Threonine 41 in β-Catenin Serves as a Key Phosphorylation Relay Residue in β-Catenin Degradation†

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ABSTRACT: β-Catenin phosphorylation at serine 45 (Ser45), threonine 41 (Thr41), Ser37, and Ser33 is critical for β-catenin degradation, and regulation of β-catenin phosphorylation is a central part of the canonical Wnt signaling pathway. β-Catenin mutations at Ser45, Thr41, Ser37, and Ser33 perturb β-catenin degradation and are frequently found in cancers. It is established that Ser45 phosphorylation by casein kinase I (CKI) initiates phosphorylation at Thr41, Ser37, and Ser33 by glycogen synthase kinase 3 (GSK3) and that phosphorylated Ser37 and Ser33 are recognized by the F-box protein β-TrCP, a component of a ubiquitin ligase complex that mediates β-catenin degradation. While the roles of Ser45, Ser37, and Ser33 are well documented, the function of Thr41 remains less defined. Here we show that Thr41 strictly acts as a phosphorylation relay residue and that the Ser-X-X-X-Ser (X is any amino acid) motif is obligatory for β-catenin phosphorylation by GSK3. β-Catenin phosphorylation/degradation and its regulation by Wnt can occur normally in the absence of Thr41 as long as the Ser-X-X-X-Ser motif/spacing is preserved. These results suggest that Thr41 functions to bridge sequential phosphorylation from Ser45 to Ser37 and provide further insights into the discrete steps and logic in β-catenin phosphorylation−degradation.

The canonical Wnt signal transduction pathway plays a central role in a diverse array of biological processes, such as the segment polarity patterning during *Drosophila* development and the formation of a dorsal−ventral axis in early *Xenopus* embryogenesis (*1*−*3*). Deregulation of the Wnt signaling pathway is associated with many human cancers, in particular colorectal carcinoma, melanoma, and heptocellular carcinoma (*4*).

The oncogene product β-catenin is a central component of the Wnt signal transduction pathway. In the absence of Wnt stimulation, cytosolic β-catenin is phosphorylated at its N-terminus by a multiprotein complex including Axin, casein kinase Iα (CKIα), glycogen synthase kinase 3 (GSK3), and adenomatous polyposis coli (APC) (*5*−*16*). Ser33 and Ser37 doubly phosphorylated β-catenin is specifically recognized by the F-box/WD40 protein β-TrCP (*17*−*22*), a component of the E3 ubiquitin ligase complex that polyubiquitinates β-catenin for degradation via the proteosome pathway (*23*, *24*). In the presence of a Wnt ligand, the activation of the cell surface Wnt receptor complex (*25*) results in the inhibition of the Axin/APC/GSK3 complex. As a result, β-catenin is no longer phosphorylated on Ser33 and Ser37 and, thus, not ubiquitinated and degraded. Stabilized β-catenin translocates into the nucleus and complexes with members of the TCF/LEF family of transcription factors (*26*−*28*), leading to the activation of Wnt/β-catenin responsive genes such as cyclin D1 and c-myc (*29*, *30*). Regulation of β-catenin phosphorylation is a central part of the Wnt/β-catenin pathway.

There are four highly conserved serine/threonine residues at the N-terminal region of β-catenin: Ser45, Thr41, Ser37, and Ser33 (Figure 1A). CKIα is responsible for Ser45 phosphorylation (*12*−*14*), which serves as a priming phosphorylation event for GSK3. The consensus phosphorylation motif for GSK3 is Ser^{1}-X-X-X-$pSer^{5}$, in which the phosphoserine (or phosphothreonine) residue at position 5 facilitates the phosphorylation of the serine/threonine residue at position 1. Ser45-phosphorylated β-catenin is subsequently phosphorylated on Thr41, Ser37, and Ser33, most likely via GSK3 (*12*−*16*). Tumorigenic mutations in β-catenin mainly occur at these phosphorylation sites (Ser45, Thr41, Ser 37, and Ser33) and the β-TrCP binding site (Ser33, Ser37, and surrounding residues) (*4*), thereby resulting in abnormal accumulation of β-catenin that may lead to cancers.

Thus, while Ser45 phosphorylation by CKIα initiates β-catenin phosphorylation, and phosphorylation of Ser33 and Ser37 by GSK3 triggers β-TrCP recognition for ubiquitination/degradation, a question remains with regard to the exact role of Thr41 phosphorylation. Does it simply serve as a phosphorylation relay between Ser45 and Ser37 or does Thr41 phosphorylation provide an additional control/check point in the processive β-catenin phosphorylation/degradation cascade? In this study, we addressed this issue by designing a series of β-catenin mutant derivatives that have Thr41 and three other residues between Ser45 and Ser37 deleted, such that the spacing between Ser45 and Ser37 is kept as four residues. We found that these deletion mutants still retain full ability to bind to β-TrCP and are properly phosphorylated and degraded. Importantly, the stability of these β-catenin derivatives is subjected to proper regulation by Wnt signal-

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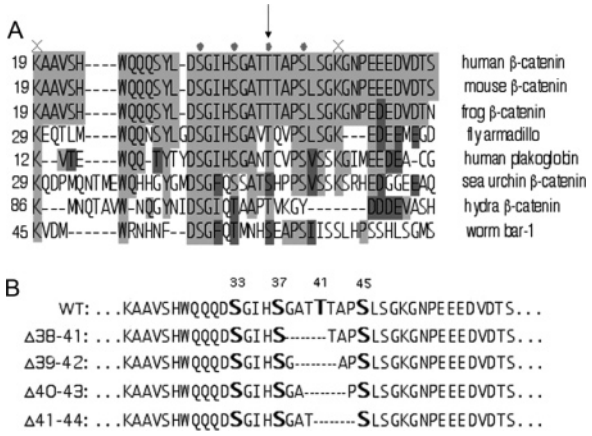

FIGURE 1: (A) Sequence alignment of the N-terminal region of β-catenin and homologues across species by the Clustal W program. Identical residues among all proteins are shown in dark gray, whereas similar residues are shown in gray. The phosphorylation sites (Ser33, Ser37, Thr41, and Ser45) in human β-catenin are marked by dots, and Thr41 is further highlighted by an arrow. Putative ubiquitination sites (lysines 19 and 49) are marked by crosses. A tyrosine residue occupies the equivalent Ser45 position in hydra β-catenin. (B) The N-terminal amino acid sequences surrounding Ser33 to Ser45 are shown for the wild-type (WT) β-catenin, Δ38−41 (deleting residues 38 to 41), Δ39−42 (deleting residues 39 to 42), Δ40−43 (deleting residues 40 to 43), and Δ41−44 (deleting residues 41 to 44). Δ39−42 and Δ40−43 resulted in the same sequence. Ser 33, Ser37, Thr41, and Ser45 in the WT β-catenin and the corresponding residues in the deletion mutants are highlighted.

ing. Finally, we demonstrate that deleting or inserting a single residue between Thr41 and Ser37 disrupts β-catenin phosphorylation/degradation. Thus, Thr41 functions primarily as a phosphorylation relay residue, and the spacing requirement in β-catenin phosphorylation by GSK3 is obligatory.

EXPERIMENTAL PROCEDURES

Plasmids. The following constructs were described: CS2+ wild-type β-catenin (Flag-tagged), CS2+ wild-type β-TrCP (myc-tagged), and CS2+ β-TrCPΔF (myc-tagged) (*18*); CS2+ β-catenin(S45A) (Flag-tagged) (*12*); CS2+ LRP6ΔN (VSVG tagged) (*32*). All β-catenin derivatives were tagged with the Flag epitope at the C-terminus and subcloned in pCS2+. The deletion and insertion mutant β-catenin constructs, Δ38−41, Δ39−42, Δ40−43, Δ41−44, Del-1, and Ins-1, were created by the QuickChange site-directed mutagenesis kit (Stratagene).

Mammalian Cell Transfection. Transfections were done in human embroyonic kidney 293T cells in six-well plates using the calcium phosphate method. Forty-eight hours after transfection, cells were lysed in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, and 1% Nonidet P-40, with a cocktail of protease inhibitors. The cell lysate were centrifuged at 14000 rpm for 10 min, and the supernatant of the cell lysates was taken for further studies.

Antibodies, Immunoprecipitation, and Western Blotting. The supernatant of cell lysates was immunoprecipitated with appropriate antibodies (1−4 μg) plus protein G beads at 4 °C for 2 h. Precipitates were washed five times with the same Nonidet P-40 lysis buffer (0.9 mL), examined by SDS−PAGE, and analyzed by Western blotting to Immobilon-P membrane (Millipore). The membranes were incubated in blocking buffer (5% nonfat dry milk in TBS buffer with 0.1% Tween-20) for 1 h at room temperature and then were incubated with primary antibodies diluted in 0.1% BSA in the TBS/Tween-20 buffer for 1 h, followed by incubation with horseradish peroxide-conjugated secondary antibodies diluted at 1:10000 in 0.1% BSA in the TBS/Tween-20 buffer for 25 min. Protein detection was performed using the ECL system (Amersham Pharmacia). The following antibodies were used (values in parentheses are the dilution ratios used for Western blotting): anti-Flag (M2) (1:1000) from Sigma (F3165), anti-myc (1:1000) from Upstate Biotechnology (06549), and anti-tubulin (1:1000) from Santa Cruz (C2206).

RESULTS

Design of β-Catenin N-Terminal Deletion Derivatives Lacking Thr41. Examination of tumor-derived mutations in β-catenin shows that N-terminal Ser33, Ser37, Thr41, and Ser45 are among major mutation "hot spots" found in cancers (*4*). Sequence alignment of the N-terminal sequence of human β-catenin with homologues from other species reveals that these Ser/Thr residues are highly conserved (Figure 1A). The importance and conservation of Ser33 and Ser37 can be explained by their requirement for recognition, upon phosphorylation, by β-TrCP, whereas the importance and conservation of Ser45 can be explained by the requirement of its priming phosphorylation by CKIα. To investigate the role of Thr41 in β-catenin phosphorylation, we constructed a series of mutants in which four residues including Thr41 were deleted between Ser37 and Ser45 (Figure 1B). In these deletion mutants, namely, Δ38−41, Δ39−42, Δ40−43, and Δ41−44, the spacing between Ser37 and Ser45 was kept four residues apart, in accordance with the phosphorylation consensus motif for GSK3. As Δ39−42 and Δ40−43 resulted in the identical amino acid sequence, only Δ39−42, along with Δ38−41 and Δ41−44, was used in the study.

Deleting Four Residues Including Thr41 Does Not Affect β-Catenin Recognition by β-TrCP. β-TrCP binding to β-catenin requires phosphorylated Ser33 and Ser37 (*12*, *17*), thus providing a faithful readout for Ser33 and Ser37 phosphorylation status. We investigated the interaction between β-TrCP and the various deletion derivatives of β-catenin in 293T cells. As a negative control, we used β-catenin(S45A), a β-catenin mutant that harbors a Ser45 to Ala mutation and thus exhibits neither Ser45 phosphorylation nor subsequent phosphorylation on Thr41/Ser37/Ser33 (*12*). All β-catenin derivatives, the wild type (WT), S45A mutant, and the deletion mutants, were expressed at comparable levels in transfected 293T cells (Figure 2). As expected, the WT β-catenin readily co-immunoprecipitated β-TrCP (Figure 2, lane 3), whereas β-catenin S45A did not (Figure 2, lane 4). In contrast to the S45A mutant, all of the deletion derivatives, Δ38−41, Δ39−42, and Δ41−44, co-precipitated β-TrCP as robustly as the WT β-catenin (Figure 2, lanes 5−7). Conversely, these deletion derivatives, such as the WT β-catenin but not the S45A mutant, were coprecipitated by β-TrCP (Figure 2). These results indicated that Ser33 and Ser37 are properly phosphorylated in these β-catenin mutants lacking Thr41 and three surrounding residues.

Deleting Four Residues Including Thr41 Does Not Affect β-Catenin Degradation. We next examined whether these

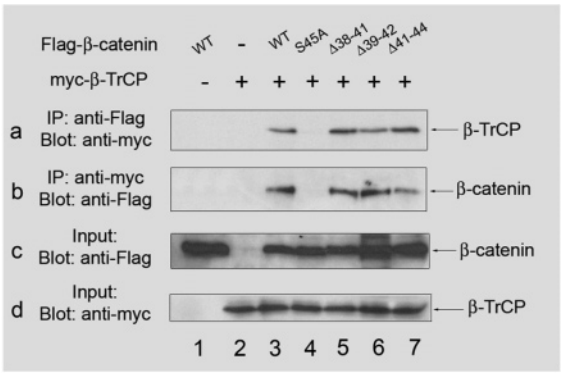

FIGURE 2: Deleting four residues including Thr41 of β-catenin does not affect its recognition by β-TrCP. Expression plasmids (1 μg each) for Flag-tagged β-catenin or indicated β-catenin derivatives and myc-tagged β-TrCP were cotransfected into 293T cells. Empty vectors were used as controls. The cell lysates were immunoprecipitated with an anti-Flag antibody, and the precipitates were immunoblotted with an anti-myc antibody (a). The cell lysates were also immunoprecipitated with the anti-myc antibody, and the precipitates were immunoblotted with the anti-Flag antibody (b). The same cell lysates were immunoblotted with the anti-Flag antibody (c) and the anti-myc antibody (d) to examine the protein expression levels.

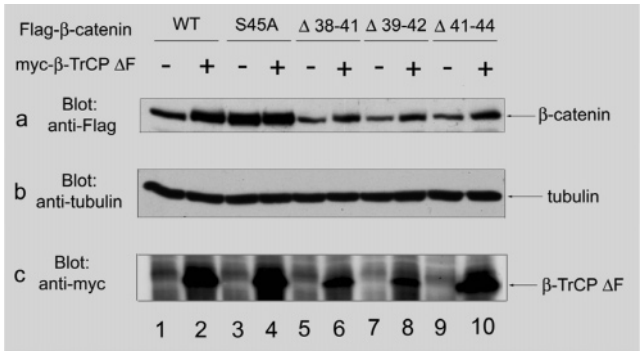

FIGURE 3: β-Catenin mutants lacking Thr41 and three surrounding residues are stabilized by a dominant negative mutant β-TrCPΔF. 0.05 μg of the expression plasmid for Flag-tagged β-catenin and indicated β-catenin mutants were cotransfected into 293T cells together with 1 μg of the plasmid for myc-tagged β-TrCPΔF or an empty vector. 48 h later cell lysates were immunoblotted with the anti-Flag antibody for β-catenin level (a), an anti-tubulin antibody as a loading control (b), or the anti-myc antibody (c) for the expression levels of β-TrCPΔF.

β-catenin deletion derivatives could be degraded via the β-TrCP-mediated ubiquitination pathway. We coexpressed these β-catenin mutants with a dominant-negative mutant β-TrCP, β-TrCPΔF (*18*). In β-TrCPΔF, the F-box motif is deleted, but the WD40-repeat domain that binds to Ser33 and Ser37 phosphorylated β-catenin is retained. β-TrCPΔF can compete with the wild-type β-TrCP for β-catenin (*18*). Indeed, expression of β-TrCPΔF effectively stabilized the WT β-catenin (Figure 3, compare lanes 1 and 2), presumably via interfering with the endogenous β-TrCP in these cells. In contrast, the β-catenin(S45A) mutant was stabilized at a high level with or without β-TrCPΔF (Figure 3, compare lanes 3 and 4), consistent with the observation that it does not bind to β-TrCP (Figure 2) and thus is not subjected to degradation. Importantly, all deletion derivatives, Δ38−41, Δ39−42, and Δ41−44, were stabilized by β-TrCPΔF (Figure 3, lanes 5−10). Thus these β-catenin deletion mutants without Thr41 (and three surrounding residues) are nonetheless properly degraded via the endogenous β-TrCP.

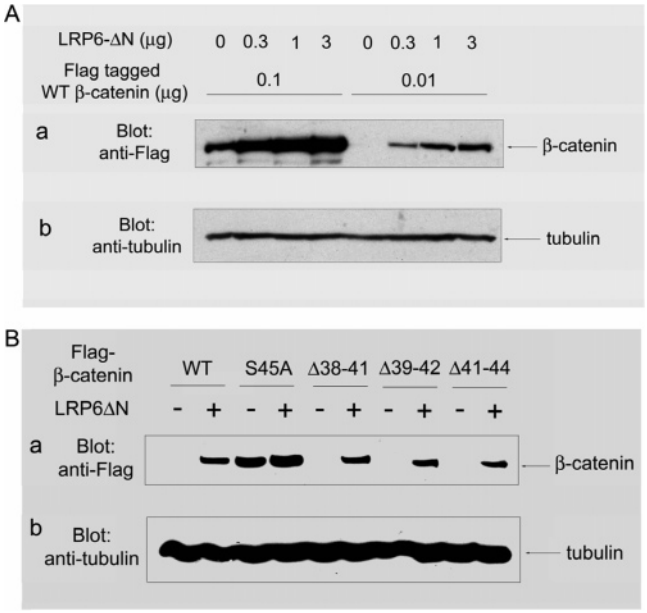

FIGURE 4: β-Catenin mutants lacking Thr41 and three surrounding residues are stabilized by a constitutively active Wnt coreceptor LRP6ΔN. (A) The WT β-catenin was stabilized by LRP6ΔN in a dose-dependent manner. Indicated amounts of expression vectors for VSVG-tagged LRP6ΔN and Flag-tagged WT β-catenin were cotransfected into 293T cells. After 48 h, cell lysates were immunoblotted with an anti-Flag antibody (a) for β-catenin level and an anti-tubulin antibody (b). (B) β-Catenin deletion mutants Δ38−41, Δ39−42, and Δ41−44 were stabilized by LRP6ΔN. 0.01 μg of expression vectors for Flag-tagged β-catenin and derivatives was cotransfected into 293T cells with 2 μg of either empty vector or VSVG-tagged LRP6ΔN. After 48 h, cytosolic extracts were immunoblotted with an anti-Flag antibody (a) and an anti-tubulin antibody (b).

β-Catenin Mutants Lacking Four Residues Including Thr41 Are Stabilized by Activated Wnt Coreceptor LRP6. We next asked whether these β-catenin mutants could be stabilized in response to the upstream Wnt signal. We employed a constitutively active Wnt coreceptor LRP6, LRP6ΔN, which others and we have shown fully activates Wnt/β-catenin signaling (*25*, *31*, *32*). We titrated the amount of transfected WT β-catenin cDNA so that β-catenin protein exhibits robust stabilization by LRP6ΔN in a dose-dependent manner (Figure 4A). We found that β-catenin deletion mutants, Δ38−41, Δ39−42, and Δ41−44, were all stabilized by LRP6-ΔN in a manner indistinguishable from that of the WT β-catenin (Figure 4B), In contrast, the β-catenin(S45A) mutant was constitutively stabilized at a high level and did not respond to LRP6ΔN (Figure 4B). Thus these β-catenin deletion mutants lacking Thr41 and three surrounding residues are fully responsive to the upstream Wnt signaling pathway.

Inserting or Deleting One Residue between Ser37 and Thr41 Prevents β-Catenin Recognition by β-TrCP and Degradation. In all β-catenin deletion mutants we tested above, the spacing between Ser37 and "Ser45" remained constant at four residues, in accordance with the consensus phosphorylation motif for GSK3. Under these conditions, Thr41 is dispensable for β-catenin phosphorylation and degradation. To further investigate whether this four-residue spacing is obligatory for GSK3 phosphorylation of β-catenin at Ser37 and Ser33, we made two additional β-catenin mutant derivatives, β-catenin(Ins-1), which harbors a glycine inser-

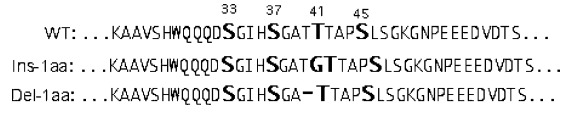

FIGURE 5: β-Catenin mutants with a single amino acid insertion (Ins-1) or deletion (Del-1) before Thr41. The amino acid sequences surrounding Ser33 to Ser45 are shown for the WT, Ins-1 (a glycine insertion before Thr41), and Del-1 (a threonine deletion before Thr41). Ser33, Ser37, Thr41, Ser45, inserted glycine, and the deletion are highlighted.

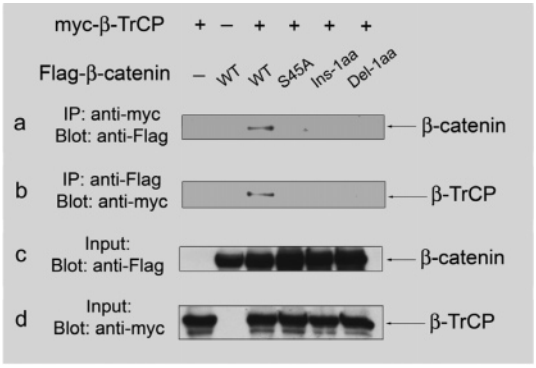

FIGURE 6: β-Catenin mutants with one amino acid insertion or deletion before Thr41 are not recognized by β-TrCP. 1 μg of expression vectors for Flag-tagged β-catenin and mutants was cotransfected into 293T cells together with 1 μg of the expression vector for myc-tagged β-TrCP or empty vector. After 48 h, the cell lysates were immunoprecipitated with an anti-myc antibody, and the precipitates were immunoblotted with an anti-Flag antibody (a). The cell lysates were also immunoprecipitated with an anti-Flag antibody, and the precipitates were immunoblotted with an anti-myc antibody (b). The cell lysates were also immunoblotted with the anti-Flag antibody (c) for β-catenin expression levels or with the anti-myc antibody (d) for the β-TrCP expression level.

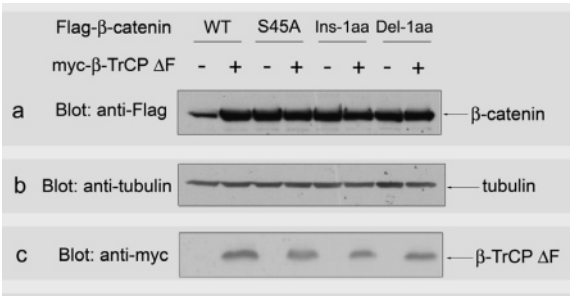

FIGURE 7: β-Catenin mutants with one amino acid insertion or deletion before Thr41 are not degradable by β-TrCP. 0.05 μg of expression vectors for Flag-tagged β-catenin or mutants was cotransfected into 293T cells together with 2 μg of the expression vector for myc-tagged β-TrCPΔF or an empty vector. After 48 h, the cell lysates were immunoblotted with an anti-Flag antibody (a), an anti-tubulin antibody (b), or an anti-myc antibody (c).

tion before Thr41, and β-catenin(Del-1), which has a single threonine deletion before Thr41 (Figure 5). Similar to β-catenin(S45A) and contrary to the WT β-catenin, neither β-catenin(Ins-1) nor β-catenin(Del-1) coprecipitated, or was coprecipitated with, β-TrCP (Figure 6), indicating phosphorylation at Ser33 and Ser37 in these two mutants did not occur. Thus changing the spacing between Thr41 and Ser37, by adding or deleting one amino acid residue, disrupts GSK3 phosphorylation of β-catenin.

We further examined whether β-catenin(Ins-1) or β-catenin(Del-1) could be degraded by the β-TrCP-mediated pathway. Contrary to the WT β-catenin, which was stabilized by β-TrCPΔF, β-catenin(Ins-1) and β-catenin(Del-1) behaved identically to β-catenin(S45A) and were maximally stabilized regardless of β-TrCPΔF (Figure 7). These results showed that neither β-catenin(Ins-1) nor β-catenin(Del-1) could be degraded via β-TrCP, fully consistent with the observation that these two mutants are not recognized by β-TrCP.

DISCUSSION

β-Catenin phosphorylation at Ser45, Thr41, Ser37, and Ser33 is essential for its degradation and is a central regulatory node in the canonical Wnt signaling pathway. Mutations of these Ser/Thr residues occur frequently in human cancers, highlighting their key roles in regulation of β-catenin stability for tissue homeostasis. Ser45 phosphorylation by CKIα primes and initiates Thr41, Ser37, and Ser33 phosphorylation by GSK3 (*12*−*14*), and phosphorylated Ser37 and Ser33 then provide the recognition site for β-TrCP (*12*, *17*) that mediates β-catenin degradation. While these studies have clarified the critical roles of Ser45, Ser37, and Ser33 in β-catenin phosphorylation and degradation, the role of Thr41, which is conserved from hydra to human and often mutated in cancers, remains less clear. To directly examine this issue, we deleted Thr41 and three surrounding residues from β-catenin such that these β-catenin mutant derivatives maintain the Ser-X-X-X-Ser spacing arrangements, a consensus motif for GSK3 phosphorylation. We found that these β-catenin mutants behave indistinguishably from the WT β-catenin; i.e., they demonstrate proper recognition by β-TrCP and thus normal Ser37 and Ser33 phosphorylation, are degraded by the β-TrCP-dependent degradation pathway, and, importantly, are stabilized by an activated Wnt coreceptor LRP6. Thus these β-catenin mutants lacking Thr41 are regulated properly both in the absence and in the presence of Wnt stimulation. We further demonstrated an obligatory requirement for the Ser-X-X-X-Ser consensus in GSK3 phosphorylation in vivo. β-Catenin mutants that harbor a single amino acid insertion or deletion in the Ser-X-X-X-Ser motif are not phosphorylated at Ser37 and Ser33 and thus not recognized by β-TrCP and not degraded. These results demonstrate that Thr41 functions primarily as a phosphorylation relay residue in processive β-catenin phosphorylation and do not seem to support the speculation that Thr41 adds an additional checkpoint after Ser45 to regulate β-catenin phosphorylation and degradation. Our study also predicts that any "spacing−shifting" mutations between Ser45 and Ser33 will lead to deregulation of β-catenin degradation and be tumorigenic.

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