Induction of Gsk3β-β-TrCP Interaction Is Required for Late Phase Stabilization of β-Catenin inCanonical Wnt Signaling*

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Background: Despite being a key component of the Wnt pathway, how Gsk3β is regulated in Wnt signaling remains elusive.

Results: Wnt stimulation induces Gsk3β-β-TrCP interaction and monoubiquitination of Gsk3β, leading to inhibition of β-catenin recruitment of β-TrCP.

Conclusion: β-Catenin stabilization in late Wnt signaling requires increased Gsk3β-β-TrCP interaction.

Significance: First evidence that Gsk3β is regulated by monoubiquitination in Wnt signaling.

A pivotal step in canonical Wnt signaling is Wnt-induced β-catenin stabilization. In the absence of Wnt, β-catenin is targeted for β-transducin repeats-containing proteins (β-TrCP)-mediated degradation due to phosphorylation by glycogen synthase kinase 3 (Gsk3). How canonical Wnt signaling regulates Gsk3 to inhibit β-catenin proteolysis remains largely elusive. This study reveals novel key molecular events in Wnt signaling: induction of Gsk3β ubiquitination and Gsk3β-β-TrCP binding. We found that Wnt stimulation induced prolonged monoubiquitination of Gsk3β and Gsk3β-β-TrCP interaction. Monoubiquitination did not cause Gsk3β degradation nor affects its enzymatic activity. Rather, increased monoubiquitination of Gsk3β/Gsk3β-β-TrCP association suppressed β-catenin recruitment of β-TrCP, leading to long-term inhibition of β-catenin ubiquitination and degradation.

The canonical Wnt (Wnt/β-catenin) signaling pathway plays a crucial role in development, tissue regeneration, stem cells, and leads to tumor formation when aberrantly activated (1). A key feature of the Wnt/β-catenin pathway is the regulated degradation of β-catenin by the β-catenin destruction complex consisting of glycogen synthase kinase 3 (Gsk3α and Gsk3β),2 casein kinase 1(CK1), adenomatous polyposis coli (APC), Axin, and β-catenin, with Axin being the scaffold protein that directly interacts with other core components of the destruction complex (2, 3). In the absence of Wnt, β-catenin is phosphorylated by Gsk3 on serine 33 and 37 and threonine 41 (which requires priming phosphorylation of β-catenin by CK1) (3); phosphorylation of β-catenin triggers its recruitment of ubiquitin E3 β-TrCP, causing its ubiquitination and proteasomal degradation (4, 5).

Many different models have been proposed to explain how Wnt signaling inhibits β-catenin degradation including: 1) Wnt induces rapid disruption of Axin/Gsk3 interactions, which diminishes β-catenin phosphorylation and causes initial stabilization of β-catenin (6). 2) Wnt abrogates β-TrCP recruitment to β-catenin and blocks β-catenin ubiquitination within the destruction complex (7). 3) Wnt induces membrane sequestration of Axin1/Gsk3 complex by binding Wnt co-receptor LRP5/6 (the low-density lipoprotein receptor-related protein 5/6) (8–10). 4) Wnt signaling inhibits Gsk3 phosphorylation of β-catenin via phosphorylated PPSSPXS motifs of Wnt coreceptor LRP6 (11, 12). 5) Wnt induces Axin dephosphorylation by protein phosphatase PP1 within the LRP6/Axin signaling complex. Dephosphorylation of Axin changes its conformation, leading to disassembly of destruction and signaling complexes (8). 6) Wnt induces Axin1 degradation, which contributes to chronic Wnt signaling (6, 13). 7) Wnt-induced sequestration of Gsk3 from the cytosol into multivesicular bodies (14) is also considered as possible mechanisms for long-term Wnt signaling, although a later study failed to confirm it (7). Overall, these models and others that are not elaborated here provide some mechanistic explanations for Wnt-induced stabilization of β-catenin; however, despite its pivotal role in the Wnt pathway, how Gsk3 is regulated by Wnt to achieve β-catenin stabilization remains a fundamental question in the field of Wnt signaling.

Post-translational modifications (PTMs) play central roles in creating a highly dynamic relay system that reads and responds to intracellular or environmental changes, by reversibly regulate protein functions, such as activity, stability, localization, and protein-protein interaction without requiring de novo protein synthesis (15). This study has investigated Wnt-induced β-catenin stabilization from an important but never explored angle-Gsk3β ubiquitination. We find that Wnt induces prolonged multi-monoubiquitination of Gsk3β and Gsk3β-β-TrCP binding. Induction of Gsk3β ubiquitination/Gsk3β-β-TrCP interaction inhibits β-catenin recruitment of β-TrCP, leading to inhibition of β-catenin ubiquitination. Together, our results have identified the induction of Gsk3β ubiquitination/Gsk3β-β-TrCP interaction as novel key molecular steps required for Wnt-induced chronic stabilization of β-catenin.

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The abbreviations used are: Gsk, glycogen synthase kinase; β-TrCP, β-transducin repeats-containing protein; CK, casein kinase; APC, adenomatous polyposis coli; CHX, cycloheximide.
Wnt Signaling Requires Increased Gsk3β-β-TrCP Binding

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids of hemagglutinin (HA)-Gsk3β and VSVG-LRP6 were obtained from Addgene. Plasmid expressing HA-tagged β-TrCP has been described previously (16). Gsk3β mutants containing lysine to arginine mutations were generated by PCR-directed mutagenesis. The Ubiquitin-Gsk3β fusion constructs were generated by fusion of ubiquitin to the N-terminal of Gsk3β (without first methionine) via a two-amino acid linker (Glu-Phe). To prevent ubiquitin fusion proteins function as ubiquitin or ubiquitin-like modifiers in the cells, the C-terminal diglycine of ubiquitin were deleted (ΔGG) in the fusion constructs. pcDNA3-Flag-ubiquitin was generated by inserting the ubiquitin coding sequence into pcDNA3-Flag, which was kindly provided by Dr. Jens Lykke-Andersen (University of California, San Diego). The sequence of shRNA oligonucleotides for Gsk3β (sense: 5′-GGACACAGAAGATTAAAGAT-3′) was reported previously (17). The annealed primers were ligated into pLKO.1 which was obtained from Addgene to construct lentiviral-based vector for Gsk3β knockdown. Four synonymous mutations were introduced into the shRNA targeting sequence of Gsk3β (sense: 5′-GGATACAGAGCTTAAAAC-3′) to generate shRNA-resistant Gsk3β. Plasmids for shRNA-resistant Gsk3β, and its mutants were generated by PCR-directed mutagenesis. All plasmid constructs were verified by DNA sequencing. pLKO.1-TRC control was obtained from Addgene. The mouse Gsk3α-specific shRNA in pLKO.1-puro was purchased from Sigma. siRNA oligonucleotides for β-TrCP1/2 have been described previously (18) and were purchased from Thermo Scientific. Control siRNA oligos were purchased from Santa Cruz Biotechnology.

Cell Culture and Production of Wnt3a-conditioned Medium—Human embryonic kidney (HEK) 293T cells and human colon cancer cell line SW480 cells were obtained from ATCC. Immortalized Gsk3β+/+ and Gsk3β−/− mouse embryonic fibroblasts (MEFs) were generously provided by James Woodgett, Ontario Cancer Institute, Canada. These cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS) in a 37 °C humidified incubator containing 5% CO2. Wnt3a-producing L cells and control L cells were obtained from ATCC and used for generating Wnt3a-conditioned medium (Wnt3a-CM) and control-conditioned medium (control-CM) according to ATCC’s instructions. In experiments involving Wnt stimulation, to ensure that cells exert a maximal response to Wnt, cells were maintained at about 40% (for MEFs) to 70% (HEK293T) confluent state before Wnt3a treatment.

Transient Transfection, RNA Interference, Lentivirus Production, and Infection—Plasmid and siRNA transient transfections were performed using PolyJet In Vitro DNA Transfection Reagent (SignaGen) according to the manufacturer’s instruction. The transfection efficiency for MEFs was about 60–70%. Gsk3β shRNA lentiviral particles were produced in HEK293T cells by transfection of the lentiviral vector expressing shRNA against Gsk3β with the third generation packaging systems (Addgene). The media containing viral particles were filtered through syringe filters and subsequently used to infect target cells. Cells lines stably expressing Gsk3β shRNA were established by puromycin selection.

Immunoblotting, Antibodies, and Reagents—Immunoblotting analysis was carried out using whole cell lysates. Anti-HA, anti-phospho-β-catenin (Ser-33/37/Thr-41), anti-ubiquitin, anti-Gsk3α, anti-β-TrCP, anti-Axin1, anti-Axin2, anti-α-tubulin, anti-LRP6, anti-phospho-LRP6 (Ser1490), and anti-Gsk3β (Cell Signaling); anti-ubiquitin (Dako); anti-FLAG M2 (Sigma); anti-ubiquitin and anti-β-catenin (BD Bioscience); anti-eIF4E, anti-Skp2 (Santa Cruz); Anti-Gsk3 recognizing Gsk3α/β was obtained from Stressgen/Enzo Life Sciences; Anti-Gsk3β for immunoprecipitation was purchased from Abcam and Bethyl Laboratories. The reagents used in this study were purchase from the indicated companies: MG-132 (Sigma) and cycloheximide (MP). SuperSignal West Pico Chemiluminescent Substrate and SuperSignal Western blot Enhancer (Thermo Scientific) were used to enhance western signal when needed.

Immunoprecipitation—Cells were washed with cold PBS twice and then lysed with M-PER buffer (Thermo) supplemented with protease inhibitor and 20 mM N-ethylmaleimide (NEM) (Sigma). The whole cell lysates were collected by centrifugation and pre-cleared with Protein G-Sepharose beads at 4 °C for 30 min. The cleared lysates were incubated with indicated antibody together with Protein G-Sepharose beads at 4 °C for 3–4 h. The immunoprecipitates were washed three times with IP lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 10% glycerol, separated by SDS-PAGE, and then immunoblotted with indicated antibodies. Input corresponded to 5% of the total lysates used for the IPs and was verified by immunoblotting.

In Vivo Ubiquitination Assay—Cells were washed with cold PBS and then lysed with IP lysis buffer containing protease inhibitor, 20 mM NEM and 1% SDS. 1% SDS was added to disassociate proteins complexed with Gsk3β. The lysates were then diluted with IP lysis buffer to bring the final SDS concentration to 0.1% and subjected to pre-clear with Protein G-Sepharose beads at 4 °C for 30 min. The cleared lysates were incubated with anti-Gsk3β, or EZView Red anti-HA, or anti-FLAG M2 affinity gel (Sigma) at 4 °C for 3–4 h. The immunoprecipitates were washed three times with RIPA buffer (Thermo), separated by SDS-PAGE, and then immunoblotted with indicated antibodies. Input corresponded to 5% of the total lysates used for the IPs and was verified by immunoblotting.

In Vitro Kinase Assay—Briefly, HEK293T cells were transfected with empty vector or plasmids expressing HA-tagged GSK3β or its mutants. Twenty-four hours after transfection, the cells were lysed with lysis buffer. The cell lysates were incubated with EZview Red anti-HA affinity gel (Sigma) at 4 °C for 3 h. The bead-bound immunoprecipitates were washed with lysis buffer for three times followed by a final kinase buffer wash, then incubated with full-length recombinant human CK1α (5 ng, SignalChem), recombinant full-length human β-catenin (200 ng, Abcam), 10 mM ATP, kinase buffer (Cell Signaling) in a total volume of 15 μl at 37 °C for 2 h. The reaction product was subjected to SDS-PAGE. Kinase activity was evaluated by immunoblotting with antibody recognizing phosphorylated β-catenin at Ser-33, Ser-37, and Thr-41.

In Vitro Binding Assay—HA-tagged Gsk3β and its mutants were generated using the TNT T7 quick-coupled transcription/translation system, and the binding reactions were performed at 37 °C for 2 h with indicated proteins in a reaction volume of 15 μl. The reaction products were subjected to SDS-PAGE and then immunoblotted with indicated antibodies.
translation kit (Promega). 15 μl out of 25 μl of the translation product was incubated with EZview Red anti-HA affinity gel (Sigma) for 1 h at 4 °C in M-PER buffer. The immunoprecipitates were extensively washed with lysis buffer and then incubated with 750 ng GST-β-TrCP (Novus) in binding buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, and 10% glycerol for 2 h. After washing with lysis buffer, beads were analyzed by SDS-PAGE, followed by immunoblotting.

Protein Stability Assay—HEK293T cells were transfected with vectors expressing wild type (wt) HA-Gsk3β or mutant proteins. 24 h after transfection, the cells were treated with or without 20 μg/ml cycloheximide (CHX) in control-CM or Wnt3a-CM for 1 h at 4 °C in M-PER buffer. The immunoprecipitation with EZview Red anti-HA affinity gel (Sigma). The reaction mixture was incubated at 37 °C for 1 h, and then subjected to SDS-PAGE followed by immunoblotting with indicated antibody. WT HA-Gsk3β and HA-Gsk3β-KKKK15/27/32/36RRRRR mutant proteins were synthesized using the TNT T7 quick-coupled transcription/translation kit (Promega). To purify in vitro translated HA-Gsk3β proteins, 5 μl out of 25 μl of the translation product were used for immunoprecipitation with EZview Red anti-HA affinity gel (Sigma). The immunoprecipitates were subjected to in vitro ubiquitination assay as described above. All experiments were performed independently at least three times.

RESULTS AND DISCUSSION

Wnt StimulationInduces Ubiquitination of Gsk3β—Gsk3β is an ubiquitination substrate (19). In HEK293T cells, the basal level of Gsk3β ubiquitination was low since ubiquitination of HA-Gsk3β could be detected by immunoblotting only when ubiquitin was overexpressed (Fig. 1A). Treatment of the Con8 rat mammary epithelial tumor cell with the dexamethasone induces ubiquitination of Gsk3β (19); we therefore tested whether Wnt stimulation could induce Gsk3β ubiquitination. Similar to the case of ectopically expressed HA-Gsk3β, the basal ubiquitination level of endogenous Gsk3β in HEK293T cells was barely detectable (Fig. 1B). Treatment of the cells with Wnt3a-CM, however, remarkably increased the level of ubiquitinated Gsk3β in a time course-dependent manner (Fig. 1B). Gsk3β is a shared pathway component, and only Axin-bound Gsk3 (<10% of cellular Gsk3) is engaged in the canonical Wnt pathway (20, 21). Based on this, we expected that less than 10% of cellular Gsk3β were ubiquitinated upon Wnt stimulation. The result of immunoblotting on immunoprecipitated samples indeed confirmed that only a small portion of cellular Gsk3β proteins were ubiquitinated (Fig. 1C).

Ubiquitination often triggers proteasomal degradation of the substrate. The accumulation of ubiquitinated Gsk3β upon Wnt stimulation could result from (1) Wnt-induced stabilization of ubiquitinated Gsk3β or (2) induction of Gsk3β ubiquitination. To discriminate between these two possibilities, we compared the levels of ubiquitinated Gsk3β in the absence and presence of proteasome inhibitor MG132. Treatment with MG132 did not affect the levels of total and ubiquitinated Gsk3β under Wnt "on" and "off" conditions (Fig. 1D), hence supporting the second possibility: induction of Gsk3β ubiquitination.

Next we characterized the ubiquitination sites in Gsk3β. Sequence analysis using online UbPred program revealed three putative ubiquitination sites in Gsk3β: lysines 15, 27, and 36. We mutated these and other lysine residues including lysines 74, 85, 86, 91, 94, 103, 122, 123, 292, and 349 to arginines (R), singly or in combination, and examined the ubiquitination of the mutants. We found that single lysine mutations slightly inhibited Gsk3β ubiquitination and a combination mutation of lysines 15, 27, 32, and 36 was required to substantially decrease Gsk3β ubiquitination (Fig. 1E). Simultaneous mutations of lysines 15, 27, 32, and 36 also greatly impaired Wnt-induced ubiquitination of Gsk3β (Fig. 1F). These results indicated that lysines 15, 27, 32, and 36 are ubiquitination sites in Gsk3β. It is also possible that these sites are required for Gsk3β ubiquitination by a mechanism other than being ubiquitin acceptor lysines (to be discussed in more detail later). Of note, all these lysine residues of Gsk3β are conserved across species including human, pig, mouse, rat, Xenopus, zebrafish, and Drosophila (Fig. 1G). Other lysine sites (i.e. lysines 74, 85, 86, 91, 94, 103, 122, 123, 292, and 349) did not appear to be involved in Gsk3β ubiquitination (Fig. 1H).

β-TrCP Is an Ubiquitin E3 Ligase Mediating Multi-monou- biquitination of Gsk3β—β-TrCP is the substrate recruitment module of the SCFβ-TrCP ubiquitin ligase supercomplex (4, 5, 22). Gsk3β is known to interact with FWD1 (F-box/WD40-repeat protein1), the mouse homologue of β-TrCP (5). Our co-immunoprecipitation assay confirmed that Gsk3β, but not elf4E (eukaryotic translation initiation factor 4E), bound to β-TrCP (Fig. 2A). We further found that siRNA knockdown of β-TrCP prevented Wnt-induced ubiquitination of endogenous Gsk3β (Fig. 2B) without affecting ubiquitination of mutant Gsk3β with lysines 15/27/32/36 mutated (Fig. 2C). These results raised a possibility that β-TrCP is an ubiquitin E3 ligase for Gsk3β. To test this, we performed a reconstituted in vitro ubiquitination assay using purified recombinant proteins: UbcH5a, UbcH5b, and UbcH7 as E2 conjugating enzymes and SCFβ-TrCP complex as the E3 ligase. The results showed that ubiquitination of Gsk3β occurred only when β-TrCP complex was added (Fig. 2D), hence proving that ubiquitination of Gsk3β is β-TrCP dependent. Recruitment of β-TrCP to its substrate, for example β-catenin, generally requires substrate phosphorylation (4, 5, 22). Consistent with this requirement, Gsk3β purified from tissue or baculovirus-infected insect cells is indeed highly phosphorylated (23).

It appeared that most of the PTM found in endogenous Gsk3β was monoubiquitination, whereas multi-monoubiquitination of Gsk3β occurred when manipulating the system in vitro or via overexpression of ubiquitin. To validate
FIGURE 1. Ubiquitination of Gsk3β is induced by Wnt. HEK293T cells were used for the studies. A, the cells were transfected with empty vector or plasmids encoding the indicated proteins and harvested 24 h after transfection. The whole cell lysates were used for immunoblotting (IB) with anti-HA. B–C, the cells were treated with Wnt3a-CM for the indicated time. Ubiquitination of Gsk3β was examined by immunoprecipitation (IP) with anti-Gsk3β followed by IB with anti-ubiquitin (B) or anti-Gsk3β (C). D, the cells were treated with or without MG132 (10 μM) in the presence of control-CM or Wnt3a-CM for 6 h. The whole cell lysates were used for IP and IB. E, the cells were transfected with empty vector or plasmid encoding the indicated proteins. Ubiquitination of Gsk3β was evaluated by IP using anti-Flag followed by IB with anti-HA. The band intensity was analyzed using the UNSCAN-IT gel-graph digitizing software. F, the cells were transfected with indicated plasmids and treated with or without Wnt3a-CM for 6 h. Ubiquitination of wt HA-Gsk3β and HA-Gsk3β-KKKK15/27/32/36RRRR was examined by IP with anti-Flag followed by IB with anti-HA. G, alignment of the amino acid region containing ubiquitination sites of Gsk3β in different species. H, ubiquitination of wt-HA-Gsk3β and HA-Gsk3β mutants was examined as described above.
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monoubiquitination of Gsk3β, we examined whether using wild type ubiquitin or methylated ubiquitin exerts the same or distinct ubiquitination patterns of Gsk3β. The in vitro ubiquitination assay result showed that using Flag-ubiquitin (9.5 kDa) and methylated ubiquitin (8.5 kDa) caused a slight difference in band size in lane 2 and lane 3, but the overall Gsk3β ubiquitination patterns in these two lanes were very similar (Fig. 2E), hence confirming monoubiquitination of Gsk3β. The size of the highest ubiquitinated bands further suggested that about six lysines within Gsk3β were modified by single ubiquitin moieties (Fig. 2E), which could explain why mutation of four lysines on Gsk3β substantially, but not completely, abrogated in vivo monoubiquitination of Gsk3β (Fig. 1E). Interestingly, mutation of lysines 15/27/32/36 appeared to be sufficient to abrogate in vitro ubiquitination of Gsk3β (Fig. 2F). This seemly discrepancy between in vivo and in vitro might result from different ubiquitination efficiencies in different ubiquitination systems.

Ubiquitination of Gsk3β Does Not Alter Gsk3β Stability and Enzymatic Activity—Protein functions are often regulated by PTMs (15). We examined whether Gsk3β stability and activity are regulated by ubiquitination. MG132 treatment did not change the levels of ubiquitinated Gsk3β (Fig. 1D), suggesting that ubiquitination does not trigger Gsk3β degradation. Consistent with it, the cycloheximide-chase assay results showed that WT HA-Gsk3β protein and HA-Gsk3β-ΔKKKK15/27/32/36RRRR mutant protein exerted similar patterns of decay following addition and continued incubation with cycloheximide in control-CM or Wnt3a-CM (Fig. 3A), implying that lack of ubiquitination does not alter Gsk3β half-life. Next we tested whether ubiquitination regulates Gsk3β enzymatic activity. To ensure that the result seen with Gsk3β-ΔKKKK15/27/32/36RRRR mutant was caused by the loss of ubiquitination but not by mutation-induced structural change of the protein, we generated ubiquitin-Gsk3β fusion protein that mimics monoubiquitinated Gsk3β by fusing one copy of the ubiquitin sequence to the N terminus of HA-Gsk3β and the mutants (Fig. 3B). The ubiquitin fusion protein approach, complementary to the ubiquitination mutant approach, has been widely used to validate the functional significance of substrate ubiquitination, for example, the role of monoubiquitination in control of p53 fate (24). shRNA knockdown of β-TrCP did not affect ubiquitin fused to Gsk3β-ΔKKKK15/27/32/36RRRR (Fig. 3C). Using the fusion protein as a tool, we performed an in vitro kinase assay. The results showed that mutant Gsk3β proteins, both KKKK15/27/32/36RRRR and ub-KKKK15/27/32/36RRRR, phosphorylated β-catenin in vitro as efficiently as WT Gsk3β (Fig. 3D). Gsk3β phosphorylates LRP6 at Ser-1490 (10). Consistent with the in vitro result, we found that overexpression of WT Gsk3β and the mutants exerted similar effects on phosphorylation of LRP6 at Ser-1490 in HEK293T cells (Fig. 3E). Together, these results indicated that the mutant proteins have proper native folding or conformation and that Gsk3β enzymatic activity is not altered by its ubiquitination status.
Ubiquitination of Gsk3β/Gsk3β-β-TrCP Binding Inhibits β-TrCP Recruitment to β-Catenin—In the absence of Wnt, phosphorylation of β-catenin by Gsk3 triggers its ubiquitination and degradation. Recent studies show that phosphorylation of β-catenin by Gsk3 undergoes two-phase regulation upon Wnt stimulation (8, 25). We also found that Wnt3a treatment caused initial inhibition (up to 1–2 h after Wnt stimulation) and subsequent restoration of β-catenin phosphorylation (Fig. 4A). Inhibition of β-catenin phosphorylation contributes to Wnt-induced stabilization and accumulation of β-catenin. However, suppression of β-TrCP recruitment to the phosphorylated β-catenin also plays an important role in Wnt-induced chronic inhibition of ubiquitination and degradation of β-catenin (7). Our co-immunoprecipitation experiments confirmed that Wnt stimulation abrogated β-catenin-β-TrCP binding (Fig. 4B). We then asked three questions: (1) Is Wnt induction of Gsk3β ubiquitination mediated by the increased Gsk3β-β-TrCP association? (2) If so, what is the underlying mechanism responsible for the induced Gsk3β-β-TrCP binding? (3) Since β-catenin and Gsk3β share the same ubiquitin E3, does the increased Gsk3β-β-TrCP binding inhibit β-catenin recruitment of β-TrCP?

The co-immunoprecipitation results showed that Gsk3β-β-TrCP binding was continuously increased by Wnt stimulation (Fig. 4C). Wnt-induction of Gsk3β-β-TrCP interaction was further confirmed via the results of the reciprocal IP and IB (Fig. 4D). It is generally believed that β-TrCP recognizes its targets in a phosphorylation-dependent manner through a conventional recognition site DpSGxxpS or through an unconventional recognition site (26, 27). We speculated that Wnt induces Gsk3β-β-TrCP interaction through two mechanisms: (i) Wnt induces Gsk3β phosphorylation at particular site(s), which triggers the recruitment of β-TrCP to Gsk3β and (ii) ubiquitination of Gsk3β in turn further increases β-TrCP binding to Gsk3β. Identification of the putative phosphorylation sites is beyond the scope of this study; therefore we have focused on the second mechanism using Gsk3β mutants as an investigating tool.

To avoid potential interference or competition from endogenous Gsk3β, we generated Gsk3β knockdown HEK293T cells (Fig. 4E), and then reconstituted the knockdown cells with HA-Gsk3β proteins. We found that in HEK293T cells, the lack of Gsk3β ubiquitination, disrupted β-TrCP binding to HA-Gsk3β KKKK15/27/32/36RRRR (Fig. 4F), without inhibiting Gsk3β/Axin1 interaction (Fig. 4G). Mimicking Gsk3β ubiquitination by fusing an ubiquitin to HA-Gsk3β KKKK15/27/32/36RRRR, remarkably enhanced β-TrCP, but not Skp2 (S-phase kinase-associated protein 2, the substrate recruitment module of the SCF-Skp2 ubiquitin ligase supercomplex), binding to HA-Ub-Gsk3β KKKK15/27/32/36RRRR (Fig. 4F). The increased interaction between HA-Ub-Gsk3β fusion protein and β-TrCP could be mediated by ubiquitin per se or ubiquitination of Gsk3β. However, HA-ubiquitin did not co-precipitate with β-TrCP (Fig. 4H). Together, these results support the notion that ubiquitination of Gsk3β, but not ubiquitin per se, increased Gsk3β affinity for β-TrCP.
We next tested if Gsk3β directly binds to β-TrCP by mixing purified human recombinant β-TrCP with in vitro synthesized wt Gsk3β or Gsk3β mutant proteins. The in vitro binding results (Fig. 4I) mirrored the in vivo binding result (Fig. 4F), confirming a direct binding between Gsk3β and β-TrCP. The in vitro binding result also raised a question why the affinity of in vitro synthesized wt HA-Gsk3β for GST-β-TrCP was substantially higher than that of HA-Gsk3β-KKKK15/27/32/36RRRR mutant protein? It is well documented that the in vitro generated proteins using rabbit reticulocyte lysates are post-translationally modified similarly as cellular proteins (28), so a potential explanation for this is that the in vitro translated Gsk3β proteins might be modified similarly as cellular Gsk3β. Consistent with this notion, Western blot analysis showed that HA-Ub-Gsk3β KKKK15/27/32/36RRRR had two bands (Fig. 4I). As indicated by the results in Fig. 3B, the upper band represented ubiquitinated HA-Ub-Gsk3β. Consistent with this notion, Western blot analysis showed upper smeared bands after prolonged exposure (data not shown), the results of IP with anti-HA (Gsk3β) followed by IB with anti-ubiquitin were inconclusive (data not shown), possibly due to that only a small proportion of
the in vitro synthesized proteins are modified. Based on this information, another possible explanation for the lack of HA-Ub-Gsk3β/H9252 KKKK15/27/32/36RRRR-β-TrCP interaction is that lysines 15, 27, 32, and 36 on Gsk3β are required for β-TrCP binding. However, given that fusing an ubiquitin to HA-Gsk3β/KKKK15/27/32/36RRRR mutant protein not only restored but also further enhanced Gsk3β/β-TrCP interaction (Fig. 4, F and D), we tend to believe that these sites are ubiquitination sites rather than β-TrCP binding sites: (1) fusion protein approach is widely used to investigate the role of post-translational modifications; and (2) β-TrCP binds to its targets through a phosphorylated motif (26, 27). Unable to definitely distinguish cause and effect between Wnt-induced Gsk3β ubiquitination and Gsk3β/β-TrCP interaction, we collectively describe these events as “Gsk3β ubiquitination/Gsk3β/β-TrCP binding.”

Wnt abrogates β-TrCP recruitment to the phosphorylated β-catenin and blocks β-catenin ubiquitination within the
degradation complex (7), but the underlying mechanisms remain elusive. We hypothesized that the increased Gsk3β-β-TrCP binding inhibits β-TrCP recruitment to β-catenin. The results showed that β-TrCP recruitment to and ubiquitination of the phosphorylated β-catenin were substantially higher in HA-Gsk3β-KKKK15/27/32/36RRRR cells than in HA-Gsk3β cells, whereas mimicking ubiquitination or restoring Gsk3β-β-TrCP binding completely prevented mutation of KKKK15/27/32/36-mediated effects (Fig. 4J), despite Wnt-induced β-TrCP expression (29) (Fig. 4K), indicating that ubiquitination of Gsk3β/Gsk3β-β-TrCP binding suppresses β-TrCP recruitment to the phosphorylated β-catenin.

The dominant paradigm of β-TrCP-mediated ubiquitination is degradation of the ubiquitinated substrate (22). To the best of our knowledge, this is the first evidence that ubiquitination does not lead to substrate degradation. Strikingly, induction of substrate ubiquitination and substrate-β-TrCP binding is used by the cells as a novel mechanism to inhibit ubiquitination and degradation of another substrate within the same complex.

Ubiquitination of Gsk3β/Gsk3β-β-TrCP Interaction Is Required for Late Wnt Signaling—We next validated the role of Gsk3β ubiquitination/Gsk3β-β-TrCP binding in Wnt/β-catenin signaling. We first conducted course experiments (up to 16 h after Wnt3a simulation) in which levels of ubiquitinated Gsk3β, phosphorylated β-catenin, and overall levels of β-catenin were determined in parallel in wt MEFs. Consistent with the result showing in Fig. 1B, we found that Wnt3a treatment induced Gsk3β ubiquitination in MEFs (Fig. 5A). Induction of monoubiquitination of Gsk3β did not correlate with Wnt-induced early phase of β-catenin accumulation; however, it coincided with Wnt-induced late phase (about 2 h after Wnt3a treatment) stabilization of β-catenin (Fig. 5A). This result indicated that Gsk3β ubiquitination is involved in chronic (but not acute) Wnt signaling.

To validate the role of Gsk3β ubiquitination/Gsk3β-β-TrCP binding in late Wnt signaling, we compared the effects of wt and mutant Gsk3β proteins on chronic β-catenin stabilization (6 h after Wnt3a stimulation). Since Gsk3α and Gsk3β are functionally redundant in Wnt signaling (30). To avoid compensation from Gsk3α, we generated Gsk3β KO/Gsk3α knockout (KD) MEF cell line and used Gsk3β KO/Gsk3α KD MEFs for the following experiments. Gsk3β knock-out and Gsk3α knockdown increased the basal level of β-catenin (Fig. 5B), presumably mediated by the inhibition of β-catenin phosphorylation due to the lack of Gsk3 (30). Reconstitution of the Gsk3β KO/Gsk3α MEFs with HA-Gsk3β-KKKK15/27/32/36RRRR mutant protein, however, inhibited Wnt-induced accumulation of β-catenin (Fig. 5C; compare lanes 11–12 to lanes 9–10). In contrast, mimicking Gsk3β ubiquitination and/or restoring Gsk3β-β-TrCP binding restored Wnt-induced β-catenin stabilization in HA-UB-Gsk3β-KKKK15/27/32/36RRRR MEFs (Fig. 5C; compare lanes 13–14 to lanes 9–10). The results of detailed time course studies showed that HA-Gsk3β, HA-Gsk3β-KKKK15/27/32/36RRRR mutant and HA-UB-Gsk3β-KKKK15/27/32/36RRRR had a similar effect on Wnt-induced regulation of β-catenin phosphorylation by Gsk3 (Fig. 5, D–F). However, Wnt-induced late phase accumulation of β-catenin correlated with the ubiquitination status of Gsk3β; while HA-Gsk3β MEFs (Fig. 5D) and HA-UB-Gsk3β-KKKK15/27/32/36RRRR MEFs (Fig. 5F) behaved like wt MEFs (Fig. 5A), Wnt-induced stabilization of β-catenin was inhibited in HA-Gsk3β-KKKK15/27/32/36RRRR MEFs (Fig. 5E). Together, these results indicated that the lack of Gsk3β ubiquitination/Gsk3β-β-TrCP binding inhibits β-catenin accumulation, and mimicking Gsk3β ubiquitination or increasing Gsk3β-β-TrCP binding restores Wnt-induced chronic β-catenin stabilization.

In addition to MEFs, we also tested the role of Gsk3β ubiquitination/Gsk3β-β-TrCP binding in human colorectal cancer cells. Colon cancer cell lines including SW480 cells are Wnt autocrine cell lines (31). In Gsk3β knockdown SW480 cells (we knocked down endogenous Gsk3β to minimize interference from wild type Gsk3β when studying the effect of Gsk3β mutants), add back of HA-Gsk3β-KKKK15/27/32/36RRRR mutant protein reduced cellular levels of β-catenin and two Wnt/β-catenin pathway downstream targets: cyclin D1 (32) and Axin2 (33), add back of the mutant protein mimicking ubiquitinated Gsk3β exerted opposite effects (Fig. 5G). Collectively, these results indicate that ubiquitination of Gsk3β/Gsk3β-β-TrCP binding is a determining factor in the activation of late Wnt signaling.

Mutations of APC gene occur in 85% of human colorectal cancer (34). The current study shows that ubiquitination of Gsk3β regulates Wnt/β-catenin signaling in HEK293T cells and MEFs (with intact APC protein) and SW480 cells (bearing truncating APC mutations), implying that regulation of Wnt signaling by Gsk3β ubiquitination is independent of APC status. Our data suggest that ubiquitination of Gsk3β provides a platform for non-Wnt pathway components, directly or indirectly, to be involved in the regulation of Wnt signaling despite the loss of functional APC. Our finding that Wnt signaling requires Gsk3β ubiquitination/Gsk3β-β-TrCP interaction could have far-reaching implications for identifying novel approaches for targeting Wnt signaling regardless of the status of APC protein.

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