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Title	Zipper-interacting protein kinase modulates canonical Wnt/beta-catenin signaling through interacting with Nemo-like kinase and T-cell factor 4
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Title: **Zipper-interacting protein kinase modulates canonical Wnt/β−catenin signaling through interacting with Nemo-like kinase and T-cell factor 4**

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Running title: Physical and functional interactions between ZIPK and NLK

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 complex formation of NLK-T-cell factor (TCF) 4. 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.

Wnts 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) family of high-mobility-group transcription factors (1, 2). Wnt stabilizes 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 (MAP3K) 7 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 NIH 3T3 cells induced apoptosis, whereas a kinase-inactive mutant, ZIPK K42A, 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, induced 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 interacted with signal transducer and activator of transcription (STAT) 3, and enhanced its transcriptional activity (9). We further demonstrated that leukemia inhibitory factor (LIF) induced threonine-265 (Thr265) phosphorylation of ZIPK (10), which is critical for its kinase activation, suggesting that LIF signaling mediates ZIPK/STAT3 activation through phosphorylation of Thr265.

In this 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 TOPFLAH, FOPFLASH, TCF4 and Wnt3a were kindly provided by Dr. S. Matsuzawa (Burnham Inst., 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 (St. Louis, MO); anti-ZIPK antibody from Beckton Dickinson (Franklin Lakes, NJ); anti-actin antibody from Millipore (Billerica, MA).

Yeast two-hybrid screen, Gal4-ZIPK 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). *S. cerevisiae* AH109 cells were transformed with pGal4-STAT4, then mated with Y187 cells containing a pretransformed mouse 11-day embryo MATCHMAKER cDNA library (Clontech, Palo Alto, CA) and approximately 2.6 x 10^6 colonies were screened as previously described (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 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 2mM L-glutamine. A human colon carcinoma cell line, Caco2 was maintained in DMEM medium 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 (non-silencing; cat. 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, Carlsbad, CA) 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 in the section above. At 24 h after transfection, the cells were left untreated or treated with LiCl (30 mM) for an additional 12h, and 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, quantitative real-time PCR (qRT-PCR), Cells were harvested and total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high-Plus-Kit (TOYOBO, Tokyo, Japan). Primers used for RT-PCR were: CYCLIN D1: 5'-GCTGCTCCTGGTGAACAAGC-3'(sense), 5'-TTCAATGAAATCGTGCGGG-3'(antisense); SURVIVIN: 5'-GGACCACCGCATCTCTACAT-3'(sense), 5'-GACAGAAAGGAAAGCGCAAC-3'(antisense); human ZIPK (hZIPK): 5'-TGCACGACATCTTCGAGAAC-3'(sense), 5'-GTTCTTGTCCAGCAGCATGA-3'(antisense); mouse ZIPK (mZIPK): 5'-TCGCACACTTTGACCTGAAG-3'(sense), 5'-CTCCGCTCAGGAGGATGTAG-3'(antisense). Quantitative real-time PCR (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, The 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; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA). *In vitro* kinase reactions were preformed as described (14). Briefly, immune complex of TCF4 were washed in kinase buffer (10mM HEPES, pH 7.4, 50mM NaCl, 0.1mM sodium orthovanadate, 5mM $MnCl_2$, 5mM $MgCl_2$) and mixed with 5μCi/ml γ-^{32}P-ATP at 25°C for 30 minutes. The products of these reactions were separated by SDS-PAGE.

Chromatin immunoprecipitation (ChIP) analysis
HeLa cells were transfected with control or ZIPK siRNA, and 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′-CACCTCCACCTCACCCCCTAAATCC-3′ and
5′-ACTCCCCTGTAGTCCGTGTGACGTT-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)-2*H*-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. The absorbances were measured at a test wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader (Bio-Rad, Hercules, CA).

Statistical analysis, The significance of differences between group means was 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 (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 over-expression, 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 co-immunoprecipitation with FLAG-tagged NLK. As shown in Fig 1D, the kinase domain of ZIPK (ZIPK KD) as well as full length of ZIPK (ZIPK FL) interacted with NLK. Using a series of deletion mutants of NLK (Fig 1E), we also determined the NLK domain involved in the interaction with ZIPK. As shown in Fig 1F, both the N-terminal (NLK N) and kinase domains (NLK KD) of NLK individually interacted 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). TOPFLASH reporter and vectors expressing ZIPK and/or NLK were transfected into 293T cells, and the cells then treated with LiCl. LiCl activated TOPFLAH activity in 293T cells (Fig 2A). In this situation, LiCl did not

activate reporter harboring mutated LEF/TCF consensus sites (FOPFLASH) (data not shown). LiCl-induced TOPFLASH activity was reduced by expression of NLK as previously described (19). Interestingly, ectopic expression of ZIPK enhanced LiCl-induced TOPFLASH activity (Fig 2A). 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 ZIPK (WT) or a kinase-dead mutant of ZIPK, ZIPK K42A (KA) were transfected into 293T cells, and the cells then treated with LiCl. LiCl-induced TOPFLASH activity was enhanced by expression of ZIPK WT and by KA (Fig 2B), 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 small interfering RNA (siRNA) to reduce the endogenous expression of ZIPK in HeLa cells. Specific siRNA for ZIPK (ZIPK#1, ZIPK#2) or a control siRNA was transfected into HeLa cells, and aliquots of cell lysates were analyzed by western blotting, which confirmed reduced expression of ZIPK (Fig 2 C). First, we determined the effects of these siRNAs on LiCl-induced TOPFLASH/FOPFLAH activation in HeLa cells. As shown in Fig 2C, 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 is specific for TOPFLAH. We then examined the effect of ZIPK siRNAs on TNF-α-induced NF-κB luciferase or IL-6 promoter luciferase. However, we could 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 TOPFLAH. Furthermore, reduced LiCl-induced TOPFLASH activation in the ZIPK-knockdown cells was restored by over-expression of a mouse ZIPK cDNA (Fig. 2D). Therefore, 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 2E), indicating that ZIPK positively regulates canonical Wnt/β–catenin-mediated transcriptional activation in HeLa cells. We further tested whether ZIPK knockdown has any effect on LiCl-induced increase of β-catenin protein levels. As shown in Fig. 2F, siRNA-mediated reduced expression of ZIPK showed no effect on 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. 2G). Therefore, the β-catenin-TCF4 complex might 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 restoration of NLK-mediated repression of Wnt signaling by ZIPK. NLK-mediated repression of Wnt signaling is initiated by a direct interaction between NLK and TCF family proteins (5). We, therefore, examined whether ZIPK directly interacts 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. 3A, a significant interaction of ZIPK with TCF4 was observed, while NLK also bound to TCF4, as described previously (5), indicating that both ZIPK and NLK associate with TCF4. Similar interactions between ZIPK and TFC4 were also observed in HeLa cells (Fig. 3A). We next determined 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, NLK and FLAG-tagged TCF4 in 293T cells, the binding of NLK to TCF4 disappeared in the presence of ZIPK (Fig 3C), suggesting that interaction between ZIPK and NLK disrupts NLK-TCF4 complex formation. This possibility is, in part, supported by the fact that TCF4 recognizes the same NLK-binding domain of ZIPK (Fig 3B). We also tested whether the kinase activity of ZIPK is 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 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, and 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 significant reduced phosphorylation of TCF4 by NLK, indicating that ZIPK-mediated disruption of NLK-TCF4 complex formation influences phopshorylation 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 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 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 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 activate β–catenin target genes, ultimately leading to cancer.

Finally we tested whether ZIPK is involved in gene expression and cell growth of colon cancer cells, using human SW480 colon carcinoma cells that carry an APC mutation. Indeed, we could observed that an anti-ZIPK antibody

co-immunoprecipitates 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 4 B). The reduced protein levels of ZIPK were observed at least for 5 days after the ZIPK siRNA treatment (data not shown). First, we determined the effects of these siRNAs on TOPFLASH/FOPFLAH 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 4 D, the expression of β–catenin/TCF-mediated genes involved in Wnt signaling, including CYCLIN D1 and SURVIVIN, was reduced by ZIPK knockdown. Furthermore, reduced these gene expressions in the ZIPK-knockdown cells was significantly restored by over-expression 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 ZIPK knockdown on cell growth of SW480 cells. As shown in Fig 5A, ZIPK knockdown in SW480 cells induced a significant decrease of cell growth, suggesting that ZIPK acts as a positive regulator in Wnt/β–catenin signaling and enhances SW480 cell growth. We also showed a growth suppression by ZIPK siRNA treatment, in other colon cancer cell lines, HCT-116 and Caco-2 (Fig. 5B 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), Bcl-9/Legless and Pygopus (22, 23), which function as co-activators to enhance transcriptional activity, whereas a second group of factors, such as NLK (5), NARF (24), ICAT (25), Sox9 (26), Chibby (27), APC (28), and P15RS (16) inhibit activity of the β-catenin-TCF4/LEF-1 complex. The mechanisms of action of the negative regulators of the β-cateninTCF4/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 (34). 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 CtBP 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 here show that ZIPK functions like a co-activator to enhance 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 a 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 and will provide insights toward the development of a novel therapeutic strategy for Wnt/β–catenin-mediated malignancies.

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Footnotes

The abbreviations used are: ZIPK, zipper-interacting protein kinase; NLK; Nemo-like kinase; TCF, T-cell factor; DAPK, death-associated protein kinase

FIGURE LEGENDS

Figure 1. ZIPK physically interact with NLK in vivo.

A. 293T cells ($1x10^7$) were transfected with HA-tagged ZIPK (10 μg) and/or Myc-tagged NLK (10 μg). The cells were lysed 48 h later in lysis buffer, immunoprecipitated with anti-HA, and blotted with anti-Myc or anti-HA antibody. TCL (1%) was blotted with anti-Myc antibody. B. HeLa cells ($3x10^7$) were lysed and immunoprecipitated with control or anti-ZIPK IgG, and blotted with anti-NLK antibody (upper panels). HeLa cells ($3x10^7$) were also lysed and 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 schematically shown. D. 293T cells ($1x10^7$ cells) were transfected with Myc-tagged ZIPK WT or its mutants (10 mg) together with or without FLAG-tagged NLK (10 μg). At 48h 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 schematically shown. F. 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged NLK WT or its mutants (10 μg) together with or without Myc-tagged ZIPK (10 μg). At 48h 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.

Figure 2. ZIPK positively regulates the Wnt-mediated transcription in HeLa cells. A. 293T cells in a 24-well plate were transfected with TOPFLAH (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 the luciferase activity. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. **p<0.01. TCL (1%) was blotted with an anti-FLAG or anti-Myc antibody. B. 293T cells in a 24-well plate were transfected with TOPFLAH (100 ng) with or without ZIPK WT or KA mutant (10, 30, 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 the 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 TOPFLAH or FOPFLAH (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 the

luciferase activity as described the above. **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, ZIPK#1 siRNA (20 pmol) and then

transfected with or without Myc-tagged mouse ZIPK (0.5 μg) together with TOPFLAH (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 the luciferase activity as described the above. **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 TOPFLAH (100 ng). At 36 h after transfection, cells were treated with Wnt3a

(culture sup. of Wnt3a-transfected COS7 cells) for an additional 8 h. The cells were harvested and assayed for

the luciferase activity as described the above. **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.   Relative intensity of β-catenin

was normalized to the actin protein of the same sample. G. HeLa cells in a 10-cm dish were transfected with

control, ZIPK#1 or ZIPK#2 siRNA, and followed by transfection with TCF4 (10 μg). At 36 h after

transfection, cells were treated with LiCl (30 mM) for an additional 2 h. Chromatin immunoprecipitation

(ChIP) assay was the performed on siRNA-transfected cells using IgG, or anti-β-catenin antibody. Chromatin

immunoprecipitation DNA was analyzed by RT-PCR with primers specific for the cyclin D1 promoter DNA.

Figure 3. ZIPK influences the interaction between NLK and TCF4. A. 293T cells (1×10^7 cells) were

transfected with FLAG-tagged TCF4 (10 μg) together with or without Myc-tagged ZIPK or FLAG-NLK (10

μg). At 48h after transfection, the cells were lysed, immunoprecipitated with anti-Myc or anti-TCF4, and

blotted with anti-Myc or anti-FLAG antibody. TCL (1%) was blotted with anti-FLAG antibody. HeLa cells

(1×10^7 cells) were transfected with FLAG-tagged TCF4 (10 μg) together with or without Myc-tagged ZIPK

(10 μg). At 48h 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)

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were transfected with FLAG-tagged TCF4 together with or without Myc-tagged ZIPK FL, KD or LZ (10 μg). At 48h 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-Myc antibody. C. 293T cells ($1x10^7$ cells) were transfected with Myc-tagged NLK (10 μg) together with FLAG-tagged TCF4 and/or Myc-tagged ZIPK (10 μg). At 48h 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 ($1x10^7$ cells) were transfected with FLAG-tagged NLK (10 μg) together with Myc-tagged TCF4 (10 μg) and/or FLAG-tagged ZIPK WT or KA (10 μg). At 48h 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 ($1x10^7$ cells) were transfected with FLAG-tagged TCF4 (10 μg) with or without Myc-tagged NLK (10 μg) and/or Myc-tagged ZIPK (10 μg). The cell were lysed and immunoprecipitated with anti-FLAG antibody and assayed for in vitro kinase activity (upper panel). The amounts of TCF4 proteins were shown to be similar by Western blotting with anti-FLAG antibody (middle panel). TCL (1%) was also blotted with anti-FLAG or anti-Myc antibody (lower panels).
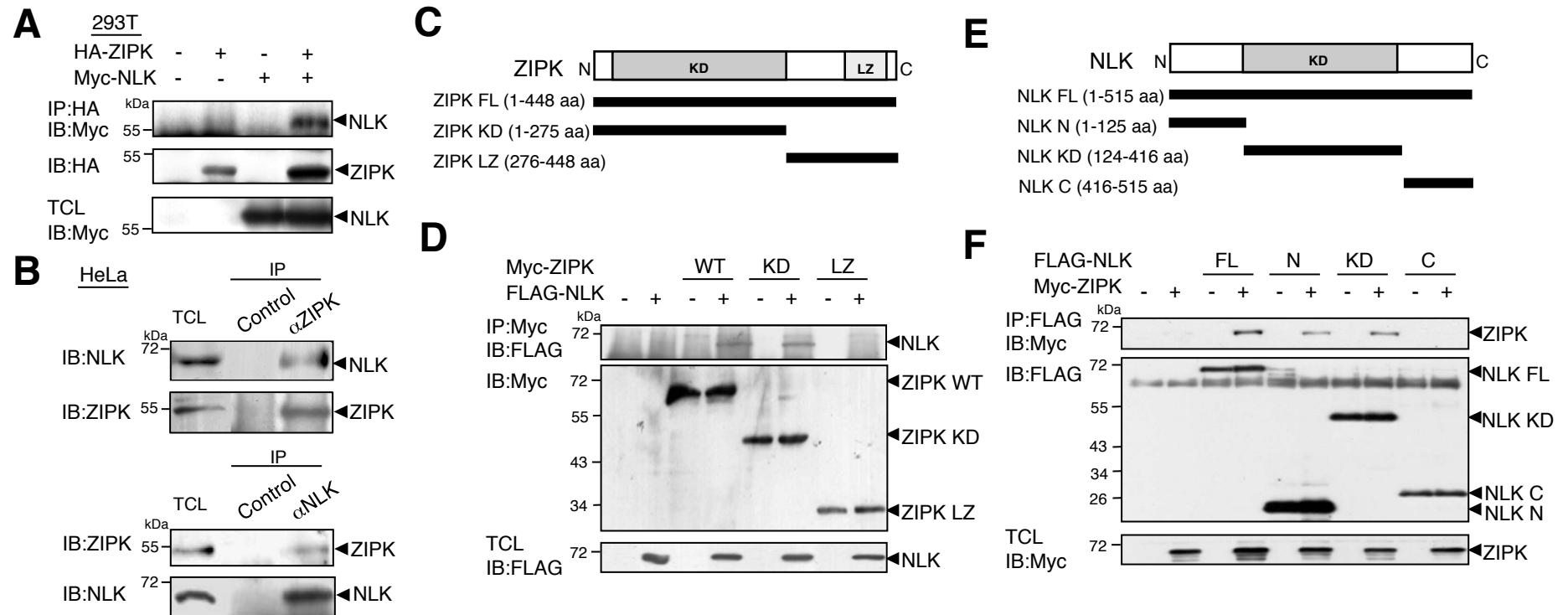
Figure 4. ZIPK knockdown influences β-catenin/TCF-mediated gene expression in SW480 colon carcinoma cells. A. SW480 cells ($3x10^7$) were lysed and immunoprecipitated with control or anti-NLK IgG, and blotted with anti-ZIPK antibody. TCL (1%) was blotted with anti-NLK or anti-ZIPK antibody. B. SW480 cells in a 24-well plate were transfected 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 in a 24-well plate were transfected with control, ZIPK#1 or ZIPK#2 siRNA (20 pmol) and then transfected with TOPFLAH or FOPFLAH (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 the luciferase activity as described the above. $*p<0.05$. D. SW480 cells in a 24-well plate were transfected

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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 the SD. E. HeLa cells in a 24-well plate were transfected with control, ZIPK#1 siRNA (20 pmol) and then transfected with or without Myc-tagged mouse ZIPK (0.5 μg). 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.
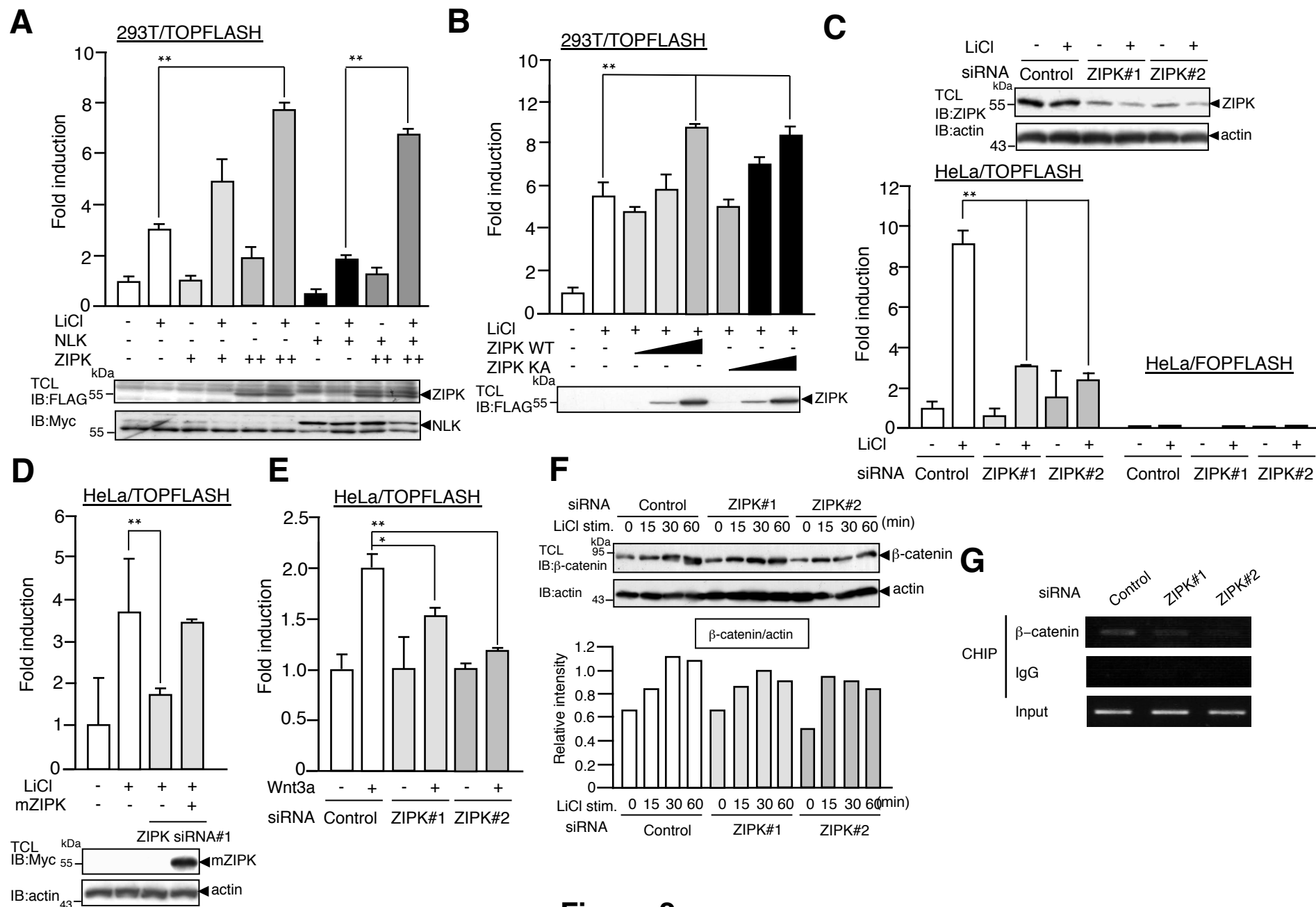
Figure 5.   ZIPK knockdown influences cell growth in colon carcinoma cells.

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 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). Approximately 60% reduction of ZIPK mRNA in HCT116 cells and 50% reduction of ZIPK mRNA in Caco2 by ZIKP siRNAs were confirmed by quantitative real-time PCR analysis (data not shown). At the indicated time point, cell viability was determined by Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results.
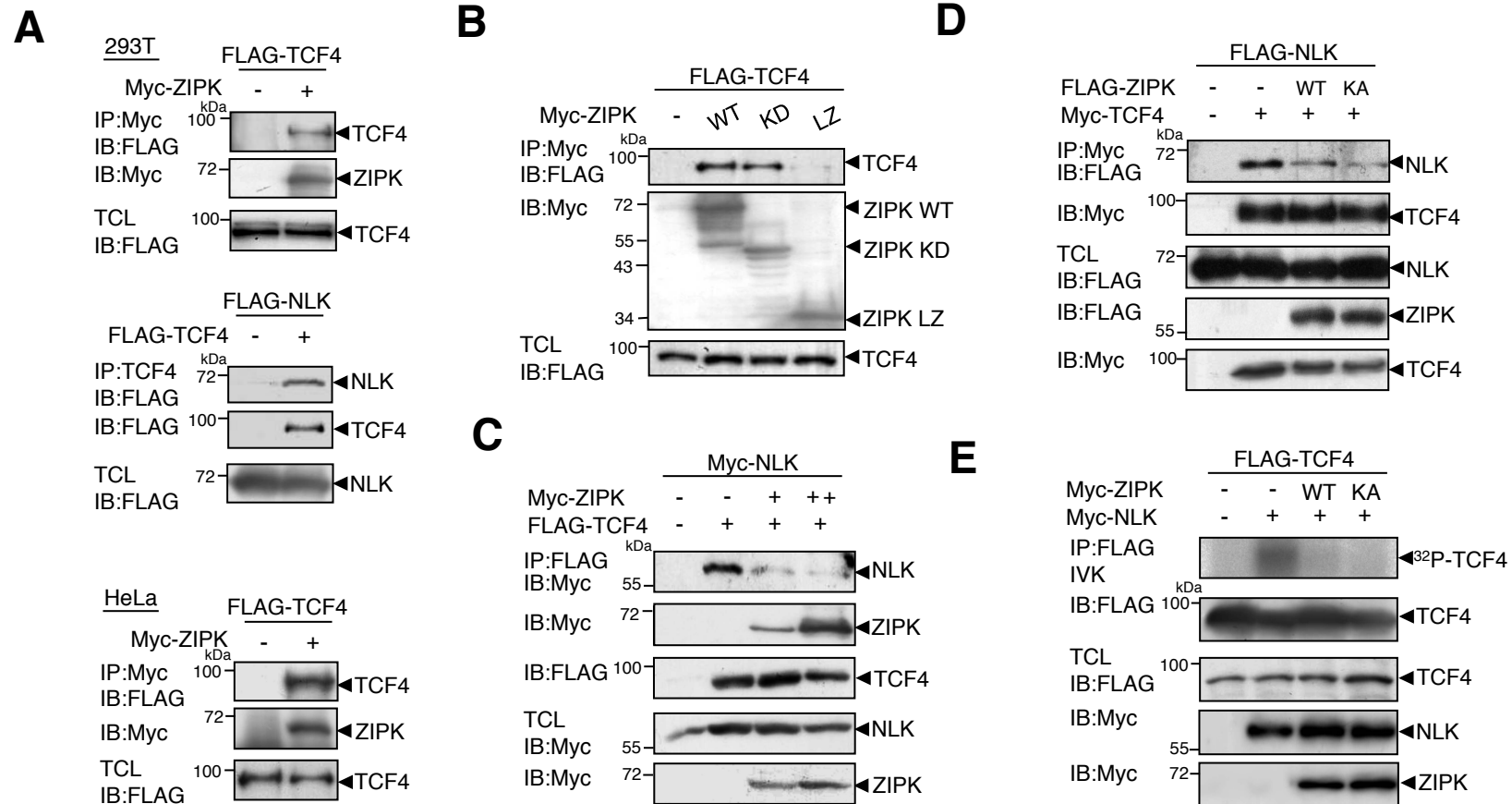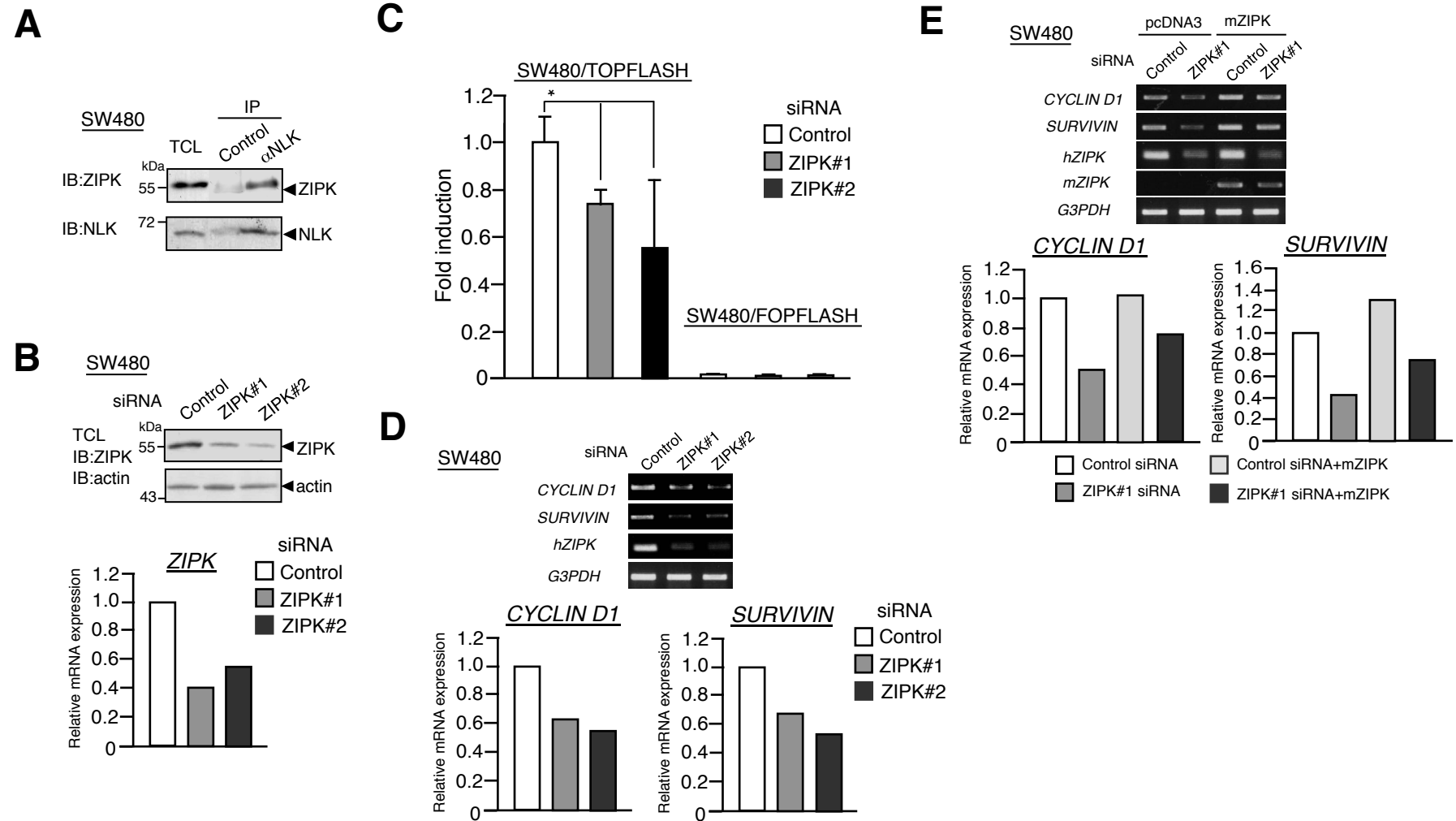
A 293T

| HA-ZIPK | - | + | - | + |
| Myc-NLK | - | - | + | + |

IP:HA
IB:Myc   55 —   ◄NLK

IB:HA   55 —   ◄ZIPK

TCL
IB:Myc   55 —   ◄NLK

B HeLa

IP

TCL   Control   αZIPK

IB:NLK   72 —   ◄NLK

IB:ZIPK   55 —   ◄ZIPK

IP

TCL   Control   αNLK

IB:ZIPK   55 —   ◄ZIPK

IB:NLK   72 —   ◄NLK

C

ZIPK N [KD   ] [LZ]C

ZIPK FL (1-448 aa)
ZIPK KD (1-275 aa)
ZIPK LZ (276-448 aa)

D

| Myc-ZIPK | | | WT | | KD | | LZ | |
| FLAG-NLK | - | + | - | + | - | + | - | + |

IP:Myc
IB:FLAG   72 —   ◄NLK

IB:Myc   72 —   ◄ZIPK WT

            55 —

            43 —   ◄ZIPK KD

            34 —   ◄ZIPK LZ

TCL
IB:FLAG   72 —   ◄NLK

E

NLK N [KD   ]C

NLK FL (1-515 aa)
NLK N (1-125 aa)
NLK KD (124-416 aa)
NLK C (416-515 aa)

F

| FLAG-NLK | | | FL | | N | | KD | | C | |
| Myc-ZIPK | - | + | - | + | - | + | - | + | - | + |

IP:FLAG
IB:Myc   72 —   ◄ZIPK

IB:FLAG   72 —   ◄NLK FL

            55 —   ◄NLK KD

            43 —

            34 —   ◄NLK C
            26 —   ◄NLK N
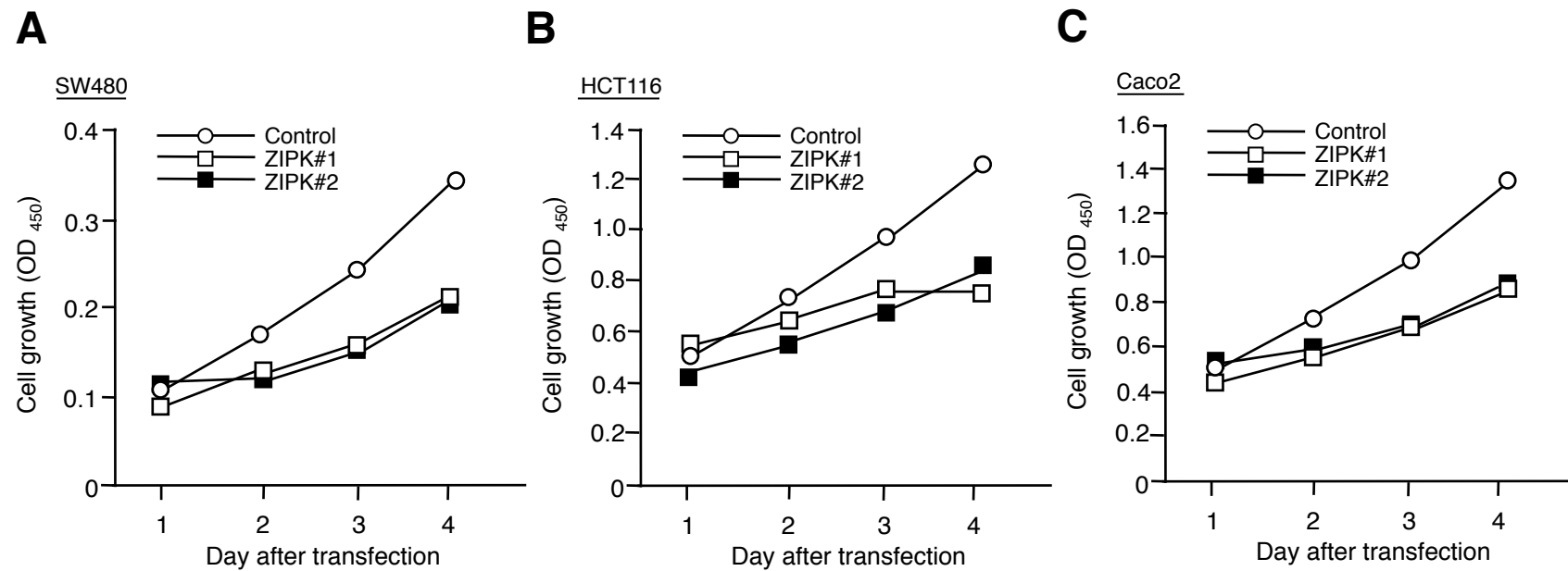
TCL
IB:Myc   72 —   ◄ZIPK

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5