

Zipper-interacting Protein Kinase (ZIPK) Modulates Canonical Wnt/ β -Catenin Signaling through Interaction with Nemo-like Kinase and T-cell Factor 4 (NLK/TCF4)*

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Zipper-interacting protein kinase (ZIPK) is a widely expressed serine/threonine kinase that has been implicated in apoptosis and transcriptional regulation. Here, we identified Nemo-like kinase (NLK) as a novel ZIPK-binding partner and found that ZIPK regulates NLK-mediated repression of canonical Wnt/ β -catenin signaling. Indeed, siRNA-mediated reduction of endogenous ZIPK expression reduced Wnt/ β -catenin signaling. Furthermore, ZIPK affected the formation of NLK-T-cell factor 4 (TCF4) complex. Importantly, ZIPK siRNA treatment in human colon carcinoma cells resulted in a reduction of β -catenin/TCF-mediated gene expression and cell growth. These results indicate that ZIPK may serve as a transcriptional regulator of canonical Wnt/ β -catenin signaling through interaction with NLK/TCF4.

Wnt are a family of secreted proteins involved in a wide range of developmental processes, including the control of asymmetric division and cell polarity. The Wnt signaling pathway regulates many developmental processes through a complex of β -catenin and the T-cell factor/lymphoid enhancer factor 1 (TCF/LEF-1)² family of high mobility group transcription factors (1, 2). Wnt stabilize cytosolic β -catenin, which then binds to TCF/LEF-1 and recruits transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression (3). This signaling cascade is conserved in vertebrates, *Drosophila*, and *Caenorhabditis elegans*. TAK1 (a kinase activated by transforming growth factor- β), mitogen-activated protein-kinase-kinase kinase 7 (MAP3K7), and MAP kinase (MAPK)-related NEMO-like kinase (NLK) have been shown to be involved in the regulation of Wnt signaling (4). TAK1 activation stimulates NLK activity and down-regulates transcriptional

activation mediated by β -catenin and TCF/LEF-1. NLK phosphorylates TCF/LEF-1 factors and inhibits the interaction of the β -catenin-TCF/LEF-1 complex with DNA (5), indicating that the TAK1-NLK pathway negatively regulates the Wnt signaling pathway.

Zipper-interacting protein kinase (ZIPK) was originally identified as a binding partner of activating transcription factor 4 (ATF4) and aggregates through its C-terminal leucine zipper (LZ) structure, thereby becoming an active enzyme (6). Ectopic expression of ZIPK in NIH3T3 cells induced apoptosis, whereas a kinase-inactive mutant, ZIPK K42A (KA), failed to induce apoptosis, indicating that ZIPK stimulates apoptosis via its kinase activity (6, 7). Previous studies showed that ZIPK, in collaboration with Daxx and Par-4, induces apoptosis from promyelocytic leukemia protein nuclear bodies (8). However, the mechanisms responsible for the activation of ZIPK and the downstream substrates that mediate its apoptotic activity remain unknown. Recently, we demonstrated that ZIPK specifically interacts with signal transducer and activator of transcription 3 (STAT3) and enhances its transcriptional activity (9). We further demonstrated that leukemia-inhibitory factor induces Thr-265 phosphorylation of ZIPK (10), which is critical for its kinase activation, suggesting that leukemia-inhibitory factor signaling mediates ZIPK/STAT3 activation through phosphorylation of Thr-265. In the current study, we focused on ZIPK as a novel transcriptional regulator of Wnt signaling and demonstrated a functional link between ZIPK and NLK-mediated repression of Wnt signaling.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—LiCl was purchased from Wako Chemicals (Osaka, Japan). Expression vectors for TOPFLASH, FOPFLASH, TCF4, and Wnt3a were kindly provided by Dr. S. Matsuzawa (Sanford-Burnham Medical Research Institute, La Jolla, CA) (11). ZIPK, NLK, and their mutants were described previously (6, 8). Anti-NLK, anti-Myc, and anti- β -catenin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG, anti-HA antibody from Sigma-Aldrich; anti-ZIPK antibody from BD Biosciences; and anti-actin antibody from Millipore (Billerica, MA).

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² The abbreviations used are: TCF, T-cell factor; ZIPK, zipper-interacting protein kinase; NLK, Nemo-like kinase; DAPK, death-associated protein kinase; LZ, leucine zipper; qRT, quantitative real-time; KD, kinase domain; KA, K42A; APC, adenomatous polyposis coli; FL, full length; TCL, total cell lysate.

Yeast Two-hybrid Screen—Gal4-ZIPK kinase domain (KD) was constructed by fusing the coding sequence for the kinase domain (amino acids 1–125) of ZIPK in-frame to the Gal4 DNA-binding domain in the pGBKT7 vector (Clontech, Palo Alto, CA) (12). *Saccharomyces cerevisiae* AH109 cells were transformed with pGal4-STAT4 and then mated with Y187 cells containing a pretransformed mouse 11-day embryo MATCHMAKER cDNA library (Clontech), and $\sim 2.6 \times 10^6$ colonies were screened as described previously (9). Plasmid DNAs derived from positive clones were extracted from the yeast and sequenced.

Cell Culture, Transfection, siRNA, and Luciferase Assays—A human cervix carcinoma cell line, HeLa, and a human embryonic kidney carcinoma cell line, 293T, were maintained in DMEM containing 10% FCS. A human colon carcinoma cell line, SW480, was maintained in L-15 medium containing 10% FCS. A human colon carcinoma cell line, HCT116, was maintained in McCoy's 5A medium containing 10% FCS with 2 mM L-glutamine. A human colon carcinoma cell line, Caco2, was maintained in DMEM containing 20% FCS. HeLa cells were transfected using jetPEI (Polyplus Transfection, Strasbourg, France) according to the manufacturer's instructions. 293T cells were transfected using a standard calcium precipitation protocol (13). The siRNAs targeting human ZIPK used in this study were as follows: ZIPK-1, 5'-CCAACAUCUCAGCCGUGAATT-3'; ZIPK-2, 5'-CCAGCUUGCCGCCCAACAATT-3'. Control siRNA was obtained from Qiagen (nonsilencing; catalog No. 1022076). HeLa or SW480 cells were plated on 24-well plates at 2×10^4 cells/well and incubated with an siRNA-Lipofectamine 2000 (Invitrogen) mixture at 37 °C for 4 h followed by the addition of fresh medium containing 10% FCS (13). HeLa cells were further transfected with or without TOPFLASH or FOPFLASH using jetPEI as described above. At 24 h after transfection, the cells were left untreated or were treated with LiCl (30 mM) for an additional 12 h and then assayed for their luciferase activities using a Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

RNA Isolation and Quantitative Real-time (qRT)-PCR—Cells were harvested, and total RNAs prepared by using IsoGen (Nippon Gene, Tokyo, Japan) were used in RT-PCR. RT-PCR was performed using the RT-PCR High-Plus kit (Toyobo, Tokyo, Japan). The primers used for RT-PCR were: cyclin D1, 5'-GCTGCTCCTGGTGAACAAGC-3' (sense) and 5'-TTCAATGAAATCGTGCGGG-3' (antisense); SURVIVIN, 5'-GGAACCCGCATCTCTACAT-3' (sense) and 5'-GACAGAAA-GGAAAGCGCAAC-3' (antisense); human ZIPK (hZIPK), 5'-TGCACGACATCTTCGAGAAC-3' (sense) and 5'-GTTCT-TGTCCAGCAGCATGA-3' (antisense); and mouse ZIPK (mZIPK), 5'-TCGCACACTTTGACCTGAAG-3' (sense) and 5'-CTCCGCTCAGGAGGATGTAG-3' (antisense). qRT-PCR analyses of the respective genes as well as the control actin mRNA transcripts were carried out using the Assay-on-Demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) (13).

Immunoprecipitation, Immunoblotting, and in Vitro Phosphorylation—Immunoprecipitation and Western blotting assays were performed as described previously (13). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer Life Sciences). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore). *In vitro* kinase reactions were performed as described (14). Briefly, immune complex of TCF4 were washed in kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 0.1 mM sodium orthovanadate, 5 mM MnCl₂, and 5 mM MgCl₂) and mixed with 5 μCi/ml [γ -³²P]ATP at 25 °C for 30 min. The products of these reactions were separated by SDS-PAGE.

Chromatin Immunoprecipitation (ChIP) Analysis—HeLa cells were transfected with control or ZIPK siRNA followed by transfection with an expression vector for TCF4. At 36 h after transfection, ChIP-enriched DNAs were prepared from HeLa cells as described previously (15, 16). Antibodies used for immunoprecipitation were control IgG or IgG for β -catenin. Primers used for the cyclin D1 promoter were: 5'-CACCTCC-ACCTCACCCCCTAAATCC-3' and 5'-ACTCCCCTGTAG-TCCGTGTGACGTT-3'. RT-PCR was used to quantify the precipitated DNA fragments.

Cell Proliferation Assay—The numbers of viable SW480 cells after the indicated treatments were measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay (Cell Counting Kit-8, Wako Pure Chemicals) (17). Briefly, 10 μl of WST-8 solution was added to the cells in each well and incubated for 2 h. Absorbency was measured at a test wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader (Bio-Rad).

Statistical Analysis—The significant differences between group means were determined by Student's *t* test.

RESULTS

Physical and Functional Interactions between ZIPK and NLK—We performed a yeast two-hybrid screen of a HeLa cDNA library using the kinase domain of ZIPK as bait (9). From a screen of 2.6×10^6 transformants, we identified several positive clones. Sequence analysis revealed that one of them encoded the N-terminal domain (amino acids 1–125) of NLK. We first examined whether NLK binds ZIPK in mammalian cells. 293T cells were transfected with HA-tagged ZIPK together with Myc-tagged NLK. Western blot analysis revealed that the immunoprecipitates with an anti-HA antibody (for ZIPK) contained NLK protein in 293T cells (Fig. 1A). To exclude the possibility that the interaction between ZIPK and NLK was due to overexpression, we examined direct binding between endogenous ZIPK and NLK in HeLa cells by co-immunoprecipitation. The immunoprecipitates with anti-ZIPK antibody, but not those with control antibody, contained endogenous NLK proteins. An anti-NLK antibody also co-immunoprecipitated ZIPK, indicating that the binding of ZIPK to NLK occurs at physiological expression levels (Fig. 1B). Next, to delineate the regions of ZIPK involved in the ZIPK-NLK interaction, various deletion constructs of Myc-ZIPK (Fig. 1C) were subjected to

Physical and Functional Interactions between ZIPK and NLK

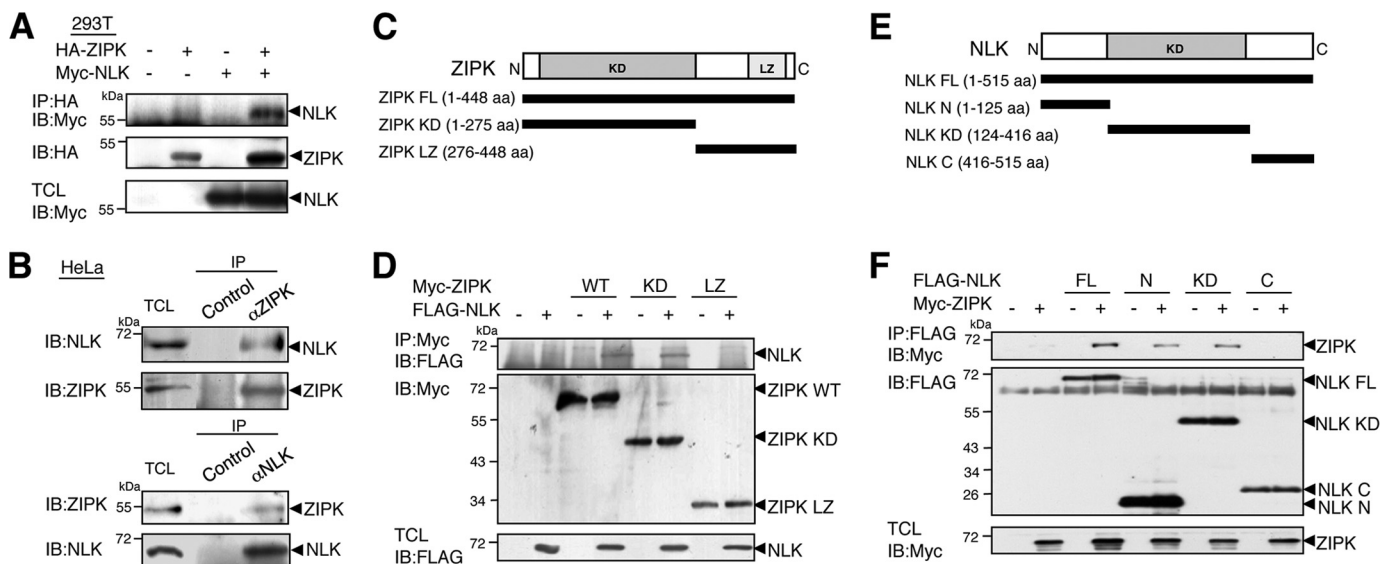


FIGURE 1. ZIPK interact physically with NLK *in vivo*. *A*, 293T cells (1×10^7) were transfected with HA-tagged ZIPK (10 μ g) and/or Myc-tagged NLK (10 μ g). The cells were lysed 48 h later in lysis buffer, immunoprecipitated with anti-HA (IP), and blotted with anti-Myc or anti-HA antibody (IB). TCL (1%) was blotted with anti-Myc antibody. *B*, HeLa cells (3×10^7) were lysed, immunoprecipitated with control or anti-ZIPK IgG, and blotted with anti-NLK antibody (upper panels). HeLa cells (3×10^7) were also lysed, immunoprecipitated with control or anti-NLK IgG, and blotted with anti-ZIPK antibody (lower panels). TCL (1%) was blotted with anti-ZIPK or anti-NLK antibody. *C*, domain structure of ZIPK and its mutant fragments are shown schematically. *D*, 293T cells (1×10^7 cells) were transfected with Myc-tagged ZIPK WT or its mutants (10 mg) together with or without FLAG-tagged NLK (10 μ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-Myc, and blotted with anti-FLAG or anti-Myc antibody. TCL (1%) was blotted with anti-FLAG antibody. *E*, domain structure of NLK and its mutant fragments are shown schematically. *F*, 293T cells (1×10^7 cells) were transfected with FLAG-tagged NLK WT or its mutants (10 μ g) together with or without Myc-tagged ZIPK (10 μ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG, and blotted with anti-Myc or anti-FLAG antibody. TCL (1%) was blotted with anti-Myc antibody. *N*, N-terminal domain; *C*, C-terminal domain.

co-immunoprecipitation with FLAG-tagged NLK. As shown in Fig. 1*D*, the kinase domain of ZIPK (ZIPK KD) as well as full-length ZIPK (ZIPK FL) interacted with NLK. Using a series of deletion mutants of NLK (Fig. 1*E*), we also determined the NLK domain involved in the interaction with ZIPK. As shown in Fig. 1*F*, both the N-terminal (NLK N) and kinase domains (NLK KD) of NLK interacted individually with ZIPK.

To clarify the physiological significance of the molecular interactions between ZIPK and NLK, we focused on the effect of ZIPK on NLK-mediated suppression of Wnt signaling. Recently, NLK has been shown to act as a negative regulator of Wnt signaling by interacting with and phosphorylating TCF/LEF-1 family proteins on two serine/threonine residues located in the central region (5). This phosphorylation by NLK inhibits DNA binding by the β -catenin-TCF complex. We first tested whether ZIPK affects Wnt/ β -catenin-mediated transcriptional activation by using the established β -catenin/LEF/TCF luciferase reporter (TOPFLASH) (18). The TOPFLASH reporter and vectors expressing ZIPK and/or NLK were transfected into 293T cells, and the cells were then treated with LiCl, which activated TOPFLASH activity in 293T cells (Fig. 2*A*). In this situation, LiCl did not activate the reporter harboring mutated LEF/TCF-consensus sites (FOPFLASH) (data not shown). LiCl-induced TOPFLASH activity was reduced by expression of NLK as described previously (19). Interestingly, ectopic expression of ZIPK enhanced LiCl-induced TOPFLASH activity (Fig. 2*A*). Furthermore, NLK-mediated reduction of LiCl-induced TOPFLASH activity was restored by ZIPK expression. We also tested whether the kinase activity of ZIPK is required for ZIPK-mediated enhanced Wnt signaling. TOPFLASH reporter and vectors containing wild-type (WT) ZIPK or a

kinase-dead mutant of ZIPK, ZIPK K42A, were transfected into 293T cells, and the cells were then treated with LiCl. LiCl-induced TOPFLASH activity was enhanced by expression of ZIPK WT and KA (Fig. 2*B*), indicating that the kinase activity of ZIPK is not necessary for ZIPK-mediated enhanced Wnt signaling. To further explore the involvement of ZIPK in Wnt signaling, we used siRNA to reduce the endogenous expression of ZIPK in HeLa cells. Specific siRNAs for ZIPK (ZIPK-1 and ZIPK-2) or a control siRNA was transfected into HeLa cells, and aliquots of cell lysates were analyzed by Western blotting, which confirmed the reduced expression of ZIPK (Fig. 2*C*). First, we determined the effects of these siRNAs on LiCl-induced TOPFLASH/FOPFLASH activation in HeLa cells. As shown in Fig. 2*C*, siRNA-mediated reduced expression of ZIPK resulted in a significant reduction of LiCl-induced TOPFLASH activation. We further tested whether the effect of ZIPK siRNAs was specific for TOPFLASH. We then examined the effect of ZIPK siRNAs on TNF- α -induced NF- κ B luciferase or IL-6 promoter luciferase. However, we did not observe any significant reduction of TNF- α -induced luciferase activities by ZIPK knockdown (data not shown), suggesting that the effect of ZIPK siRNAs is specific for TOPFLASH. Furthermore, reduced LiCl-induced TOPFLASH activation in the ZIPK knockdown cells was restored by overexpression of a mouse ZIPK cDNA (Fig. 2*D*). Therefore, we concluded that ZIPK increases LiCl-induced TOPFLASH activation. We also examined the effects of these siRNAs on Wnt3a-induced TOPFLASH activation in HeLa cells. siRNA-mediated reduced expression of ZIPK also resulted in a significant reduction of Wnt3a-induced TOPFLASH activation (Fig. 2*E*), indicating that ZIPK positively regulates canonical Wnt/ β -catenin-mediated transcriptional acti-

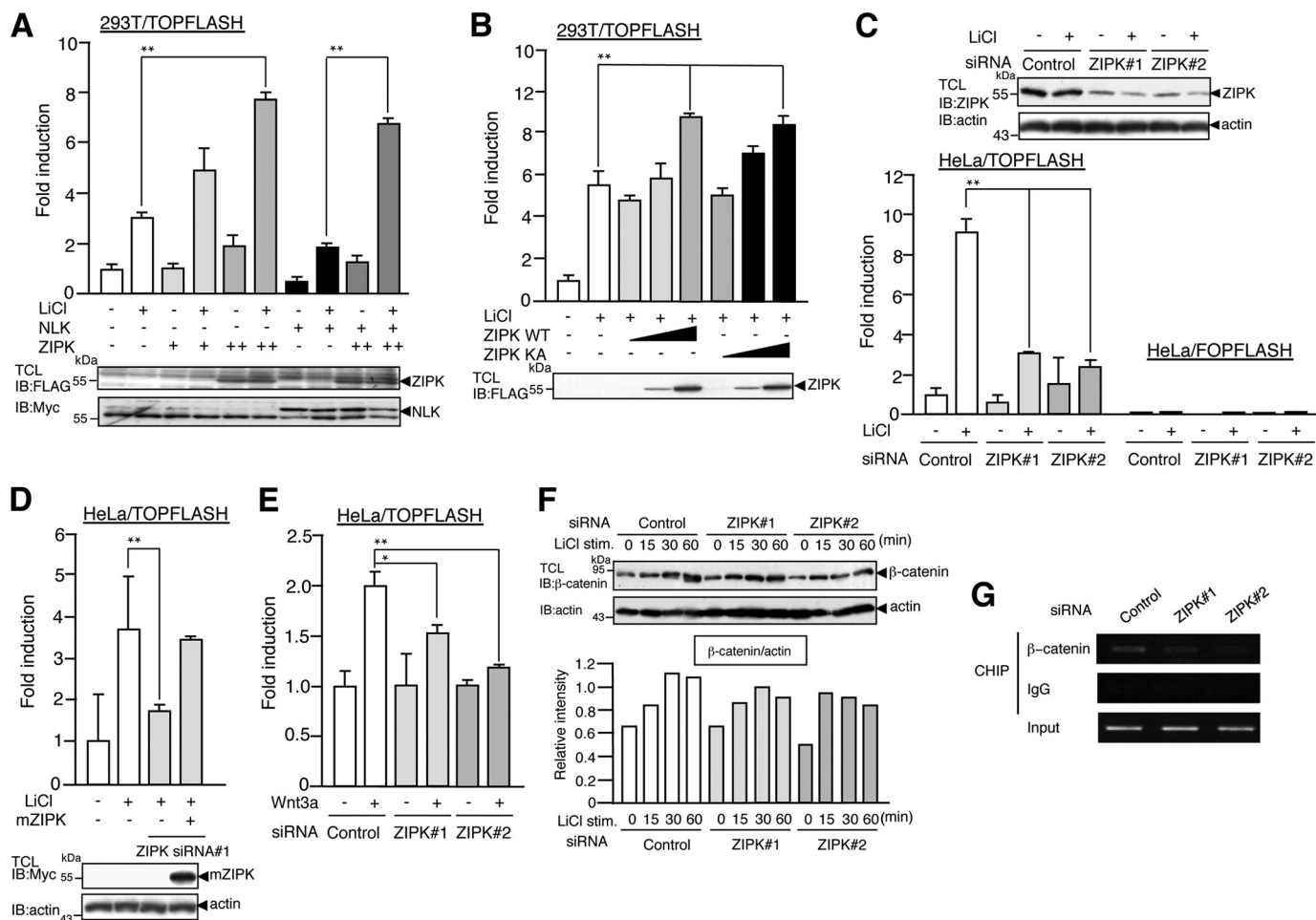


FIGURE 2. ZIPK positively regulates Wnt-mediated transcription in HeLa cells. *A*, 293T cells in a 24-well plate were transfected with TOPFLASH (100 ng) with or without ZIPK and/or NLK (+, 10 ng; ++, 100 ng) using jetPEI (Polyplus Transfection). At 36 h after transfection, cells were treated with LiCl (30 mM) for an additional 12 h. The cells were harvested and assayed for luciferase activity. The results are indicated as -fold induction of luciferase activity from triplicate experiments, and the error bars represent S.D. **, $p < 0.01$. TCL (1%) was blotted with an anti-FLAG or anti-Myc antibody (*IB*). *B*, 293T cells in a 24-well plate were transfected with TOPFLASH (100 ng) with or without ZIPK WT or KA mutant (10, 30, and 100 ng) using jetPEI. At 36 h after transfection, cells were treated with LiCl (30 mM) for an additional 12 h. The cells were harvested and assayed for luciferase activity as described the above. **, $p < 0.01$. TCL (1%) was blotted with an anti-FLAG antibody. *C*, HeLa cells in a 24-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA (20 pmol) and then transfected with TOPFLASH or FOPFLASH (100 ng). At 36 h after transfection, cells were treated with LiCl (30 mM) for an additional 12 h. The cells were harvested and assayed for luciferase activity as described in *A*. **, $p < 0.01$. TCL (1%) was blotted with anti-ZIPK or anti-actin antibody. *D*, HeLa cells in a 24-well plate were transfected with control or ZIPK-1 siRNA (20 pmol) and then transfected with or without Myc-tagged mouse ZIPK (0.5 μ g) together with TOPFLASH (100 ng). At 36 h after transfection, cells were treated with LiCl (30 mM) for an additional 12 h. The cells were harvested and assayed for luciferase activity as described in *A*. **, $p < 0.01$. TCL (1%) was blotted with anti-Myc or anti-actin. *E*, HeLa cells in a 24-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA (20 pmol) and then transfected with TOPFLASH (100 ng). At 36 h after transfection, cells were treated with Wnt3a (culture supernatant of Wnt3a-transfected COS-7 cells) for an additional 8 h. The cells were harvested and assayed for luciferase activity as described in *A*. **, $p < 0.01$. *F*, HeLa cells in a 24-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA (20 pmol). At 36 h after transfection, cells were treated with LiCl (30 mM) for the indicated periods. Cells were then lysed, and TCL (1%) was blotted with anti- β -catenin or anti-actin antibody. Densitometric quantification of the above results was also shown. The relative intensity of β -catenin was normalized to the actin protein of the same sample. *G*, HeLa cells were transfected in a 10-cm dish with control, ZIPK-1, or ZIPK-2 siRNA followed by transfection with TCF4 (10 μ g). At 36 h after transfection, cells were treated with LiCl (30 mM) for an additional 2 h. A ChIP assay was then performed on siRNA-transfected cells using IgG or anti- β -catenin antibody. ChIP DNA was analyzed by RT-PCR with primers specific for the cyclin D1 promoter DNA.

vation in HeLa cells. We further tested whether ZIPK knockdown would have any effect on the LiCl-induced increase of β -catenin protein levels. As shown in Fig. 2*F*, siRNA-mediated reduced expression of ZIPK showed no effect on the LiCl-induced increase of β -catenin protein levels. We also investigated the recruitment of β -catenin to the cyclin D1 promoter region with ChIP analysis. When we transfected with TCF4 in control or ZIPK siRNA-treated HeLa cells, LiCl-induced TCF4-mediated binding of β -catenin on the cyclin D1 promoter was markedly reduced in ZIPK siRNA-transfected HeLa cells (Fig. 2*G*). Therefore, the β -catenin-TCF4 complex may be a direct target of ZIPK in the nucleus.

ZIPK Influences Complex Formation of NLK-TCF4—To further understand the molecular mechanisms responsible for ZIPK-mediated enhancement of Wnt signaling, we focused on the restoration of NLK-mediated repression of Wnt signaling by ZIPK. NLK-mediated repression of Wnt signaling is initiated by a direct interaction between the NLK and TCF family proteins (5). We therefore examined whether ZIPK interacts directly with TCF4. Expression vectors for Myc-tagged ZIPK or NLK with FLAG-tagged TCF4 were transfected into 293T cells. The cells were lysed, and the lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-Myc antibody. As shown in Fig. 3*A*, a significant interaction of

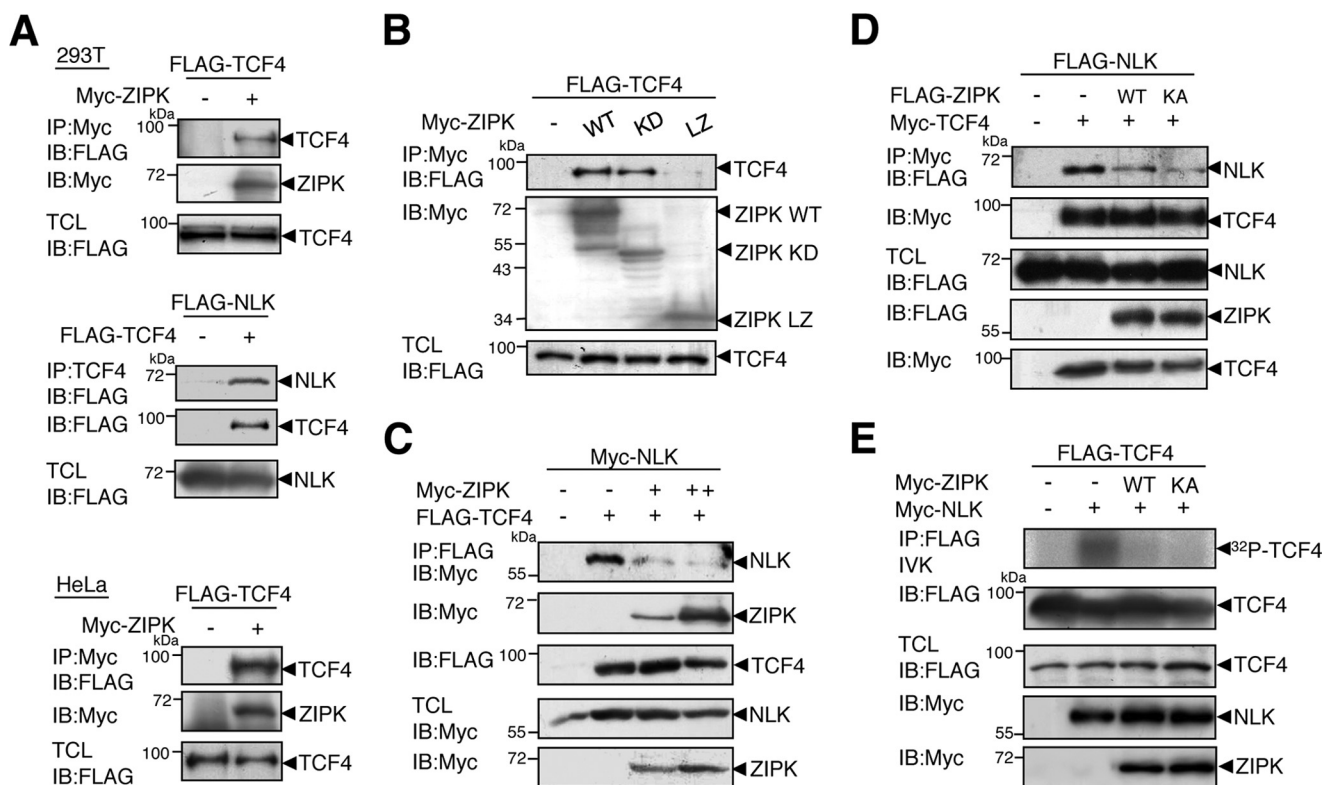


FIGURE 3. ZIPK influences the interaction between NLK and TCF4. *A*, 293T cells (1×10^7 cells) were transfected with FLAG-tagged TCF4 ($10 \mu\text{g}$) together with or without Myc-tagged ZIPK or FLAG-NLK ($10 \mu\text{g}$). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-Myc or anti-TCF4 (IP), and blotted with anti-Myc or anti-FLAG antibody (IB). TCL (1%) was blotted with anti-FLAG antibody. HeLa cells (1×10^7 cells) were transfected with FLAG-tagged TCF4 ($10 \mu\text{g}$) together with or without Myc-tagged ZIPK ($10 \mu\text{g}$). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-Myc, and blotted with anti-FLAG or anti-Myc antibody. TCL (1%) was blotted with anti-FLAG antibody. *B*, 293T cells (1×10^7 cells) were transfected with FLAG-tagged TCF4 together with or without Myc-tagged ZIPK FL, KD, or LZ ($10 \mu\text{g}$). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG, and blotted with anti-FLAG or anti-Myc antibody. TCL (1%) was blotted with anti-Myc antibody. *C*, 293T cells (1×10^7 cells) were transfected with Myc-tagged NLK ($10 \mu\text{g}$) together with FLAG-tagged TCF4 and/or Myc-tagged ZIPK ($10 \mu\text{g}$). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG, and blotted with anti-Myc or anti-FLAG antibody. TCL (1%) was blotted with anti-Myc antibody. *D*, 293T cells (1×10^7 cells) were transfected with FLAG-tagged NLK ($10 \mu\text{g}$) together with Myc-tagged ZIPK FL, KD, or LZ ($10 \mu\text{g}$) and/or FLAG-tagged ZIPK WT or KA ($10 \mu\text{g}$). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-Myc, and blotted with anti-FLAG or anti-Myc antibody. TCL (1%) was blotted with anti-FLAG or anti-Myc antibody. *E*, 293T cells (1×10^7 cells) were transfected with FLAG-tagged TCF4 ($10 \mu\text{g}$) with or without Myc-tagged NLK ($10 \mu\text{g}$) and/or Myc-tagged ZIPK ($10 \mu\text{g}$). The cells were lysed and immunoprecipitated with anti-FLAG antibody and assayed for *in vitro* kinase activity (top panel). The amounts of TCF4 proteins were shown to be similar by Western blotting with anti-FLAG antibody (second panel from top). TCL (1%) was also blotted with anti-FLAG or anti-Myc antibody (bottom three panels).

ZIPK with TCF4 was observed, whereas NLK also bound to TCF4 as described previously (5), indicating that both ZIPK and NLK associate with TCF4. Similar interactions between ZIPK and TCF4 were also observed in HeLa cells (Fig. 3A). Next, to determine the regions of ZIPK involved in the ZIPK-TCF4 interaction, various deletion constructs of Myc-ZIPK (Fig. 1C) were subjected to co-immunoprecipitation with FLAG-tagged TCF4. As shown in Fig. 3B, the kinase domain but not the LZ domain of ZIPK interacted with TCF4. These findings allowed us to investigate whether ZIPK affects complex formation between NLK and TCF4. When we co-expressed Myc-tagged ZIPK and NLK and FLAG-tagged TCF4 in 293T cells, the binding of NLK to TCF4 disappeared in the presence of ZIPK (Fig. 3C), suggesting that the interaction between ZIPK and NLK disrupts NLK-TCF4 complex formation. This possibility is, in part, supported by the fact that TCF4 recognized the same NLK-binding domain of ZIPK (Fig. 3B). We also tested whether the kinase activity of ZIPK was required for ZIPK-mediated disruption of NLK-TCF4 complex formation. When we co-expressed FLAG-tagged NLK, Myc-tagged TCF4, and FLAG-tagged ZIPK WT or KA in 293T cells, the binding of NLK to

TCF4 decreased in the presence of either ZIPK WT or KA (Fig. 3D), suggesting that the kinase activity of ZIPK is not required for ZIPK-mediated disruption of NLK-TCF4 complex formation. We further tested the effect of ZIPK on the phosphorylation of TCF4 by NLK. Expression vectors for Myc-tagged NLK or ZIPK WT or KA together with those for FLAG-tagged TCF4 were transfected into 293T cells. The cells were lysed, immunoprecipitated with an anti-FLAG antibody, and subjected to *in vitro* kinase assay. As shown in Fig. 3E, a robust phosphorylation of TCF4 by NLK was observed. Importantly, co-expression of ZIPK WT or KA with NLK resulted in a significantly reduced phosphorylation of TCF4 by NLK, indicating that ZIPK-mediated disruption of NLK-TCF4 complex formation influences phosphorylation of TCF4 by NLK.

ZIPK Acts as an Endogenous Positive Regulator of Colon Cancer Cells—As described above, the Wnt signal is mediated by β -catenin, a transcription factor that is normally degraded in the cytosol by the ubiquitin-proteasome system. Phosphorylation of β -catenin by a large protein complex composed of adenomatous polyposis coli (APC) protein, Axin, and glycogen synthase kinase 3b (GSK3b) initiates the ubiquitylation and

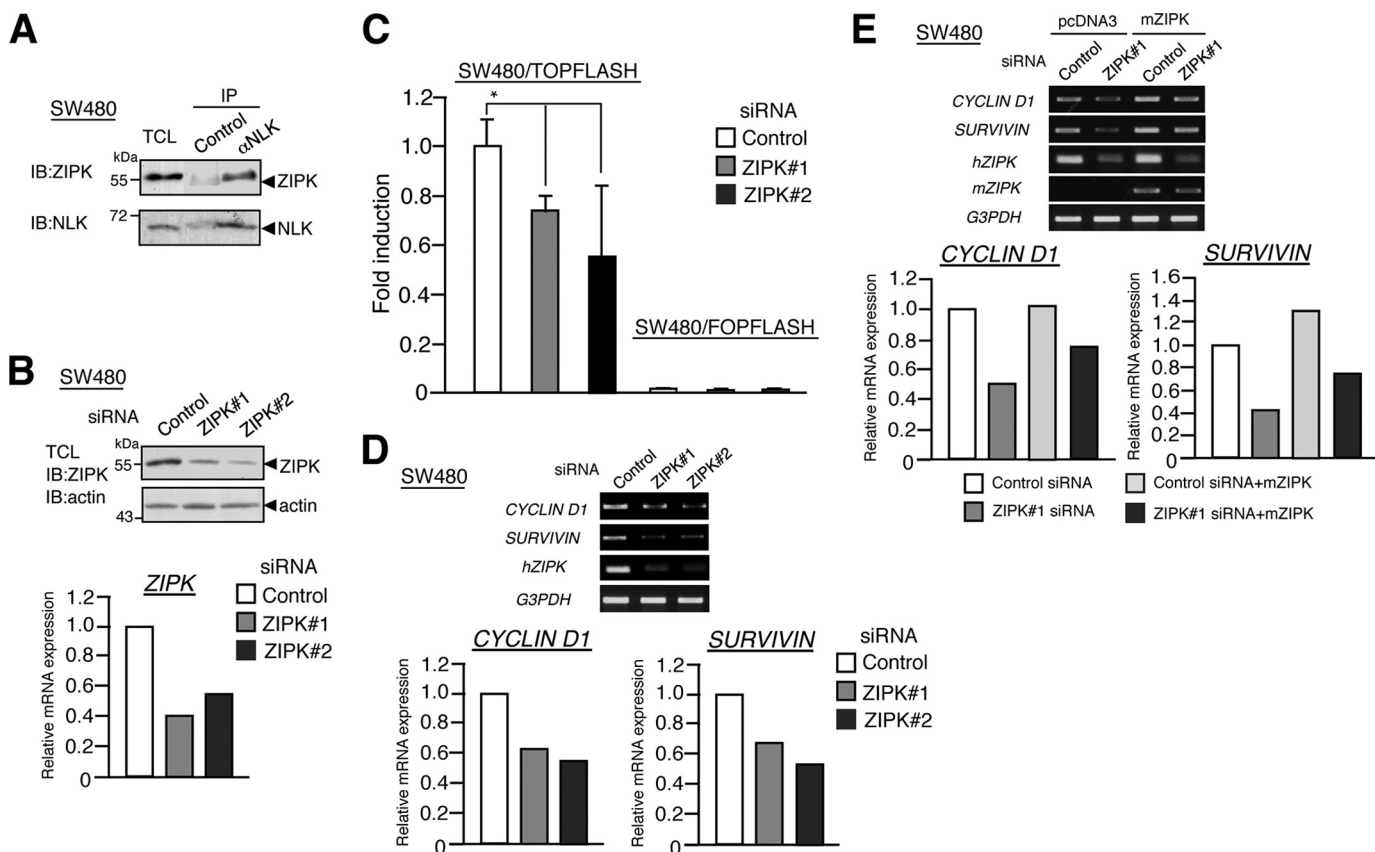


FIGURE 4. ZIPK knockdown influences β -catenin/TCF-mediated gene expression in SW480 colon carcinoma cells. *A*, SW480 cells (3×10^7) were lysed and immunoprecipitated with control or anti-NLK IgG, and blotted with anti-ZIPK antibody (IB). TCL (1%) was blotted with anti-NLK or anti-ZIPK antibody. *B*, SW480 cells were transfected in a 24-well plate with control, ZIPK-1, or ZIPK-2 siRNA. At 24 h after transfection, TCL (1%) was blotted with anti-ZIPK or anti-actin antibody. Total RNA samples isolated from these cells were also quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of these mRNA normalized to that of an actin internal control and are expressed relative to the value of control siRNA-treated samples. Shown is a representative experiment, which was repeated at least three times with similar results. *C*, SW480 cells were transfected in a 24-well plate with control, ZIPK-1, or ZIPK-2 siRNA (20 pmol) and then transfected with TOPFLASH or FOPFLASH (100 ng). At 24 h after transfection, cells were treated with LiCl (30 mM) for an additional 12 h. The cells were harvested and assayed for luciferase activity as described in Fig. 2A. *, $p < 0.05$. *D*, SW480 cells in a 24-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA (20 pmol). Total RNA samples isolated from these cells were also subjected to RT-PCR analysis using cyclin D1, SURVIVIN, hZIPK, or G3PDH primers. These mRNA expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of these mRNA normalized to that of an actin internal control and are expressed relative to the value of control siRNA-treated samples. Results are representative of three independent experiments, and the error bars represent S.D. *E*, HeLa cells in a 24-well plate were transfected with control or ZIPK-1 siRNA (20 pmol) and then transfected with or without Myc-tagged mouse ZIPK (0.5 μ g). Total RNA samples isolated from these cells were also subjected to RT-PCR analysis using cyclin D1, SURVIVIN, hZIPK, mZIPK, or G3PDH primers. These mRNA expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis. Data represent the levels of these mRNA normalized to that of an actin internal control and are expressed relative to the value of control siRNA-treated samples. Shown is a representative experiment, which was repeated at least three times with similar results.

proteasomal degradation of β -catenin (1, 2). Upon Wnt signaling, phosphorylation of β -catenin is inhibited, resulting in the accumulation and translocation of β -catenin into the nuclei, thereby inducing the expression of several genes such as *c-myc* and cyclin D. Mutations in APC, Axin, and β -catenin, resulting in abolished β -catenin ubiquitylation, are found in many human cancers (1, 2), indicating that the inappropriate activation of Wnt signaling plays an important role in human cancers. Most colorectal cancers have mutations of APC or β -catenin that result in the stabilization of β -catenin and activation of β -catenin target genes, ultimately leading to cancer.

Finally we tested whether ZIPK was involved in gene expression and cell growth of colon cancer cells by using human SW480 colon carcinoma cells that carry an APC mutation. Indeed, we observed that an anti-ZIPK antibody co-immunoprecipitated NLK (Fig. 4A), indicating that the binding of ZIPK to NLK occurs at physiological expression levels in SW480 cells. A specific siRNA for ZIPK-1, ZIPK-2, or a control siRNA was

transfected into SW480 cells, and aliquots of cell lysates were analyzed by Western blotting and qRT-PCR, which confirmed reduced expression of ZIPK (Fig. 4B). The reduced protein levels of ZIPK were observed for at least 5 days after the ZIPK siRNA treatment (data not shown). First, we determined the effects of these siRNAs on TOPFLASH/FOPFLASH activation in SW480 cells. As shown in Fig. 4C, siRNA-mediated reduced expression of ZIPK resulted in a significant reduction of TOPFLASH activation in SW480 cells. We next examined the effect of these siRNAs on β -catenin/TCF-mediated gene expression by RT-PCR and qRT-PCR. As shown in Fig. 4D, the expression of β -catenin/TCF-mediated genes involved in Wnt signaling, including cyclin D1 and SURVIVIN, was reduced by ZIPK knockdown. Furthermore, the reduced expression of these genes in the ZIPK knockdown cells was significantly restored by overexpression of a mouse ZIPK cDNA (Fig. 4E). Therefore, ZIPK positively regulates β -catenin/TCF-mediated gene expression in SW480 cells. We further examined the effect of

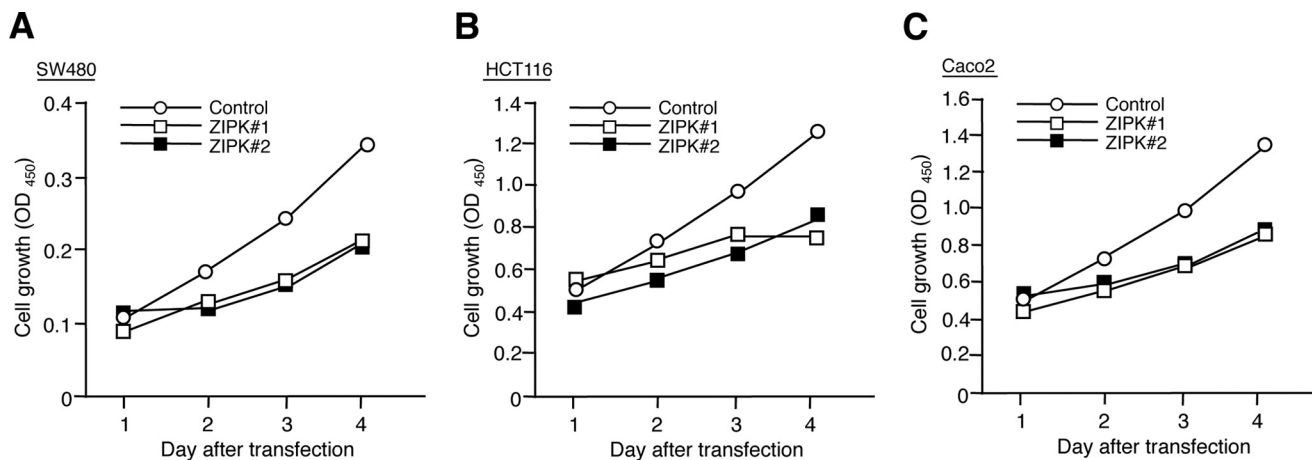


FIGURE 5. ZIPK knockdown influences cell growth in colon carcinoma cells. A, SW480 cells in a 6-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA. At 24 h after transfection, siRNA-transfected cells were harvested and seeded into 96-well plates (5×10^3 /well). At the indicated time point, cell viability was determined by using Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results. B and C, HCT116 (B) or Caco2 (C) cells in a 6-well plate were transfected with control, ZIPK-1, or ZIPK-2 siRNA. At 24 h after transfection, siRNA-transfected cells were harvested and seeded into 96-well plates (5×10^3 /well). An approximately 60% reduction of ZIPK mRNA in HCT116 cells and 50% reduction of ZIPK mRNA in Caco2 by ZIPK siRNAs was confirmed by quantitative real-time PCR analysis (data not shown). At the indicated time point, cell viability was determined by using Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results.

ZIPK knockdown on the growth of SW480 cells. As shown in Fig. 5A, ZIPK knockdown in SW480 cells induced a significant decrease in cell growth, suggesting that ZIPK acts as a positive regulator in Wnt/ β -catenin signaling and enhances SW480 cell growth. We also showed growth suppression by ZIPK siRNA treatment in other colon cancer cell lines (HCT-116 and Caco-2) (Fig. 5, B and C).

DISCUSSION

In the present study, we have shown that ZIPK regulates Wnt/ β -catenin-mediated transcription and gene expression via interaction with NLK. Canonical Wnt/ β -catenin signaling is tightly regulated by a variety of interacting proteins. Recently, many factors have been demonstrated to interact with the β -catenin-TCF4/LEF-1 complex, which includes a group of factors such as TATA-binding protein (20), Pontin52 (21), and Bcl-9/Legless and Pygopus (22, 23), which function as co-activators to enhance transcriptional activity; a second group of factors, such as NLK (5), NARF (24), ICAT (25), Sox9 (26), Chibby (27), APC (28), and P15RS (16), inhibits the activity of the β -catenin-TCF4/LEF-1 complex. The mechanisms of action of the negative regulators of the β -catenin-TCF4/LEF-1 complex are diverse. For example, NLK phosphorylates TCF4 to mediate the dissociation of the β -catenin-TCF4/LEF-1 complex from DNA (5). NARF, an NLK-associated ring finger protein, mediates the ubiquitylation and degradation of TCF4/LEF-1 (24). ICAT (inhibitor of catenin) inhibits the interaction of β -catenin with TCF4 (25). Sox9 interacts with β -catenin to promote phosphorylation and translocation of β -catenin from the nucleus (26). Chibby, a nuclear β -catenin-associated antagonist of Wnt signaling, competes with LEF-1 for binding with β -catenin (27). APC enhances the ability of C-terminal binding protein to inhibit the transcriptional activity of the β -catenin-TCF4/LEF-1 complexes (28). P15RS, a p15INK4b-related gene involved in G1/S progression, interacts with both β -catenin and TCF4 and inhibits the formation of the β -catenin-TCF4 complex (16). We show here that ZIPK functions like a co-ac-

tivator to enhance the activity of the β -catenin-TCF4/LEF-1 complex by competing with NLK on TCF4.

ZIPK is also known as DAPK3. ZIPK and DAPK1 constitute members of a family of death-associated protein kinases that includes DAPK2, DRAK1, and DRAK2 (29), all of which are implicated in executing apoptosis. Furthermore, evidence that the gene encoding DAPK may function as a tumor suppressor has been presented (30, 31). These findings demonstrate the negative regulatory roles of DAPK family proteins. Therefore, a novel function of ZIPK as a positive regulator in Wnt signaling appears attractive. The kinase domain of ZIPK shows strong homology to that of death-associated protein kinase 1 (DAPK1) (6). We examined the effect of siRNA-mediated reduced expression of DAPK1 or DAPK2 on LiCl-induced TOPFLASH activation in HeLa cells. siRNA-mediated reduced expression of DAPK1 or DAPK2 resulted in no significant reduction of LiCl-induced TOPFLASH activation (data not shown), suggesting that ZIPK specifically regulates canonical Wnt/ β -catenin-mediated transcriptional activation in HeLa cells.

Recently, it has been demonstrated that another DAPK family kinase, DRAK2, has lymphoid-specific expression and plays an essential role in maintaining the survival of T cells activated under specific contexts (32). Moreover, DAPK has also been shown to be a positive regulator of mammalian target of rapamycin signaling in response to growth factor activation (33), suggesting that DAPK family kinases may affect not only apoptosis but also cell survival.

Further detailed work will be required to clarify the molecular mechanisms of ZIPK-mediated modulation of Wnt/ β -catenin signaling. It will also provide insights into the development of a novel therapeutic strategy for Wnt/ β -catenin-mediated malignancies.

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