ISCI, Volume *6*

Supplemental Information

Understanding How Wnt Influences Destruction Complex Activity and β-Catenin Dynamics

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Supplementary Information:

Transparent Methods:

Mammalian Cell Culture and treatment with Wnt-3A and MG132

Human embryonic kidney (HEK293T) cells were purchased from the American Type Culture Collection (ATCC CRL-11268) and cultured in Dulbecco's Modified Eagle Medium (High-glucose DMEM; Gibco 11995065) supplemented with 10% HI-FBS (Heat-Inactivated Fetal Bovine Serum; Gibco 10438018) and 1% penicillin-streptomycin (Gibco) (hereon referred to as D-10 media) in a humidified incubator at 37°C with 5% CO_2. Cultured mammalian cells were treated with recombinant human Wnt-3A (R&D Systems; 5036-WN) at a final concentration of 750-1000 ng/ml for the indicated time periods. Proteasomal inhibitor MG132 (Sigma-Aldrich; M7449) treatment was done at a working concentration of 20 μM for the time periods mentioned in the article.

Antibodies

The following antibodies were used for immunoblotting and immunoprecipitation; anti-β-catenin (BD Transduction Laboratories; #610153), anti-phospho-β-catenin antibody (Ser33/37/Thr41) (Cell Signaling Technology; #9561), anti-phospho-β-catenin antibody (Ser45) (Cell Signaling Technology; #9564), anti-APC (Cell signaling Technology, #2504), anti-APC (Abcam; #ab15270), anti-Axin1 (Cell Signaling Technology; #2087), monoclonal rabbit IgG isotype control (Abcam; #ab172730), anti-LRP6 (C5C7) antibody (Cell Signaling Technology; #2560), anti-α-tubulin (Abcam; #ab52866), anti-Histone H3 (Abcam; #ab1791), anti-biotin (Abcam; #ab53494) and anti-GAPDH (Abcam; #ab8245).

Harvesting cells and preparation of whole-cell lysates and cytoplasmic and nuclear extracts for time series experiments

HEK293T cells were cultured in 6-well tissue culture plates (Corning) at an initial concentration of 1.5 million cells per well and allowed to grow 80-90% confluent after approximately 36-48 h. After experimentation, the cells were washed in ice-cold DPBS (Corning) twice and lysed in a lysis buffer of DPBS containing 0.5% digitonin (pH 7.5) (Kim *et al.*). The cells were incubated on ice for 30-45 min for complete lysis and then harvested using cell scrapers. The residual debris in the lysate was spun down after centrifugation at 13,000 g for 15 min. The supernatant was used as the whole-cell lysate for further analysis.

To obtain the cytoplasmic and nuclear extract of HEK293T cells, the above supernatant was incubated with Concanavalin A-Sepharose 4B (GE Healthcare) beads for 60 min at 4°C. After incubation, the beads were spun down at 3000g for 5 mins. The cytoplasmic and nuclear fractions of the lysates were in the supernatant and the membrane-associated proteins were bound to the Concanavalin A beads. The whole-cell lysates and cytoplasmic and nuclear fractions of the lysates were denatured after the addition of LDS Sampling Buffer (Thermo Scientific) at 100°C for 10-15 mins and then processed for immunoblotting (western blots).

BONCAT and detection of newly synthesized protein

Methionine-free, cystine-free Dulbecco's Modified Eagle Medium (DMEM) containing 4 mM of L-Azidohomoalanine or L-AHA (Anaspec; #AS-63669) was used for the unnatural amino acid tagging experiments. HEK293T cells were cultured in 15 cm tissue culture dishes (Corning) at an initial concentration of 15 million cells per dish for 36-48 hours in D-10 media. After the cells were treated with Wnt-3A for 0.5 hours, the D-10 media was removed and the cells were washed twice with DPBS followed by one wash with methionine-free, cystine-free Dulbecco's Modified

Eagle Medium (DMEM). The cells then treated with L-AHA containing DMEM (containing 20 μM MG-132 and Wnt-3A as mentioned in the article) for 2.5 hours. The cells were then washed twice with ice-cold DPBS (Corning) and lysed using 2% SDS in DPBS containing phosphatase (Sigma-Aldrich) and protease inhibitor (Thermo Scientific) cocktails at concentrations prescribed by the manufacturer(s). The lysates were subsequently denatured by boiling at 100°C for 15 mins. To reduce the viscosity of the protein lysates, the samples were treated with benzonase nuclease (Sigma Aldrich; #E1014) at 37°C for 30 mins with constant mixing. 10 mg of total processed lysates were reacted with 150 μM DBCO-sulfo-biotin for 3-5 hours at room temperature. The reaction was quenched with excess L-AHA and passed through PD-10 (GE Healthcare) desalting columns to remove unreacted DBCO-sulfo-biotin and other low molecular weight compounds. The biotin-tagged proteins were then quantified using a semi-quantitative dot-blot (as per Dieterich *et al.*). The biotin-tagged proteins were finally extracted by incubating with Streptavidin-agarose beads (Thermo Scientific) for 2 hours at room temperature. Streptavidin beads were then washed with 2% SDS in PBS 5-6 times to remove the non-biotin tagged proteins. The protein mixture was then denatured using the LDS Sample Loading Buffer (Thermo Scientific) at 100°C for 10-15 min and loaded onto 4-12% Bis-Tris Gels for further analysis.

Co-Immunoprecipitation (Co-IP)

HEK293T cells were cultured in 15-cm tissue culture dishes (Corning) at an initial concentration of 15 million cells per dish and incubated in D-10 media for approximately 36-48 hours till 80% confluency. After experimentation, the cells were washed in ice-cold DPBS (Corning) twice and lysed in ~2 ml of lysis buffer containing 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol (for anti-APC IP) or 150 mM NaCl, 30 mM Tris (pH 7.5), 1 mM EDTA, 1% Triton X-100, 10% Glycerol (for anti-Axin1 IP). Lysis buffers were supplemented

with 0.1 mM PMSF, 0.5 mM DTT, phosphatase inhibitor cocktails 2 &3 (at 1:100 dilution) (Sigma) and Halt protease inhibitor cocktail (at 1:100 dilution) (Thermo Scientific). The cells were then incubated on ice for 30-45 mins, harvested and spun down at speeds mentioned earlier. After being spun down at 13,000 g for 15 mins, cell lysates were pre-cleared by incubating with Rabbit IgG isotype control antibody (Abcam, #ab172730) for 4 hours followed by Pierce Protein A Magnetic beads (Thermo Scientific) for 2 hours at 4°C. A magnetic rack (MagnaRack; Thermo Scientific) was then used to separate out the magnetic beads and the supernatant was then incubated overnight with the appropriate antibody at 4°C with constant mixing. The lysate was then incubated in Protein A magnetic beads for 2 hours at 4°C with constant mixing. The magnetic beads were then separated using the MagnaRack, washed 5 times with TBS containing 0.1% Tween-20 and then by 1x TBS. The beads were then denatured in 2x LDS Sampling Buffer with 2-mercaptoethanol by heating at 95-100°C for 15 mins. The magnetic beads were again separated and the supernatant containing the immunoprecipitated proteins were analyzed using immunoblotting.

SDS-PAGE Gel Electrophoresis and Immunoblotting

Proteins in the lysates were resolved using SDS-PAGE in 3-8% Tris-Acetate (for detecting APC) and 4-12% Bis-Tris Polyacrylamide gels (for all other proteins) in OWL P8DS (Thermo Scientific). The proteins were then transferred to a nitrocellulose membrane using electrophoretic transfer in the Trans-Blot Turbo Transfer System (Bio-Rad).

For immunoblotting, the membrane was blocked in a blocking buffer (TBS, 0.1% Tween-20, 5% non-fat dry milk or BSA; per antibody manufacturer's recommendation) for 1 hour at room temperature. The membrane was then incubated overnight at 4°C in primary antibody diluted in 5% BSA in TBST (TBS, 0.1% Tween-20). The dilution used for different antibodies was

optimized for individual applications, using the manufacturer's recommendation as the starting point. After the primary antibody incubation, the membrane was washed three times with TBST for 5 min each and then incubated in secondary antibody diluted in the blocking buffer for 1 hour at room temperature. For total β-catenin detection, an HRP-conjugated anti-mouse antibody (Jackson Immunoresearch) was used at a dilution of 1:10000. In all other cases, an HRP-conjugated anti-rabbit antibody (Thermo Scientific) was used at a dilution of 1:3000-1:5000. The protein bands in the membrane were then detected in a ChemiDoc MP Imaging system (Bio-Rad) using the SuperSignal West Femto Maximum Sensitivity Substrate. Quantitation of the protein concentration from the detected protein bands was carried out by using the analysis tools provided in the Image Lab Software (Bio-Rad) and validated using the ImageJ software in the previously ascertained linear dynamic range of concentrations for respective proteins.

Modeling β-catenin dynamics

B_0, B_1, B_2, and B_3 represent unphosphorylated β-catenin, CK1-phosphorylated β-catenin, GSK3-phosphorylated β-catenin, and ubiquitylated β-catenin, respectively(Hernandez et al., 2012) respectively. S represent the rate of synthesis of β-catenin, $[B_i]$ denote concentrations (i = 0, 1, 2, 3), and k_{deg} and $k_{j,}$ (j = ±1, ±2, ±3) denote rate constants. The phosphorylation and degradation of β-catenin is modeled using the following dynamical equations (Hernandez et al., 2012):

$$\frac{d[B_0]}{dt} = S - k_1[B_0] + k_{-1}[B_1] \quad \text{(S1)}$$

$$\frac{d[B_1]}{dt} = k_1[B_0] - (k_2 + k_{-1})[B_1] + k_{-2}[B_2] \quad \text{(S2)}$$

$$\frac{d[B_2]}{dt} = k_2[B_1] - (k_3 + k_{-2})[B_2] + k_{-3}[B_3] \quad \text{(S3)}$$

$$\frac{d[B_3]}{dt} = k_3 B_2 - (k_{deg} + k_{-3})[B_3] \quad \text{(S4)}$$

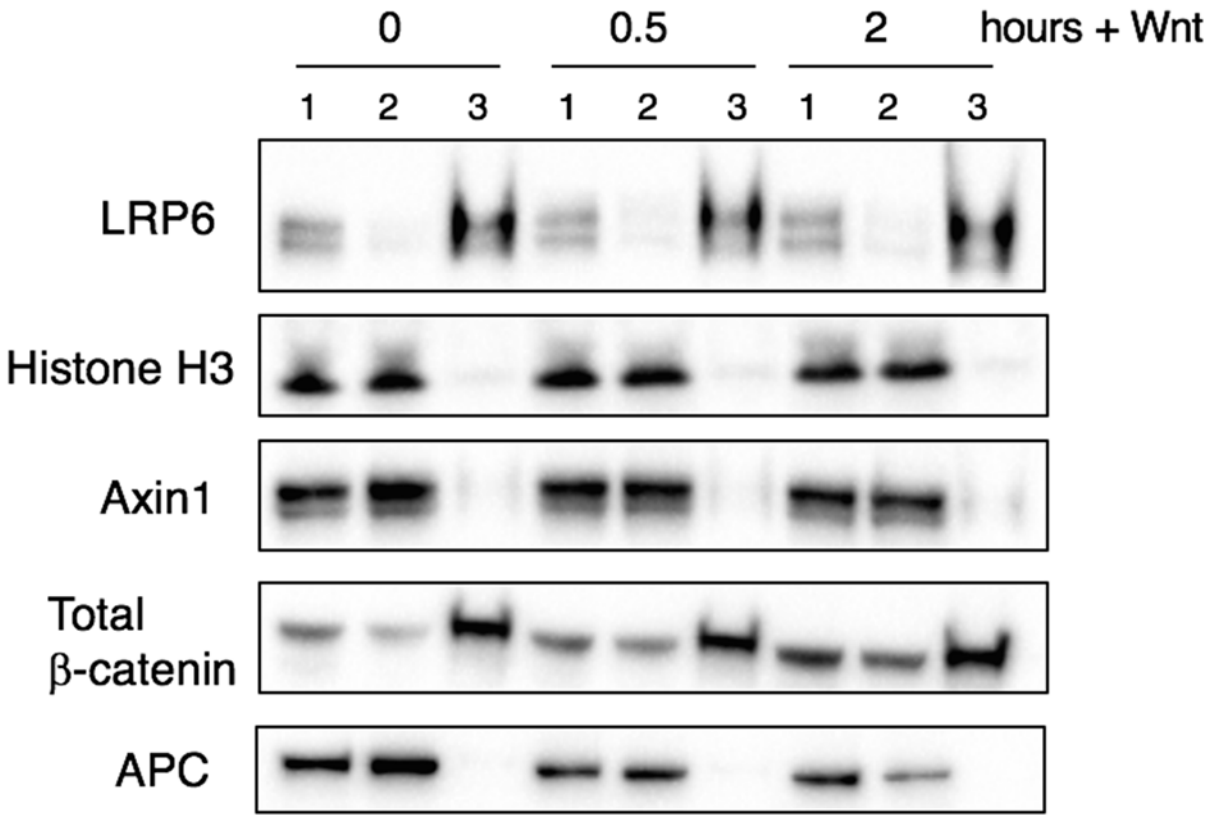

Figure S1, related to Fig. 1. Characterization of protein fractions after pull-down using Concanavalin A-Sepharose 4B beads at different time points following Wnt-3A stimulation. Within each time point, Lane 1 shows the protein in whole cell lysates, Lane 2 shows the flow-through after treatment with Concanavalin A-Sepharose 4B beads, and Lane 3 shows proteins eluted from the beads.

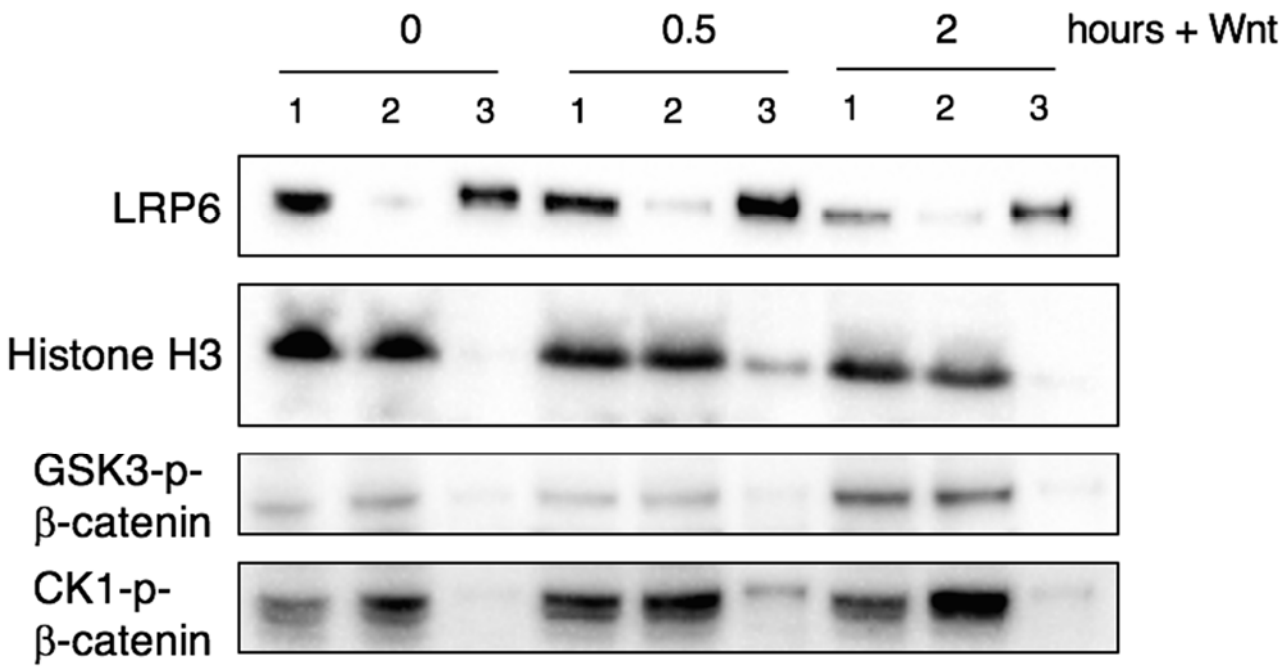

Figure S2, related to Fig. 2. Characterization of protein fractions after pull-down using Concanavalin A-Sepharose 4B beads at different time points following Wnt-3A stimulation. Within each time point, Lane 1 shows the protein in whole cell lysates, Lane 2 shows the flow-through after treatment with Concanavalin A-Sepharose 4B beads, and Lane 3 shows proteins eluted from the beads.

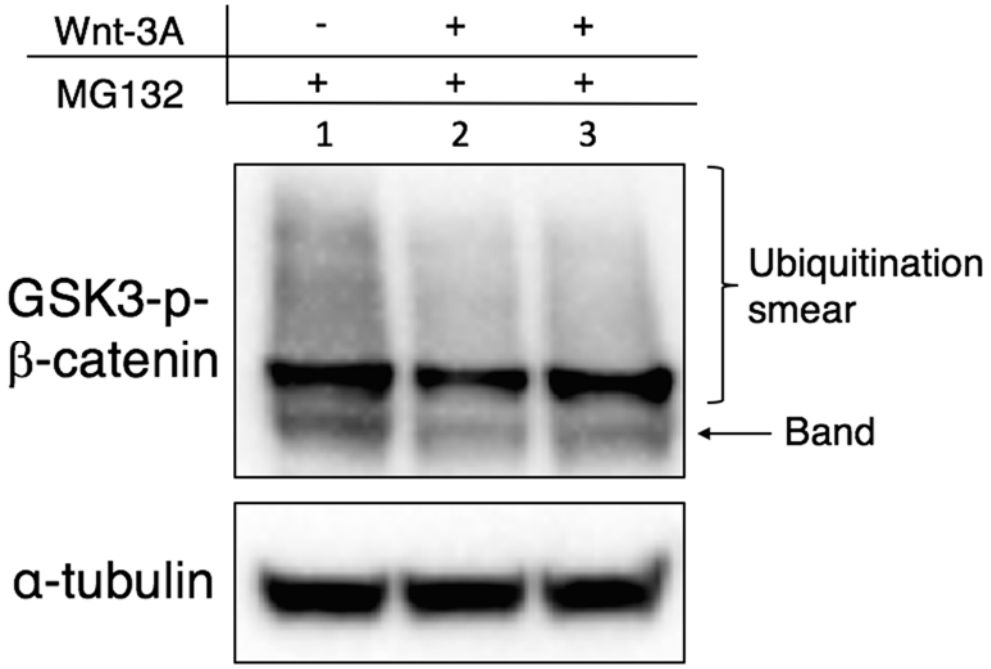

Figure S3, related to Fig. 3. Ubiquitinated GSK3-p-β-catenin levels in presence of Wnt signaling. Lane 1 and Lane 2 shows ubiquitinated GSK3-p-β-catenin levels in absence and presence of Wnt-3A respectively. The protein loading in Lane 3 was adjusted to compare ubiquitination levels for the same amount of GSK3-p-β-catenin in absence and presence of Wnt-3A respectively.