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Identification of a Novel Role of ZMIZ2 Protein in Regulating the Activity of the Wnt/β-Catenin Signaling Pathway*[S]

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Background: ZMIZ2 is a transcriptional coactivator, but its biological role has not been fully investigated.
Results: We demonstrate a promotional role of ZMIZ2 in β-catenin-mediated transcription and cell growth using a variety of biologically relevant *in vitro* and *in vivo* approaches.
Conclusion: ZMIZ2 is a coactivator of the Wnt/β-catenin signaling pathway.
Significance: This study explores a novel mechanism for PIAS-like proteins in regulating Wnt signaling pathways.

ZMIZ2, also named ZIMP7, is a protein inhibitor of activated STAT (PIAS)-like protein and a transcriptional coactivator. In this study, we investigated the interaction between ZMIZ2 and β-catenin, a key regulator of the Wnt signaling pathway. We demonstrated that the expression of exogenous ZMIZ2 augments TCF (T cell factor) and β-catenin-mediated transcription. In contrast, shRNA knockdown of ZMIZ2 expression specifically represses the enhancement of TCF/β-catenin-mediated transcription by ZMIZ2. Using Wnt3a-conditioned medium, we demonstrated that ZMIZ2 can enhance Wnt ligand-induced TCF/β-catenin-mediated transcription. We also showed a promotional role of ZMIZ2 in enhancing β-catenin downstream target gene expression in human cells and in *Zmiz2* null (*Zmiz2*$^{-/-}$) mouse embryonic fibroblasts (MEFs). The regulatory role of Zmiz2 in Wnt-induced TCF/β-catenin-mediated transcription can be restored in *Zmiz2*$^{-/-}$ MEFs that were infected with adenoviral expression vectors for Zmiz2. Moreover, enhancement of Zmiz2 on TCF/β-catenin-mediated transcription was further demonstrated in *Zmiz2* knockout and *Axin2* reporter compound mice. Furthermore, the protein-protein interaction between ZMIZ2 and β-catenin was identified by co-immunoprecipitation and *in vitro* protein pulldown assays. We also observed recruitment of endogenous ZMIZ2 onto the promoter region of the *Axin 2* gene, a β-catenin downstream target promoter, in a Wnt ligand-inducible manner. Finally, a promotional role of ZMIZ2 on cell growth was demonstrated in human cell lines and *Zmiz2* knockout MEFs. Our findings demonstrate a novel interaction between ZMIZ2 and β-catenin and elucidate a novel mechanism for PIAS-like proteins in regulating Wnt signaling pathways.

The protein inhibitor of activated STAT (PIAS)[2] proteins were originally identified as repressors of the STAT transcription factors (1). Multiple lines of evidence have shown that they can function as transcriptional coregulators to modulate the activity of a diverse set of transcription factors such as p53, Smads, and nuclear hormone receptors (2–5). Members of the PIAS family contain a highly conserved extended SP-RING domain, also named the Msx-interacting zinc finger domain (6). This motif appears to be important for interactions with target proteins and is highly similar to the RING finger domain present in E3 ubiquitin ligases (5). Indeed, numerous studies have implicated a role for the PIAS proteins in the ubiquitin-like sumoylation pathway, where they appear to enhance SUMO conjugation of target proteins through the Msx-interacting zinc finger domain (4, 7, 8).

ZMIZ1 and ZIMZ2, originally named ZIMP10 and ZIMP7, are PIAS-like proteins that were originally identified as androgen receptor-interacting proteins (9, 10). They both contain an extended SP-RING domain, in common with other PIAS proteins (11). In addition to this domain, ZMIZ1 and ZMIZ2 proteins also contain a strong intrinsic transactivation domain through which they augment the transcriptional activity of nuclear hormone receptors and other transcription factors (10, 12–16). An ortholog of ZMIZ1 and ZMIZ2, called *tonalli* (*tna*), has been identified in *Drosophila* and genetically interacts with the ATP-dependent SWI/SNF and Mediator complexes (11). ZMIZ2 has been shown to interact with Brg-1 and BAF57, components of the mammalian SWI/SNF complexes (9). ZMIZ proteins have recently been implicated to play a role in tumorigenesis. A t(9;10)(q34;q22.3) translocation between the *ZMIZ1* and *ABL1* genes was found in B cell acute lymphoblastic leukemia (17). Ectopic expression of Zmiz1 in mice induces oncogenic transformation in cutaneous squamous cells (18). An interaction between the ZMIZ1 and NOTCH1 pathways has been implicated in promoting c-MYC activity in acute T lymphoblastic leukemia (19). Multiple lines of evidence suggest that there is no functional redundancy between ZMIZ1 and ZMIZ2 proteins during mouse early development (12, 20). Therefore, it is necessary to precisely assess the biological role of the two ZMIZ proteins in embryogenesis and tumorigenesis.

Wnt/β-catenin signaling plays a critical role in development and tumorigenesis (21). In the canonical pathway, secreted Wnt ligands bind to the coreceptors Frizzled and Lrp5/6 and regulate the stability of β-catenin, a key component of Wnt

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[2] The abbreviations used are: PIAS, protein inhibitor of activated STAT; ZMIZ2, zinc finger MIZ domain-containing protein 2; SUMO, small ubiquitin-like modifier; CM, conditioned medium; MEF, mouse embryonic fibroblast; Luc, luciferase; E10.5, embryonic day 10.5; TCF, T cell factor.

signaling (22). In the absence of a Wnt signal, β-catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3β, axin, and adenomatous polyposis coli (23–26). These proteins promote the phosphorylation of serine and threonine residues in the N-terminal region of β-catenin and, thereby, target it for degradation by the ubiquitin proteasome pathway (27). Wnt signaling inhibits this process and leads to the accumulation of β-catenin in the nucleus, in which β-catenin forms transcriptionally active complexes with members of the Lef/Tcf family of transcription factors (28). Increasing experimental evidence suggests that β-catenin functions as a primary coactivator by recruiting additional transcriptional coregulators in the Wnt signaling pathway (29, 30). Therefore, it is important to identify and define the additional coactivators that regulate the transcriptional activity of β-catenin.

We recently searched for ZMIZ2-interacting proteins and identified a convergence of the Wnt/β-catenin and ZMIZ2 pathways. Using different *in vitro* and *in vivo* approaches, we demonstrated that ZMIZ2 physically interacts with β-catenin. Through the interaction, ZMIZ2 augments β-catenin-mediated transcription and cell growth. The enhancement of ZMIZ2 on β-catenin is induced further by Wnt3a-CM. Further analyses of mouse embryonic fibroblasts (MEFs) isolated from *Zmiz2* knockout embryos showed that loss of Zmiz2 reduces both β-catenin-mediated transcription and cell proliferation. Alteration of the expression of Axin2, a downstream target gene of β-catenin, was observed in *Zmiz2* knockout and *Axin2* reporter compound mice. Down-regulation of other β-catenin downstream targets, such as CD44 and c-Jun, were also observed in Zmiz2 null mice. These data provide the first line of evidence demonstrating an interaction between the Wnt/β-catenin and ZMIZ signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Cultures, Lentivirus and Adenovirus Production, and Transient Transfections—The human embryonic kidney cell line HEK293 was maintained in DMEM supplemented with 5% FCS (HyClone, Denver, CO). The LNCaP and LAPC4 cell lines were maintained as described previously (31). Transient transfections were carried out using a Lipofectamine 2000 kit (Invitrogen). Approximately 1.5×10^4 cells were seeded into a 48-well plate 16 h before transfection. Approximately 300 ng of total plasmid DNA and 0.5 μl of Lipofectamine 2000/well were used in the transfection, as described previously (32). To generate shRNA lentiviruses, pLenti-shRNA vectors, pCMV-dR8.91, and pMD2.G-VSVG plasmids were cotransfected into HEK293T cells at a ratio of 3:2:1 using a Lipofectamine kit as described previously (33, 34). The media were replaced at 6 h post-transfection and then collected 36–40 h later. The viral supernatant was centrifuged briefly to remove cellular debris and stored at −80 °C. Lentivirus infection was carried out in the presence of 6 mg/ml Polybrene. To produce ZMIZ2 expression adenoviruses, we followed the protocol described by Luo *et al.* (35). Briefly, PacI-digested recombinant adenoviral plasmids were transfected into HEK293 cells using a Lipofectamine kit. Recombinant adenoviruses were collected by repeated freeze/thaw cycles after 14 days. The viruses were then used to infect HEK293 cells for virus amplification. Viral supernatant was collected after 2–3 days. The infected HEK293 cells showed the cytopathic effect resulting from virus amplification.

Luciferase Reporter Assays—Wnt3a-CM or control medium (L-CM) was prepared according to a previous report (36). Transient transfection and luciferase assays were performed using Topflash (pGL3-OT, OT-Luc) and Foflash (pGL3-OF, OF-Luc) luciferase reporters as described previously (32). The luciferase activity from individual transfections was measured in a Monolight 3010 luminometer (Pharmingen), normalized by β-galactosidase activity in the same samples, and reported as relative light units. The relative light units were determined from three independent transfections. The results are presented as the mean ± S.D. of triplicate transfections.

RNA Isolation and RT Quantitative PCR Assay—Wnt3a-CM or L-CM was added into cells, and total RNA was isolated 6 h after induction using RNA-Bee (TEL-TEST, Inc., Friendswood, TX). Reverse transcription was carried out following our previous report (37). Briefly, cDNA was synthesized from 1 μg of total RNA with 9 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) using 0.1 μM oligo(dT) primer in a total volume of 20 μl. For quantitative PCR, cDNA samples were mixed with SYBR qPCR Super Mix Universal (Invitrogen) and specific primers in the MX 3005P thermocycler (Stratagene) according to the protocol of the manufacturer. Relative mRNA levels were calculated by the ΔΔ C(T) method as reported by Livak *et al.* (38). Reactions were done in triplicate, and the values were normalized by GAPDH expression levels. Primers for human GAPDH (5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′), human cyclin D1 (5′-CAATGACCCCGCACGATTTC-3′ and 5′-CATGGAGGGCGGATTGGAA-3′), human c-Myc (5′-GTCAAGAGGCGAACACACAAC-3′ and 5′-TTGGACGGACAGGATGTATGC-3′), human Axin2 (5′-TACACTCCTTATTGGGCGATCA-3′ and 5′-TTGGCTACTCGTAAAGTTTTGGT-3′), mouse GAPDH (5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3′), mouse c-Myc (5′-CCCTATTTCATCTGCGACGAG-3′ and 5′-GAGAAGGACGTAGCGACCG-3′), mouse Axin2 (5′-ATGAGTAGCGCCGTGTTAGTG-3′ and 5′-GGGCATAGGTTTGGTGGACT-3′), mouse c-Jun (5′-ACTCGGACCTTCTCACGTC-3′ and 5′-GGTCGGTGTAGTGGTGATGT-3′), mouse cyclin D1 (5′-GCGTACCCTGACACCAATCTC-3′ and 5′-ACTTGAAGTAAGATACGGAGGGC-3′), and mouse CD44 (5′-TCGATTTGAATGTAACCTGCCG-3′ and 5′-CAGTCCGGGAGATACTGTAGC-3′) were synthesized and used in the quantitative PCR reactions.

Immunoprecipitation and Western Blotting—pcDNA3-FLAG-ZMIZ2, alone or with the pcDNA3-β-catenin plasmid, was transfected into HEK293 cells. Transfected cells were cultured for 48 h and then harvested in a buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 2 mM $MgCl_2$, 50 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 25 mM NaF. Lysates were clarified by incubation on ice and centrifugation for 5 min. 400 μl of clarified lysate from each sample was precleared for 20 min with 10 μl of protein-A/G-agarose beads (Pierce). Precleared lysates were then incubated with pre-equilibrated protein-A/G-agarose beads with either normal mouse IgG or FLAG monoclonal

antibody (Sigma) at 4 °C for 3 h. The beads were washed three times in 500 μl of lysis buffer and eluted by boiling in SDS-PAGE sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher & Schüll) and blocked in TBS-T (50 mM Tris-HCl, 150 mM NaCl, and 0.08% Tween 20) with 5% dry nonfat milk. Membranes were probed with FLAG (Sigma), ZMIZ2 (9), β-catenin (BD Biosciences), GFP (Invitrogen), or tubulin (Santa Cruz Biotechnology) antibody at the appropriate dilutions. Anti-rabbit or mouse IgG conjugated to horseradish peroxidase were used as secondary antibodies (Promega). Detection was performed with ECL reagents according to the protocol of the manufacturer using ECL Hyperfilm (Amersham Biosciences).

GST Pulldown Assays—Expression and purification of GST fusion proteins were performed as described previously (31, 39). The full-length ZMIZ2 proteins were generated and labeled *in vitro* by the TNT-coupled reticulocyte lysate system (Promega) with [^{35}S]methionine. Equal amounts of GST fusion proteins coupled to glutathione-Sepharose beads were incubated with the radiolabeled proteins at 4 °C for 2 h in a modified binding buffer (20 mM Tris-HCl (pH 7.8), 180 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 50 μM $ZnCl_2$, 10% glycerol, 0.1% Nonidet P-40, 0.05% dry nonfat milk, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Beads were carefully washed three times with 500 μl of binding buffer and then analyzed by SDS-PAGE, followed by autoradiography.

ChIP Assays—HEK293 cells were treated with L-CM or Wnt3a-CM and then incubated at 37 °C for 1 h. Subsequently, cells were treated with formaldehyde and subjected to ChIP analysis as described previously (14). Briefly, cells were collected and washed sequentially with cold PBS, wash buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)), and wash buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)). Cells were then lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris (pH 8.1), and protease inhibitors). The chromatin was sheared to an average size of 800 bp by sonication, diluted 10-fold in ChIP dilution buffer (2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 1% Triton X-100), subjected to immunoprecipitation with either an anti-ZMIZ2 antibody or anti-β-catenin antibody (BD Biosciences, catalog no. 610254) overnight at 4 °C, and recovered with protein-A/G-agarose beads (Pierce). The immunoprecipitates were serially washed with different TSE (Triton/SDS/EDTA)-based buffers, and eluted for PCR analysis. The immunocomplexes were eluted from the beads through incubation with 10× bead volume of elution buffer (1% SDS and 0.1 M $NaHCO_3$). Cross-links were reversed by incubating the elution samples at 65 °C for 6 h, and chromatin DNA fragments were purified with a PCR purification kit (Qiagen). The above samples and inputs were analyzed by PCR using specific primers for the human Axin2 promoter (40, 41), 5′-GGCTGCGCTTTGATAAGGTC-3′ and 5′-CCCGAAATCCATCGCTCTGA-3′, respectively. Axin2 PCR parameters were as follows: 95 °C for 5 min and then 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s. The samples were also analyzed with primers for the mouse GAPDH promoter region, 5′-CGGTGCGTGCCCAGTTG-3′ and 5′-GCGACGCAAAAGAAGATG-3′, as controls (42).

Mouse Embryonic Fibroblasts—Mice heterozygous for the *Zmiz2* gene were mated, and the female mice were sacrificed at E10.5. The embryos were isolated in cold PBS and then incubated in 500 μl of trypsin (0.25%) for 30 min at 37 °C with intermittent agitation. The embryos were disrupted by pipetting and added to at least a 3× volume of DMEM containing 10% FBS and 1% penicillin/streptomycin. The cells were directly plated into 6-well plates, allowed to adhere overnight, and then used for a quantitative PCR assay. To determine MEF genotypes, embryo yolk sacs isolated during the dissection were digested, and genomic DNA was extracted as described in our previous reports (20, 43) and used for genotyping with appropriate primers for wild-type or mutant *Zmiz2* alleles. Three primers were used to identify wild-type and *Zmiz2*-deleted alleles, including the common forward primer 5′-TGAGGGTTCCTGGCTTCCTATGAT-3′, the wild-type reverse primer 5′-AGCTGGTTAAGGCAGGGTCAGAAG-3′, and the and deleted reverse primer 5′-AGCGGGAAGGCTGTAGAGGTT-3′. PCR was performed as follows: 5 min at 95 °C and then 35 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final step at 72 °C for 5 min.

β-Galactosidase Staining—$Zmiz2^{+/-}$:$Axin2^{+/+}$ females were mated with $Zmiz2^{+/-}$:$Axin2^{LacZ/+}$ males and then sacrificed at E10.5 to isolate embryos. DNA samples were isolated from yolk sacs for genotyping. The embryos were washed three times with PBS at room temperature, fixed in 0.2% glutaraldehyde fix solution at 4 °C for 30 min, and then washed three times at room temperature for 15 min in washing buffer (0.1 M phosphate buffer, 2 mM $MgCl_2$, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate) prior to staining with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution (washing buffer with 5 mM potassium ferrocyanide and 5 mM potassium ferrocyanide) at room temperature for 60 min. The embryos were then washed three times in washing buffer at room temperature for 10 min each before images were taken. $Axin2^{LacZ/+}$ mice were genotyped using the standard Axin2tm1Wbm/J genotyping protocol from The Jackson Laboratory (strain 009120). All animal experiments performed in this study were approved by the ethics committee of the Administrative Panel on Laboratory Animal Care at Stanford University.

MTS Cell Viability Assay—Approximately 2000 cells/well were plated and cultured in the absence or presence of either Wnt3a-CM or L-CM, which was prepared as described previously (36), and then harvested at different time points. Cell growth assays were carried out using an MTS cell proliferation assay kit (Promega). Cell numbers were determined by absorbance at 490 nm as suggested by the manufacturer.

Statistical Analyses—We presented the data as the mean ± S.D. We made comparisons between groups using a two-sided Student's t test. $p < 0.05$ and $p < 0.01$ were considered significant.

RESULTS

ZMIZ2 Regulates β-Catenin/TCF-mediated Transcription—Our previous microarray data suggest a potential involvement of ZMIZ proteins in regulating Wnt/β-catenin-mediated transcription. Both ZMIZ1 and ZMIZ2 proteins contain a strong intrinsic transactivation domain through which they augment

the transcriptional activity of nuclear hormone receptors and other transcriptional factors (10, 12–16). We first tested the effect of ZMIZ proteins on β-catenin/TCF-mediated transcription. Plasmids expressing T cell factor 1 (TCF1), ZMIZ1, ZMIZ2, and either the Topflash (pGL3-OT) reporter containing a multiple β-catenin/TCF binding motif or Fopflash (pGL3-OF) containing a multiple-mutated motif were transfected into HEK293 cells. Only 1-fold induction of transcriptional activity in pGL3-OT above pGL3-OF was observed without cotransfection of TCF1, ZMIZ1, and ZMIZ2 (Fig. 1*A*). Expression of TCF1 alone showed about a 4-fold induction of pGL3-OT activity above the base line. Coexpression of β-catenin with TCF1 enhanced the induction nearly 10-fold. Intriguingly, coexpression of ZMIZ2 with TCF1 showed about an 8-fold induction above the base line. However, there was no further increase when ZMIZ1 was cotransfected with TCF1. Most importantly, the greatest induction of the pGL3-OT reporter was observed in the samples cotransfected with TCF1, β-catenin, and ZMIZ2 proteins, which is significantly different from the samples transfected with TCF1 and β-catenin or ZMIZ2 proteins only ($p < 0.05$). No significant change was observed in the samples that were transfected with the Fopflash (pGL3-OF) reporters. These results provide the first line of evidence demonstrating that ZMIZ2, rather than ZMIZ1, enhances TCF1/β-catenin-mediated transcription. Next, we used plasmids expressing β-catenin, ZMIZ2, or scrambled shRNA to further confirm the augmentation of ZMIZ2 on TCF1/β-catenin-mediated transcription. Expression of β-catenin shRNA reduces the activity of pGL3-OT reporters in the samples transfected with only the TCF1 expression vector ($p < 0.05$), although ZMIZ2 shRNA showed a mild effect in a similar experimental setting. Coexpression of β-catenin or ZMIZ2 shRNA with β-catenin or ZMIZ2 expression vectors specifically reduces β-catenin or ZMIZ2-induced transcriptional activity of pGL3-OT reporters, respectively ($p < 0.01$, Fig. 1*B*). No effect was observed in the samples transfected with the pBS-U6 vector containing scrambled shRNA. There is also a modest reduction of PGL3-OT activity in the samples transfected with ZMIZ2 shRNA and the β-catenin expression vector. We then tested the effect of ZMIZ2 on β-catenin-mediated transcription in the presence of TCF4. Although expression of either β-catenin or ZMIZ2 with TCF4 showed a 4- to 5-fold induction above the base line, coexpression of β-catenin, ZMIZ2, and TCF4 produced an ~10-fold induction of pGL3-OT reporter activity (Fig. 1*C*). Transfection of β-catenin or ZMIZ2 shRNA expression vectors resulted in a knockdown effect on the transcriptional activity of β-catenin or ZMIZ2 with TCF4 ($p < 0.01$, Fig. 1*C*). These data further demonstrate an enhancement of ZMIZ2 on TCF/β-catenin-mediated transcription.

We next tested the effect of ZMIZ2 on the cyclin D1 promoter, a downstream target gene of the Wnt/β-catenin signaling pathway in HEK 293 cells (44). Expression of ZMIZ2 showed a dosage-dependent induction of cyclin D1 promoter/reporter activity, but no effect was observed in samples transfected with ZMIZ1 expression vectors. A significant increase in cyclin D1 promoter activity was shown in the samples transfected with 20 ng of ZMIZ2 expression vectors ($p < 0.01$).

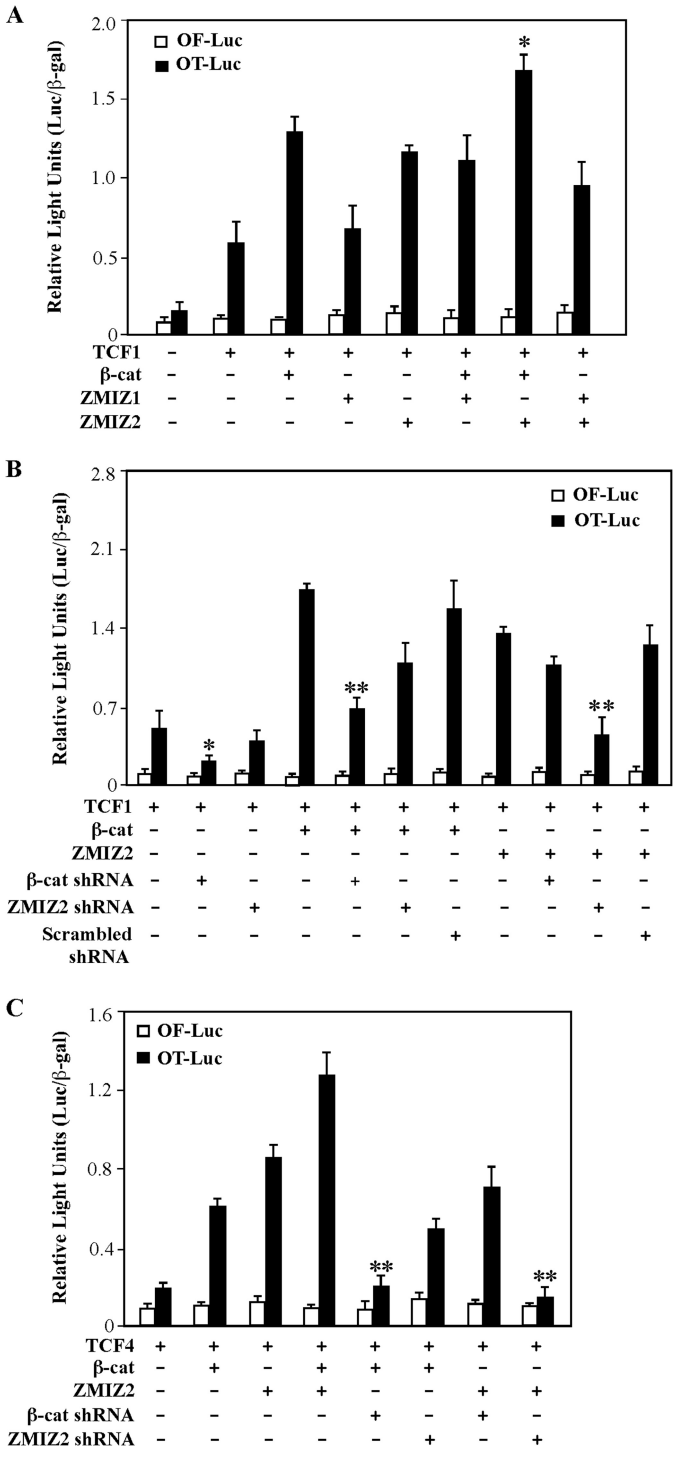

FIGURE 1. **ZMIZ2 controls β-catenin/TCF-mediated transcription.** *A*, 100 ng of pGL3-OT (*OT-Luc*) or pGL3-OF (*OF-Luc*), 25 ng of pcDNA3-β-gal, 5 ng of TCF1 expression vector, 5 ng of β-catenin (*β-cat*), 10 ng of pcDNA3-FLAG-ZMIZ1, and 10 ng of pcDNA3-FLAG-ZMIZ2 were transfected into HEK293 cells. Cells were cultured for 24 h, and luciferase and β-gal activities were measured. *B*, 100 ng of pGL3-OT or pGL3-OF, 25 ng of pcDNA3-β-gal and/or 5 ng of TCF1 expression vector, 5 ng of β-catenin, 10 ng of pcDNA3-FLAG-ZMIZ2, and other shRNA constructs, as indicated in the figures, were transfected into HEK293 cells. Luciferase and β-gal activities were measured. Similar experiments were repeated with the TCF4 expression vector in HEK293 cells *C*, luciferase activity is reported as relative light units (luciferase/β-galactosidase) and represented as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ (see text).

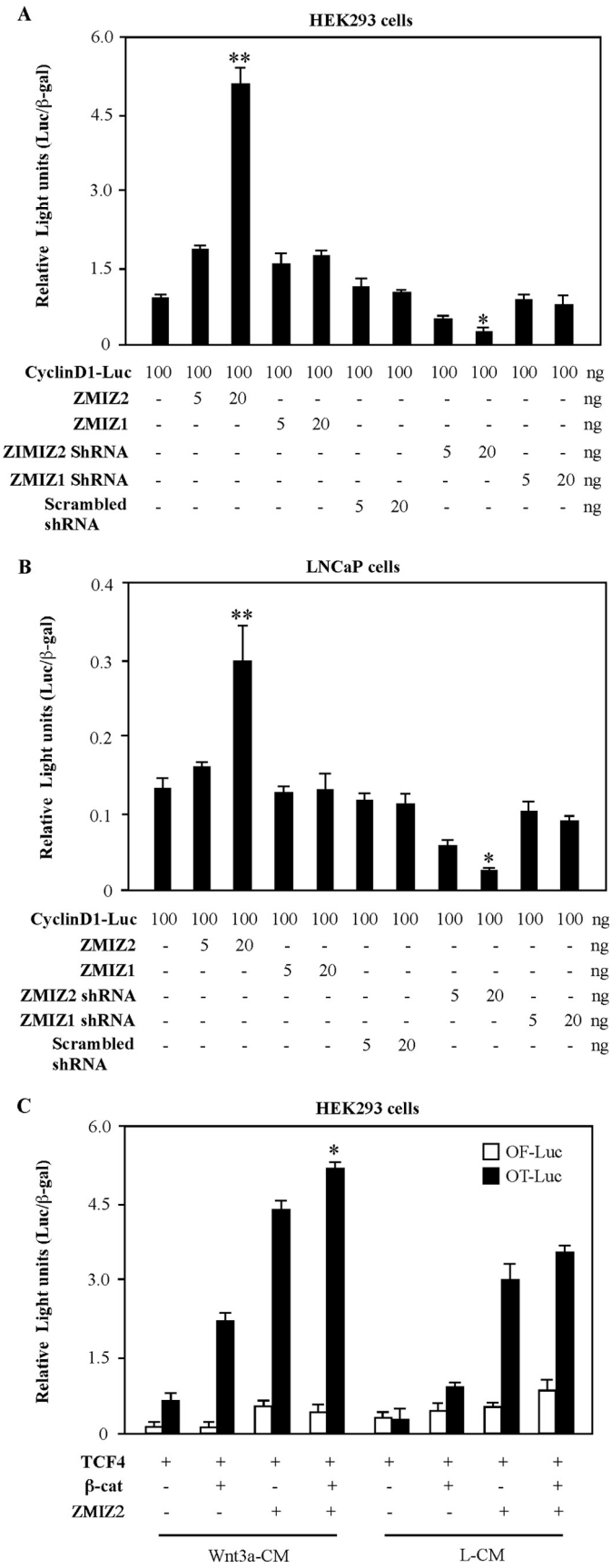

FIGURE 2. **ZMIZ2 regulates cyclin D1 promoter activity and Wnt-induced β-catenin/TCF-mediated transcription.** *A*, 100 ng of pGL3-Cyclin D1 promoter (*Cyclin D1-Luc*), 25 ng of pcDNA3-β-gal, and various amount of pcDNA3-FLAG-ZMIZ2, pcDNA3-FLAG-ZMIZ1, and other shRNA constructs, as indicated, were transfected into HEK293 cells. Cells were cultured for 24 h in the regular media, and luciferase and β-gal activities were measured. Similar experiments were repeated with LNCaP cells (*B*). *C*, 100 ng of pGL3-OT (*OT-Luc*) or pGL3-OF (*OF-Luc*), 25 ng of pcDNA3-β-gal, 5 ng of TCF4 expression vector, 5 ng of β-catenin, and 10 ng of pcDNA3-FLAG-ZMIZ2 were transfected into HEK293 cells. Cells were cultured for 24 h, washed, and incubated with either Wnt3a-CM or L-CM for another 24 h. The cells were harvested, and luciferase and β-gal activities were measured. Luciferase activity is reported as relative light units (luciferase/β-galactosidase) and represented as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ (see text).

Expression of ZMIZ2 shRNA expression vectors reduced endogenous cyclin D1 promoter activity mainly regulated by β-catenin. The samples with 20 ng of ZMIZ2 shRNA showed a significant knockdown effect in comparison with other samples ($p < 0.05$, Fig. 2*A*). Similarly, no significant effect was observed in samples transfected with either ZMIZ1 or scrambled shRNA vectors (Fig. 2*A*). We then repeated the above experiments in the LNCaP prostate cancer cell line. We observed a similar dosage-dependent induction of ZMIZ2, but not of ZMIZ1, on β-catenin-mediated transcription of the cyclin D1 promoter ($p < 0.01$, Fig. 2*B*). Transfection of plasmids expressing ZMIZ2 and β-catenin shRNA sequences also showed a specific knockdown effect on ZMIZ2 and β-catenin-induced cyclin D1 promoter activity in LNCaP cells ($p < 0.05$), respectively. Taken together, these results suggest that ZMIZ2 can enhance the activity of endogenous β-catenin on the cyclin D1 promoter.

It has been suggested that the Wnt ligands can induce the nuclear translocation of β-catenin and facilitate the transcription of Wnt target genes (45). To determine whether enhancement of β-catenin by ZMIZ2 is required by Wnt ligand induction, we performed transient transfection experiments with L-CM or Wnt3a-CM using pGL3-OT and -OF reporters in HEK293 cells. Expression of β-catenin or ZMIZ2 with TCF4 showed an increase in the activity of pGL3-OT in comparison to samples with expression of only TCF4 (Fig. 2*C*). Coexpression of β-catenin, ZMIZ2, and TCF4 produced the highest activity of pGL3-OT. The addition of Wnt3a-CM in the above experiments further induced the activity of pGL3-OT in comparison with the samples transfected with different expression vectors in the presence of L-CM ($p < 0.05$). There was no significant change in the activity of pGL3-OF in the presence of either L-CM or Wnt3a-CM. These results suggest that ZMIZ2 enhances Wnt-induced β-catenin-mediated transcription.

ZMIZ2 Enhances the Transcription of Endogenous TCF/β-Catenin and Target Genes—We examined the effect of ZMIZ2 on the expression of endogenous TCF/β-catenin downstream target genes. We infected HEK293 cells with either ZMIZ2 shRNA or GFP shRNA lentiviruses and then evaluated endogenous ZMIZ2 expression using Western blot analysis. A specific knockdown of ZMIZ2 expression was observed in the samples infected with ZMIZ2 shRNA viruses (Fig. 3*A*). Total RNA samples were isolated from the cells infected with lentiviruses and cultured in the presence of L-CM or Wnt3a-CM. Expression of the endogenous β-catenin target genes cyclin D1, c-Myc, and Axin2 was examined using real-time quantitative RT-PCR assays (Fig. 3*B*). Approximately 0.5- to 1-fold induction of c-Myc, Axin2, and cyclin D1 transcription was observed in HEK293 cells infected with GFP shRNA viruses as controls in the presence of Wnt3a-

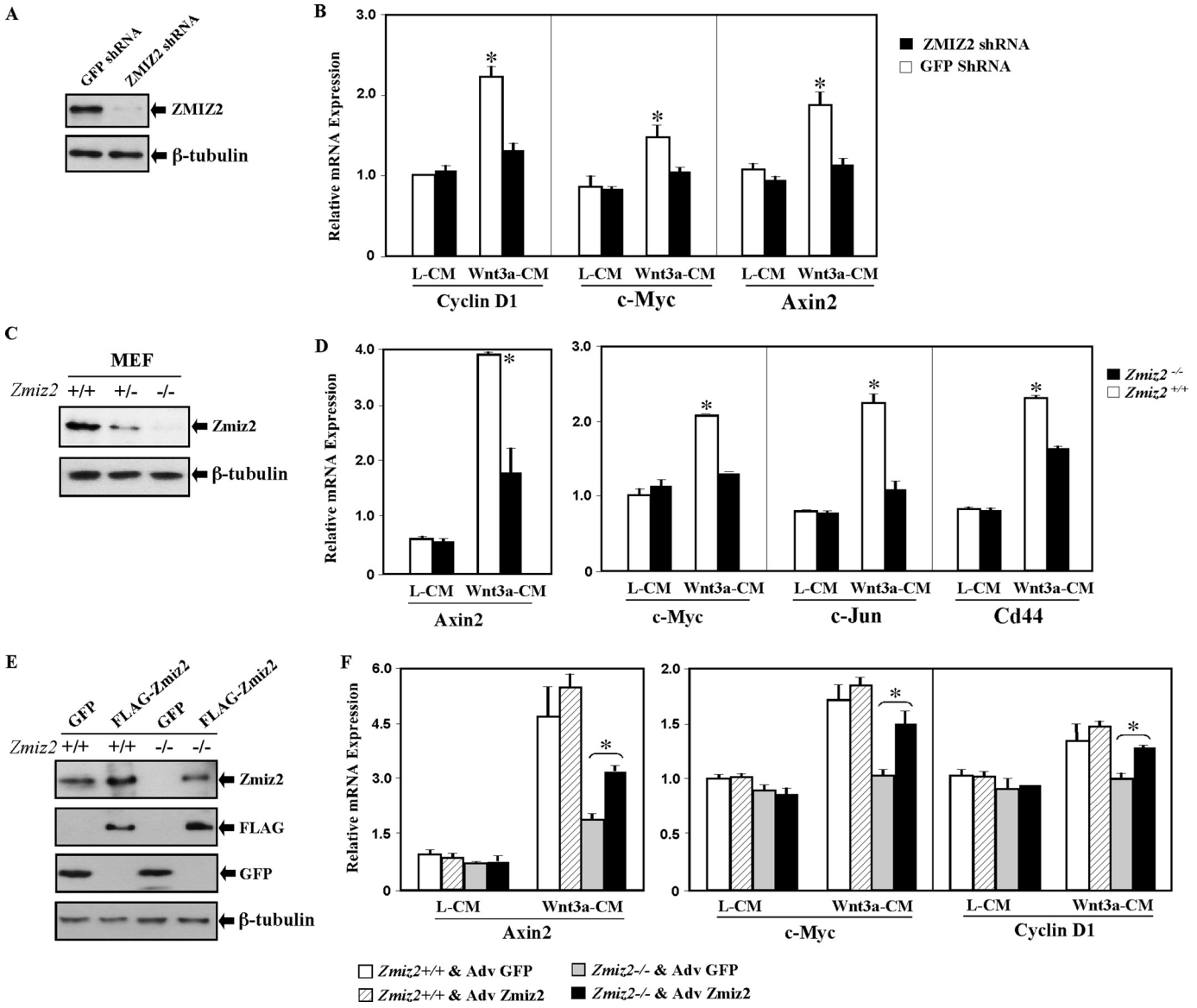

FIGURE 3. **ZMIZ2 is required for the expression of β-catenin target genes in response to Wnt3a.** HEK293 cells were infected with a lentivirus encoding shRNA against ZMIZ2 or GFP. The infected cells were cultured for 72 h and then harvested for Western blot assays with either anti-ZMIZ2 or anti-tubulin antibody (*A*) or incubated for a further 6 h in the presence of Wnt3a-CM or L-CM and then harvested for quantitative RT-PCR (*B*). The levels of cyclin D1, c-Myc, and Axin2 were normalized to that of GAPDH mRNA. *C*, MEFs were prepared from different genotype embryos at E10.5. Whole cell lysates were analyzed by Western blot assays with either anti-ZMIZ2 or anti-tubulin antibody. *D*, MEFS were cultured in the presence of Wnt3a-CM or L-CM for 6 h and then harvested for quantitative RT-PCR. The levels of Axin2, c-Myc, c-Jun, and Cd44 were normalized to that of GAPDH mRNA. *E* and *F*, different genotypes of MEFs were infected with an adenovirus expressing GFP (*Adv GFP*) or FLAG-ZMIZ2 (*Adv Zmiz2*). The infected cells were cultured for 24 h, harvested for Western blot assays with anti-ZMIZ2, anti-FLAG, anti-GFP, or anti-tubulin antibody *(E)*, or incubated for a further 6 h in the presence of Wnt3a-CM or L-CM and then harvested for quantitative RT-PCR (*F*). The levels of Axin2, c-Myc, and cyclin D1 were measured and normalized to that of GAPDH mRNA. The relative mRNA levels from each sample are presented as the mean ± S.D. of triplicate reactions. *, $p < 0.05$ (see text).

CM. The induction of the three target genes was almost fully diminished in cells infected with ZMIZ2 shRNA viruses ($p < 0.05$, Fig. 3*B*). These results demonstrate a promotional role of ZMIZ2 in inducing endogenous β-catenin target gene expression. To further test the regulatory role of ZMIZ2 *in vivo*, we developed Zmiz2 knockout mice in which the *Zmiz2* gene locus is disrupted. We isolated MEFs from E10.5 embryos and examined endogenous Zmiz2 expression in these cells. Expression of Zmiz2 is fully lacking or reduced in $Zmiz2^{-/-}$ or $Zmiz2^{+/-}$ MEFs (Fig. 3*C*). RNA samples were isolated from the different genotype MEFs, cultured in either L-CM or Wnt3a-CM, and used to assess the expression of Wnt target genes using real-time quantitative RT-PCR assays. There was no significant difference in expression of the *Axin2, c-Myc, c-Jun*, and *CD44* genes between RNA samples isolated from $Zmiz2^{-/-}$ or $Zmiz2^{+/+}$ MEF cultures with L-CM. Although an increase in expression of the target genes was observed in both the $Zmiz2^{-/-}$ and $Zmiz2^{+/+}$ MEF samples in the presence of Wnt3a-CM, a significant elevation in the expression of these genes only appeared in $Zmiz2^{+/+}$ *versus* $Zmiz2^{-/-}$ MEFs ($p < 0.05$, Fig. 3*D*). These data further support our previous observation that ZMIZ2 enhances Wnt ligand induced β-catenin-mediated transcription.

To further confirm the significance of Zmiz2 in β-catenin-mediated transcription, we infected Zmiz2 expression adeno-

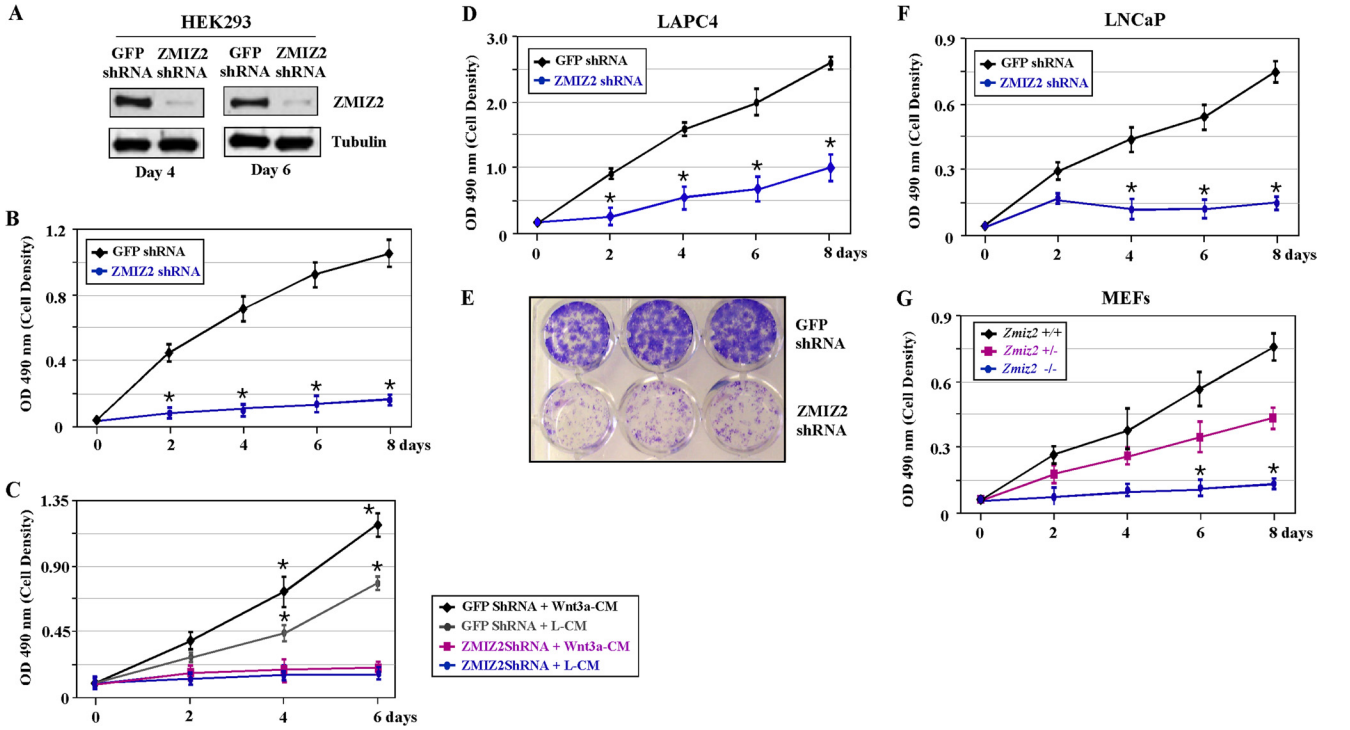

FIGURE 4. **Loss of ZMIZ2 inhibits cell growth.** *A*, HEK293 cells were infected with a lentivirus encoding shRNA against ZMIZ2 or GFP. The infected cells were cultured for 4 and 6 days and then analyzed by Western blot assays with either anti-ZMIZ2 or anti-tubulin antibody. Infected HEK293 cells were seeded into 96-well plates and cultured in the regular media (*B*) or in the presence of Wnt3a-CM or L-CM (*C*). Then, cell growth was measured every other day by MTS assay. ZMIZ2 shRNA or GFP shRNA encoding lentivirus-infected LAPC4 cells (*D*) and LNCaP cells (*F*) were cultured, and then cell growth was measured by MTS assay. *E*, for the colony formation assay, ~500 LAPC4 cells infected with either GFP shRNA or ZMIZ2 shRNA were seeded in 6-well plates. After 12 days, cells were fixed and stained with crystal violet. *G*, MTS cell assay using E10.5 MEFs isolated from *Zmiz2* heterozygous intercrosses ($^{+/+}$, $n = 3$; $^{+/-}$, $n = 4$; $^{-/-}$, $n = 3$). The data represent the mean ± S.D. of three independent experiments. *, $p < 0.05$ (see text).

viruses into Zmiz2 null MEFs and assessed whether exogenous Zmiz2 expression could restore β-catenin-mediated transcription in Zmiz2 null cells. The specific expression of exogenous Zmiz2 was detected in Zmiz2 null MEFs that were infected with Zimz2 expression adenoviruses (Fig. 3*E*). Using quantitative PCR approaches, we measured the expression of β-catenin downstream target genes in both $Zmiz2^{+/+}$ and $Zmiz2^{-/-}$ MEFs in the presence of Wnt3-CM or L-CM. We observed that, in presence of Wnt3a-CM, expression of exogenous Zmiz2 proteins can restore β-catenin downstream target expression in $Zmiz2^{-/-}$ MEFs. $Zmiz2^{-/-}$ MEFs infected with adenoviral expression vectors for Zmiz2 and GFP showed significant differences in Axin2, c-Myc, and cyclin D1 expression ($p < 0.05$, Fig. 3*F*). These results provide additional lines of evidence demonstrating the promotional role of Zmiz2 in regulating Wnt ligand induced β-catenin-mediated transcription.

ZMIZ2 Enhances Cell Growth—Next, we investigated the role of ZMIZ2 in the regulation of cell growth. We infected either ZMIZ2 or GFP shRNA lentiviruses into HEK293 cells and examined endogenous ZMIZ2 expression 4 and 6 days post-infection. A substantial reduction of ZMIZ2 expression was observed at both time points (Fig. 4*A*). We measured cell growth using MTS assays. The number of ZMIZ2 shRNA lentivirus-infected cells was significantly reduced at days 2, 4, 6, and 8 in comparison with those infected with control GFP shRNA viruses ($p < 0.05$, Fig. 4*B*). Because we observed the promotional role of ZMIZ2 on Wnt ligand-induced β-catenin-mediated transcription, we next examined the effect of ZMIZ2 on cell growth in the presence of Wnt3a-CM or L-CM. Wnt3a-CM increased the number of GFP shRNA-infected cells significantly at days 4 and 6 in comparison to cells treated with L-CM ($p < 0.05$), although samples infected with ZMIZ2 shRNA viruses showed less cell numbers than samples infected with GFP viruses in the presence of Wnt 3a-CM or L-CM ($p < 0.05$, Fig. 4*C*). Intriguingly, there was no increase in ZMIZ2 shRNA-infected cells in the presence of L-CM or Wnt3a-CM, implicating an important role of ZMIZ2 in promoting cell growth.

Next, we examined the role of ZMIZ2 in the prostate cancer cell lines LAPC4 and LNCaP. Knockdown of endogenous ZMIZ2 in LAPC4 cells significantly reduced cell numbers at days 2, 4, 6, and 8 in comparison with cell samples that were infected with GFP shRNA viruses ($p < 0.05$, Fig. 4*D*). Using similar approaches, we examined the effect of ZMIZ2 on LNCaP cell growth. Cells infected with ZMIZ2 shRNA lentiviruses were significantly decreased in comparison with those infected with the control GFP shRNA viruses. There was a significant difference between these two groups at days 4, 6, and 8 ($p < 0.05$, Fig. 4*F*). We then assessed the growth-promoting effect of ZMIZ2 using colony formation assays. Approximately 500 LAPC4 cells infected with either GFP or ZMIZ2 shRNA were seeded in 6-well plates. After 12 days, cells were fixed and stained with crystal violet. There were more and larger colonies in the cell samples infected with GFP shRNA viruses in comparison with cells with ZMIZ2 shRNA viruses (Fig. 4*E*). The cells infected with control GFP shRNA viruses had a signifi-

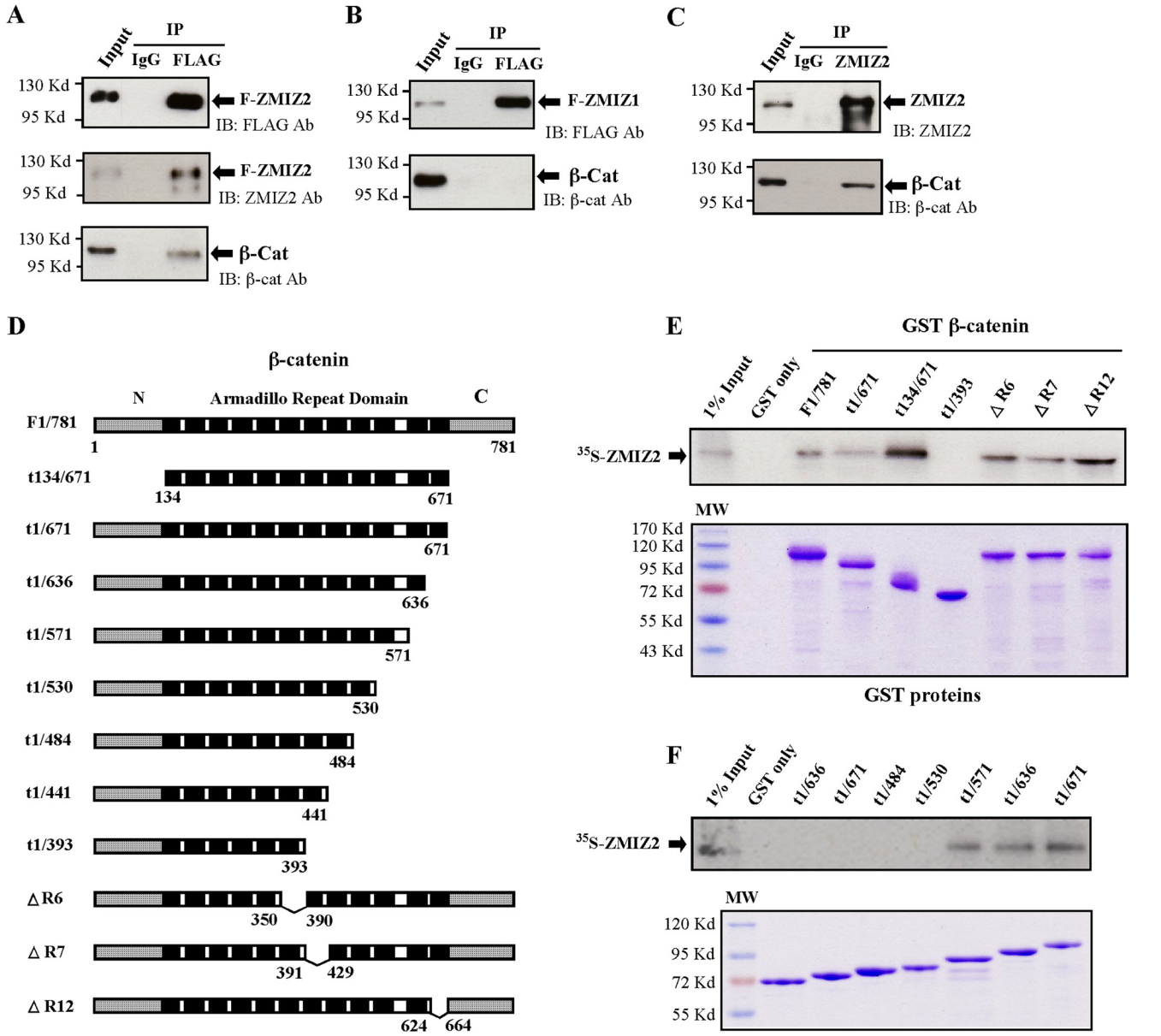

FIGURE 5. **Physical interaction between ZMIZ2 and β-catenin *in vitro* and in intact cells.** *A*, HEK293 cells were transfected with pcDNA3-FLAG-ZMIZ2 and pcDNA3-β-catenin. Cell lysates were then immunoprecipitated (*IP*) with the anti-FLAG antibody (*Ab*) or normal mouse IgG and then analyzed on SDS-PAGE with anti-FLAG, anti-ZMIZ2, or anti-β-catenin (*β-cat*) antibody. *IB*, immunoblot. *B*, HEK293 cells were transfected with pcDNA3-FLAG-ZMIZ1 and pcDNA3-β-catenin. Cell lysates were immunoprecipitated with the anti-FLAG antibody or normal mouse IgG and then analyzed on SDS-PAGE with anti-FLAG or anti-β-catenin antibody. *C*, whole cell lysates of HEK293 cells were immunoprecipitated with the anti-ZMIZ2 antibody or normal rabbit IgG and analyzed on SDS-PAGE with anti-ZMIZ2 or anti-β-catenin antibody. *D*, GST fusion proteins containing full length with both the amino terminus (*N*) and the carboxy terminus (*C*) regions and different deletion mutants of β-catenin were constructed. *E* and *F*, equal amounts of GST fusion proteins containing full-length and different deletion mutants of β-catenin were used to pull down *in vitro*-translated [^{35}S]methionine-labeled full-length ZMIZ2. GST protein alone was used as a negative control and analyzed with other fusion proteins with molecular weight (*MW*) markers. Materials bound to glutathione-Sepharose beads were subjected to SDS-PAGE and autoradiography. Equal amounts of GST fusion proteins were resolved in SDS-PAGE and stained with Coomassie Blue for measuring expression.

cantly greater number of colonies containing 50 or more cells than those infected with ZMIZ2 shRNA viruses ($p < 0.01$). Finally, we assessed the promotional role of ZMIZ2 in cell growth using MEFs generated from E10.5 *Zmiz2*$^{-/-}$, *Zmiz2*$^{-/+}$, or *Zmiz2*$^{+/+}$ embryos. A significant reduction in cell growth was observed in *Zmiz2*$^{-/-}$ MEFs when compared with wild-type or heterozygous MEFs at days 4, 6, and 8 ($p < 0.05$, Fig. 4*G*). Taken together, our data demonstrate that either knockdown or deletion of endogenous ZMIZ2 expression in mouse MEFs or human cancer cells reduces cell growth, implicating a promoting role of ZMIZ2 in cell growth.

ZMIZ2 Interacts with β-Catenin—To understand the mechanism underlying ZMIZ2 augmentation of β-catenin-mediated transcription, we first performed immunoprecipitation assays to examine a potential interaction between ZMIZ2 and β-catenin. We transfected plasmids expressing FLAG-tagged ZMIZ2 and β-catenin into HEK293 cells. Whole cell lysates were isolated and subjected to immunoprecipitation with normal IgG or an anti-FLAG antibody. Input and immunoprecipitate samples were then analyzed by Western blot analysis (Fig. 5*A*). Expression of FLAG-tagged ZMIZ2 was detected by both FLAG and ZMIZ2 antibodies in immunoprecipitates pulled down by

FLAG antibodies (Fig. 5*A*, *top* and *center panels*). Importantly, expression of β-catenin was also detected in FLAG antibody immunoprecipitate but not in normal IgG immunoprecipitate (Fig. 5*A*, *bottom panel*). These results demonstrate an interaction between the ZMIZ2 and β-catenin proteins. Because ZMIZ1 and ZMIZ2 share significant sequence similarity, we extended our investigation to test the interaction between ZMIZ1 and β-catenin using similar immunoprecipitation approaches. However, we did not observe β-catenin proteins in immunoprecipitates with cotransfected FLAG-tagged ZMIZ1 (Fig. 5*B*). Next, we evaluated the interaction between endogenous ZMIZ2 and β-catenin proteins in intact cells. Both endogenous ZMIZ2 and β-catenin proteins were detected in the whole-cell lysates of HEK293 cells (Fig. 5*C*, *top* and *bottom panels*). However, we only detected these endogenous proteins in immunoprecipitates pulled down with ZMIZ2 antibody but not normal IgG, suggesting a specific interaction between endogenous ZMIZ2 and β-catenin.

Using GST pull-down experimental approaches, we further analyzed the physical interaction between ZMIZ2 and β-catenin. A series of GST fusion proteins with the full-length β-catenin and different truncation and internal deletion mutants were generated and immobilized onto a glutathione-Sepharose matrix (Fig. 5*D*). Equal amounts of GST-β-catenin fusion proteins were used in the binding assays with [^{35}S]methionine-labeled ZMIZ2 protein bound to the GST fusion protein containing full-length β-catenin and its mutants lacking N-terminal, C-terminal, and armadillo repeats 7–12 (Fig. 5*E*, *bottom panel*) and then subsequently analyzed by SDS-PAGE and detected by autoradiography. The most pronounced interaction was observed between ZMIZ2 and GST-β-catenin containing the full armadillo repeats (amino acids 134–671). However, there was no binding between ZMIZ2 and the GST-β-catenin deletion mutant lacking the region between armadillo repeats 7–12 (amino acids 1–393). Using three internal deletion mutants of GST-β-catenin, which lack armadillo 6, 7, or 12, we further assessed their binding abilities with [^{35}S]-methionine-labeled ZMIZ2 proteins. Interestingly, all three mutants appeared to retain an interaction with ZMIZ2 proteins. To further map the interaction region of β-catenin with ZMIZ2, we generated a series of C-terminal deletion mutants that lack a single armadillo repeat between repeats 6–12. Although deletion of repeats 12, 11, and 10 retains the binding activity, further deletion of repeats 9–7 fully abolishes the interaction between β-catenin and ZMIZ2 (Fig. 5*F*, *top panel*). Taken together, these data suggest that the context of armadillo repeats between repeats 6–9 is critical for β-catenin to interact with ZMIZ2.

ZMIZ2 Is Recruited onto the Endogenous Axin2 Promoter and Induces Axin2 Expression in Vivo—To further examine the role of ZMIZ2 in regulating endogenous Wnt/β-catenin signaling *in vivo*, we crossed *Zmiz2*$^{+/-}$ mice with the *Axin2*$^{LacZ/+}$ reporter strain to generate *Zmiz2* knockout and *Axin2* reporter compound mice. Axin2 is a downstream target of the canonical Wnt signaling pathway and, therefore, has been frequently used to assess Wnt activity. In the *Axin2*$^{LacZ/+}$ reporter strain, the endogenous *Axin2* gene is replaced with an NLS (nuclear localization signal)-LacZ reporter gene under the control of the

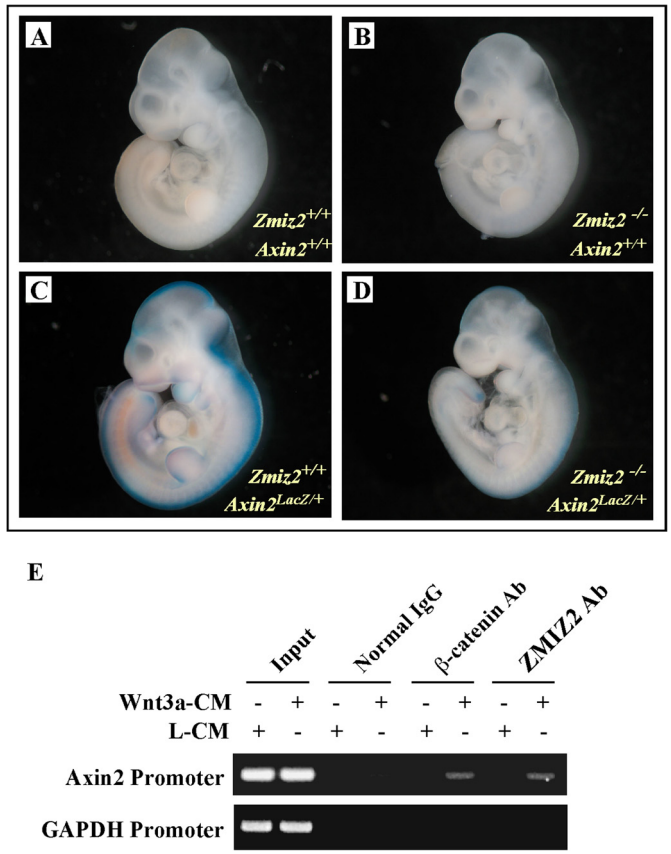

FIGURE 6. **Alteration of the Axin2 expression in Zmiz2 knockout mice.** β-Galactosidase staining of E10.5 embryos using the Wnt activity reporter *Axin2*$^{LacZ/+}$. Reduced staining was observed dorsally and in the forebrain, midbrain, hindbrain, and mandibular branches of *Axin2*$^{LacZ/+}$:*Zmiz2*$^{-/-}$ (*D*) embryos as compared with *Axin2*$^{LacZ/+}$:*Zmiz2*$^{+/+}$ (*C*) embryos. No staining was detected in *Axin2*$^{+/+}$:*Zmiz2*$^{-/-}$ (*B*) and *Axin2*$^{+/+}$:*Zmiz2*$^{+/+}$ (*A*) embryos. *E*, HEK293 cells were cultured in the presence of Wnt3a-CM or L-CM for 1 h. Cells were then cross-linked with formaldehyde, sheared by sonication, and immunoprecipitated with anti-ZMIZ2 antibody, anti-β-catenin antibody, or normal rabbit IgG. Primers specific to the β-catenin/TCF-binding sites (−60, 60 bp before the transcriptional initiation site) in the Axin2 promoter were used to PCR-amplify the eluted chromatin. Primers specific to the GAPDH promoter were used as a control to monitor immunoprecipitation specificity.

endogenous Axin2 promoter/enhancer (46). We assessed *Axin2*^{LacZ} reporter expression during embryonic development using different genotype embryos ($n = 4$). β-Gal staining was detected at E10.5 in *Zmiz2*$^{+/+}$:*Axin2*$^{LacZ/+}$ and *Zmiz2*$^{-/-}$:*Axin2*$^{LacZ/+}$ embryos (Fig. 6, *C* and *D*). However, the staining in *Zmiz2*$^{-/-}$:*Axin2*$^{LacZ/+}$ embryos appears much weaker than in *Zmiz2*$^{+/+}$:*Axin2*$^{LacZ/+}$ embryos (Fig. 6, *D versus C*). The areas of intense staining include the forebrain, midbrain, hindbrain, and mandibular brachial arches. The staining displayed widespread dorsal expression, including the tail bud. Craniofacial staining was apparent. Interestingly, the embryo forelimbs and hind limbs also displayed significant staining. There was no staining in both *Zmiz2*$^{-/-}$:*Axin2*$^{+/+}$ and *Zmiz2*$^{+/+}$:*Axin2*$^{+/+}$ embryos (Fig. 6, *A* and *B*). These results suggest that deletion of Zmiz2 in mouse embryos reduces endogenous β-catenin downstream target expression.

To further assess the mechanism underlying ZMIZ2 enhancing Axin2 expression, ChIP assays were performed to detect the occupancy of ZMIZ2 on Axin2 β-catenin-regulated promoters. HEK293 cells were grown in the presence of Wnt3a-CM or

L-CM. Soluble chromatin was prepared after formaldehyde treatment of the cell cultures, and specific antibodies against ZMIZ2 or β-catenin were used to immunoprecipitate ZMIZ2- or β-catenin-bound genomic DNA fragments. The genomic DNA was analyzed by PCR using specific pairs of primers spanning the β-catenin/TCF-binding sites in the Axin2 promoter (40, 41). The recruitment of both ZMIZ2 and β-catenin was detected within the region of the *Axin2* promoter that contains the functional β-catenin/TCF-binding site in samples cultured with Wnt3a-CM (Fig. 6*E*, *top panel*). There was no recruitment in samples treated with L-CM and within the GAPDH promoter used as a control (Fig. 6*E*, *bottom panel*). These results implicate that ZMIZ2 can be recruited onto β-catenin-involved transcriptional complexes within the Axin2 promoter in response to Wnt3a-CM induction.

DISCUSSION

ZMIZ2 is a PIAS-like protein and shares significant sequence similarity with its homolog, ZMIZ1, particularly within their C-terminal regions (9, 13). These two ZMIZ proteins contain an intrinsic transactivation domain and function as transcriptional coactivators (9, 10). It has been shown that there are different tissue distribution profiles of ZMIZ1 and ZMIZ2, suggesting distinct roles for these proteins in regulating the expression of different target genes. Data from our previous microarray and ChIP-on-chip experiments suggested a potential role of ZMIZ2 on regulating Wnt/β-catenin downstream target genes. In this study, we used both *in vitro* and *in vivo* approaches to demonstrate that ZMIZ2, rather than ZMIZ1, physically interacts with β-catenin and enhances Wnt/β-catenin-mediated transcription and cell growth. Identifying ZMIZ2 in promoting Wnt/β-catenin-mediated transcription and cell growth explores a novel role for ZMIZ proteins in development and tumorigenesis.

In this study, we performed a series of experiments to assess the effect of ZMIZ2 as a transcriptional coactivator on β-catenin-mediated transcription. We observed that expression of exogenous ZMIZ2 or knockdown of endogenous ZMIZ2 affects β-catenin-mediated transcription on both the Topflash (pGL3-OT) and cyclin D1 promoters. The augmentation of ZMIZ2 on β-catenin-mediated transcription occurs in the presence of either TCF1 or TCF4. The regulation of ZMIZ2 on Wnt/β-catenin was also induced by Wnt ligands through Wnt3a-CM. Moreover, we also demonstrated that ZMIZ2 enhances the expression of endogenous β-catenin downstream target genes in both HEK293 cells and MEFs. Furthermore, we showed that expression of Zmiz2 can restore Wnt3a-CM induced β-catenin-mediated transcription in Zmiz2 null MEFs using rescue assays. Finally, the enhancement by Zmiz2 was observed in endogenous Axin2 expression, a downstream target of β-catenin, in *Zmiz2* knockout and *Axin2* reporter compound mice. A decreased expression of Cd44 and c-jun, two downstream targets of β-catenin, was also shown in Zmiz2 null mouse embryos in comparison to wild-type controls (supplemental Fig. 1). These multiple lines of experimental evidence demonstrate an enhancement of ZMIZ2 in Wnt/β-catenin-induced transcription. ZMIZ2 harbors a strong intrinsic transactivation domain within its C terminus (9). Therefore, ZMIZ2 may act as a transcriptional coactivator to augment β-catenin facilitated transcription through this domain. Our finding is consistent with previous observations showing that ZMIZ proteins function as transcriptional coactivators of the androgen receptor, Smad3, and p53 (10, 13, 14).

The Wnt/β-catenin-mediated signaling pathways play a critical role in early development. The interaction between ZMIZ2 and Wnt/β-catenin signaling pathways identified in this study implicates the potential mechanisms for ZMIZ2 in early development. It has been shown that TnaA protein encoded by the *tonalli* (*tna*) gene in *Drosophila* is the ortholog of ZMIZ proteins (11). Expression of the Zmiz proteins has been observed in the developing mouse embryo (20, 47). At E7.5, *Zmiz2* possesses restricted expression in the primitive streak, but at midgestation it expresses in the spinal cord and brain lobules. Interestingly, the dynamics of *Zmiz2* expression extend from anterior to posterior as development proceeds, suggesting a particular transcriptional organization. A recent report has shown that both the *Zmiz1* and *Zmiz2* genes initiate their transcription at early stages in the embryonic male gonad, reaching their peak at 13.5 days post-coitus, which coincides with the process of sex-specific germ cell mitotic arrest (48). Particularly, Zmiz2 is expressed in germ cells of the embryonic gonad and the adult testis and localizes to nuclear areas in meiotic spermatocytes. Interestingly, deletion of Zmiz2 in mice shows no significant defect during embryonic development.[3] However, β-catenin null mice showed severe defects during early embryonic development (49, 50). These results suggest that other coregulators and modulators of β-catenin may be able to compensate for the lack of Zmiz2 regulation on β-catenin activity spatiotemporally during the course of embryonic development. Therefore, investigation of the expression and activation of other β-catenin regulators in Zmiz2 knockout mice may provide useful information regarding the interactive regulatory contexts on activating Wnt/β-catenin signaling during embryogenesis.

Increasing cellular β-catenin has been suggested as a key event in tumorigenesis, as shown by mutations in β-catenin and destruction complex components in tumor cells that inhibit the normal degradation of β-catenin (45, 51). In this study, we also demonstrated that ZMIZ2 enhances the cell growth of HEK293 kidney cells and both LNCaP and LAPC4 prostate cancer cells. This observation is consistent with the effect of ZMIZ2 in enhancing Wnt/β-catenin-mediated transcription and suggests the biological importance of ZMIZ2 in the interaction of the Wnt/β-catenin signaling pathways. Interestingly, a critical role of ZMIZ proteins in tumorigenesis has recently emerged. Specifically, ectopic expression of Zmiz1 in mice induces oncogenic transformation in cutaneous squamous cells (18). An interaction between the ZMIZ1 and NOTCH1 pathways has been implicated in promoting c-MYC activity in acute T lymphoblastic leukemia (19). Recently, we also explored the biological significance of *ZMIZ2* in human tumorigenesis in a pilot experiment. We examined the expression and cellular localization of *ZMIZ2* protein in human prostate cancer specimens. We observed that *ZMIZ2* was stained in the nucleus of luminal

[3] S. H. Lee, D. T. Johnson, and Z. Sun, unpublished data.

cells of normal prostatic glands. However, there was increased *ZMIZ2* staining in the nuclei of tumor cells in several prostate specimens that we tested (data not shown). These results provide the first line of evidence demonstrating that the expression of *ZMIZ2* is elevated in human prostate cancer cells. Intriguingly, mutations in β-catenin, adenomatous polyposis coli, and other components of the destruction complex are very rare in prostate cancer samples (52–54). However, many studies have demonstrated a promoting role of β-catenin in prostate cancer development and progression (55–57). These data suggest that other distinct pathways and mechanisms may regulate cellular β-catenin in prostate tumorigenesis. Therefore, further investigation of aberrant expression and activity of ZMIZ2 in prostate cancer cells should be carried out. It may implicate the dysregulation of the Wnt/β-catenin signaling pathway by ZMIZ2 in inducing cell growth that may directly contribute to the initiation and progression of prostate cancer.

The finding that ZMIZ2 enhances Wnt/β-catenin-mediated transcription suggests a novel molecular mechanism underlying cross-talk between PIAS or PIAS-like proteins and Wnt/β-catenin signaling. β-catenin serves as a scaffold that mediates the assembly of the specific and general transcriptional complexes (58–60). In this study, we have shown that ZMIZ2 physically binds to β-catenin. Using *in vitro* protein pull-down experiments, we found that the region spanning the armadillo repeats 7–9 in β-catenin is critical for binding to ZMIZ2. Given the results from both the biochemical and functional approaches in this study, it appears that ZMIZ2 may interact with β-catenin, TCFs, and other transcriptional coactivators to enhance transcription (61). It has been shown that PIAS proteins can function as SUMO-1 E3 ligases to facilitate sumoylation of steroid receptors and other transcription factors (4). In this study, we also investigated the possible role or involvement of ZMIZ2 in the sumoylation of β-catenin and TCFs. Coexpression of ZMIZ2 and β-catenin and/or TCF4 in the presence of SUMO-1 in cells showed that there is no significant change on the status of β-catenin and/or TCF4 sumoylation (data not shown). The results suggest that the regulatory role of ZMIZ2 on β-catenin transcription may not be mediated through the sumoylation of these proteins.

Accumulating evidence has implicated critical roles for both ZMIZ proteins and Wnt/β-catenin signaling pathways in development and tumorigenesis. The identification of the interaction between ZMIZ2 and β-catenin presented experimental evidence implicating a novel molecular mechanism underlying a significant regulatory process for the cross-talk between these two regulators. ZMIZ2 binds to β-catenin and functions as a transcriptional activator to induce Wnt/β-catenin-mediated cell growth and possible oncogenic transformation. Further effort to investigate this regulation should enhance our knowledge of the Wnt/β-catenin signaling pathway and the role of ZMIZ proteins in early development and tumorigenesis.

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