Supporting Information

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SI Text

SI Results

Overexpression of Nucleolar Phosphoprotein 140 Reduced the Stability of Procaspase-2 and β-Catenin. Procaspase-2 and β-catenin are substrates of casein kinase 2 (CK2) and play significant roles in apoptosis (1) and the Wnt-signaling pathway (2–4), respectively. To examine the effect of nucleolar phosphoprotein 140 (Nopp140) on CK2-dependent phosphorylation of β-catenin, the level of phosphorylation of purified β-catenin by CK2 was examined. Phosphorylation of β-catenin by CK2 was effectively detected by the antibodies (Fig. S6*A*, lane 2). In the presence of Nopp140, however, phosphorylation of β-catenin was significantly reduced (Fig. S6*A*, lane 3). These results suggested that the phosphorylation of β-catenin by CK2 was effectively inhibited by Nopp140. To investigate whether overexpression of Nopp140 affects CK2-dependent phosphorylation of procaspase-2 and β-catenin, the cellular levels of procaspase-2 and β-catenin were monitored in HeLa cells transfected with pNopp or siNopp (Fig. S6*C*). When Nopp140 was overexpressed, the levels of procaspase-2 and β-catenin were significantly reduced, and a decrease of only 20–40% in the level of these proteins was observed relative to the untreated cells (Fig. S6*C*, second lanes of the second and third panels). In contrast, the levels of procaspase-2 and β-catenin were slightly increased or unchanged when Nopp140 was knocked down by siNopp (Fig. S6*C*, third lanes of the second and third panels), thereby suggesting that knockdown of Nopp140 hardly affected the endogenous levels of CK2α and GAPDH (Fig. S6*C*, first and last panels). These results indicate that overexpression of Nopp140 suppressed the catalytic activity of endogenous CK2, and this suppression enhanced the maturation of caspase-2 and degradation of β-catenin.

Activation of the Wnt-signaling pathway has been implicated in the development of colon cancer (5, 6), and an elevated level of β-catenin has been observed in colorectal tumors (7). To examine the effect of Nopp140 on the Wnt-signaling pathway, the levels of β-catenin and cyclin D1, which were induced by Wnt (8), were examined in the SW480 cells transfected with pNopp (Fig. S6*D*). Overexpression of Nopp140 (Fig. S6*D*, lane 2 of the first panel) significantly reduced the endogenous levels of β-catenin (Fig. S6*D*, lane 2 of the second panel). In addition, the level of cyclin-D1 decreased more dramatically when Nopp140 was overexpressed (Fig. S6*D*, lane 2 of the third panel). When Nopp140 was knocked down (Fig. S6*D*, lane 3 of the first panel), cyclin-D1 expression was much higher than in the untreated cells (Fig. S6*D*, lane 3 of the third panel). When these cells were treated with Wnt (Fig. S6*D*, lanes 4–6 of every panel), the expression of cyclin-D1 was significantly enhanced compared with the levels in the untreated cells (Fig. S6*D*, third panel). The levels of β-catenin and cyclin-D1 in the cells treated with Wnt were also reduced by overexpression of Nopp140 (Fig. S6*D*, lane 5 of the second and third panels). These results indicate that overexpression of Nopp140 reduces the levels of β-catenin, which results in reduced expression of cyclin-D1.

TRAIL-Mediated Apoptosis in Human Colon Cancer Cells Enhanced by Overexpression of Nopp140. Down-regulation of CK2 enhanced TRAIL-mediated apoptosis by activation of procaspase-2 into caspase-2, which is required for the processing of procaspase-8 (1). To examine whether overexpression of Nopp140 affects the maturation of procaspase-2, the level of procaspase-2 in TRAIL-sensitive colon cancer cells (SW480) transfected with pNopp or siNopp was measured by Western blot. Overexpression of Nopp140 enhanced the processing of procaspase-2 in SW480 cells (Fig. S6*E*, lane 2 of third panel) as was observed in the HeLa cells (Fig. S6*C*). The levels of procaspase-2 were not significantly affected when the expression of Nopp140 was suppressed (Fig. S6*E*, lane 3 of third panel). The endogenous level of CK2α in colon cancer cells (SW480 and HT29) was 1.5-fold higher than that in HeLa cells (Fig. S6*B*, top panel), whereas the endogenous levels of Nopp140 in TRAIL-sensitive HeLa and SW480 cells were more than 4-fold higher than those in TRAIL-resistant HT29 cells (Fig. S6*B*, middle panel). The effect of Nopp140 on the survival rate of these cells was also examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In the TRAIL-sensitive SW480 and HeLa cells, treatment with TRAIL reduced the survival rate to 56% and 70%, respectively (Fig. S6 *E* and *F*, first and second columns). When Nopp140 was overexpressed in these cells, treatment with TRAIL reduced the survival rate of the transfected SW480 and HeLa cells to 36% and 52%, respectively (Fig. S6 *D* and *F*, third and fourth columns). The effect of Nopp140 overexpression on the sensitivity of TRAIL was further examined in the colon cancer cell line HT29, which is resistant to TRAIL (9, 10). The growth rate of the HT29 cells was not affected by TRAIL (Fig. S6*G*, first and second columns), but the survival rate of the HT29 cells transfected with pNopp was reduced to 70% by treatment with TRAIL (Fig. S6*H*, third and fourth columns). These results show that the processing of procaspase-2 is enhanced by overexpression of Nopp140, which also enhances the TRAIL sensitivity of HeLa, SW480, and HT29 cells.

SI Discussion

Nopp140 is mainly localized to the nucleolus, but a certain fraction of Nopp140 can be found in the cytoplasm because it has the ability to shuttle between the two locations (11). However, cytosolic Nopp140 may not be present in sufficient quantities to affect CK2 because the activity of CK2 and the levels of procaspase-2 and β-catenin were not altered when Nopp140 was knocked down by siNopp (Fig. 5). When Nopp140 was overexpressed, the level of cytosolic Nopp140 increased and eliminated the activity of CK2, which is responsible for the phosphorylation of procaspase-2 and β-catenin. Alternatively, overexpressed Nopp140 in the nucleus would prevent phosphorylation of β-catenin by nuclear CK2 and increase the levels of β-catenin. The inhibitory activity of Nopp140 on CK2 and the high levels of CK2 in various cancer cells suggest that the levels of Nopp140 might be related to cancer development. When the gene expression patterns of Nopp140 were analyzed from the microarray database containing the expression profiles of human genes in carcinoma, adenoma, and normal colon cells, the expression of Nopp140 in human benign colon tumor cells was similar or enhanced relative to normal colon cells (Fig. S7). In contrast, the expression of Nopp140 in malignant colorectal cancer cells was dramatically lower than that in normal cells, thereby suggesting that reduced expression of Nopp140 in transformed cells might correlate with cancer development (Fig. S7).

SI Materials and Methods

Materials. Protein kinase CK2 and α-casein were purchased from New England Biolabs and Sigma, respectively. D-*myo*-Inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) was obtained from Calbiochem. Polyclonal antibodies against Nopp140 were prepared from mice immunized with purified Nopp140. Peptides were purchased from Peptron. All other consumables were of reagent grade.

Preparation of Proteins and Peptides. Full-length and C terminus regions of Nopp140 were prepared as previously described (12). The phosphorylated form of Nopp140 was prepared by extensive phosphorylation of the purified recombinant Nopp140 using CK2 as previously described (13). Synthetic peptides representing different regions of Nopp140 were purchased from Peptron.

The $CK2\alpha_{1-335}$ gene was amplified by the PCR. The forward and reverse oligonucleotide primers were: 5′-G CTA TAT **CAT ATG** TCG GGA CCC GTG CCA AGC AG-3′ and 5′-G CTA ATT **GGA TCC** TCA ACC CAT TCG AGC CTG GTC CTT CAC-3′, respectively. The bases in bold represent the NdeI and BamHI cleavage sites. The amplified DNA was digested with NdeI and BamHI and was then inserted into the NdeI/BamHI-digested expression vector pET-21a(+) (Novagen). The plasmid was transformed into *Escherichia coli* BL21(DE3) star cells for protein expression. The cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani medium containing 100 μg/mL ampicillin and the protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were grown at 291 K for 12 h after isopropyl-β-D-thiogalactopyranoside induction and were harvested by centrifugation at 4,611 × *g* (V10004A rotor; Vision) for 30 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer A (20 mM Tris·HCl, pH 8.5, 200 mM NaCl, and 5 mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and was homogenized with an ultrasonic processor and a microfluidizer M-110P (Microfluidics). The crude cell extract was centrifuged at 15,777 × *g* (V506CA rotor; Vision) for 30 min at 277 K. The supernatant was loaded onto a HiTrap 5-mL Heparin HP column (GE Healthcare), which was previously equilibrated with buffer A, and the protein was eluted using a linear gradient of 0.3–1 M NaCl in buffer A. Further purification was performed by gel filtration on a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with buffer A. The homogeneity of the purified protein was assessed by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% (wt/vol) SDS (14). The protein solution was concentrated to about 14.1 mg/mL using a centrifugal filter unit (Sartorius Stedim). The protein concentration was estimated by measuring the absorbance at 280 nm and using the calculated molar extinction coefficient of 61,770 $M^{-1}{\cdot}cm^{-1}$ (ProtParam; web.expasy.org/protparam).

Cell Lines and Culture Conditions. The siRNA of Nopp140 was purchased from Santa Cruz Biotechnology. DRB was purchased from Sigma. Human TRAIL and β-catenin were obtained from ProSpec-Tany TechnoGene and SignalChem, respectively. Nopp140 was expressed in *E. coli*, purified, and phosphorylated using a previously described protocol (15). The human cervical cancer HeLa cell line and human colon cancer cell lines SW480 and HT29 were obtained from the Korean Cell Line Bank. The SW480 and HT29 cells were cultured in RPMI 1640 medium containing 10% (vol/vol) FBS, and the HeLa cells were grown in DMEM containing 10% (vol/vol) FBS at 37 °C. Transfection of the cells with siRNAs or plasmids was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western Blot Analysis. The endogenous expression levels of various proteins in the cell lysates were analyzed by Western blot analysis. The cultured cells were incubated in lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture, 1 mM PMSF, and phosphatase inhibitor mixture) at 4 °C for 1 h, and 20 μg of proteins from the lysates were loaded into each well of a 12% SDS/PAGE gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore). The blotted membranes were treated with blocking buffer (PBS buffer containing 5% skim milk and 0.1% Tween 20) and immunostained with antibodies specific for the following antigens: phosphoserine (Sigma), phosphothreonine (Abcam), CK2α (Santa Cruz Biotechnology), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology), Nopp140 (Santa Cruz Biotechnology), β-catenin (Sigma), cyclin-D1 (Sigma), and procaspase-2 (Santa Cruz Biotechnology).

Transfection, Cell Viability Assay, and Wnt-Signaling Assay. For transfection, ~0.4×10^5 cells of HeLa, SW480, or HT29 were plated in the wells of 96-well plates and transfected with 0.2 μg of the mammalian expression vector of Nopp140 (pNopp) or 4 pmol of Nopp140-specific siRNA (siNopp), using Lipofectamine 2000 and incubated for 24 h at 37 °C. For treatment with TRAIL, the medium in each well was replaced with 100 μL of fresh medium with or without 500 ng/mL TRAIL, and incubated for 48 h at 37 °C. The viability of the TRAIL-treated cells was determined by adding 100 μL of the medium containing 0.5 mg/mL MTT. After 1 h of incubation at 37 °C, the wells were washed with PBS buffer. Dimethyl sulfoxide (200 μL) was then added to each well, and the optical density of the solution was measured at 490 nm. The effect of Wnt treatment on SW480 and HeLa cells was examined by measuring the expression levels of cyclin-D1 after treatment with 100 ng/mL recombinant human Wnt-3a (R&D Systems) for 6 h at 37 °C. The cell lysates were then analyzed by Western blot. All experiments were performed in triplicate.

Gene Expression Profile of Nopp140. The gene expression profiles of Nopp140 in adenoma and carcinoma cells from colon cancer patients were analyzed using the microarray data from the National Center for Biotechnology Information's Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). This database contains the mRNA levels of Nopp140 in carcinoma, tubular adenoma, and normal colon mucosa epithelial cells microdissected from colon tubular adenomas containing focal adenocarcinomas.

CK2 Assay. The endogenous activity of CK2 in HeLa cells transfected with pNopp140 or siNopp was measured using the Kinase-Glo substrate (Promega). The cells were incubated with lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture, 1 mM PMSF, and phosphatase inhibitor mixture) for 1 h at 4 °C with mild agitation, and the insoluble materials were removed by centrifugation at 15,777 × *g* for 30 min at 4 °C. In the CK2 assay, the lysate (7 μg of the protein) was mixed with 3 μg of α-casein and 10 μM ATP in the CK2-reaction buffer (20 mM Tris·HCl, pH 7.5, 50 mM KCl, and 10 mM $MgCl_2$), and incubated at 30 °C for 30 min. For the negative control, 50 μM DRB was incubated with α-casein. After the reaction was completed, 1 vol of the Kinase-Glo substrate (Promega) was added to the reaction mixture and further incubated for 10 min at 30 °C. The mixture was then diluted with 20 vol of 50 mM Tris·HCl (pH 7.5), and the luminescence intensity at 405 nm of the reaction mixtures was measured using a Triad LT plate reader (Dynex Technologies). All experiments were conducted in triplicate. The CK2-dependent phosphorylation of purified β-catenin was examined by Western blot analysis. Briefly, 1 μg of β-catenin was incubated with 5 μg of CK2α in the presence or absence of Nopp140 (10 μg) in CK2-reaction buffer (20 mM Tris·HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM ATP) for 1 h at 37 °C. The proteins in the reaction mixture were then separated by 10% SDS/PAGE, and the phosphorylation of β-catenin was examined by Western blot using anti-phosphoserine (Sigma) and anti-phosphothreonine (Abcam) antibodies.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were performed using MicroCal ITC200 instruments (GE Healthcare) at 22 °C. The 44 μM CK2α wild type, K74E, K76E, and K77E mutants, which are prepared in 50 mM sodium phosphate (pH 7.4), were degassed at 22 °C before measurements. Using a microsyringe, 2 μL of 1.32 mM IP_6 solution was added at intervals of 180 s to the CK2α solution in the cell with gentle stirring.

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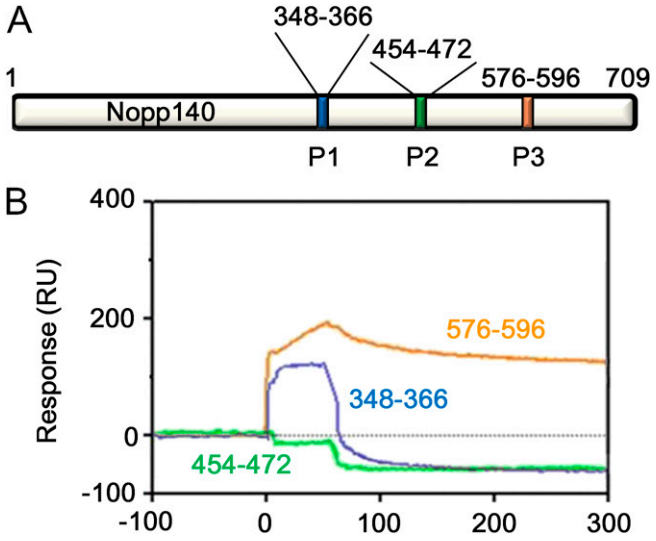

Fig. S1. First screening of interaction site of Nopp140 with CK2α using synthetic peptides and surface plasmon resonance (SPR) analysis. (*A*) Schematic representation of full-length Nopp140 protein and location of three peptides for first screening. (*B*) SPR sensorgrams of the first screening results using three peptides, residues 348–366, 454–472, and 576–596.

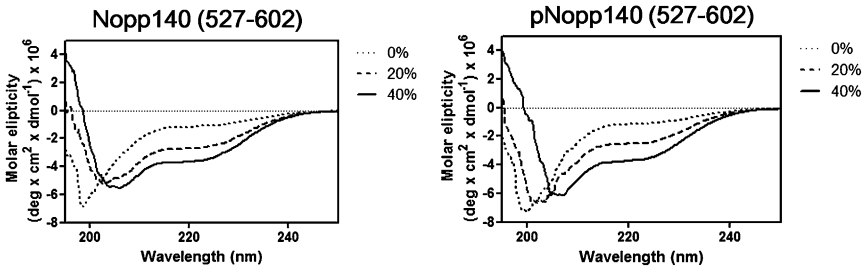

Fig. S2. Far-UV circular dichroism spectra of Nopp140 (527–602) and phosphorylated Nopp140. Following the addition of trifluoroethanol (TFE), increasing proportions of helical structure become evident in the far-UV circular dichroism spectrum of Nopp140 (527–602). Dotted line, Nopp140 in buffer; dashed line, Nopp140 in 20% TFE; black line, Nopp140 in 40% TFE.

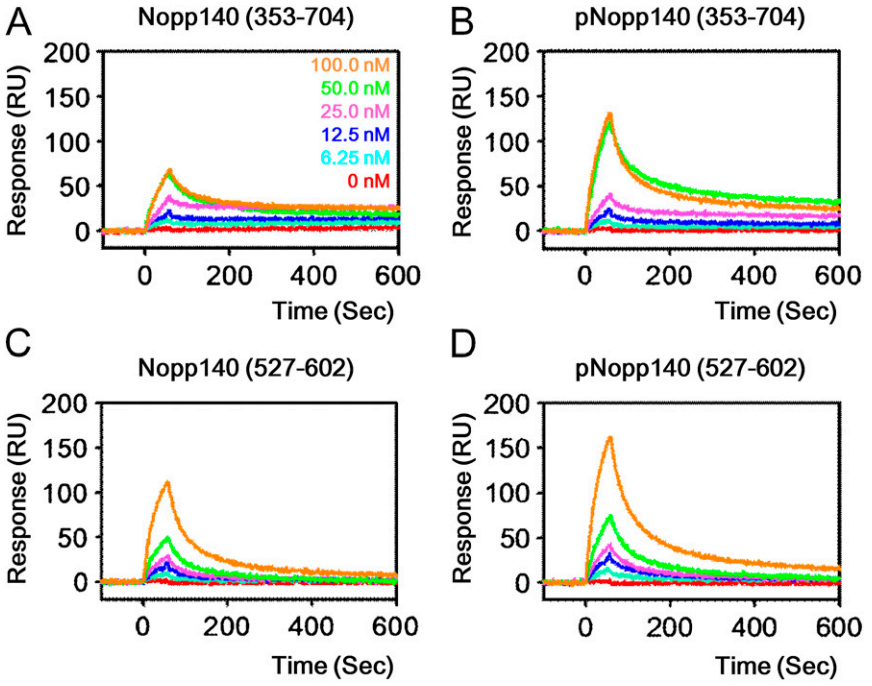

Fig. S3. SPR analysis of the interaction between CK2α wild-type and Nopp140 fragments. The binding sensorgrams of CK2α with Nopp140 (353–704) (*A*), CK2α with phosphorylated Nopp140 (353–704) (*B*), CK2α with Nopp140 (528–602) (*C*), and CK2α with phosphorylated Nopp140 (528–602) (*D*). All sensorgrams were measured using ProteOn XPR36 protein interaction array system (Bio-Rad). CK2α solutions with various concentrations were passed continuously over the Nopp140-immobilized chip at a rate of 100 μL/min. Regeneration of the surface was carried out with 1 M NaCl solution for 30 s.

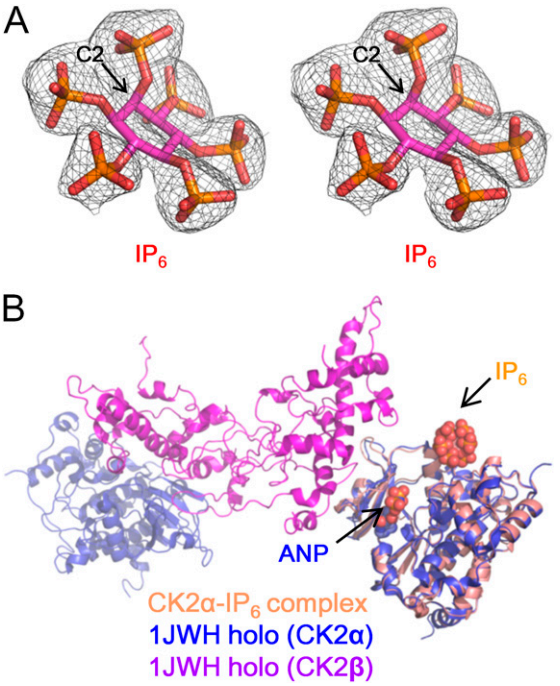

Fig. S4. Electron density map of IP_6 and the IP_6 binding site on the CK2 holoenzyme. (*A*) Stereo electron density map of IP_6 (1.0 σ). (*B*) $CK2\alpha_{1-335}$–IP_6 complex (colored in orange) was superimposed over the CK2 holoenzyme (PDB ID code 1JWH). CK2α and CK2β of the CK2 holoenzyme were colored in blue and purple, respectively. IP_6 is shown in the space-filling model.

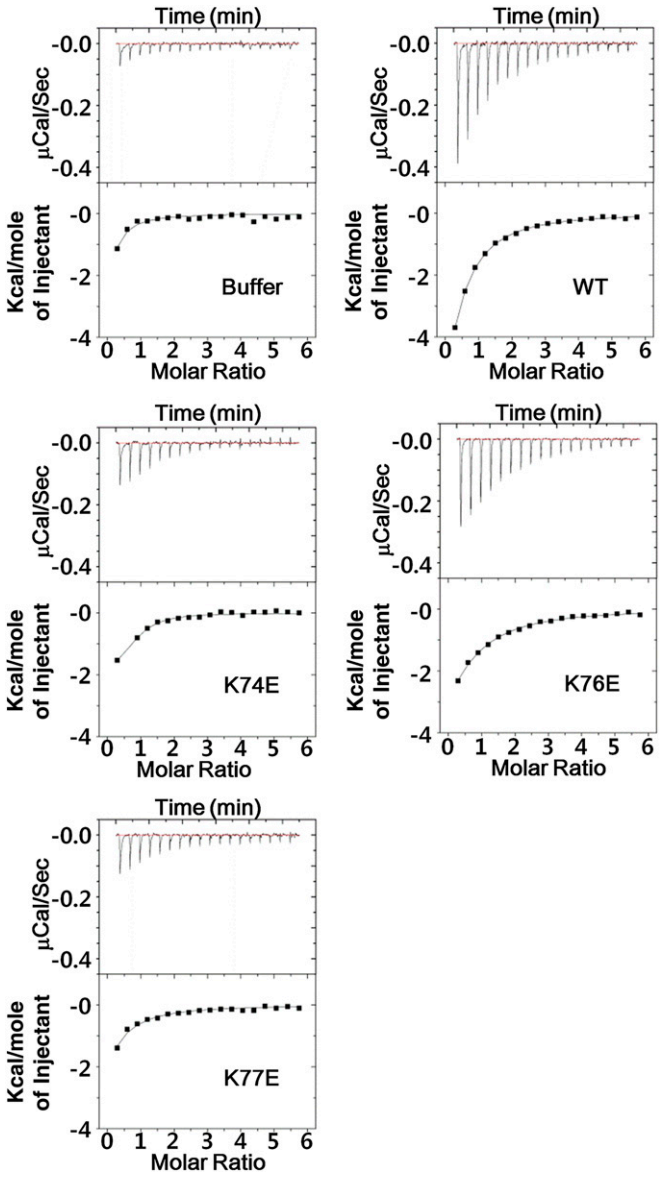

Fig. S5. ITC of IP_6 into CK2α solution. The wild-type CK2α and its mutants (K74E, K76E, and K77E) were titrated against IP_6. Superimposed ITC enthalpy plots for the binding of CK2α to IP_6 is shown in Fig. 3*D*. The effect of buffer addition was examined by titrating IP_6 solution (injectant) to the sample cell containing buffer without CK2α protein (*Top Left*).

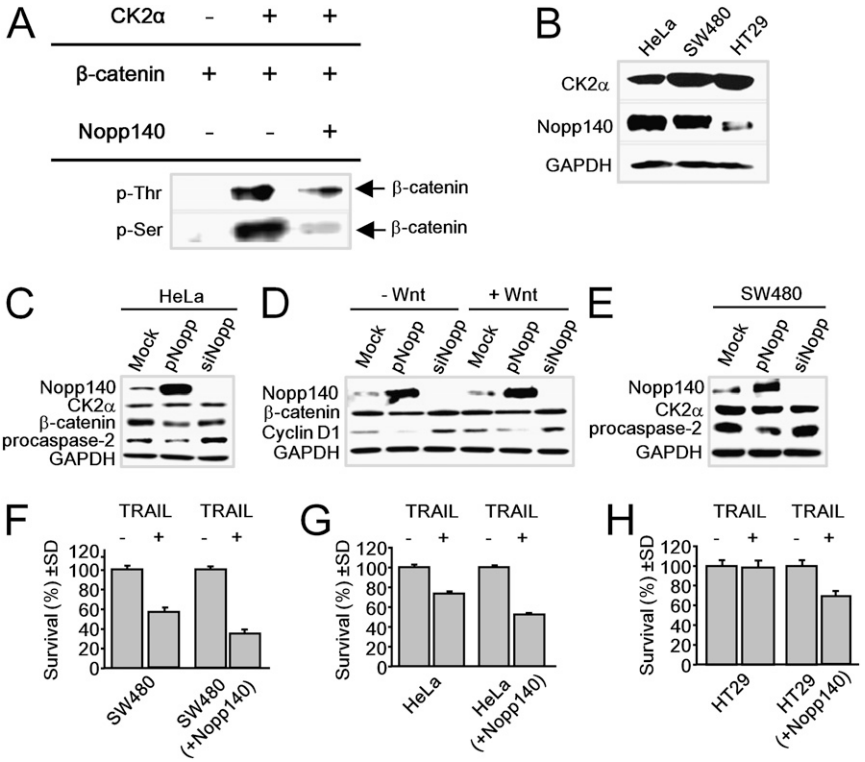

Fig. S6. Wnt-signaling and TRAIL-mediated apoptosis are regulated by Nopp140-controlled CK2. (*A*) Approximately 1 μg of β-catenin was incubated with 5 μg of CK2α in the presence or absence of Nopp140 (10 μg) in CK2 reaction buffer (20 mM Tris·HCl, 1 mM ATP, 50 mM KCl, and 10 mM $MgCl_2$, pH 7.4) for 1 h at 37 °C. The proteins in the reaction mixture were separated by 10% SDS/PAGE, and the level of phosphorylation of β-catenin was examined by Western blot using anti-phosphothreonine (p-Thr) and anti-phosphoserine (p-Ser) antibodies. (*B*) The lysates of HeLa, SW480, and HT-29 cells were immunoblotted with antibodies specific to the following antigens: CK2α, Nopp140, and GAPDH. (*C*) The effect of Nopp140 on the cellular stability of procaspase-2 and β-catenin. The lysates of HeLa cells transfected with pNopp or siNopp were analyzed by Western blot using antibodies against Nopp140, CK2α, β-catenin, procaspase-2, and GAPDH. (*D*) Nopp140 reduces the cellular stability of β-catenin and cyclin-D1 expression in Wnt-sensitive SW480 cells. SW480 cells were transfected with pNopp or siNopp, and then incubated with or without recombinant human Wnt-3a. The lysates of these cells were analyzed by Western blot using antibodies against Nopp140, β-catenin, cyclin-D1, and GAPDH. (*E*) Nopp140 reduces the cellular level of procaspase-2 in TRAIL-sensitive SW480 cells. The lysates of the SW480 cells (20 μg of proteins) transfected with pNopp or siNopp were immunoblotted with antibodies specific to the following antigens: Nopp140, CK2α, procaspase-2, and GAPDH. Effect of Nopp140 on TRAIL-mediated apoptosis in human cancer cells. (*F*) TRAIL-sensitive SW480, (*G*) TRAIL-sensitive HeLa, (*H*) and TRAIL-resistant HT29 cells were transfected with pNopp or siNopp, and treated with 500 ng/mL TRAIL for 48 h. The viability of these cells was then measured using the MTT assay. The relative optical density at 490 nm of the TRAIL-treated cells compared with the untreated cells was used as the survival rate. All experiments were performed in triplicate.

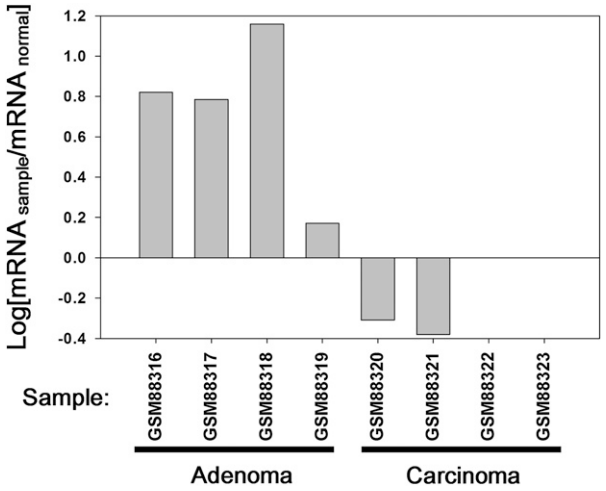

Fig. S7. Gene expression profile of Nopp140 in normal human colon and carcinoma cells. The expression levels of Nopp140 in various human tissue samples were obtained from the National Center for Biotechnology Information's Gene Expression Omnibus. The levels of Nopp140 in benign adenoma (GSM88316–GSM88319) or carcinoma (GSM88320–GSM88323) are presented as the log value of their relative ratios compared with the level of Nopp140 in normal tissue. The mRNA levels of Nopp140 in the two carcinoma samples (GSM88322 and GSM88323) were undetectable.

Table S1. Statistics for data collection and refinement

Dataset	CK2α–IP_6 complex
A. Data collection statistics	
X-ray source	PLS BL-7A
X-ray wavelength, Å	0.97928
Space group	$P2_1$
a, *b*, *c*, Å	70.14, 56.57, 95.54
β, °	96.14
Resolution range, Å	50–2.4
Total/unique reflections	89,357/29,130
Completeness, %	98.4 (99.7)*
Average $I/\sigma(I)$	24.7 (3.5)*
R_{merge},† %	8.2 (46.9)*
B. Model refinement statistics	
Resolution range, Å	27.6–2.4
R_{work}/R_{free},‡ %	23.5/24.8
Number/average *B*-factor, $Å^2$	
Protein nonhydrogen atoms	5,522/48.1
Water oxygen atoms	105/46.4
Ligand molecules	2 IP_6/78.1
rms deviations from ideal geometry	
Bond lengths, Å	0.006
Bond angles, °	1.123
Protein-geometry analysis	
Ramachandran favored, %	96.9 (628/648)
Ramachandran allowed, %	3.1 (20/648)
Ramachandran outliers, %	0.0 (0/648)

*Values in parentheses refer to the highest resolution shell (2.44–2.40 Å).

†$R_{merge} = \Sigma_{hkl}\Sigma_i \mid I_i(hkl) - \langle I(hkl)\rangle \mid / \Sigma_{hkl}\Sigma_i I_i(hkl)_i$, where $I(hkl)$ is the intensity of reflection *hkl*, Σ_{hkl} is the sum over all reflections, and Σ_i is the sum over i measurements of reflection *hkl*.

‡$R = \Sigma_{hkl} \mid |F_{obs}| - |F_{calc}| \mid / \Sigma_{hkl} |F_{obs}|$, where R_{free} was calculated for a randomly chosen 10% of reflections, which were not used for structure refinement, and R_{work} was calculated for the remaining.