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Investigation of the molecular mechanism of δ-catenin ubiquitination: Implication of β-TrCP-1 as a potential E3 ligase

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ABSTRACT

Ubiquitination, a post-translational modification, involves the covalent attachment of ubiquitin to the target protein. The ubiquitin–proteasome pathway and the endosome–lysosome pathway control the degradation of the majority of eukaryotic proteins. Our previous study illustrated that δ-catenin ubiquitination occurs in a glycogen synthase kinase-3 (GSK-3) phosphorylation-dependent manner. However, the molecular mechanism of δ-catenin ubiquitination is still unknown. Here, we show that the lysine residues required for ubiquitination are located mainly in the C-terminal portion of δ-catenin. In addition, we provide evidence that β-TrCP-1 interacts with δ-catenin and functions as an E3 ligase, mediating δ-catenin ubiquitin–proteasome degradation. Furthermore, we prove that both the ubiquitin–proteasome pathway and the lysosome degradation pathway are involved in δ-catenin degradation. Our novel findings on the mechanism of δ-catenin ubiquitination will add a new perspective to δ-catenin degradation and the effects of δ-catenin on E-cadherin involved in epithelial cell–cell adhesion, which is implicated in prostate cancer progression.

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1. Introduction

δ-Catenin or NPRAP, a member of the p120-catenin subfamily of armadillo proteins, was first identified through its interaction with presenilin-1 [1]. It is known to play a vital role in Cri-du-chat syndrome, a form of mental retardation, and in severe autism [2], and has also been found to be overexpressed in various human tumors. Several lines of evidence indicate that δ-catenin may play a pivotal role in cognitive function. For example, severe learning deficits and abnormal synaptic plasticity were found in δ-catenin-deficient mice [3]. δ-Catenin has also been implicated in the cell–cell interaction and signaling transduction in cancers including prostate [4-6], lung [7-10], ovarian [11], brain [12] and colorectal tumors [13]. Our previously published data shows that δ-catenin promotes angiogenesis through the stabilization of HIF-1α, activating VEGF in the CWR22Rv-1 prostate cancer cell line [14]. We have also demonstrated that δ-catenin promotes E-cadherin processing, resulting in the activation of β-catenin-mediated oncogenic signals [15]. In order to elucidate ways in which to downregulate and maintain the proper protein levels of δ-catenin in cells, we investigated the degradation pathways of δ-catenin, which are based on its ubiquitination.

δ- and β-catenin belong to different subfamilies of armadillo (ARM) proteins, however, these proteins may also exhibit similar signaling functions such as binding to the juxta-membranous region of E-cadherin [15]. A recent report illustrated that δ-catenin elevates total β-catenin protein levels and increases its nuclear distribution, thus activating β-catenin/LEF1-mediated and androgen receptor-mediated transcription [15]. Further, our publication illustrates that δ-catenin itself interacts with LEF-1 and may negatively regulate its transcriptional activity depending on its subcellular localization [16]. From these publications it can be suggested that δ- and β-catenin share similar binding partners in signaling pathways. As illustrated in the Wnt signaling pathway, β-TrCP-1 associates with phosphorylated β-catenin, regulating its activity in cells [17], and ubiquitinated β-catenin undergoes proteasomal degradation [18,19]. We hypothesize that δ-catenin may also be ubiquitinated and degraded via a similar pathway.

It has been reported that GSK-3 phosphorylates δ-catenin, negatively regulating the δ-catenin protein, and thus affecting its stability [20]. However, the mechanism by which the degradation takes place is not clear enough to explain the regulation of δ-catenin stability by GSK-3. Moreover, another relatively unexplored area is the mechanism by

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which δ-catenin is ubiquitinated. In the present report, we found that δ-catenin can be degraded through two pathways, the ubiquitin–proteasome pathway and the lysosome degradation pathway. In the ubiquitin–proteasome pathway, β-TrCP-1 interacted with δ-catenin as a potential E3 ligase, mediating δ-catenin ubiquitination for degradation. Furthermore, the ubiquitination sites within δ-catenin were found to be lysine residues, mainly at Lys^{1049} and Lys^{1158}. Therefore, investigation into δ-catenin ubiquitination will provide a better understanding of the effects of δ-catenin on E-cadherin involved in epithelial cell–cell adhesion, which is implicated in cancer progression.

2. Materials and methods

2.1. Materials

2.1.1. Plasmids

Preparation of the constructs for N-terminally GFP-tagged full length δ-catenin (FL), T1078A δ-catenin, δ-catenin (△N85–325) in pEGFP-C1 have been previously described [21,22]. The deletion constructs of δ-catenin (1–1070, 1–690, 691–1040, Δ1040–1070, and Δ1070–1140) and the mutant constructs of δ-catenin, in which lysine residue(s) are substituted to arginine, were generated by a PCR-based EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, Korea). All constructs were confirmed by sequencing. The C-terminally GFP-tagged δ-catenin (N3) and the untagged δ-catenin are in the pEGFP-C1 and pEBG vector, respectively. The cytomegalovirus promoter-derived mammalian expression vector (pCS4-3Myc), separately containing β-TrCP-1, β-TrCP-2, Smurf-2, and Fbw-7, was provided by Professor Kwang Youl Lee (Chonnam National University). The HA-tagged GSK-3β wild type (hereafter referred to as GSK-3β WT) was kindly donated by Kang-Yeol Choi (Yonsei University, Seoul, Korea). We generated a HA-tagged GSK-3β wild type construct (hereafter also referred to as GSK-3β WT) in the cytomegalovirus promoter-derived mammalian expression vector (pCS4-3HA) by PCR amplification.

2.1.2. Antibodies

The antibodies were purchased from commercial companies as follows: anti-δ-catenin (BD Bioscience and Upstate Biotechnology, #611537), anti-GFP (Clontech, Abcam, and Sigma), anti-β-catenin (Sigma, #A5441), anti-β-actin (Santa Cruz Biotechnology and Sigma), anti-Myc (Cell signaling, #2276), anti-GSK3α/β (Cell signaling, #5676) and anti-phospho-GSK3α/β (Cell signaling, #9331S). The HA epitope, which was obtained using media from 12CA5 hybridoma cells, was provided by Professor Lee (Chonnam National University).

3. Methods

3.1. Cell culture and transfection

The Bosc23 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. CWR22Rv-1 (human prostate cancer cell line) cells were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Bosc23 cells were transfected using calcium phosphate or PEI (polyethylenimine), while CWR22Rv-1 cells were transfected with Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

3.2. ALLN, chloroquine, and cycloheximide treatment

3.2.1. ALLN proteasome inhibitor treatment

In order to detect the ubiquitinated δ-catenin, blocking the degradation of δ-catenin using a proteasome inhibitor is a common method. The peptide ALLN (N-Ac-Leu-Leu-Nile-CHO or calpain inhibitor I), from Life Science (#1834-5), inhibits proteasome-mediated proteolysis, which can lead to an accumulation of proteins that are usually degraded via this pathway [23]. The ALLN peptide was dissolved in DMSO to a stock concentration of 2 μM and kept at −20 °C until use. The transfected cells were treated with 10 μM (diluted in warm media followed by gentle mixing) for different periods of time as indicated. Following the treatment, the cells were harvested at the same time.

3.2.2. Chloroquine lysosome inhibitor treatment

To further study the degradation pathway of δ-catenin, lysosome inhibitor (Chloroquine) treatment was performed to inhibit the lysosome degradation pathway. Chloroquine powder (Sigma, #C6628-25G) was dissolved in distilled water at a stock concentration of 50 mg/ml and kept at −20 °C. Prior to treatment, the stock was diluted with media, followed by gentle mixing, to a working concentration of 50 μg/ml, and the cells were treated for different time periods. All transfected cells were cultured for 24 h and then harvested with lysis buffer.

3.3. Immunoblotting and immunoprecipitation

For immunoblotting, transfected cells were harvested with lysis buffer (MLB: 10% glycerol, 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). The protein concentration of the cell lysates for each sample was quantified using a BCA assay kit (Pierce, Rockford, IL). Equal amounts of protein sample (10 or 20 μg) were solubilized and boiled at 95 °C with SDS sample buffer (0.1 M Tris–HCl, pH 6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1.43 M β-mercaptoethanol) for 2 min, and then loaded onto an appropriate Tris-Glycine gel and subjected to electrophoresis. Proteins were separated by molecular weight and transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Millipore, Billeria, MA), which was activated with methanol before use. The membrane was cut according to the target proteins' molecular weights and reactivated with methanol. The cut membranes were blocked with 5% skimmed milk followed by probing with primary antibodies as indicated. Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies, followed by visualization with enhanced chemiluminescence (ECL) western blotting detection reagents (Millipore, Billerica, MA) and detection by LAS-4000 (Fujifilm).

Immunoprecipitation was performed using lysates from transfected cells. Cells were lysed in MLB lysis buffer as described above. Lysates were incubated with primary antibodies (applied amount was dependent on the data sheet) for 16 h at 4 °C and pulled down with protein G sepharose (GE Healthcare, Uppsala, Sweden). The immunoprecipitated proteins were denatured at 95 °C for 2 min with 15 ml 2× sample buffer (0.1 M Tris–HCl, pH 6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1.43 M β-mercaptoethanol) and analyzed by western blotting as described above. All the transfected cells were harvested 36 h after transfection. 20 μg of each lysate was subjected to immunoblotting, while 1 g was used for immunoprecipitation.

3.4. LC/MS/MS analysis

The captured proteins were released with the elution buffer (0.1 M glycine·HCl at pH 2.5), and each eluted fraction was neutralized with 0.5 M Tris, pH 9.5. Eluted proteins in dried fractions were denaturated at 90 °C for 10 min in a 50 mM ammonium bicarbonate (ABC) buffer. After denaturation, the samples were reduced with 10 mM dithiothreitol (DTT) and alkylated with 100 mM iodoacetamide for 20 min each. Trypsin was added and incubated at 37 °C for 12 h.

The tryptic peptides were analyzed by nano-UPLC (Waters, Manchester, UK) and tandem mass spectrometry using Q-Tof Premier (Waters). The peptides were injected into a 2 cm × 180 μm trap column and separated by a 25 cm × 75 μm nanoACQUITY™ C18 column (Waters) using the LC system. Mobile phase A was composed of water with 0.1% formic acid (FA) and mobile B phase was composed of 0.1% FA in

acetonitrile. The peptides were separated with a gradient of 3–45% mobile phase B over 30 min at a flow rate of 300 nl/min.

MS/MS data were converted into peak lists using Distiller (Matrix Science, London, UK;version 2.0). All MS/MS samples were analyzed using Mascot (Matrix Science; version 2.3). Mascot was set up to search the UniProtKB/Swiss-Prot protein knowledgebase release 2015_05 for ubiquitinated peptides, assuming trypsin as the digestion enzyme with a parent ion mass tolerance of 0.1 Da and a fragment ion tolerance of 0.05 Da. Three missed cleavages were allowed during trypsin digestion. To detect ubiquitinated peptides, a residual GG tag on the modified lysine was selected as a fixed modification and oxidation (Met), N-terminal acetylation and carbamidomethylation (Cys) were selected as variable modifications.

4. Results

4.1. The ubiquitination sites are located in the C-terminus of δ-catenin

It is known that phosphorylation is the most common degron that provides the substrate for ubiquitination. Our previous study showed that glycogen-synthase kinase-3 (GSK-3) negatively affects the stability of δ-catenin via ubiquitin–proteasome proteolysis in fibroblasts, suggesting that δ-catenin ubiquitination occurs in a GSK-3 phosphorylation-dependent manner [20]. However, the molecular mechanism underlying this ubiquitination was not clear enough to explain the regulation of δ-catenin degradation by GSK-3. Therefore, we characterized the ubiquitin lysine sites in δ-catenin. Sequence analysis revealed that there are 55 lysine residues in δ-catenin (Table 1). Firstly, we narrowed down the ubiquitination region of δ-catenin using N-terminal deletions (δ-catenin △N85–325; δ-catenin 691–1040) and C-terminal deletions (δ-catenin 1–1070; δ-catenin 1–1040; δ-catenin 1–690). We found that δ-catenin △N85–325 and 1–1070 can be significantly ubiquitinated, as shown by one major band in immunoblotting (Fig. 1A) and immunoprecipitation (Fig. 1B). However, the ubiquitinated δ-catenin band could not be detected with the other two deletions, δ-catenin 1–690 and 691–1040 (Fig. 1C), meaning that δ-catenin 1–1040 could not be ubiquitinated. To confirm this observation, the ubiquitination of δ-catenin 1–1040 was also attempted, and shown not to be possible (data not shown). Taken together, the ubiquitination sites were shown to be located in δ-catenin 1040–1070. Moreover, there is another hypothesis that more ubiquitination sites remain in δ-catenin 1070–1247.

To test the two possibilities, we counted the number of lysine residues in the different δ-catenin constructs and analyzed the positions of these lysine residues at the C-terminus of δ-catenin (residues 1040–1247) by the Gene Runner program (Table 1). Sequence analysis revealed two putative lysine residues in 1040–1070, Lys^{1049} and Lys^{1050}. To highlight the ubiquitination sites in δ-catenin 1040–1070, we firstly made point mutations of full length (FL) δ-catenin. The single mutant (δ-catenin FL K1050R) was a substitution of a lysine with an arginine, while the double point mutant (δ-catenin FL KK1049/1050RR) contained both substitutions as shown in the schematic diagram (Fig. 2A). We then examined whether the two mutants could be ubiquitinated. The results illustrate that the single point mutant did not block δ-catenin ubiquitination (Fig. 2B), while the double point mutant slightly inhibited δ-catenin ubiquitination (Fig. 2C). Therefore, we can conclude that the effects of these two point mutants on the ubiquitination of δ-catenin differ, implying that Lys^{1049} is among the multi-ubiquitination sites within δ-catenin, and that there are more ubiquitin lysine residues located in δ-catenin 1070–1247.

In order to elucidate the remaining ubiquitin lysine residues in δ-catenin 1070-1247, point mutations were carried out on δ-catenin deletions (δ-catenin 1–1140 and δ-catenin 1–1200), with substitutions of Lys^{1049} and Lys^{1050} with arginine, indicated as δ-catenin 1–1140 (KR) and 1–1200 (KR) in the schematic diagram (Fig. 2A). It was also investigated whether these two new deletion mutants could be targeted for ubiquitination. The results show that δ-catenin 1–1140 (KR) and 1–1200 (KR) could be significantly ubiquitinated (Fig. 2D). To confirm that the ubiquitination sites of δ-catenin were within δ-catenin 1040–1070, we deleted this whole region and named the construct δ-catenin Δ1040–1070. However, data indicate that δ-catenin Δ1040–1070 could also be slightly ubiquitinated (Fig. 2E). Thus, it is clear that there are further ubiquitin lysine residues other than Lys^{1049}.

Due to the involvement of the three C-terminal segments of δ-catenin 1070–1247 (δ-catenin 1070–1140, 1140–1200, 1200–1247; Table 1) in ubiquitination, δ-catenin 1070–1140 was initially focused on. There are 4 lysine residues in this area (Table 1). To facilitate the investigation, we deleted the entire 1070–1140 and point mutated the double lysine residues (KK1049/1050RR) on δ-catenin FL and δ-catenin 1–1200, named δ-catenin FLΔ1070–1140 (KR) and δ-catenin 1–1200Δ1070–1140 (KR), respectively (Fig. 2A). These deletion mutations were used in the following experiments, and the results indicate that δ-catenin FLΔ1070–1140 (KR) and 1–1200Δ1070–1140 (KR) could also be ubiquitinated (Fig. 2F and G), suggesting that δ-catenin 1070–1140 may not contain the major ubiquitination sites and that there are further ubiquitin lysine residues contained in δ-catenin 1140–1200 and/or in δ-catenin 1200–1247. We can conclude that the ubiquitination sites are located in the C-terminal portion of δ-catenin.

In order to map more accurate ubiquitination sites in δ-catenin in cells, we performed LC/MS/MS analysis. To identify ubiquitination sites, ubiquitinated protein was digested with trypsin. Two ubiquitinated sites were identified by mass shifts of modified lysine residue. ESI-MS/MS spectrum of one ubiquitinated peptide (1046–1067, SLYKKDGWSQ YHFVASSSTIER) was detected at 901.7731 m/z (mass to charge ratio), showing modified lysine residue (Fig. 3A). The other ubiquitinated peptide (1148–1173, NSYGAPAEDIKQNQVSTQPVPQEPSR) was detected at 985.4735 m/z (triple charge, Fig. 3B top panel), and 1477.7102 m/z (double charge, Fig. 3B bottom panel). Taken together, our LC/MS/MS analysis data strongly suggest that Lys1049 and Lys1158 are two major ubiquitination sites in δ-catenin. In order to confirm this, we constructed δ-catenin mutant having both deleted the entire 1070-1140 and point mutated the 3 lysine residues (KKK1049/1050/1158RRR) and tested its ubiquitination level. As shown in Fig. 3C, the δ-catenin mutant showed noticeably decreased but still detectable levels of ubiquitinated δ-catenin. However, unexpectedly, δ-catenin mutant having both deleted the entire 1070-1140 and point mutated the 5 lysine residues (KKKKK1049/1050/1158/1174/1214RRRRR) did not abolish its ubiquitination as shown in Fig. 3D. Therefore, we cannot rule the possibility that δ-catenin may contain additional minor ubiquitination site(s), other than its major Lys^{1049} and Lys^{1158} ubiquitination sites.

Table 1
Number of lysine residues in different δ-catenin constructs and in the region between 1040–1247 of δ-catenin.

δ-Catenin constructs or δ-catenin region	Number of lysine residues[a]	Position	
δ-Catenin full length (1–1247)	55		
δ-Catenin (△85–325)	52		
δ-Catenin (1–1070)	48	–	
δ-Catenin (1–1040)	46		
δ-Catenin (1–690)	21		
δ-Catenin (691–1040)	25		
1040–1070 of δ-catenin	2	Lys^{1049}	Lys^{1050}
1070–1140 of δ-catenin	4	Lys^{1106} Lys^{1125}	Lys^{1109} Lys^{1132}
1140–1200 of δ-catenin	2	Lys^{1158}	Lys^{1174}
1200–1247 of δ-catenin	1	Lys^{1214}	

[a] The lysine residues in δ-catenin are based on the mouse δ-catenin sequence.

4.2. β-TrCP-1 interacts with δ-catenin and functions as an E3 ubiquitin ligase, facilitating δ-catenin degradation via the ubiquitin–proteasome pathway

In order to identify potential E3 ubiquitin ligases for δ-catenin, we examined the effects of two types of E3 ligases on δ-catenin protein

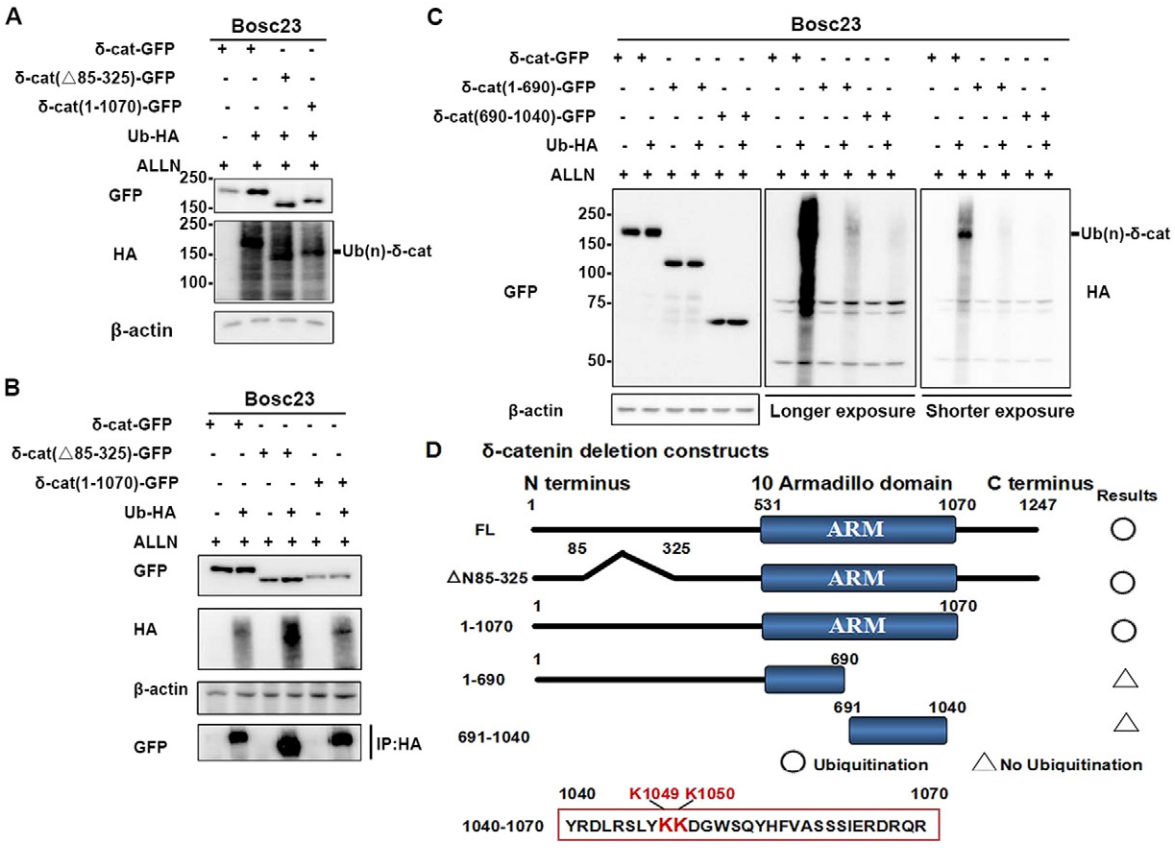

Fig. 1. The ubiquitination sites are located in δ-catenin 1040–1070. (A–B) Bosc23 cells were transfected with the indicated GFP-tagged deletions and treated with the proteasome inhibitor ALLN (10 μM) 4 h before harvesting. Cell lysates were subjected to immunoblotting (IB) and immunoprecipitation (IP) with the indicated antibodies. Anti-GFP was used to detect δ-catenin full length and the deletions. (C) Bosc23 cells were transfected with the indicated plasmids. Transfected cells were treated with ALLN for 4 h prior to lysis. Cell lysates were subjected to IB with an anti-GFP antibody. β-actin was used as a loading control. (D) The schematic representation shows the different δ-catenin deletions, and the sequence of δ-catenin 1040–1070 is shown in the bottom red frame.

levels. One type is the HECT domain E3 ligase family (including Smurf2), and the other type is the RING finger E3 ligase family that includes β-TrCP-1, β-TrCP-2, and Fbw-7. All the E3 ligases tested in this study have been proven to downregulate β-catenin protein levels, thus β-catenin was used as a positive control (Fig. 4A, middle panel). We investigated the ability of these E3 ligases to modulate δ-catenin, and found that the protein levels of δ-catenin and β-catenin were reduced dramatically by β-TrCP-1 (Fig. 4A, the upper panel). It is well-known that β-TrCP-1 is a specific E3 ligase of β-catenin, mediating ubiquitin–proteasome degradation in the Wnt signaling pathway. Therefore, further detailed study was conducted to investigate the function of β-TrCP-1 and how it affects δ-catenin. In Bosc23 cells, overexpressed β-TrCP-1 significantly reduced the protein levels of total δ-catenin and ubiquitinated δ-catenin in the absence of ALLN [Fig. 4B (i)], while the downregulation of total δ-catenin and ubiquitinated δ-catenin by β-TrCP-1 were abolished in the presence of the proteasome inhibitor ALLN, as shown in the schematic representation [Fig. 4B (ii) and 4D (ii)], suggesting that β-TrCP-1 downregulates δ-catenin through the ubiquitin–proteasome-mediated degradation pathway. To confirm this conclusion, we overexpressed β-TrCP-1 in an increasing manner in Bosc23 cells. The results show a similar pattern that the levels of total δ-catenin and ubiquitinated δ-catenin were downregulated without ALLN treatment, while these were increasingly upregulated with ALLN treatment, with increasing expression of β-TrCP-1 [Fig. 4D (i)]. In addition, δ-catenin protein levels were dramatically decreased by β-TrCP-1 upon co-transfection with ubiquitin [Fig. 4B (i) and 4D (i); upper panel]. The pattern can be clearly seen in the figures [Fig. 4B (ii) and D (ii)], suggesting that abundant ubiquitin is necessary for δ-catenin degradation through β-TrCP-1. Above all, we conclude that β-TrCP-1 downregulates δ-catenin via the ubiquitin–proteasome degradation pathway.

Moreover, the results shown in Fig. 4 suggest the possibility that β-TrCP-1 is a potential E3 ligase for δ-catenin. To affirm this possibility, we examined the interaction between δ-catenin and β-TrCP-1. EGFR and β-catenin were used as positive controls, as EGFR interacts with δ-catenin and β-catenin binds to β-TrCP-1. The results of IP and reverse IP show that δ-catenin was co-immunoprecipitated with β-TrCP-1 (Fig. 5A and B). Together, β-TrCP-1 interacts with δ-catenin and functions as an E3 ubiquitin ligase, facilitating δ-catenin degradation via the ubiquitin–proteasome pathway. To prove GSK3 dependent β-TrCP-1 interaction with δ-catenin, we tested the interaction between β-TrCP-1 and δ-catenin mutant (Thr1078 to Ala) where its major GSK-3 phosphorylation site was point-mutated. As we expected, the interaction of δ-catenin mutant (T1078A) with β-TrCP-1 was significantly weaker than that of wild type δ-catenin. Along the same line, we have also observed that the ubiquitination of δ-catenin mutant (T1078A) by β-TrCP-1 was significantly weaker than that of wild type δ-catenin (Fig. 5C).

4.3. δ-Catenin can take part in both the ubiquitin–proteasome pathway and the lysosome degradation pathway

Several reports have illustrated that different types of ubiquitination lead substrates to different degradation pathways [24,25]. Thus, further study regarding the degradation pathway of δ-catenin was conducted with the lysosome inhibitor chloroquine (CQ), which is known to prevent EGFR from lysosomal degradation. Therefore endogenous EGFR in CWR22RV-1 cells was detected as a positive control. We treated RV/C (CWR22RV-1/C: stable GFP-expressing prostate cancer cell; as a

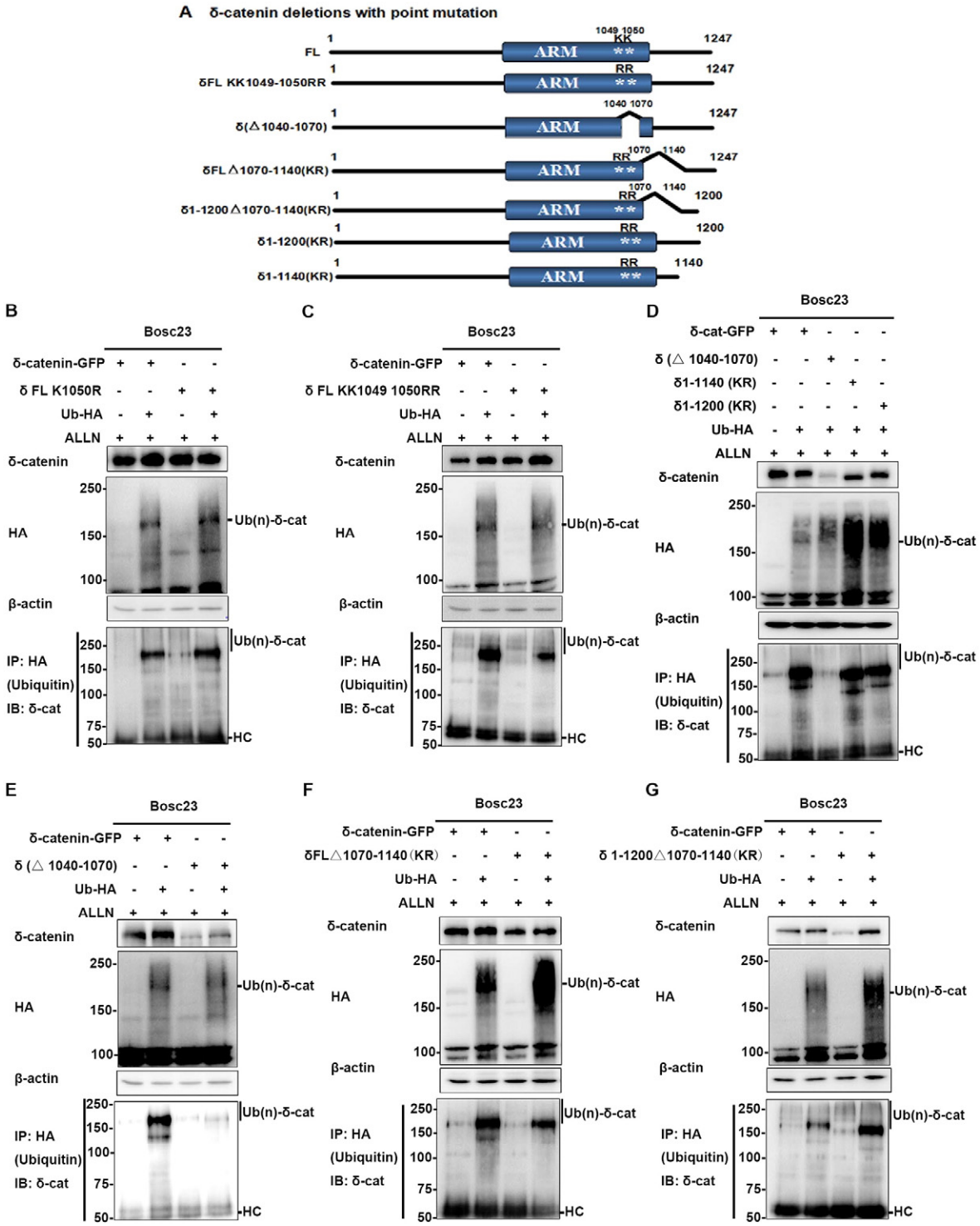

Fig. 2. More ubiquitination sites are located in δ-catenin 1070–1247. (A) The schematic representation of the double point mutant (δ-catenin FL KK1049 1050RR) and deletion constructs of δ-catenin: δ-catenin Δ1040–1070, δ-catenin FL Δ1070–1140 (KR) and δ-catenin 1–1200 Δ1070–1140 (KR), δ-catenin 1–1200 (KR), δ-catenin 1–1140 (KR). (B–G) Bosc23 cells were transfected with the indicated plasmids of δ-catenin constructs and the cell lysates were subjected to immunoprecipitation with anti-HA and then followed by immunoblotting with anti-δ-catenin. Heavy chain (HC) was indicated in figures (the bottom panel).

negative control) and RV/δ (CWR22RV/δ: stable δ-catenin-expressing prostate cancer cell) cells with increasing concentrations of chloroquine to investigate whether a lysosome inhibitor can prevent the degradation of δ-catenin. The results indicate that the lysosome inhibitor worked well, enhancing both EGFR and δ-catenin protein levels (Fig. 6A). Furthermore, Bosc23 cells were transiently transfected with δ-catenin and then treated with CQ at the indicated concentrations. The results show that δ-catenin protein levels were increased in a concentration-dependent manner [Fig. 6B (i)]. δ-catenin-expressing cells were treated with chloroquine for different periods of time. Dramatically increasing levels of δ-catenin were detected [Fig. 6B (ii)], thus we can conclude that the lysosome inhibitor blocked δ-catenin degradation. To elucidate the manner in which the lysosome inhibitor affects δ-catenin degradation, we investigated the ubiquitination of

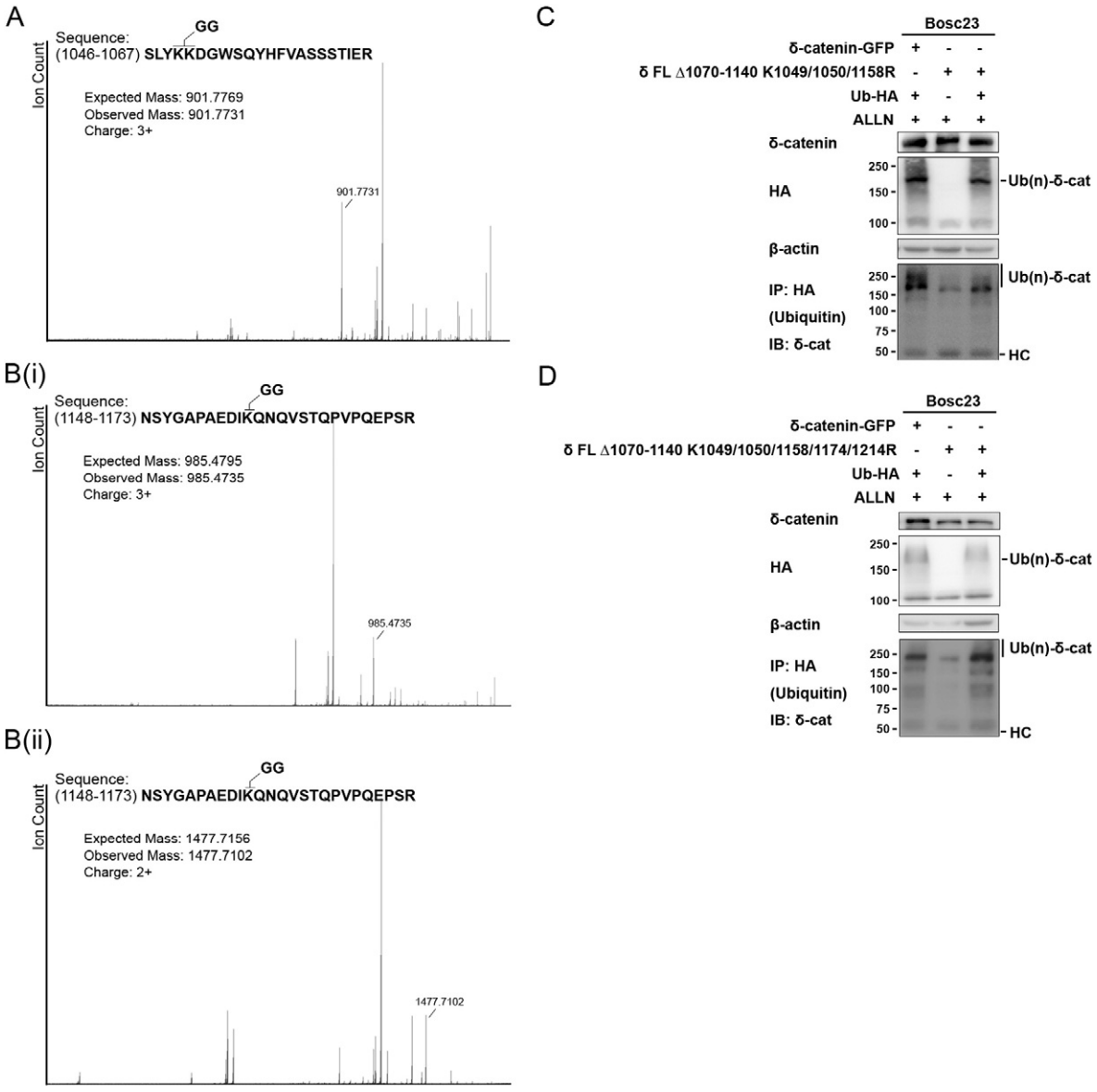

Fig. 3. Ubiquitination of δ-catenin at Lys[1158] and Lys[1049] or Lys[1050] residues are identified by LC/MS/MS analysis. (A and B) LC/MS/MS analysis of ubiquitinated δ-catenin peptide. For the analysis, protein was prepared by immunoprecipitation with anti-δ-catenin Ab for the lysates from Bosc23 cells which was transfected with δ-catenin plasmids and treated with ALLN. (C and D) Bosc23 cells were transfected with the indicated plasmids of δ-catenin constructs, and the cell lysates were subjected to immunoprecipitation with anti-HA and followed by immunoblotting with anti-δ-catenin Ab.

δ-catenin with and without CQ treatment. As seen in Fig. 6B and C, ubiquitination of δ-catenin was induced in response to the lysosome inhibitor; therefore, ubiquitinated δ-catenin also undergoes lysosomal degradation. Previous results have indicated that β-TrCP-1 downregulates δ-catenin via the ubiquitin–proteasome degradation pathway, which strongly suggests that δ-catenin ubiquitination can take place via two degradation pathways. Thus, there are two main questions to be asked through the investigation into the mechanism of δ-catenin ubiquitination; (1) which of the two is the major pathway mediating δ-catenin degradation? and (2) can β-TrCP-1 also induce δ-catenin via lysosomal degradation? We compared ubiquitinated δ-catenin treated with ALLN and CQ. Ubiquitinated δ-catenin in the presence of a proteasome inhibitor was slightly increased compared with that in the presence of a lysosome inhibitor and further increased by co-treatment with a lysosome inhibitor (Fig. 6D). However, in contrast to β-TrCP-1-mediated downregulation of δ-catenin in ALLN treatment, β-TrCP-1 did not significantly enhance the ubiquitination of δ-catenin when there was chloroquine treatment as shown in Fig. 6E and F. Therefore, the degradation of δ-catenin is regulated by both the ubiquitin–proteasome pathway and the lysosome degradation pathway, and β-TrCP-1 mainly leads to its proteasome degradation pathway.

δ-Catenin is a family member of the ARM repeat proteins, which binds to E-cadherin. In the process of ubiquitination, δ-catenin is firstly phosphorylated by GSK-3β mainly at Thr[1078], and the phosphorylated δ-catenin can then be ubiquitinated at the C-terminus via interaction with β-TrCP-1, an E3 ubiquitin ligase. Ubiquitinated δ-catenin undergoes degradation via two pathways, the ubiquitin–proteasome pathway and the lysosome degradation pathway. Among these, β-TrCP-1 mainly leads to the proteasome degradation pathway.

5. Discussion

In the present study, we investigated the molecular mechanism of δ-catenin ubiquitination. Firstly, we found that the lysine residues required for ubiquitination are located mainly at the C-terminus of δ-catenin. In addition, we provide evidence that β-TrCP-1 interacts

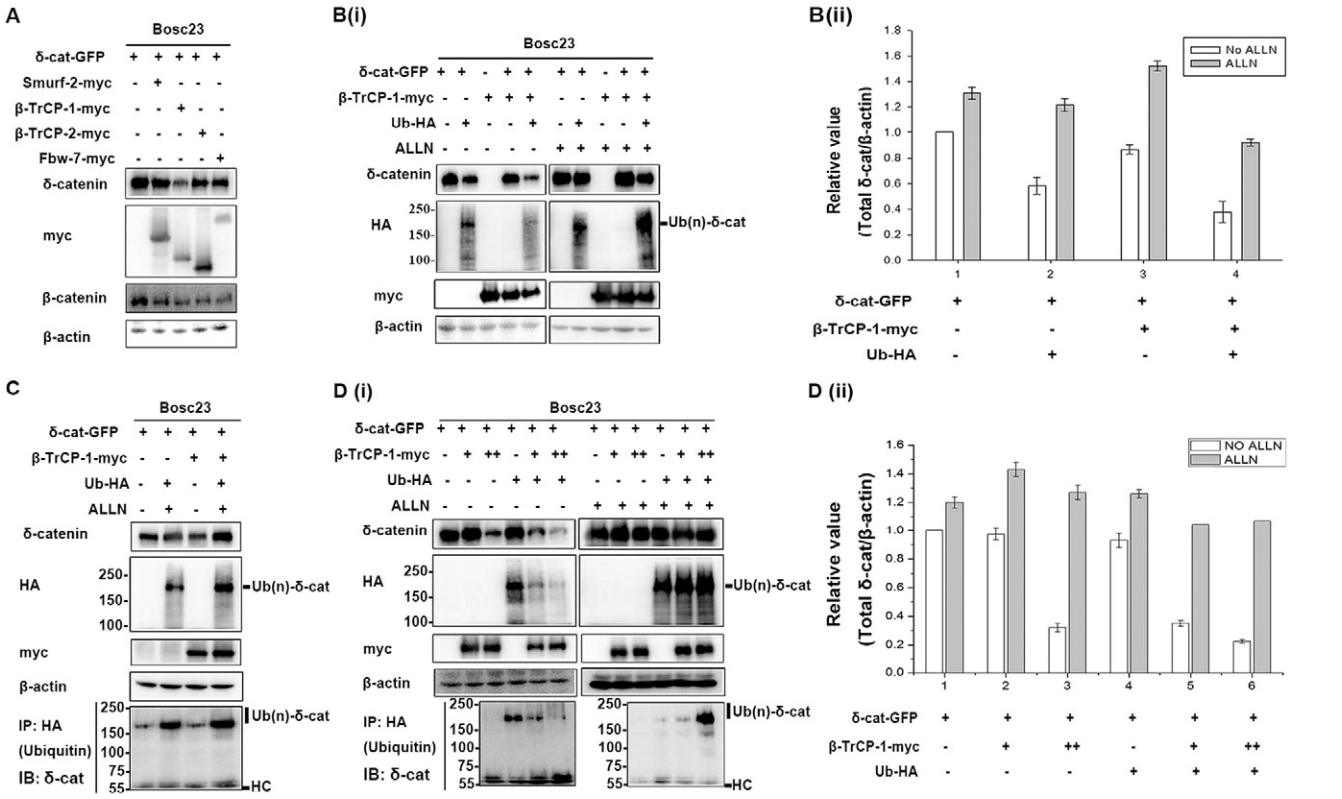

Fig. 4. β-TrCP-1 downregulates δ-catenin and facilitates its ubiquitination.(A) Bosc23 cells were transfected with Myc-tagged E3 ligases (Smurf-2-myc, β-TrCP-1-myc, β-TrCP-2-myc, Fbw-7-myc) and δ-catenin-GFP. Immunoblotting was performed with different antibodies. (B) Bosc23 cells were transfected with β-TrCP-1-myc, Ub-HA, and δ-catenin-GFP. Cell lysates were subjected to immunoblotting with the indicated antibodies. Schematic representation shows the total δ-catenin protein levels in the presence and absence of ALLN (panel ii). (C) Bosc23 cells were transfected with the indicated plasmids. Cell lysates were subjected to IP with anti-HA, followed by IB with anti-δ-catenin. (D) Bosc23 cells were transfected with increasing concentrations of β-TrCP-1 and other plasmids as indicated, and the cell lysates were then evaluated by IP with anti-HA, followed by IB with anti-δ-catenin. Heavy chain (HC) is also shown, and β-actin was used as a loading control.

with δ-catenin and functions as an E3 ubiquitin ligase, facilitating δ-catenin ubiquitination. Furthermore, we demonstrate that ubiquitinated δ-catenin can undergo degradation via two pathways, the ubiquitin–proteasome pathway and the lysosome degradation pathway (Fig. 7).

It is known that p120-ctn and β-catenin are the two major proteins in the armadillo (ARM) repeat protein family [1]. δ-Catenin is a member of p120-catenin subfamily. In addition, δ-catenin, p120-catenin, and β-catenin have been proven to share many characteristics such as the binding partners, E-cadherin and PS-1. Several reports have proven that β-catenin is earmarked for Lys^{48}-linked polyubiquitination and proteasomal degradation [18,24,26]. Our previous publication reported that glycogen-synthase kinase-3 (GSK-3) phosphorylates δ-catenin and negatively regulates δ-catenin protein levels via polyubiquitination–proteasome degradation [20]. Here, we provide evidence that β-TrCP-1, an E3 ligase, interacts with δ-catenin and mediates ubiquitin–proteasome degradation (Figs. 4 and 5), suggesting that δ-catenin shares a similar degradation pathway with that of β-catenin. However, the higher molecular weight δ-catenin was not as obvious as with endogenous β-catenin. One possible explanation for the weakly detected higher molecular weight δ-catenin is that the ubiquitination machinery becomes less efficient when δ-catenin is overexpressed. Furthermore, our results demonstrate that δ-catenin can also undergo lysosomal degradation (Fig. 6), which implies that two degradation pathways are involved. Therefore, there is one interesting question related to the machinery involved in δ-catenin degradation; which is the major pathway mediating δ-catenin degradation? Meanwhile, the degradation pathway is also related to the ubiquitination sites and the type of δ-catenin ubiquitination. Thus, in order to elucidate the major degradation pathway, we also investigated the lysine residues of δ-catenin that are involved in ubiquitination.

The ubiquitination region of δ-catenin was narrowed down using different point mutants and deletions (Fig. 2A). The results suggest that the C-terminus of δ-catenin is essential for ubiquitination, δ-catenin 1040–1247. Interestingly, our LC/MS/MS analysis data also strongly suggest that Lys^{1049} and Lys^{1158} are two major ubiquitination sites in δ-catenin. It is well-known that the members of the ARM repeat family (including δ-catenin, β-catenin, and p120-catenin) are the substrates for GSK-3β, and the consensus motif for GSK-3β-phosphorylation is S/TxxxS/T (in single-letter amino-acid code, where x is any amino acid). It has been reported that the GSK-3β consensus phosphorylation site in β-catenin is necessary for ubiquitination. Meanwhile, the ubiquitination machinery and GSK-3β use essentially the same sites in β-catenin. Moreover, both β-TrCP-1 binding sites for ubiquitination and CK-1/GSK-3β phosphorylation sites of β-catenin have been reported to be located in the N-terminus [27]. Our previous data has illustrated that GSK-3 phosphorylates δ-catenin, and the Thr^{1078} residue in δ-catenin is one of the multiple phosphorylation sites used by GSK-3 [20], which differs from β-catenin. In addition, despite the fact that the ubiquitination sites of p120-ctn are unknown, the phosphorylation residues used by GSK-3 have been proven to be Ser^{252} and Thr^{310} [28], which is also different from that of δ-catenin and β-catenin. Moreover, it is known that phosphorylation is the most common degron that triggers the ubiquitination of substrates. All evidence implies that the ubiquitination and phosphorylation regions of the ARM repeat family members are different; however, the ubiquitination sites are located in close proximity to the phosphorylation sites contained in the GSK-3β consensus motif. That is, these family members are firstly phosphorylated by GSK-3β, and the phosphorylated substrates are then ubiquitinated at lysine residues that are closest to the phosphorylation sites. Even though both our

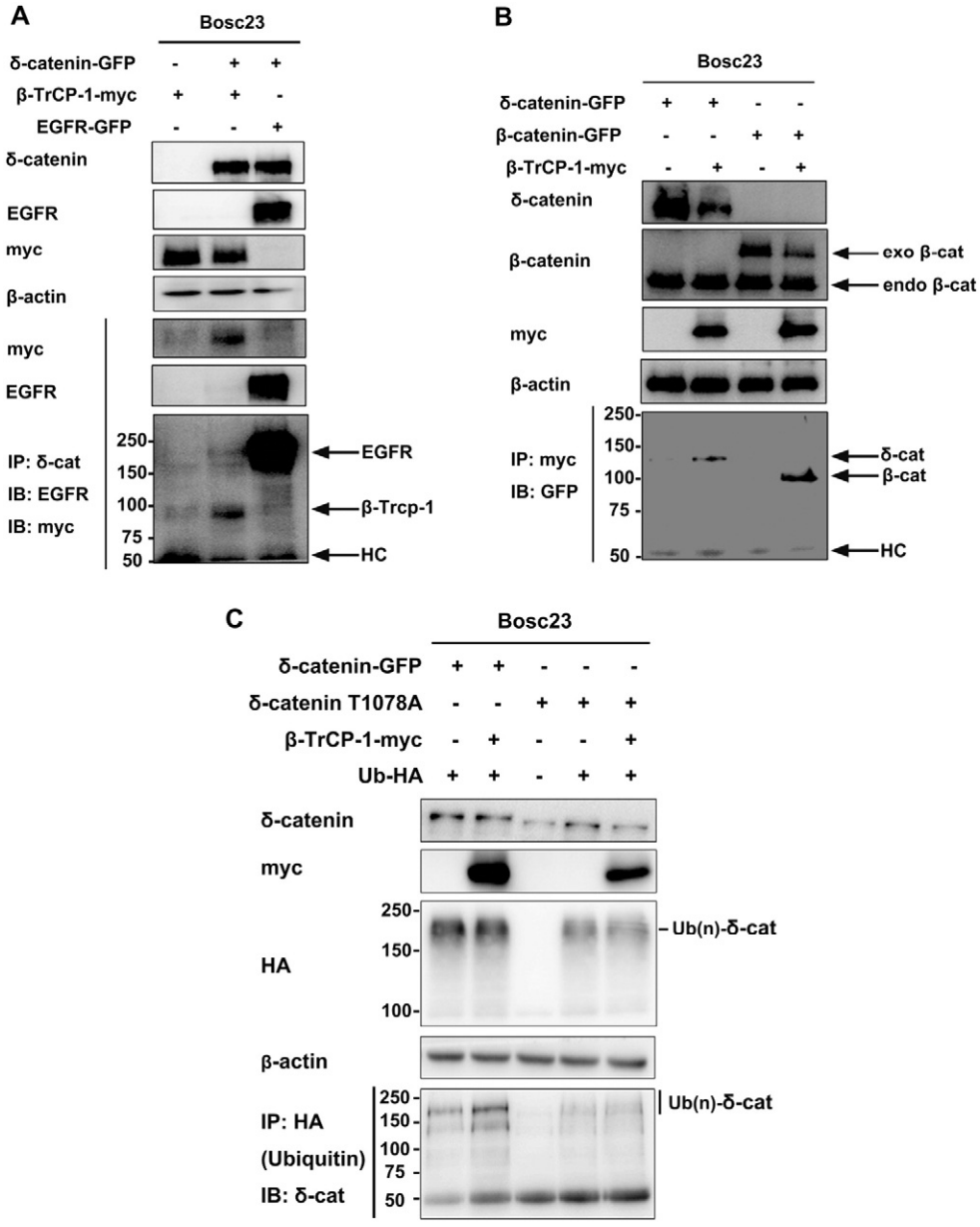

Fig. 5. β-TrCP-1 interacts with δ-catenin. (A) Bosc23 cells were transfected with δ-catenin-GFP, β-TrCP-1-Myc, and EGFR-GFP. Cell lysates were used for IP with anti-δ-catenin, followed by IB with the indicated antibodies. δ-catenin, EGFR, β-TrCP-1, and heavy chain (HC) detected by IP are indicated by arrows (bottom panel). (B) Bosc23 cells were transfected with δ-catenin-GFP, β-TrCP-1-Myc, and β-catenin-GFP. Cell lysates were subjected to IP with a Myc antibody, followed by IB with an anti-GFP antibody, which can detect GFP-tagged δ-catenin and β-catenin (arrows). β-actin was used as a loading control. (C) Bosc23 cells were transfected with indicated plasmids and the cell lysates were subjected to immunoprecipitation with anti-HA and then followed by immunoblotting with anti-δ-catenin Ab.

point mutants and LC/MS/MS analysis data strongly suggest that both Lys^{1049} and Lys^{1158} are two major ubiquitination sites on δ-catenin, unfortunately, δ-catenin mutant having both deleted the entire 1070–1140 and point mutated the 3 lysine residues (KKK1049/1050/1158RRR) showed noticeably decreased but still detectable levels of ubiquitinated δ-catenin (Fig. 3C), ruling out the possibility that δ-catenin contains only two ubiquitination sites, Lys^{1049} and Lys^{1158}. Moreover, δ-catenin mutant having both deleted the entire 1070-1140 and point mutated the 5 lysine residues (KKKKK1049/1050/1158/1174/1214RRRRR) did not abolish its ubiquitination (Fig. 3D). The first possibility for our inconsistent data among δ-catenin constructs is that δ-catenin construct which deletes 4 lysines between 1070 and 1140 and point mutates 5 lysines (KKKKK 1049/1050/1158/1174/1214RRRRR) may activate alternative ubiquitination site(s) which may be not or less happened under normal surroundings. The second possibility for this discrepancy is due to their conformational change in deletion constructs. The homology modeling of δ-catenin based on the structure of p120 catenin suggests that the residues 1040–1060 are predicted to compose the C-terminal helix cap of ARM domain in δ-catenin. Therefore, our both 691–1040 and 1–1040 constructs may have unstable folding of ARM domain and form partial aggregations of the expressed proteins in the cell, which might interfere with the efficient ubiquitination of the constructs. If this is the case, we may underestimate the ubiquitination of δ-catenin at N-terminal of 1040. However, of note is that our LC/MS/MS analysis with full-lengh wild-type δ-catenin revealed ubiquitination of δ-catenin at Lys^{1049}/Lys^{1050} and Lys^{1158}. Together with deletion and point mutation data, these strongly suggest that both Lys^{1049} and Lys^{1158} are indeed major ubiquitination sites of δ-catenin. However, as we currently cannot rule out above mentioned possibilities, we should draw our conclusion as δ-catenin contains two major ubiquitination sites at Lys^{1049} and Lys^{1158} but may include more ubiquitination site(s) among the residues N-terminal of 1040. Further

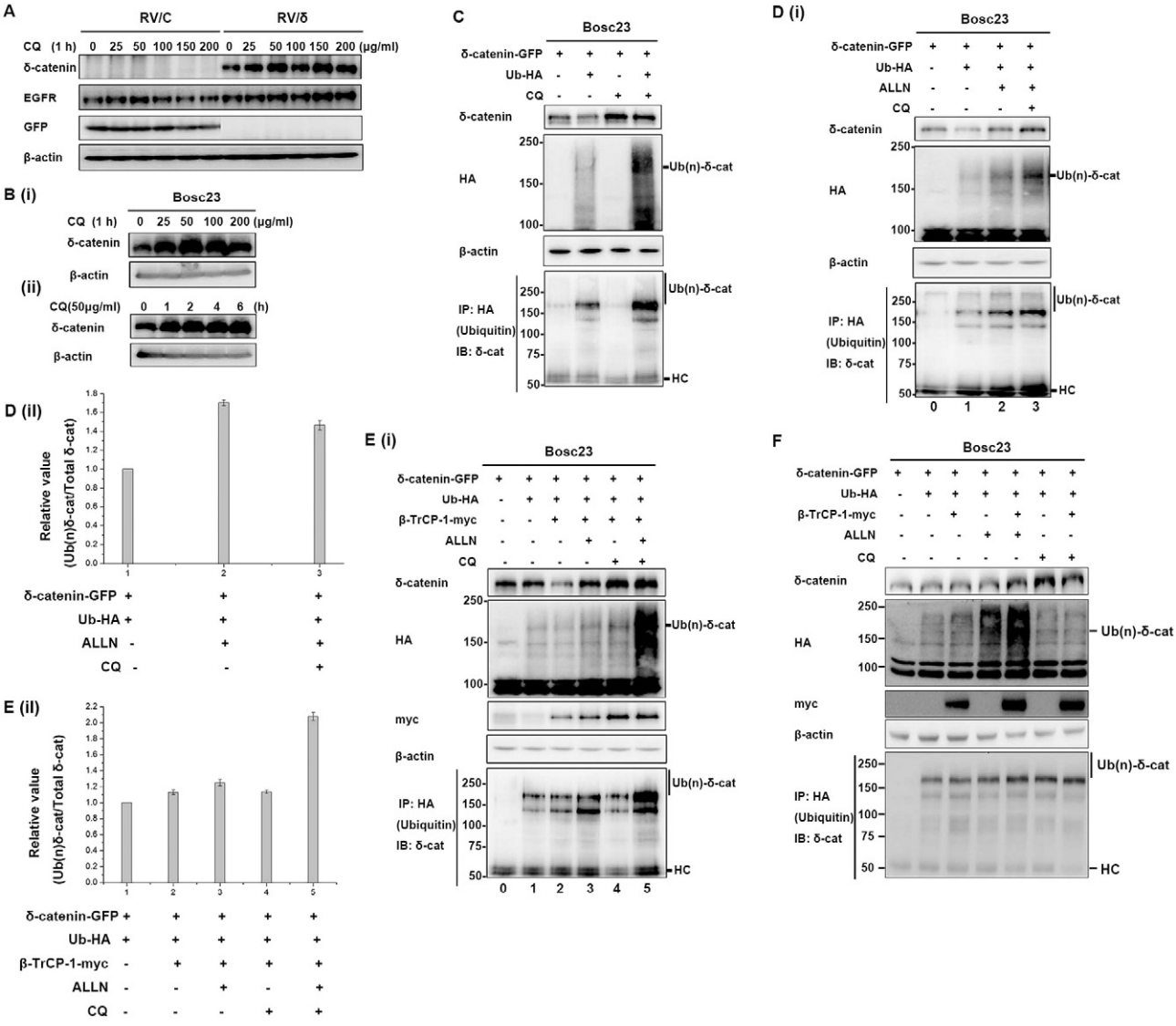

Fig. 6. δ-catenin also undergoes lysosomal degradation. (A) To determine the effects of Chloroquine (CQ) on exogenous δ-catenin, CWR22RV-1 cells were treated with the indicated concentrations for 1 h prior to lysis. Endogenous EGFR was detected by an anti-EGFR antibody. Stable GFP- and δ-catenin-expressing RV/C and RV/δ cells were subjected to IB with an anti-δ-catenin and an anti-GFP antibody, respectively. (B) Bosc23 cells were transfected with δ-catenin and treated with different concentrations of CQ for 1 h prior to lysis (upper panel). δ-catenin-GFP proteins were detected by anti-δ-catenin (panel i). δ-catenin-expressing Bosc23 cells were treated with CQ (50 μg/ml) for the indicated duration. The levels of δ-catenin were compared by IB using anti-δ-catenin (panel ii). β-actin was used as a loading control. (C-F) Bosc23 cells were transfected with the indicated plasmids and then treated with ALLN (10 μM) and CQ (50 μg/ml) 4 h prior to lysis. The cell lysates were subjected to IB and IP. Ubiquitinated δ-catenin was detected by IP using anti-HA (IP: HA), followed by IB with anti-δ-catenin (bottom panel). The levels of ubiquitinated δ-catenin were compared [D (ii) and E (ii)]. Heavy chain (HC) is shown in the figures (bottom panel).

study with respect to mapping is required in order to pinpoint all ubiquitin lysine residues among the multiple lysine residues of δ-catenin.

Several interesting phenomena observed in this study are worthy of discussion. (1) Low protein expression from the plasmid DNA encoding the Armadillo repeat portion of δ-catenin/NPRAP has been reported, for unknown reasons [29]. In our study, the expression of the deletions, δ-catenin 1–1040-GFP and δ-cat △1040–1070-GFP (10 Armadillo repeat of δ-catenin-GFP: 531–1070) were also low, for unknown reasons. (2) When β-TrCP-1 was co-transfected with ubiquitin, the protein levels of β-TrCP-1 were slightly decreased in the absence of ALLN (proteasome inhibitor) and CQ (lysosome inhibitor), however, recovered in the presence of proteasome and/or lysosome inhibitors [Figs. 4 and 5E(ii), middle panel]. It appears that β-TrCP-1 can also be degraded via the ubiquitin–proteasome pathway and the lysosome degradation pathway. However, β-TrCP-1, a RING type E3 ligase, transfers charged ubiquitin to the substrate directly from E2s. We were curious as to whether β-TrCP-1 could be ubiquitinated, and we found that it could be (data not shown). (3) What is the binding region of δ-catenin to β-TrCP-1? It has been reported that b-catenin N-terminal 1-130 fragment interacts with β-TrCP-1, and Ser^{33}/Ser^{37} in β-catenin are located in a DSGxxS motif (in single-letter amino-acid code, where x is any amino acid). The DSGxxS motif represents a signal sequence that is common to a given subset of substrates that rely upon β-TrCP-1 for their targeted degradation [27]. Despite the fact that δ-catenin lacks the DSGxxS destruction motif, δ-catenin can also be targeted by β-TrCP-1, through an unconventional recognition site as demonstrated in the case of cyclin D1 and β-TrCP-1 association [30]. Thus, further experiments are required to identify the recognition sites in δ-catenin used by β-TrCP-1.

In this study, investigation into the mechanism of δ-catenin ubiquitination has added a new perspective to δ-catenin degradation and provided a better understanding of the effects of δ-catenin on E-cadherin involved in epithelial cell–cell adhesion, which is implicated in cancer progression.

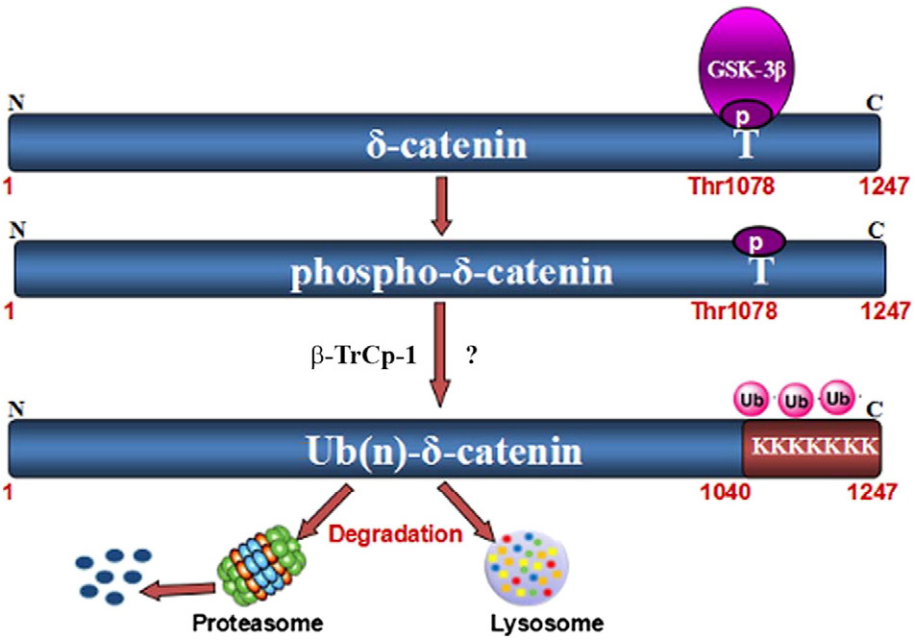

Fig. 7. Schematic representation depicts the mechanism of δ-catenin ubiquitination, in which two degradation pathways are involved.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

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