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90K Glycoprotein Promotes Degradation of Mutant β-Catenin Lacking the ISGylation or Phosphorylation Sites in the N-terminus[1,2]

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Abstract

β-Catenin is a major transducer of the Wnt signaling pathway, which is aberrantly expressed in colorectal and other cancers. Previously, we showed that β-catenin is downregulated by the 90K glycoprotein via ISGylation-dependent degradation. However, the further mechanisms of β-catenin degradation by 90K-mediated ISGylation pathway were not investigated. This study aimed to identify the β-catenin domain responsible for the action of 90K and to compare the mechanism of 90K on β-catenin degradation with phosphorylation-dependent ubiquitinational degradation of β-catenin. The deletion mutants of β-catenin lacking N- or C-terminal domain or mutating the N-terminal lysine or nonlysine residue were employed to delineate the characteristics of β-catenin degradation by 90K-mediated ISGylation pathway. 90K induced Herc5 and ISG15 expression and reduced β-catenin levels in HeLa and CSC221 cells. The N-terminus of β-catenin is required for 90K-induced β-catenin degradation, but the N-terminus of β-catenin is not essential for interaction with Herc5. However, substituting lysine residues in the N-terminus of β-catenin with arginine or deleting serine or threonine residue containing domains from the N-terminus does not affect 90K-induced β-catenin degradation, indicating that the N-terminal 86 amino acids of β-catenin are crucial for 90K-mediated ISGylation/degradation of β-catenin in which the responsible lysine or nonlysine residues were not identified. Our present results highlight the action of 90K on promoting degradation of mutant β-catenin lacking the phosphorylation sites in the N-terminus. It provides further insights into the discrete pathway downregulating the stabilized β-catenin via acquiring mutations at the serine/threonine residues in the N-terminus.

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Introduction

90K is a glycoprotein present at increased concentrations in the serum of individuals with various cancers or viral infections. It was originally identified as a tumor-associated antigen released into the culture medium by human breast cancer cells and was named 90K after its molecular mass. 90K has an N-glycosylated N-terminal signal peptide, meaning that it is secreted into the extracellular matrix via the secretory pathway [1]. The glycoprotein is also implicated in immune defense and immune regulation. 90K is present in human serum, biological fluids, and epithelial tissues; in addition, tumor cells within human neoplastic tissues show strong upregulation of 90K, whereas normal tissues show weak or no expression [2]. In breast cancer patients, a high level of 90K (>11 μg/ml of serum) is a poor prognostic factor [3], whereas high expression on immunohisto-

Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal cancer; GFP, green fluorescent protein; CM, conditioned medium

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chemical analysis of non–small cell lung cancer correlates with a poor prognosis for both disease-free and overall survival [2]. 90K is also overexpressed in prostate cancer [4] and colorectal cancer (CRC) compared with normal tissues [5]. By contrast, 90K also has positive effects. For example, tumor cells engineered to overexpress 90K show reduced tumorigenic potential in nude mice [6] and syngeneic mice [7]; however, the lack of 90K expression in laryngeal cancer is associated with the presence of known markers of aggressiveness and shorter survival [1].

β-Catenin, a component of the cadherin-catenin complex, is a major effector/transcription factor of the canonical Wnt signaling pathway. Wnt binds the Frizzeled receptor and phosphorylates Disheveled (Dvl) and other adenomatous polyposis coli (APC) complexes, leading to the accumulation of β-catenin [8]. Accumulated β-catenin associates with members of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family to regulate specific target genes such as c-myc, cyclin D1, CD44, and Axin2 [9]. Over 90% of CRC patients harbor mutations in APC or β-catenin, leading to β-catenin accumulation in the nucleus; thus, β-catenin plays an important role during progression of CRC [10,11]. In normal cells, β-catenin is degraded by two APC-dependent proteasomal degradation pathways, one involving GSK3β and the other involving p53-Siah-1 [12,13].

Previously, we showed that β-catenin undergoes 90K-mediated degradation via an ISG15-dependent proteasomal degradation pathway [7] and that proteasomal degradation of β-catenin by 90K does not require APC and is independent of GSK3β and Siah; these findings suggest the presence of another ubiquitination pathway for 90K-mediated β-catenin degradation. Here, we used deletion mutants lacking the N- or C-terminal domains of β-catenin or mutating the N-terminal lysine or nonlysine residue to characterize the β-catenin degradation by 90K-mediated ISGylation pathway. We also asked whether the mechanism of 90K on β-catenin degradation has any advantage on the regulation of β-catenin expression compared with phosphorylation-dependent degradation of β-catenin by Axin/APC/GSK3β complex, a central part of Wnt/β-catenin pathway. We found that removing all lysine, serine, and threonine residue-containing domains from the N-terminus of β-catenin does not affect 90K-induced β-catenin degradation. It indicated that 90K glycoprotein promotes degradation of mutant β-catenin lacking the ISGylation or phosphorylation sites in the N-terminus, which acquires stability from phosphorylation-dependent degradation and is very commonly found in CRC tissues. This action of 90K on β-catenin degradation has advantage on the regulation of β-catenin expression compared with phosphorylation-dependent degradation of β-catenin by Axin/APC/GSK3β complex, a central part of Wnt/β-catenin pathway.

Material and Methods

Plasmids and Antibodies

The construction of full-length, ΔN, ΔC, and ARM β-catenin tagged with green fluorescent protein (GFP) has been described previously [14]. All other β-catenin constructs were generated using a polymerase chain reaction–based EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, Korea). All constructs were confirmed by sequencing. The ISG15 E1 and E2 enzyme, Ube1L and UbcH8, respectively, expression plasmids were kindly provided by Prof. Keun Il Kim (Sookmyung Women's University, Seoul, Korea).

The following antibodies were used in the present study: anti–β-catenin (#9582; Cell Signaling Technology), anti-Herc5 (H00051191-A01; Abnova), anti-ISG15 (sc-69,701; Santa Cruz Biotechnology), anti-actin (A2066; Sigma), anti-myc (M047–3; MBL), and anti-GFP (sc-8344; Santa Cruz Biotechnology).

Cell Culture, Transfection, and Reagents

HEK293T (human embryonic kidney) and HeLa (human cervical cancer) cell lines were purchased from the Korean Cell Line Bank (Seoul, South Korea), and CSC221 (human colorectal adenocarcinoma–enriched cancer stem cell) cell line was purchased from the BioMedicure (San Diego, CA). HEK293T and HeLa cells used in the study were authenticated by a commercial service (Korean Cell Line Bank) with short tandem repeat profiling. HEK293T, HeLa, and CSC221 cells were maintained in RPMI or DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. HEK293T cells stably expressing a 90K-myc were generated as previously described [7]. Transfection of siRNAs was performed using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. The sequence of the 90K siRNA was 5′-ACAAATGTCTATCAACTT-TAA-3′. Herc5 siRNA was purchased from the Thermo Fisher Scientific (Waltham, MA).

Preparation of 90K-Conditioned Medium

90K-conditioned medium (90K/CM) was prepared as previously described [7]. Briefly, HEK293T cells were stably transfected with a 90K-myc expression plasmid and incubated in serum-free culture medium. After 4 days, the medium was collected and filtered to remove cells and debris. Control conditioned medium was obtained from HEK293T cells transfected with a mock vector. The concentration of 90K in 90K/CM (521.5 ng/ml) was determined by enzyme-linked immunosorbent assay [7]. The verified conditioned media were then used to treat cells (volume used = 10% of the volume of the culture medium).

Immunoblotting and Immunoprecipitation

Immunoprecipitation and immunoblotting were performed as previously described [15]. Briefly, cells were harvested after washing with ice-cold phosphate buffered saline and lysed in an ice-cold lysis buffer. Immunoprecipitation was performed by incubating cell lysates with an appropriate primary antibody for 16 hours at 4°C. Immunoprecipitated complexes were then pulled down using protein G-Sepharose beads (GE Healthcare). Cell lysates or immunoprecipitated proteins were resolved by electrophoresis and transferred to PVDF membranes. Proteins were visualized using appropriated primary antibody followed by horseradish peroxidase–conjugated secondary antibody. Blots were developed with the enhanced chemiluminescence solution. Images were obtained and analyzed by a LAS4000 luminescent image analyzer (Fuji Photo Film). All results are representative of at least three independent experiments.

The study protocol was reviewed and approved by the Institutional Review Board of Chonnam National University Medical School Research Institution (2013-19).

Statistical Analysis

Experimental differences were tested for statistical significance by using analysis of variance followed by Tukey honestly significant difference *post hoc* test or Student's *t* test. All statistical tests were

two-sided, and *P* values of less than .05 were considered statistically significant. Statistical analysis was performed with PASW Statistics 20 (SPSS, an IBM Company, Chicago, IL) software.

Results

N-Terminus of β-Catenin for 90K-Induced β-Catenin Degradation

We previously reported that glycoprotein 90K suppresses the Wnt/β-catenin signal in colorectal cancer tissues by promoting ISGylational (ISG15-conjugation) degradation of β-catenin [7]. 90K treatment increases expression of ISG15 mRNA in 293T, HCT116, and Caco2 cells and promotes association of the HECT E3 ligase, Herc5, with β-catenin. Here, we examined the effects of 90K on HeLa (cervical cancer) and CSC221 (colorectal adenocarcinoma–enriched cancer stem cell) cells. As shown in Figure 1*A*, 90K/CM treatment significantly induced Herc5 and ISG15 expression and significantly reduced β-catenin levels in both cell lines, indicating that 90K treatment can promote ISGylational degradation of β-catenin in cervical cancer cells and CRC cells–enriched cancer stem cell, in addition to CRC cells.

Next, we used several β-catenin deletion mutants to identify the β-catenin domain responsible for 90K-induced degradation. The N- and C-terminal domains of β-catenin are sensitive to mild protease digestion, and both play a role in mediating protein-protein interactions [16]. Also, the central structural core comprising 12 armadillo repeats forms the binding surface for β-catenin ligands [16]. Therefore, three mutants were constructed: ΔN86 (lacking the N-terminal 1-86 aa), ΔC (lacking the C-terminal 659-781 aa), and ARM (lacking both the N- and C-termini) [14]. We found it interesting that deleting the N-terminal 86 aa of β-catenin completely abolished the ability of 90K to degrade β-catenin (Figure 1*B*). We transfected cells stably overexpressing 90K with si-90K and used them to examine the effect of 90K/CM on the β-catenin deletion mutants. β-Catenin deletion mutants lacking the N-terminus showed no response to 90K silencing (Figure 1*C*). This clearly suggests that the N-terminal 86 aa of β-catenin are required for 90K-induced β-catenin degradation.

N-Terminus of β-Catenin for Herc5 Interaction

Because deletion of the N-terminal 86 aa of β-catenin completely abolished the effect of 90K, the N-terminus may either contain residue(s) required for ISG15-conjugation or play a crucial role in the association with ISG15 E3 ligase. As shown in Figure 2*A*, Herc5 is essential for 90K-induced β-catenin degradation. To determine whether the N-terminus of β-catenin is required for the interaction with Herc5 (the E3 ligase required for ISG15-conjugation to β-catenin), we performed an immunoprecipitation assay to measure the interaction between Herc5 and full-length (FL) or ΔN86 mutants of β-catenin. To rule out the involvement of unexpected possibility in mediating Herc5 interaction, ISG15 E1 and E2 enzyme, Ube1L and UbcH8, respectively, were overexpressed. We observed a clear interaction between Herc5 and ΔN86-β-catenin (Figure 2). These results suggest that the N-terminal 86 aa of β-catenin are not essential for its interaction with the E3 ligase, Herc5.

N-Terminal Mutation or Deletion of β-Catenin for 90K-Induced β-Catenin Degradation

Because Herc5 interacts with both FL- and ΔN86-β-catenin, N-terminal β-catenin likely harbors residues directly involved in the conjugation of ISG15. Therefore, to identify the residue(s) in the N-terminus of β-catenin that is(are) responsible for ISG15 conjugation, we compared the N-terminal aa sequence of β-catenin with the sequences published in previous studies reporting the consensus motif required for conjugation of ISG15. ISG15 conjugation occurs on the ε-amine group of lysine residues [17] or on cysteine residues [18]. As shown in Figure 3*A*, we identified two lysine residues (Lys-19 and Lys-49); however, the 86 aa N-terminus of β-catenin contains no cysteine residues. Although not reported for ISG15 conjugation, noncanonical residues such as serine or threonine are modified by ubiquitin even though lysine residues are the favored target for ubiquitin conjugation [19]. We identified nine serine residues (Ser-23, Ser-29, Ser-33, Ser-37, Ser-45, Ser-47, Ser-60, Ser-71, and Ser-73) and six threonine residues (Thr-3, Thr-40, Thr-41, Thr-42, Thr-59, and Thr-75) within the 86 aa N-terminus of β-catenin (Figure 3*A*).

To examine whether the N-terminal lysine residues of β-catenin are responsible for 90K-induced β-catenin degradation, we substituted Lys-19 and/or Lys-49 with arginine. Surprisingly, the introduction of single or double substitutions did not abrogate the ability of 90K to degrade β-catenin, suggesting that the lysine residues within the N-terminus of β-catenin are not the sole target for ISG15-conjugation (Figure 3*B*).

Next, to examine whether the nonlysine residues within the N-terminus of β-catenin are responsible for 90K-induced β-catenin degradation, deletion mutations were introduced into the domain containing serine or threonine residues. Three mutants harboring specific deletions at sites between Lys-19 and Lys-49 [Δ19-31 (deletion includes Lys-19, Ser-23, and Ser-29), Δ32-39 (deletion includes Ser-33 and Ser-37), and Δ40-48 (deletion includes Thr-40, Thr-41, Thr-42, Ser-45, and Ser-47)] were generated. As shown in Figure 3*C*, deletion of the abovementioned serine or threonine residues had no effect on 90K-induced β-catenin degradation. Mutants Δ59-60 (including Thr-59 and Ser-60) and Δ71-75 (including Ser-71, Ser-73, and Thr-75) harbored specific deletions at sites beyond Lys-49 within β-catenin. Figure 3*D* also shows that deletion of N-terminal β-catenin domains beyond Lys-49 did not affect 90K-induced β-catenin degradation. Because none of the arginine mutations or deletion mutations affected 90K-induced β-catenin degradation, we generated two other deletion mutants lacking either aa 1-48 (ΔN48) or aa 49-86 (ΔN49–85) of β-catenin. Again, these deletions had no effect on 90K-induced β-catenin degradation (Figure 3*E*), suggesting that 90K-induced β-catenin degradation would still occur even if β-catenin harbored either half of its N-terminus.

Next, we assumed that 90K-induced β-catenin degradation would occur if there was a lysine or serine residue at the N-terminus of β-catenin and asked whether the N-terminal 19-75 aa domain, which contains mostly lysine, serine, and threonine residues, is required for 90K-induced β-catenin degradation. To examine this, we generated deletion mutants lacking 19-75 aa (Δ19-75), Δ19-74 (harboring Thr-75), and Δ20-75 (harboring Lys-19). All the deletion mutants were degraded by 90K (Figure 4*A*). These mutants contained the Thr-3 residue; therefore, we introduced the T3A mutation. Surprisingly, all the β-catenin mutants were still degraded by 90K (Figure 4*B*). Thus, even though the N-terminus of β-catenin plays an essential role in 90K-induced β-catenin degradation, the specific residues responsible for this activity remain unknown. At least from Figure 4*C*, the results suggest that degradations of these β-catenin mutants are dependent on Herc5 and implicate that β-catenin N-terminus is not responsible for Herc5 interaction.

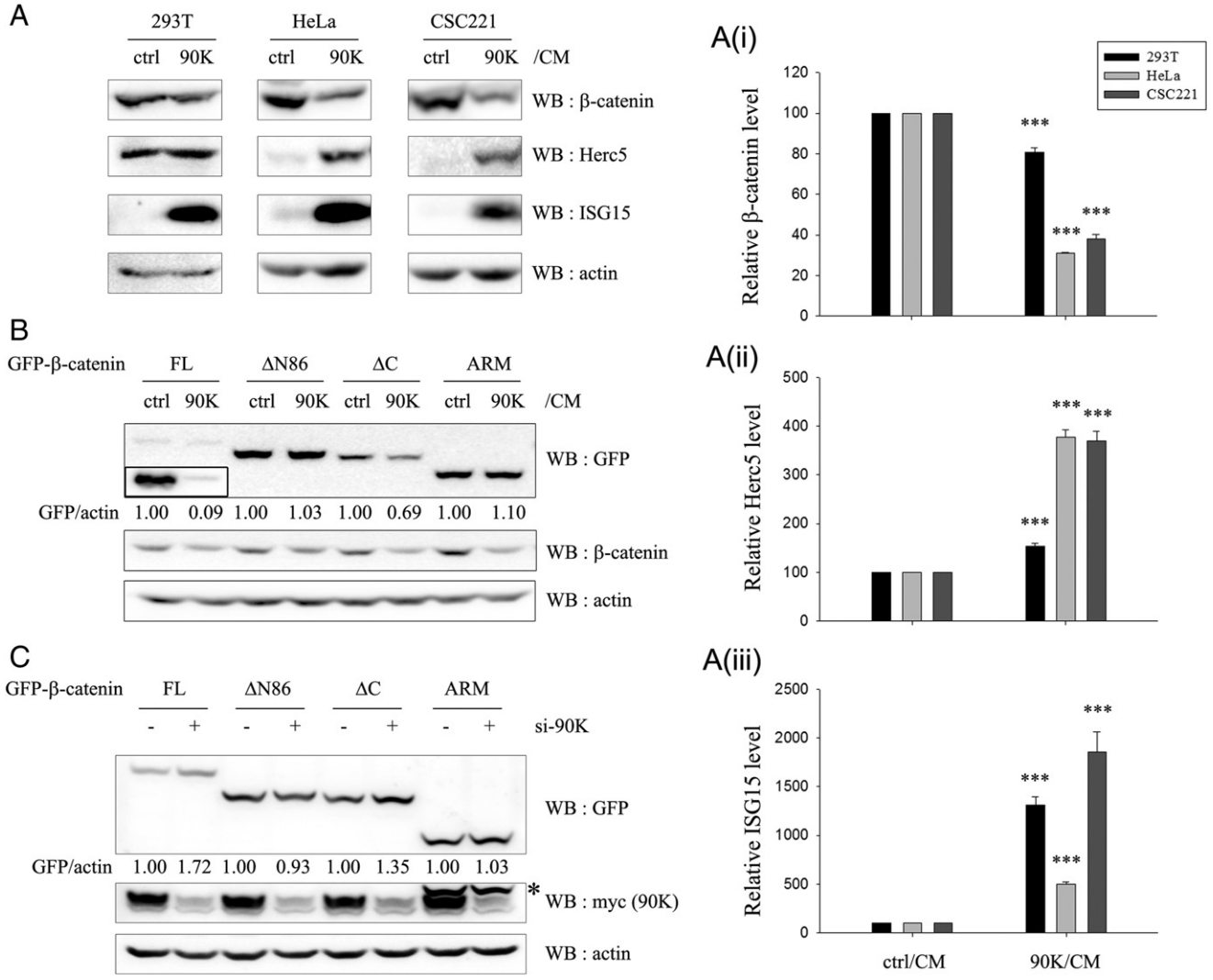

Figure 1. N-Terminus of β-catenin is required for 90K-induced β-catenin degradation. (A) 90K/CM induced Herc5 and ISG15 expression and reduced β-catenin levels in HEK293T, HeLa, and CSC221 cells. Cells were treated with either control conditioned medium (ctrl/CM) or 90K/CM for 48 hours, followed by immunoblotting with antibodies against β-catenin, ISG15, Herc5, or actin (loading control). These endogenous signals were measured by densitometry in triplicate experiments, and the fold changes of relative β-catenin level [A(i)], Herc5 level [A(ii)], and ISG15 [A(iii)] level compared with actin were depicted as a bar graph among three cell lines (right side). Each bar represents mean ± SD for triplicate samples. The asterisk (*) indicates a significant difference between ctrl/CM and 90K/CM groups (***$P < .001$). (B, C) Deleting the N-terminal 86 amino acids of β-catenin abolished the ability of 90K to degrade β-catenin. GFP-tagged deletion mutants of β-catenin lacking the N-terminus (ΔN86: deletion of aa 1-86), C-terminus (ΔC: deletion of aa 659-781), or both the N- and C-termini (ARM) were used to determine the domain responsible for 90K-induced β-catenin degradation. The exogenous β-catenin levels (GFP-β-catenin) were measured by densitometry in triplicate experiments, and the mean fold changes of relative GFP-β-catenin level (GFP/actin) compared with actin between ctrl/CM and 90K/CM groups were indicated below each gel lane. (B) The β-catenin deletion mutants were transfected into HEK293T cells, which were then treated with either ctrl/CM or 90K/CM, followed by immunoblotting with antibodies against GFP (exogenous β-catenin), endogenous β-catenin, and actin. Reductions in endogenous β-catenin levels are shown as a positive control for the effects of 90K. The levels of FL- and ΔC-β-catenin decreased in 90K/CM-treated cells, whereas those of ΔN86- and ARM-β-catenin remained constant. For the effect of 90K on FL-β-catenin, another enhanced image is shown as an inset. (C) The β-catenin deletion mutants were transfected into stable 90K-myc expressing HEK293T cells together with either scrambled or 90K-specific siRNA, followed by immunoblotting with antibodies against GFP (exogenous β-catenin), myc (90K-myc), and actin. The levels of FL- and ΔC-β-catenin increased in si-90K transfected cells. The asterisk indicates ARM β-catenin mutants, which are shown after immunoblotting of the membrane with an anti-GFP antibody.

Discussion

Here, we examined the effects of 90K on β-catenin degradation and tried to identify the β-catenin domain(s) responsible. The major findings were as follows: 1) 90K induced Herc5 and ISG15 expression and reduced β-catenin levels in cervical cancer cells and CRC cells–enriched cancer stem cell; 2) the N-terminus of β-catenin is required for 90K-induced β-catenin degradation; 3) the N-terminus of β-catenin is not essential for interaction with Herc5; and 4) substituting lysine residues in the N-terminus of β-catenin with arginine or deleting serine or threonine residue-containing domains from the N-terminus does not affect 90K-induced β-catenin degradation.

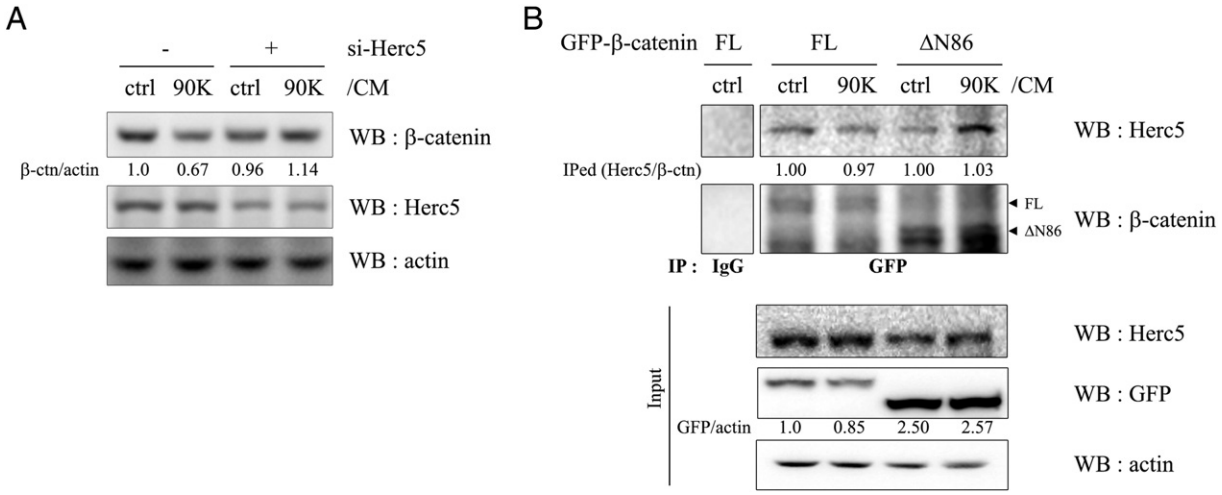

Figure 2. β-Catenin degradation by 90K is Herc5 dependent, and ΔN86-β-catenin interacts with Herc5. (A) Herc5 silencing diminishes 90K-induced β-catenin degradation. HEK293T cells transfected with either scramble or Herc5 siRNA were incubated for 24 hours and then treated with ctrl/CM or 90K/CM, followed by immunoblotting with antibodies against endogenous β-catenin, Herc5, and actin. The endogenous β-catenin levels were measured by densitometry in triplicate experiments, and the mean fold changes of relative β-catenin level (β-ctn/actin) compared with actin between ctrl/CM and 90K/CM groups were indicated below each gel lane. (B) Both FL-β-catenin and ΔN86-β-catenin interact with Herc5. HEK293T cells transfected with FL or ΔN86 deletion mutant of β-catenin together with Ube1L and UbcH8 were treated with either ctrl/CM or 90K/CM, followed by immunoprecipitation with an anti-GFP antibody. Resolved bound proteins were then immunoblotted with antibodies against Herc5 and β-catenin. Total cell lysates were immunoblotted with the corresponding antibodies to show the input amount of each protein. The immunoprecipitated Herc5 and β-catenin levels were measured by densitometry in triplicate experiments, and the mean fold changes (Herc5/β-ctn) between ctrl/CM and 90K/CM groups were indicated below each gel lane.

The N-terminus of β-catenin plays essential roles in Axin-, GSK3-, CK1-, and APC-mediated destruction of β-catenin [20]. Here, we showed that the N-terminus of β-catenin is also required for 90K-induced degradation of β-catenin. Because a ΔN86 mutant of β-catenin retained the ability to interact with Herc5, the E3 ligase required for β-catenin ISGylation, the N-terminal 86 aa of β-catenin should harbor ISG15-conjugation site(s), but at the same time, mutations at the Ser/Thr residues within the N-terminal 86 aa of β-catenin did not affect the binding of deletion mutants with Herc5 and thereby 90K could degrade these deletion mutants of β-catenin (Figures 3 and 4). Unfortunately, we were unable to map the lysine, serine, and/or threonine residues within the N-terminus of β-catenin that are involved in ISG15 conjugation. The lysine residues responsible for ISG15 conjugation have been identified in a few proteins [17], for example, K92 of UbC13, K136 of UbCH6, K117 of EFP, K134/222 of 4EHP, K2467 of filamin B, K193/360/366 of IRF3, K41 of the NS1 protein of influenza A virus, K139/324 of p63, K69/159 of PKR (double-stranded RNA-activated protein kinase), and K164/168 of PCNA. However, multiple domains appear to be involved in ISGylation of target proteins such as HIF-1a [21] and p53 [22]. It is noteworthy that, even though K19/49 residues reside within the N-terminal 86 aa of β-catenin, none of our single or double arginine mutations blocked or inhibited 90K-induced β-catenin degradation (Figure 3*B*).

ISG15 conjugation occurs primarily between the C-terminal glycine of ISG15 and the ε-amino group of a lysine side chain within the substrate protein [23]. However, conjugation of ISG15 can occur via a disulfide bridge between the Cys87 residue of Ubc13 and the Cys78 at the hinge region of ISG15 [18]. It is likely that canonical ubiquitin conjugation occurs between the C-terminal Gly76 of ubiquitin and the ε-amino group of a lysine residue within the target substrate [19]. Also, ubiquitin can be "noncanonically" conjugated to target proteins through a thiol ester bond with the –SH group of Cys or via an ester bond with the hydroxyl group of Ser or Thr [19]. Alternatively, ubiquitin conjugation may occur via the α-amino group of any N-terminal residue within the target protein [24]. Of note, "noncanonical" conjugation of ISG15 to a target protein is also possible. Because neither single nor double arginine mutations at K19/49 residues prevented 90K-induced β-catenin degradation, we generated various deletion mutants of β-catenin to selectively remove all or some of the candidate Ser or Thr residues. Unexpectedly, the levels of all of the deletion mutants were reduced after 90K treatment (Figure 3). The finding that the levels of ΔN86-β-catenin were not decreased by 90K treatment ruled out the possibility of conjugation to the α-amino group of N-terminal residues within each of the mutants. Our present results suggest that complex regulatory mechanisms underlie the ISGylational degradation of β-catenin by 90K, and two possibilities remain: 1) multiple residues are involved in 90K-induced conjugation of ISG15 to β-catenin, or 2) alternative activations of lysine or nonlysine residues occur when mutations are introduced into the N-terminus of β-catenin. Currently, either possibility is likely to regulate β-catenin degradation.

β-Catenin mutations are commonly found in a variety of cancers, and sequencing showed that approximately 9% of these cancer tissue samples have somatic mutations in the N-terminal β-TrCP-binding motif of the CTNNB1 gene [25], which results in no longer phosphorylation on Ser33, Ser37, or Ser45 residue and not ubiquitinated and degraded and thus, stabilized β-catenin. We previously observed that when constitutively active S37A β-catenin was cotransfected with increasing doses of 90K in 293T cells, TOPFLASH activity was gradually decreased with

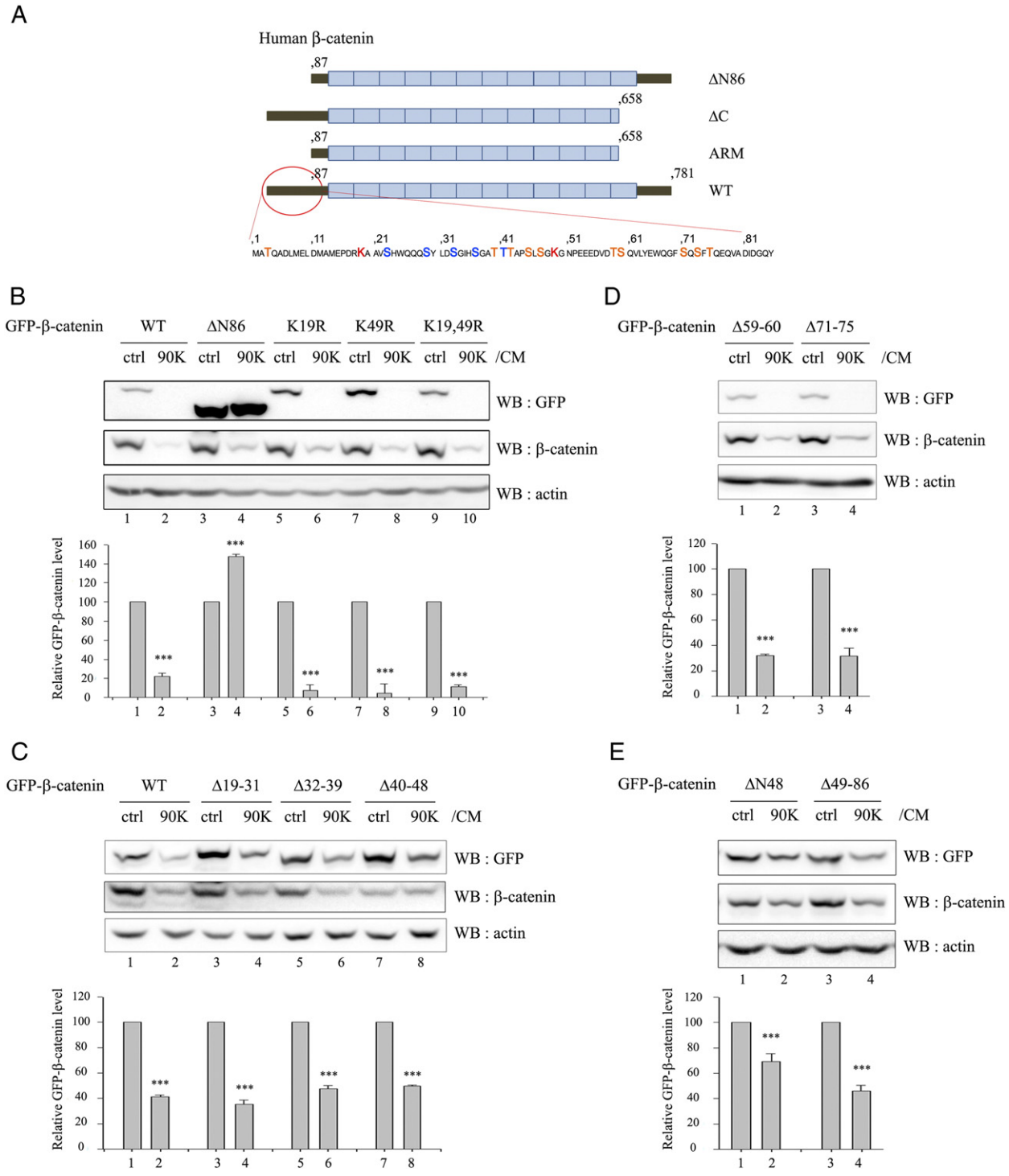

Figure 3. Substituting N-terminal lysine residues with arginine or deleting N-terminal serine- or threonine-containing domains from β-catenin. (A) Schematic illustration of the β-catenin deletion mutants and the amino acid sequence of the N-terminus of β-catenin. Lysine (K, red), serine (S, orange), and threonine (T, orange) residues within the N-terminus of β-catenin are shown in colors. Serine and threonine residues known to be targets for GSK-3β phosphorylation are shown in blue. (B-E) Substitution of N-terminal lysine residues with arginine or deleting N-terminal domains containing serine or threonine residues does not affect 90K-induced β-catenin degradation. The β-catenin mutants were transfected into HEK293T cells, which were then treated with either ctrl/CM or 90K/CM, followed by immunoblotting with antibodies against GFP (exogenous β-catenin), endogenous β-catenin, and actin. Decreases in endogenous β-catenin levels are shown as a positive control for the effects of 90K. The exogenous β-catenin levels (GFP-β-catenin) were measured by densitometry in triplicate experiments, and the fold changes of relative GFP-β-catenin level compared with actin were depicted as a bar graph below the immunoblot data. Each bar represents mean ± SD for triplicate samples, and number under each gel corresponds to each bar graph. The asterisk (*) indicates a significant difference between ctrl/CM and 90K/CM groups (**$P < .01$; ***$P < .001$). (B) Introduction of arginine mutations does not affect 90K-induced β-catenin degradation. (C) Deleting serine- and threonine-containing domains between Lys-19 and Lys-49 does not affect 90K-induced β-catenin degradation. (D) Deleting serine- and threonine-containing domains beyond Lys-49 does not affect 90K-induced β-catenin degradation. (E) 90K-induced β-catenin degradation occurs if β-catenin harbors either half of its N-terminal domain.

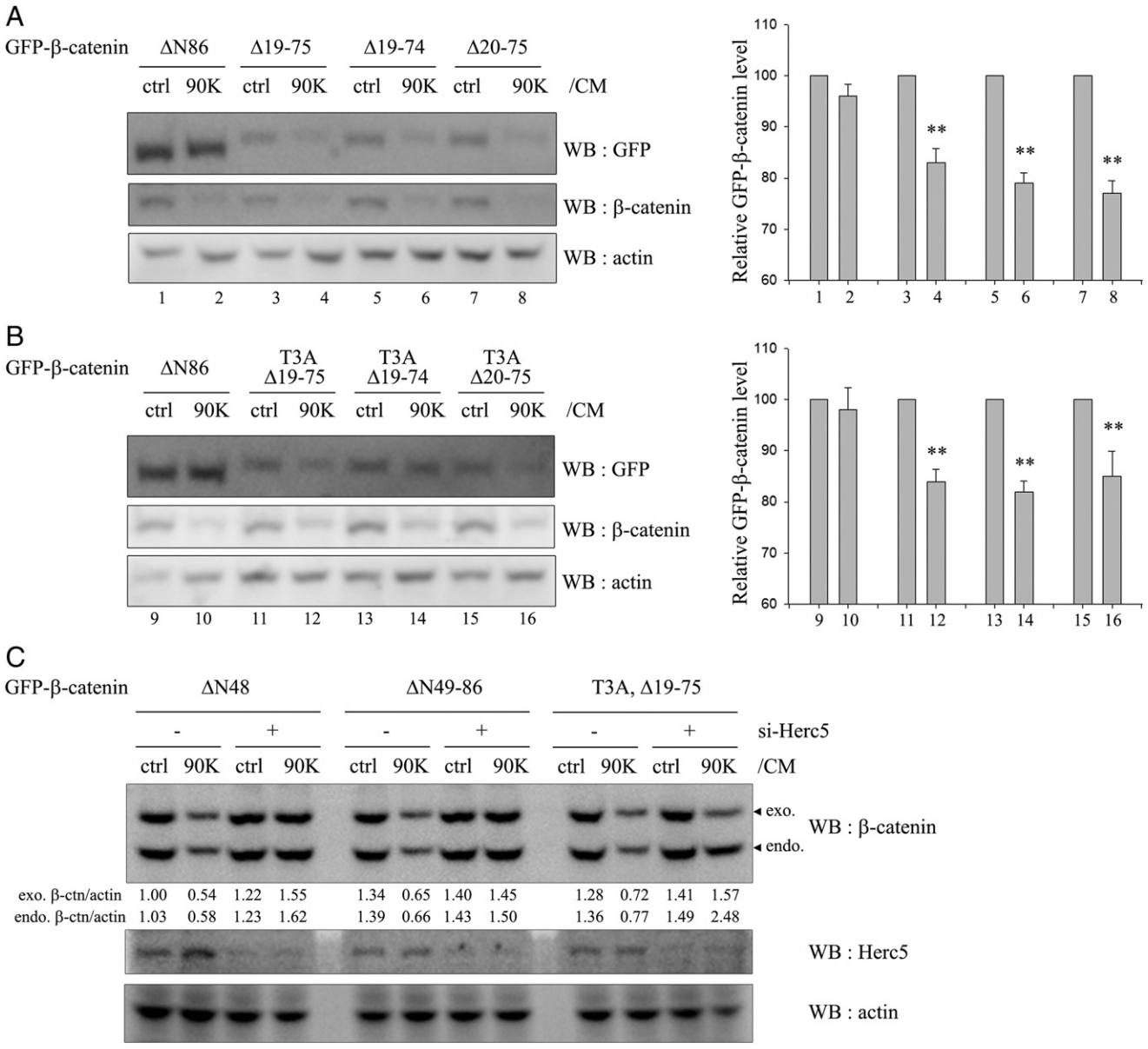

Figure 4. Removing all lysine, serine, and threonine residue-containing domains from the N-terminus of β-catenin does not affect 90K-induced β-catenin degradation. (A, B) The β-catenin mutants were transfected into HEK293T cells, which were then treated with either ctrl/CM or 90K/CM, followed by immunoblotting with antibodies against GFP (exogenous β-catenin), endogenous β-catenin, and actin. Decreases in endogenous β-catenin levels are shown as a positive control for the effects of 90K. (A) Deleting N-terminal 19-75 aa domain of β-catenin does not affect 90K-induced β-catenin degradation. (B) Removing all lysine, serine, and threonine residue-containing domains from the N-terminus of β-catenin does not affect 90K-induced β-catenin degradation. (C) The degradations of β-catenin mutants are dependent on Herc5. HEK293T cells transfected with either scramble or Herc5 siRNA were incubated for 24 hours and then transfected with β-catenin mutants. Then, cells were treated with ctrl/CM or 90K/CM, followed by immunoblotting with antibodies against β-catenin, Herc5, and actin. The exogenous and endogenous β-catenin levels were measured by densitometry in triplicate experiments, and the mean fold changes of relative β-catenin level (exo. and endo. β-ctn/actin) compared with actin between ctrl/CM and 90K/CM groups were indicated below each gel lane.

reduced β-catenin level [7]. Our present results indicated that 90K also promotes degradation of two β-catenin mutants harboring specific deletions at sites Δ32-39 (deletion includes Ser-33 and Ser-37) and Δ40-48 (deletion includes Thr-40, Thr-41, Thr-42, Ser-45, and Ser-47), which deprive phosphorylation-dependent degradation. Thus, these results indicated that 90K-mediated degradation of β-catenin via an ISG15-dependent proteasomal degradation pathway could downregulate the stabilized β-catenin escaped from GSK3β-dependent phosphorylation and provide further insights into the discrete pathway overcoming acquired resistance to phosphorylation-dependent β-catenin degradation via the mutations of the serine/threonine residues in the N-terminus.

Recently, we reported that immunohistochemical expression of 90K was significantly lower in colon cancer tissues from stage IV and its metastatic liver tissues than in that from stage I [7]. Thus, considering these frequent loss-of-function mutations in the N-terminal of β-catenin and suppressed 90K tissue level in advanced colorectal cancer patients, 90K-mediated ISGylation/degradation of

β-catenin may regulate the β-catenin level to some extent only when the tissue level of 90K is sufficiently high to maintain ISG15 conjugation to β-catenin on multiple residues or on alternatively activated lysine or nonlysine residues. Future studies should be conducted to identify the further detailed mechanisms underlying 90K-mediated ISGylation of β-catenin.

In conclusion, 90K promotes degradation of mutant β-catenins lacking the ISGylation or phosphorylation sites in the N-terminus, which acquires stability from phosphorylation-dependent degradation. This mechanism of 90K on β-catenin degradation has advantage on the regulation of β-catenin expression compared with phosphorylation-dependent degradation of β-catenin by Axin/APC/GSK3β complex, a central part of Wnt/β-catenin pathway.

Author Contributions

H. K. and K. K. K. conceived and designed the experiments; S. Y. P. and S. Y. performed the experiments; S. Y. P., S. Y., H. K., and K. K. K. analyzed the data; and H. K. and K. K. K. wrote the manuscript.

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