRNAs were from Dharmacon. Pre-designed oligonucleotides included scrambled siRNA (D-001810-01), siRNAs specific for VPS34 (D-005250-04) and Nedd4 (from Dr Edward Leithe, Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway). siRNA oligonucleotides were complexed with Lipofectamine™ RNAiMax transfection reagent (Invitrogen) in DMEM without serum, penicillin or streptomycin, and added to cells at 50 % confluency in penicillin- and streptomycin-free DMEM, at a final siRNA concentration of 70 nM. After 16 h, cells were washed twice in DMEM and maintained in DMEM for a total of 96 h post-transfection [19].

CHX (cycloheximide) chase

HeLa cells were transfected where indicated and cultured for 20 h. After a 10 h incubation with 25 μg/ml CHX, in the presence or absence of 10 μM lactacystine or 20 μM MG132, the cells were lysed and analysed by SDS/PAGE (4–20 % gels) and immunoblotting to assess the amount of undegraded protein. Actin or tubulin were used as the loading controls.

Immunoblotting and antibodies

HeLa cells were lysed in buffer [25 mM Hepes, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1 mM DTT (dithiothreitol), 0.5 % Nonidet P40 and 1:100 protease inhibitor mix (Roche Applied Science)]. After centrifugation for 5 min at 20000 ***g***, the whole cell lysate was collected and subjected to SDS/PAGE. The proteins were transferred on to Immobilon-P membranes (Millipore) and visualized after immunoblotting using the Supersignal West Pico Substrate kit (Pierce). Rabbit polyclonal antibodies against VPS34 and Beclin 1 were purchased from Cell Signaling Technology. Rabbit anti-Nedd4 antibody was a gift from Dr Edward Leithe. Mouse anti-β-actin and mouse anti-α-tubulin antibodies were from Sigma–Aldrich, whereas mouse anti-HA antibody was from Roche. The mouse monoclonal antibody against the c-Myc epitope was from the 9E10 hybridoma [19a]. Secondary antibodies were all from Jackson Immunoresearch Laboratories, and included anti-mouse HRP (horseradish peroxidase) and anti-rabbit HRP.

Immunoprecipitation

HeLa cells in 10-cm-diameter culture dishes were lysed in ice-cold lysis buffer (20 mM Hepes, pH 7.2, 2 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA and 0.1 % Triton X-100) containing inhibitors [10 mM *N*-ethylmaleimide, mammalian protease inhibitor mixture, phosphatase inhibitor cocktail I and II (Sigma–Aldrich)]. The lysates were cleared by centrifugation at 4 °C for 15 min at 10000 ***g***. The pre-cleared lysate (1 mg of total cell extract) was transferred to a new tube and incubated with 1 μg of antibody for 90 min end-over-end at 4 °C (total volume 500 μl). Protein A–Sepharose (30 μl; GE Healthcare Bio-Sciences AB) was added, followed by an incubation for 60 min at 4 °C. The Protein A slurry bound to the antibody and associated protein complexes was pelleted by centrifuging for 30 s at 25000 ***g***. The pellet was washed three times with 1 ml of lysis buffer. After the final wash, 30 μl of 2× Laemmli sample buffer supplemented with 50 mM DTT was added and the samples were boiled for 5 min at 95 °C.

In vivo ubiquitination

HeLa cells were grown on 10-cm-diameter dishes in the presence of 20 μM MG132 and were transiently transfected with HA–Ub (3 μg) and 1 μg Myc–Beclin 1 as well as 1 μg of Nedd4, Myc–Nedd4, Myc–Nedd4-2 or Myc–AIP4 as indicated. To detect ubiquitinated Beclin 1, cells were scraped in 1 ml Triton X buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.4, 5 mM EDTA, 1 mM $CaCl_2$, 1 % Triton X-100, 5 % glycerol, 10 mM NaF and 10 mM sodium orthovanadate with protease inhibitors and 10 mM *N*-ethylmaleimide). Lysates were subjected to immunoprecipitation.

Expression and purification of recombinant proteins

His-tagged fusion proteins were expressed in *Escherichia coli* BL21 cells after induction with IPTG (isopropyl β-D-thiogalactopyranoside). The cells were lysed in lysis buffer (25 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.5 % Triton X-100, 10 % glycerol and 10 mM imidazole). The cell lysates were cleared by 14000 rev./min centrifugation at 4 °C for 20 min, and the cleared lysates was incubated with Ni-NTA (Ni^{2+}-nitrilotriacetate) agarose beads at 4 °C with rotation for 2 h. The Ni^{2+}-beads were washed three times with lysis buffer containing 20 mM imidazole. The bead-bound Nedd4 was eluted by incubation of the beads with elution buffer (25 mM Tris/HCl,

pH 8.0, 50 mM NaCl, 10 % glycerol and 250 mM imidazole) at 4 °C with rotation for 15 min. The eluted Nedd4 was dialysed overnight in dialysis buffer (25 mM Tris/HCl, pH 8.0, 100 mM NaCl and 2 mM $MgCl_2$) [20]. GST-fusion proteins were expressed in *E. coli* BL21 cells and purified by affinity purification with glutathione–agarose beads as described previously [17].

In vitro ubiquitination

The *in vitro* ubiquitination experiments were based on methods described previously [20,21] with minor modifications. The assay is based on a reaction mixture (15 μl) containing 0.1 μg of GST–Beclin 1, 0.1 μg of E1, 0.5 μg of UbcH5a, 5 μg of Ub and buffer (2 mM ATP, 25 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2.0 mM $MgCl_2$ and 1 mM DTT) For an activation of Nedd4, a 1.25 mM $CaCl_2$ solution (5 μl) was added to the His–Nedd4 (0.1 μg in 10 μl) before subjecting both to the assay reaction mixture. The reaction was initiated by addition of Nedd4 to the reaction mixture, carried out at 22 °C for 20 min and stopped by adding 25 μl of 2× SDS/PAGE sample buffer.

Yeast two-hybrid assay

The yeast reporter strain L40 was transformed as described previously [22] with the yeast two-hybrid plasmids pLexA and pGAD, and grown on synthetic medium lacking tryptophan and leucine at 30 °C. The double transformants obtained were grown at 30 °C for 16 h in liquid synthetic medium. Lysates from these cells were prepared and subsequently subjected to β-galactosidase assays as described previously [23].

RESULTS

Nedd4 interacts with Beclin 1

To obtain potential clues for the regulation of Beclin 1 stability, we utilized various bioinformatic algorithms to analyse the protein sequence. Using tools for multiple sequence alignments, we identified a sequence resembling a PY motif (LPxY), a potential target for WW domains, in Beclin 1 (Figure 1A). The motif is well conserved from yeast to humans and, in humans, Beclin 1 is located downstream of the ECD at amino acid positions 349–352. Interestingly, the PY motif is also conserved in a plant homologue of Beclin 1, but is located in the N-terminal portion of the protein (results not shown). Both the canonical LPxY as well as the LPxF variant found in Beclin 1 of *Brugia malayi* represent sequence motifs that have been described to function as binding sites for Nedd4/Rsp5-like Ub-isopeptide ligases in several proteins [24,25]. The general concept is that the PY motif of the target protein has to interact specifically with the WW domains found in the Nedd4-like ligase in order to become ubiquitiated [26]. We adressed the question of whether human Beclin 1 is capable of interacting with Nedd4-like ligases by testing its interaction with the WW domains of Nedd4, Nedd4-2 and AIP4 in a yeast two-hybrid assay (Figure 1B). Beclin 1 was found to interact with all three variants of this conserved interaction module, with Nedd4(WW) displaying the strongest affinity in this assay. In contrast, none of the three WW-motifs were able to interact with VPS34, the major binding partner of Beclin 1, indicating that the observed interaction between WW domains and Beclin 1 is specific. These results provide evidence that Beclin 1 is a ligand for WW motifs. On the basis of the observation that Nedd4(WW) gave the strongest interaction in the yeast two-hybrid assay, we tested if the full-length proteins interact in human cells (Figure 1C). For this purpose, we transiently transfected

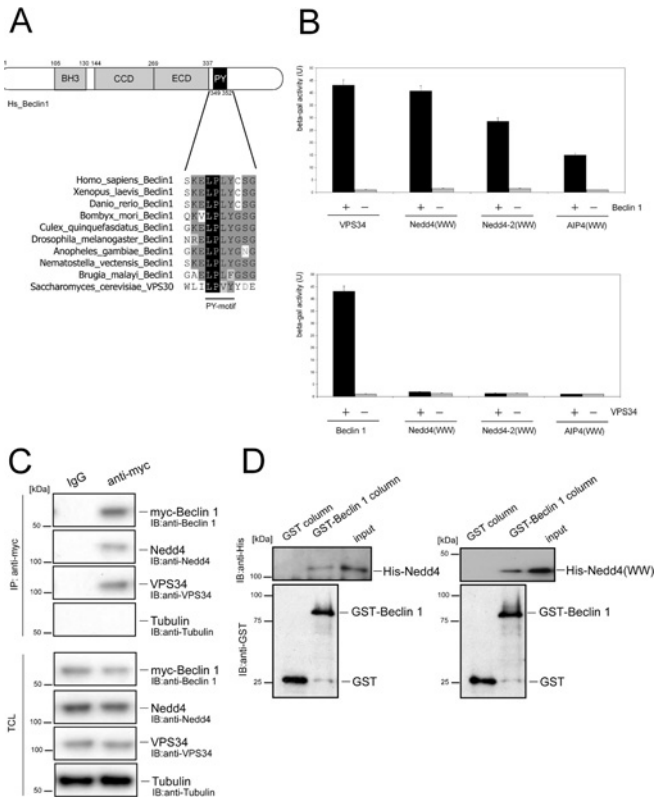

Figure 1 Beclin 1 interacts with Nedd4

(**A**) Human Beclin 1 has been described as containing a BH3 domain, a CCD and an ECD. Amino acid sequence alignments suggest that Beclin 1 contains a PY motif in addition. (**B**) The interaction of the WW fragments of Nedd4, Nedd4-2 and AIP4 were tested against Beclin 1 (upper panel) as well as VPS34 (lower panel) in yeast two-hybrid assays by measuring β-galactosidase activity. (**C**) Myc–Beclin 1 was transfected into HeLa cells and immunopreciptated (IP) via the Myc tag. VPS34 as well as Nedd4, but not tubulin, could be co-isolated with Beclin 1. (**D**) Recombinant GST-Beclin 1 and His-Nedd4 as well as His-Nedd4(WW) were expressed heterologously in *E. coli* cells. The full-length as well as the WW fragment of Nedd4 were co-eluted with GST–Beclin 1, but not with GST alone from GSH columns. IB, immunoblot; TCL, total cell lysate.

HeLa cells with Myc-tagged Beclin 1 and untagged Nedd4 and performed co-immunoprecipitation experiments via the Myc tag. As a result, a fraction of Nedd4 was co-isolated along with the known binding partner VPS34, indicating that Beclin 1 and Nedd4 can associate *in vivo*. In order to elucidate whether the observed association indeed represents a direct interaction, we purified GST–Beclin 1 and His-tagged Nedd4 as well as the WW-domain fragment from *E. coli* (Figure 1D). Using a glutathione column, we were able to co-elute GST–Beclin 1 with His–Nedd4 or His–Nedd4(WW). In contrast, none of the Nedd4 proteins could be co-eluted with GST alone, strongly suggesting a direct association of Beclin 1 with the WW fragment of Nedd4. In summary, these results establish Nedd4 as a novel Beclin 1-interacting protein.

The stability of Beclin 1 is reciprocally regulated by Nedd4 and VPS34

The association of Beclin 1 with the ligase Nedd4 suggested that the regulation of the protein concentration of Beclin 1 might be linked to ubiquitination events. To investigate this, we blocked protein synthesis by the addition of CHX to the cells and monitored the protein level of Beclin 1 depending on time and different inhibitors of proteasomal protein degradation (Figure 2A). The results show that the level of Beclin 1, in

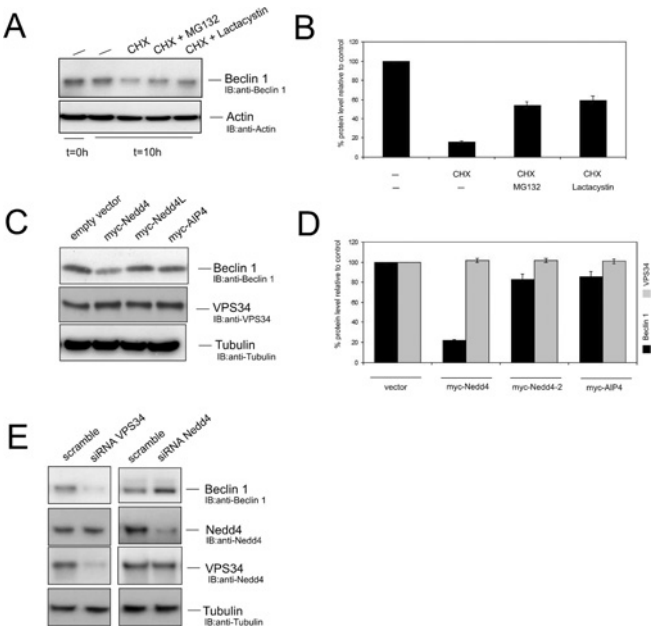

Figure 2 VPS34 and Nedd4 regulate the stability of Beclin 1

(**A**) CHX was added to the indicated samples in order to block protein synthesis. The observed partial turnover of Beclin 1 could be stopped in part by the proteasomal inhibitors MG132 and lactacystin. (**B**) Quantification of Beclin 1 protein levels for each experimental condition. Numbers represent protein levels expressed as the percentage of those from untreated cells and normalized to actin. Error bars represent means ± S.E.M. on the basis of results from three independent experiments. (**C**) HeLa cells overexpressing transiently transfected Myc–Nedd4, Myc–Nedd4-2 or Myc–AIP4 were lysed and analysed for the stability of Beclin 1. (**D**) Quantification of Beclin 1 and VPS34 protein levels for each experimental condition. Numbers represent protein levels expressed as the percentage of those from cells with empty vector and normalized to tubulin. Error bars represent means ± S.E.M. on the basis of results from three independent experiments. (**E**) HeLa cells treated with the indicated siRNA were lysed and proteins were detected by immunoblotting. siRNA directed against VPS34 induced the destabilization of Beclin 1, whereas the siRNA against Nedd4 stabilized Beclin 1.

contrast with the control protein actin, was decreased 10 h after the addition of CHX and that this basal turn-over could be blocked to a certain extent by supplementing the cells with the proteasomal inhibitors MG132 or lactacystin (Figures 2A and 2B). Since Beclin 1 could interact with the WW domains of Nedd4, Nedd4-2 and AIP4 (Figure 1B), we tested if the steady-state concentration of Beclin 1 could be affected by overexpression of the full-length versions of these three HECT ligases (Figure 2C). Although overexpression of Nedd4-2 and AIP4 had only a marginal effect on the Beclin 1 level, overexpression of Nedd4 significantly reduced the Beclin 1 concentration. In contrast, the steady-state level of VPS34 remained unchanged in all experiments (Figures 2C and 2D). Similar results were obtained in CHX assays, where overexpression of Nedd4 reduced the half-life of Beclin 1 but not VPS34 compared with the cells expressing empty vector (results not shown).

Since Beclin 1 binds both VPS34 and Nedd4, we investigated the functional relationship of endogenous VPS34 and Nedd4 by siRNA treatment (Figure 2E). siRNA-mediated depletion of VPS34 reduced the level of Beclin 1, as previously reported [19], whereas Nedd4 levels remained unaffected. The depletion of Nedd4 resulted in a moderate increase in the level of Beclin 1, but had no effect on VPS34. Taken together, the data suggest that the steady-state concentration of Beclin 1 is controlled by antagonizing factors. Although the major binding partner VPS34 stabilizes Beclin 1, the HECT ligase Nedd4 promotes its degradation by the proteasome.

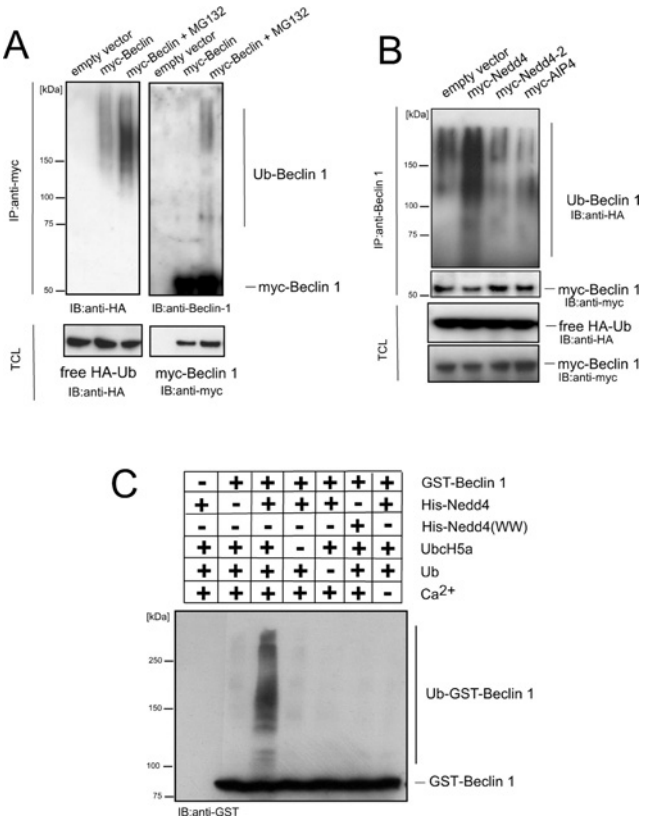

Figure 3 Beclin 1 is ubiquitinated by Nedd4

(**A**) HeLa cells were transfected with HA–Ub, Myc–Beclin 1 or empty pcDNA3 plasmid. The proteasomal inhibitor MG132 was added to the cells where indicated. Myc–Beclin 1 was immunoprecipitated (IP) using the anti-Myc antibody. The samples of the IP eluate as well as of the total cell lysate (TCL) were analysed by SDS/PAGE and immunoblotting (IB) with anti-HA and anti-Beclin 1 antibodies. (**B**) Beclin 1 was immunoprecipitated from cells expressing the ligases Myc–Nedd4, Myc–Nedd4-2 and Myc–AIP4 as well as empty vector. Samples were subjected to SDS/PAGE and analysed by anti-HA and anti-Myc antibodies. (**C**) Heterologously expressed and purified GST–Beclin 1 was subjected to *in vitro* ubiquitination assays. UbcH5a served as the Ub-conjugating enzyme, whereas His–Nedd4 served as the Ub ligase. The dependency of Beclin 1 ubiquitination on the activity of Nedd4 was tested by using the Nedd4 WW-domain fragment, which lacks the catalytic HECT domain, or by omitting the Ca^{2+} ions, which are required for releasing Nedd4 from its autoinhibition. Modified and unmodified GST–Beclin 1 species were visualized by the anti-GST antibody.

Beclin 1 is ubiquitinated by Nedd4

The finding that the concentration of Beclin 1 is influenced by the HECT ligase Nedd4 strongly suggested that Beclin 1 is post-translationally modified with Ub. To test this hypothesis we transiently transfected cells with HA–Ub and isolated Myc–Beclin 1. Analysis of the precipitates revealed that several slower migrating Beclin 1 species could be detected with the Beclin 1 antibody (Figure 3A). These signals were significantly enhanced when proteasomal activity was inhibited with MG132. The immunoblot stained with the anti-HA antibody to detect ubiquitinated species displayed signals that correspond to the Beclin 1-positive high-molecular mass species. Additionally, these signals become more intense in samples derived from MG132-treated cells. Taken together, these data indicate that Beclin 1 is ubiquitinated *in vivo*.

In order to identify the E3 Ub ligase responsible for the ubiquitination of Beclin 1, we overexpressed Nedd4, Nedd4-2 and AIP4 in human cells, added MG132 and analysed the effect on the ubiquitination of Beclin 1 (Figure 3B). The enhanced expression of Nedd4 resulted in an increase of the ubiquitinated Beclin 1

species, whereas Nedd4-2 and AIP4 had no significant effect under the chosen conditions.

In order to exclude the possibility that Nedd4 might act indirectly on Beclin 1, we investigated whether Nedd4 can directly ubiquitinate Beclin 1 *in vitro* (Figure 3C). For this purpose we used heterologously expressed GST–Beclin 1, His_6-tagged Nedd4, purified E1 Ub-activating enzyme and Ub. UbcH5a was used as an E2 Ub-conjugating enzyme, because it is known to co-operate with Nedd4 [27]. The *in vitro* Ub assay revealed a direct ubiquitination of Beclin 1 by Nedd4 (Figure 3C). Beclin 1 was not modified when Nedd4 was auto-inhibited by the absence of Ca^{2+} or when a truncated version of Nedd4 consisting of the WW fragment only was included in the assay, indicating that the enzymatically active HECT domain of Nedd4 is required for the ubiquitination of Beclin 1. In conclusion, these results identify Nedd4 as an E3 Ub ligase for Beclin 1.

Mutation of the PY motif affects ubiquitination and stability of Beclin 1

In order to verify that the putative PY-motif in Beclin 1 indeed serves as the binding site with Nedd4, we mutated Tyr^{352} to an alanine residue. The Beclin 1(Y352A) mutant displayed a significantly reduced interaction with the WW domain of Nedd4 in yeast two-hybrid assays (Figure 4A). As the binding to VPS34 was unaffected, we conclude that the PY motif functions as a specific Nedd4-binding site. In line with this, Beclin 1(Y352A) proved to be more stable than the wild-type protein in CHX chase assays (Figure 4B). Furthermore, the Y352A mutation reduced the ubiquitination of Beclin 1 significantly (Figure 4C). The fact that the modification of mutated Beclin 1 was not completely blocked indicates that the point mutation does not completely inhibit the interaction to Nedd4, which would be in agreement with Figure 4(A). However, the presented data suggest that the PY motif in Beclin 1 is a functional link to Nedd4-mediated ubiquitination.

Nedd4 modifies Beclin 1 with Lys^{11}- and Lys^{63}-linked polyUb chains

The Ub moieties within a polyUb chain can be linked via different lysine residues of Ub. Different Ub linkages are believed to result in various conformations of Ub chains and are thought to create a range of molecular signals [28]. In order to define the Ub chain linkage of Beclin 1, we expressed HA-tagged versions of single lysine mutants of Ub that allow the formation of Ub chains via one lysine residue only. After co-transfecting Nedd4 with HA-tagged fusions of Ub, Ub-K0, -Lys^6-, -Lys^{11}-, -Lys^{29}-, -Lys^{48}- and -Lys^{63}-only constructs in MG132-treated cells, we performed immunoprecipitation of Myc–Beclin 1 (Figure 5A). We expected, as described previously [29], to find Ub-positive signals even in the samples of the UbK0-expressing cells. As the endogenous Ub is still present, HA–Ub is only statistically added to chains of different length. However, the incorporation of Ub forms that are incompatible with the investigated E3/substrate combination will stop chain elongation and thus result in a lower abundance of Ub chains. In the case of Ub–Beclin 1, only the Ub-Lys^{11} and Ub-Lys^{63} samples displayed Ub-positive signals similar to the wild-type Ub sample, whereas all other Ub mutants mainly suppressed the formation of Ub chains. These data suggest that Beclin 1 can be modified by Lys^{11}- and Lys^{63}-linked polyubiquitin chains. This enhanced generation of both chain types was not visible when Nedd4-2 or AIP4 instead of Nedd4 were overexpressed (results not shown), indicating that the modifications of Beclin 1 are catalysed by Nedd4.

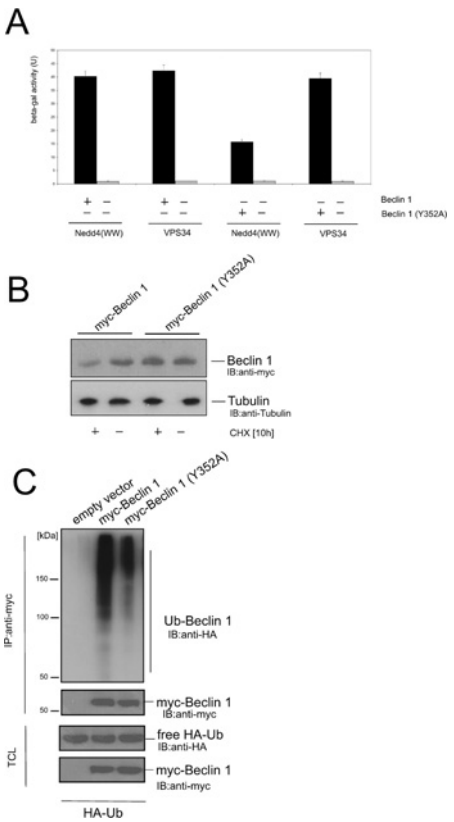

Figure 4 The mutation of the PY motif interferes with Nedd4-mediated ubiquitination

(**A**) The Y352A mutantion of Beclin 1 reduces the interaction with Nedd4, but not with VPS34, in yeast two-hybrid assays. (**B**) The Y352A mutant is more stable in CHX chase assays than wild-type Beclin 1. (**C**) HeLa cells transfected with Nedd4, Myc-tagged versions of Beclin 1 as well as HA–Ub were subjected to immunoprecipitation with anti-Myc antibodies. Analysis of the samples revealed that the Y352A mutant was less modified than the Beclin 1 with an intact PY motif. IB, immunoblot; IP, immunoprecipitation.

Degradation of Beclin 1 is mediated by Lys^{11}-linked polyUb chains

To further understand the relationship between Beclin 1, VPS34 and Nedd4-mediated ubiquitination, we analysed the VPS34-depletion-induced instability of Beclin 1 in further detail (Figure 5B). As described previously (Figure 2E), the siRNA-mediated reduction of VPS34 protein levels results in destabilization of endogenous Beclin 1. This effect was still observed when Myc–Beclin 1 was co-expressed with Nedd4 and HA–Ub fusions. Interestingly, although the level of Myc–Beclin 1 was significantly reduced, the ubiquitination of Myc–Beclin 1 was enhanced when the proteasome was inhibited simultaneously. This indicates that the decrease of Beclin 1 is accomplished by its polyubiquitination and proteasomal degradation.

In order to determine if this proteasomal turnover of Beclin 1 is mediated by Lys^{11}- or Lys^{63}-linked Ub-chains, Ub–Beclin 1 was isolated from cells expressing the indicated Ub mutants. Although Lys^{63}-linked Ub-chains could be found on Beclin 1 in the presence of VPS34 (Figure 5B), they did not mediate the enhanced ubiquitination of Beclin 1 when VPS34 was depleted and Beclin 1 was destabilized. The expression of Ub-Lys^{63} in VPS34 siRNA-treated cells reduced the formation of the ubiquitinated Beclin 1 species compared with expression of wild-type Ub. However, Ub-Lys^{63} did not rescue the breakdown of Beclin 1, as there was still enough endogenous Ub to promote degradation. In contrast, as only samples of cells expressing

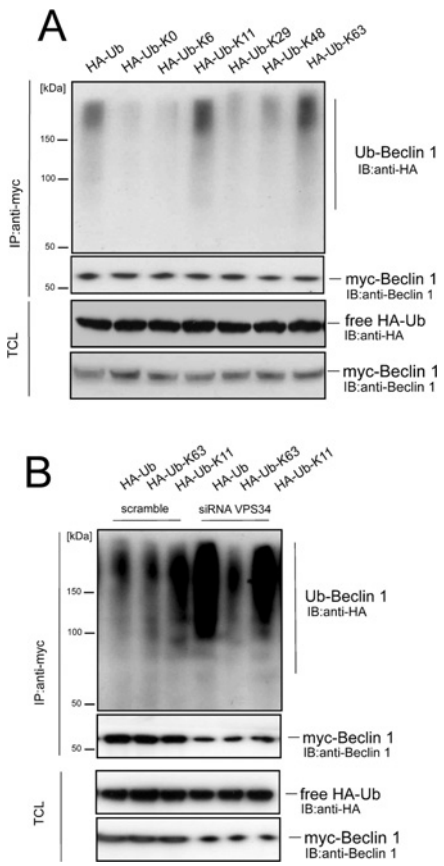

Figure 5 Beclin 1 is polyubiquitinated with Lys^{11}- and Lys^{63}-linked chains, and the former mediates its degradation

(**A**) Myc–Beclin 1 was immunoprecipitated from cells expressing Nedd4, HA–Ub or single lysine-only mutants of Ub. Samples were analysed after immunoblotting with anti-HA and -Myc antibodies. Only Ub-Lys^{11} and Ub-Lys^{63} allowed modification signals similar to the wild-type Ub. (**B**) HeLa cells were transfected with siRNA against VPS34 in order to deplete VPS34 and destabilize Beclin 1. Myc–Beclin 1 was immunoprecipitated from cells expressing Nedd4, HA–Ub, Lys^{63} only or Lys^{11} only versions after MG132-treatment. Samples were analysed with anti-HA and anti-Myc antibodies. Only Ub-Lys^{11} allowed modifications with similar intensity to the wild-type Ub. IB, immunoblot; IP, immunoprecipitation; TCL, total cell lysate.

Ub-Lys^{11} showed a ubiquitination pattern comparable with the wild-type Ub, the Lys^{11}-linked polyubiquitin chains mediated the enhanced ubiquitination of Beclin 1 induced by the depletion of VPS34. These results suggest that Nedd4 catalyses the Lys^{11}-linked polyubiquitination of Beclin 1 in order to regulate its turnover. This Lys^{11}-linked poyubiquitination and degradation are enhanced when the stabilizing interaction partner VPS34 is depleted.

DISCUSSION

Owing to the important functional role of Beclin 1 in the context of PI3K-III mediated signalling, the availability of active Beclin 1 is governed by several layers of regulation [5,6]. In the present study we present evidence suggesting a novel mode of regulation of Beclin 1, namely via Nedd4-catalysed polyubiquitination and subsequent proteasomal degradation.

We have established that Beclin 1 is a ligand for WW domains, as it contains an evolutionarily conserved PY motif and interacts in yeast two-hybrid assays with the WW domain of Nedd4, and to a lower extent with the WW motifs of Nedd4-2 and AIP4. The interaction between PY and WW domains is believed to be very specific, and the fact that Beclin 1 interacts with all three tested WW domains hints at a topic which is intensively discussed for other targets of Nedd4-like ligases as well, namely the question of substrate specificity and redundancy of ligases [30]. There are examples of proteins that bind to evolutionarily distant Nedd4 family members, such as N4WBP5, which associates with the WW domains of six different Nedd4-like proteins [31]. Additionally, it also has to be taken into account that sequences outside of the WW domain may contribute to substrate specificity [32] as well as the quality of the binding. This is indicated by the observation that the Y352A mutation of Beclin 1 does not completely abolish the interaction with Nedd4.

In summary, the data from our *in vivo* ubiquitination and stability experiments suggest that Nedd4 can be regarded as the main HECT ligase implicated in Beclin 1 ubiquitination. The reciprocity with which Nedd4 and VPS34 influence the stability of Beclin 1 is of interest. A possible molecular explanation could be derived from the situation where the ECD and the PY motif are in close proximity. This could hypothetically hint at a sterical competition of VPS34 and Nedd4 for this region of Beclin 1, suggesting that VPS34 might dynamically reduce the accessibility of the PY motif to a certain extent. Similar modes of regulation have been observed for other proteins. For instance, the PY motifs of ENaC (epithelial Na^+ channel) and ACK1 (activated Cdc42-associated tyrosine kinase 1) are recognized by N4WBP5A or WWox respectively, which in turn stabilize their interaction partner by reducing the probability of an interaction to Nedd4-like ligases [33,34].

Cross-linking studies have suggested that only 50 % of mammalian VPS34 is bound to Beclin 1, whereas the entire pool of Beclin 1 may be associated with the kinase [35]. It is conceivable that the regulation of VPS34 stability is independent of Beclin 1, whereas the stability of Beclin 1 itself depends on its major interaction partner VPS34. Thus VPS34 functions either indirectly as an assembly factor or directly as a constant binding partner in order to stabilize Beclin 1.

siRNA-mediated depletion of VPS34 results in a reduced level of Beclin 1 [19]. Our finding that Nedd4 mediates this degradation via Lys^{11}-linked polyubiquitin chains adds to the recent and relatively short literature on this type of post-translational modification. In general, Lys^{11}-linked chains are reported to function as targeting factors for proteasomal degradation [36]. The known examples are connected to cell cycle control as well as some parts of the ERAD (endoplasmic-reticulum-associated degradation) pathway [36,37]. Beclin 1 is the first known tumour suppressor shown to be targeted by this pathway, which raises the theoretical possibility of targeting Lys^{11}-linked polyubiquitination in order to maintain tumour suppressor functions.

Although we could define a functional link between Nedd4 and the Lys^{11}-linked ubiquitination of Beclin 1, which is especially evident under conditions when VPS34 is depleted and Beclin 1 is destabilized, we found that the Lys^{63}-linked chains are not required under these conditions. However, similar to the Lys^{11}-linked chains, the Lys^{63}-linked chains could be attached to Beclin 1 to participate in the regulation of the steady-state concentration of Beclin 1 when VPS34 is not destabilized. Furthermore, Lys^{63}-linked chains catalysed by Rsp5 on different substrates can serve as targeting signal for proteasomal degradation [29]. However, most of the literature on the Lys^{63}-linked chains focuses on non-proteasomal functions such as targeting to the lysosome [38]. A recent study described ubiquitination of Beclin 1 with a Lys^{63}-linked chain by the RING-type ligase TRAF6 [18]. The chain is attached to the BH3 domain of Beclin 1 in response to pro-inflammatory stimuli. This non-degradative mechanism was proposed to function as a pro-autophagic signal,

as it reduced the interaction with the autophagy inhibitor Bcl-2. Thus, although TRAF6-catalysed ubiquitination of Beclin 1 supports its function non-proteolytically, at least the Lys^{11}-linked ubiquitination catalysed by Nedd4 results in its degradation. This functional distribution is reminiscent of another target of TRAF6 and Nedd4-like ligases, the nerve growth factor receptor TrkA. Although both TRAF6 and Nedd4-2 support internalization and signalling of TrkA, TRAF6-dependent ubiquitination is supposed to function non-proteolytically, whereas Nedd4-2-mediated ubiquitination results in the degradation of this plasma membrane receptor [39,40].

The results showing Nedd4-mediated degradation of Beclin 1 in the present study point to a proteolysis-based regulation of Beclin 1. As the entire pool of Beclin 1 is not turned over rapidly, the degradation is likely to be context-dependent. For instance, this process could well represent a quality control mechanism which is significantly enhanced when the main interaction partner VPS34 is depleted. Further work will have to be performed to address the question of how the Nedd4-catalysed Ub chains are linked to the context-dependent physiological function of Beclin 1 and the PI3K-III complex during autophagy, endocytic trafficking, signalling, cytokinesis and, as a possible consequence thereof, the potential concentration-dependent positive or negative effects of Beclin 1 on tumorigenesis.

AUTHOR CONTRIBUTION

Harald Platta carried out the experimental work. Hilde Abrahamsen contributed to the *in vivo* ubiquitination assays and Sigrid Thoresen contributed to the siRNA experiments. All authors were involved in the experimental designs and data analysis. Harald Platta and Harald Stenmark wrote the manuscript.

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