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Cancer Research

VentX, a Novel Lymphoid-Enhancing Factor/T-Cell Factor–Associated Transcription Repressor, Is a Putative Tumor Suppressor

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Abstract

Lymphoid-enhancing factor/T-cell factors (LEF1/TCF) are a high-mobility group of transcriptional factors that play essential roles in cell fate determination during early embryogenesis and ontogenesis. Aberrant activations of LEF1/TCF-mediated transcription have been implicated in a variety of malignancies. Our recent studies on vertebrate embryogenesis identified Xom, a homeobox protein of the bone morphogenetic protein 4 pathway, as a novel LEF/TCF-associated transcriptional modulator. Here, we report that VentX, a human Xom homologue, is a LEF/TCF-associated inhibitor of canonical Wnt/β-catenin signaling and a negative regulator of cell proliferation. VentX is predominately expressed in hematopoietic cells, and its expression is significantly downregulated in chronic lymphocytic leukemia. Altered expression of VentX is associated with corresponding changes of LEF/TCF target oncogenes such as cyclin D1, suggesting a potential role of VentX in the clinical behavior of hematopoietic malignancies. *Cancer Res; 70(1); 202–11. ©2010 AACR.*

Introduction

Canonical Wnt/β-catenin signaling plays a critical role in cell fate determination during early embryogenesis and has been implicated in a large variety of cancers (1, 2). Besides solid tumors, such as colorectal cancers, canonical Wnt signaling is also involved in oncogenesis of hematopoietic malignancies, such as myeloid and lymphocytic leukemia (3–7).

Lymphoid-enhancing factor/T-cell factor (LEF/TCF) transcriptional factors are high-mobility group box-containing transcriptional factors that function as the nuclear transcriptional mediators of canonical Wnt/β-catenin signaling (8–10). The LEF1/TCF factors possess little intrinsic transcriptional activity (11); rather, LEF/TCF-mediated transcription is tightly controlled by their associated factors (12). In a noninduction state, LEF1/TCFs are associated with transcriptional repressors such as Groucho and CtBP (13–17). In the presence of Wnt signaling, LEF/TCFs form a complex with β-catenin that drives the expression of LEF/TCF downstream genes, including well-established oncogenes such as cyclin D1 and C-myc (11, 18–20).

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During early embryogenesis, the β-catenin/LEF/TCF signaling of the dorsal organizing center is antagonized by the bone morphogenetic protein 4 (BMP4)/Xom signaling of the ventral signaling center (21, 22). Xom (also known as Vent-2, Xbr-1, and Vox) was identified as a homeobox transcriptional mediator of BMP4 and an essential constituent of the ventral signaling center (21, 23–25). In attempting to identify the molecular mechanisms underlying the integration of dorsal and ventral signaling in the establishment of dorsal-ventral asymmetry, we found that Xom is a novel LEF/TCF-associated transcription factor (26). To explore the implications of these findings in oncogenesis, we recently identified VentX as a human Xom homologue through sequence homology search and functional analysis. VentX was originally cloned by Moretti and colleagues (27) as the first human Xom/Vent homologue from a human bone marrow (BM) cDNA library. Originally named VentX2, it was recently renamed VentX to distinguish it from the seven pseudogenes (28).

We report here that VentX is a LEF/TCF-associated factor that represses canonical Wnt/β-catenin signaling by disrupting the formation of the β-catenin/LEF/TCF complex. Tissue expression profiling showed that VentX is expressed predominately in the peripheral leukocytes of healthy individuals, including mature circulating B cells. We found that VentX expression is significantly reduced in peripheral circulating lymphocytes of chronic lymphocytic leukemia (CLL) patients. Gain-of-function and loss-of-function experiments identified VentX as an inhibitor of cell proliferation. Altered expression of VentX in primary CLL cells is associated with corresponding changes in the Wnt/β-catenin/LEF/TCF target oncogenes, such as cyclin D1, suggesting a potential role for VentX in the pathogenesis of hematologic malignancies.

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Materials and Methods

Promoter-luciferase assay. For a typical promoter-luciferase experiment, 2×10^5 cells were seeded onto a 12-well culture plate 24 h before transfection. A plasmid encoding the gene of interest (1 μg), luciferase reporter construct (0.3 μg), and supplementary vector control (in an amount calculated to ensure equal amount of total DNA in each well) were mixed with liposome transfection reagent (3 μL, TransIT, Mirus) and transfected into cultured cells following the manufacturer's instructions. Luciferase activity was measured 48 h after transfection using a TR717 Microplate luminometer (Applied Biosystems). TOPflash and FOPflash plasmids were generous gifts from Dr. Bert Vogelstein (Johns Hopkins University), and the cyclin D1 promoter-luciferase construct was a generous gift from Dr. Anil Rustgi (University of Pennsylvania). The methods of *Xenopus* embryonic manipulation and Goosecoid (Gsc) promoter-luciferase have been reported previously (29).

RNA isolation, reverse transcription-PCR, and real-time PCR. Total RNA was extracted by the Trizol method. The final RNA concentration was determined by A_{260} measurement. First-strand cDNA was synthesized with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Briefly, 3 μg of total RNA from each sample were used for reverse transcription (RT) reaction, and 1 μL of the RT product was used for PCR reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The identities of PCR products were verified by sequencing. Real-time PCR was performed using LightCycler System (Roche) and LightCycler FastStart DNA Master SYBR Green I according to the manufacturer's instructions. The relative levels of gene expression were calculated by the following formula: relative gene expression = $2 - \Delta C_d$ (ΔC_d = cycle of the specific gene − cycle of the reference *GAPDH* gene). Each test was performed in triplicate. The primers for VentX amplification were 5′-AAGGCAATTAGGCGCTGCTT-3′ (forward) and 5′-ACAGAACAACTGAGTCCTCCA-3′ (reverse). The primers for cyclin D1 real-time PCR were 5′-GTTCGTGGCCTCTAAGATG-3′ (forward) and 5′-TTGTTCACCAGGAGCAGC-3′ (reverse). The primers for GAPDH were 5′-AGAACGGGAAGCTTGTCATC-3′ (forward) and 5′-GCCTTCTCCATGGTGGTG-3′ (reverse).

Immunofluorescence and coimmunoprecipitation. For immunofluorescence experiments, green fluorescent protein (GFP)–VentX and myc-TCF4 were cotransfected into HCT116 cells with TransIT. TCF4 was stained with anti-myc antibody followed by secondary Alexa Fluor 568–labeled goat anti-mouse antibody (Invitrogen). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI) and images were taken with confocal microscopy at the core facility of Children's Hospital Boston. The detailed protocol for coimmunoprecipitation was published previously (26).

Cell culture, cell isolation, and cDNA array. Nalm6, Nalm16, Reh, RS11, H Sultan, and CLL samples were generous gifts from L.E. Silberstein's lab at Children's Hospital Boston. 293T cells, Jurkat, and TALL cells were generous gifts from Dr. R. Blumberg's lab at Brigham and Women's Hospital. The other solid tumor cell lines were generous gifts from Dr. Michael Freeman's lab at Children's Hospital. HCT116 and HepG2 were purchased from the American Type Culture Collection. All the cell lines were maintained in RPMI 1640, DMEM, McCoy's 5A, or Eagle's MEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.

Leukocytes from healthy individuals were isolated from discarded leukopaks from healthy anonymous blood donors at Brigham and Women's Blood Donation Center. BM cells were isolated from discarded femoral heads after hip replacement surgery at the Biomedical Research Institute Musculoskeletal Research Center, Brigham and Women's Hospital. Experiments with human materials were performed in accordance with guidelines approved by the institutional review committee of Brigham and Women's Hospital. Total peripheral leukocytes were obtained from leukopaks after lysis of RBCs. T cells, B cells, granulocytes, and monocytes were isolated with MCAS microbeads specifically labeled with CD3, CD19, CD15, and CD14 antibodies, respectively (Miltenyi Biotec). Human B-cell subsets were isolated from BM and peripheral blood. BM mononuclear cells were isolated after Ficoll separation. CD19 MCAS beads were used to isolate the B lineage cells. The enriched B lineage cells were stained with κ-FITC (from BD Pharmingen) and CD19-phycoerythrin (PE; from Ebioscience) and sorted by a BD FACSAria cell sorter at Dana-Farber Fluorescence-Activated Cell Sorting (FACS) Core Facility. The early B lineage cell population included both pro-B and pre-B cells ($CD19^+$, κ chain negative), whereas the late B-lineage cell population included immature B and mature B cells ($CD19^+$, κ chain positive; ref. 30). Peripheral circulating B cells were obtained from total peripheral leukocytes and sorted with CD19-PE antibody and regarded as mature B cells. The $CD5^+$ subset of peripheral B cells from healthy donors was obtained by methods of CD19 MCAS microbead isolation followed by sorting with CD5-PE antibody.

PrimeExpress II Human Normal Tissue cDNA Panel was purchased from PrimGen.

Transient transfection and establishment of VentX short hairpin RNA stable cell lines. For HCT116 and 293T cells, all transient transfections were performed with liposomal TransIT reagent following the manufacturer's instructions. For Reh and Nalm16 cells, transient transfection was performed with electroporation using the Cell Line Nucleofector Kit V (Amaxa Biosystems). VentX short hairpin RNA (shRNA) plasmids were obtained from OriGene Technologies: construct 1, 5′-CAAATCTGCCTGCGCCGGAGAGGACCATG-3′; construct 3, 5′-TTCAGAATCGCCGCATGAAACACAAACGG-3′. To establish VentX knockdown cell lines, Nalm16 cells were transfected with VentX shRNA. Forty-eight hours after transfection, the VentX knockdown cells were selected with 500 ng/mL puromycin treatment for 4 wk. Puromycin was removed 24 h before functional tests.

Cell count, MTS viability assays, and [^{3}H]Thymidine incorporation assays. For VentX overexpression experiments,

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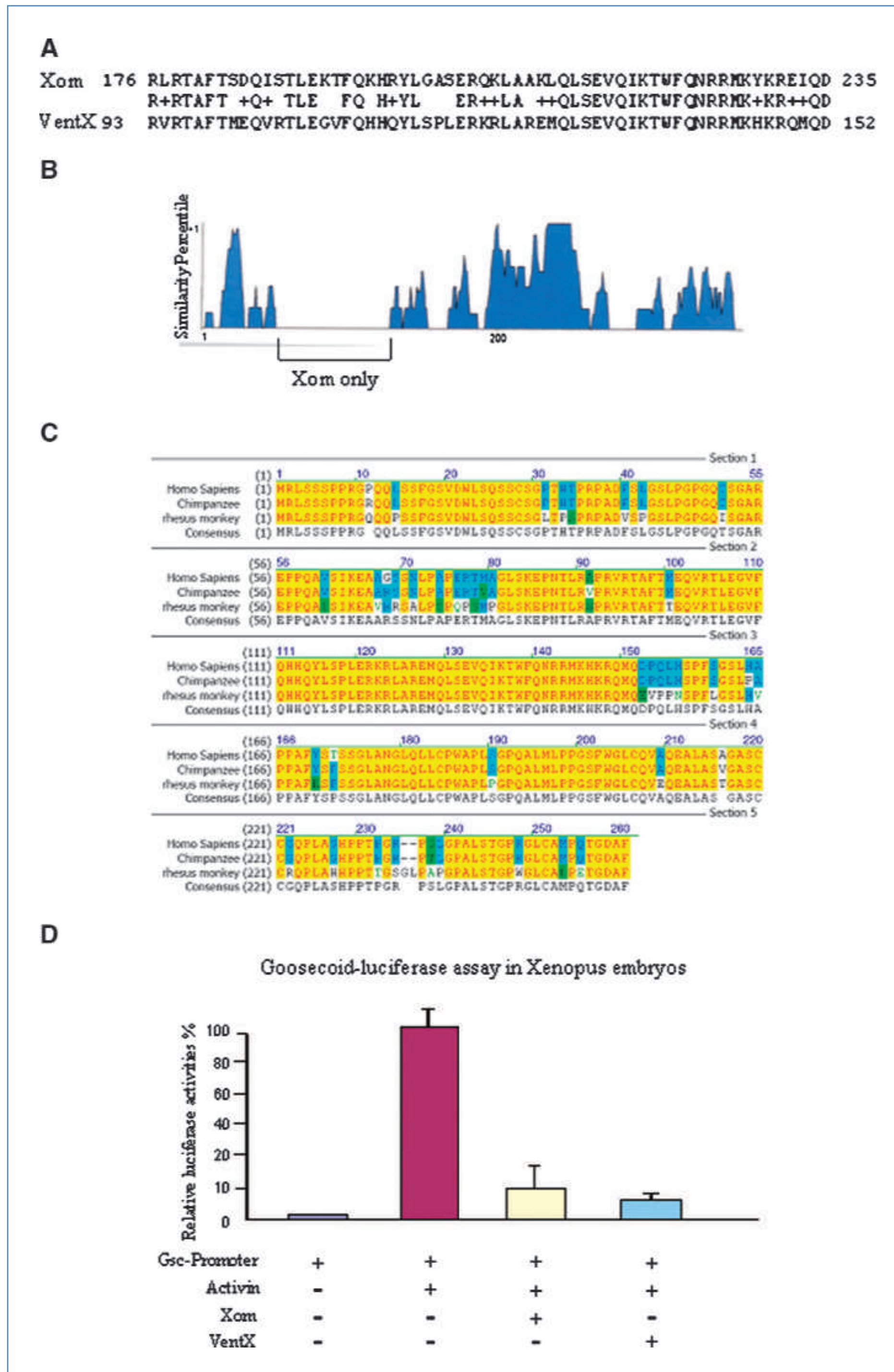

Figure 1. VentX is a human Xom homologue. *A,* amino acid sequence alignment of Xom and VentX homeodomain, which share 68% identical and 85% similar amino acid sequence. *B,* similarity plotting of Xom and VentX polypeptides using Vector NTI. The *Y* axis indicates the similarity percentile, and the *X* axis indicates the amino acid position. *C,* amino acid sequence alignment of VentX predicted chimpanzee and monkey VentX homologue. *D,* VentX inhibits transactivation of Gsc promoter by activin. Gsc promoter-luciferase construct and activin mRNA were coinjected into five *Xenopus* embryos at the two-cell stage with or without VentX mRNA as indicated. Gsc luciferase activity in embryos injected only with Gsc promoter and activin was designated 100% activity. *Columns,* mean; *bars,* SD.

cells were transfected with plasmids encoding GFP-VentX or GFP. Twenty-four hours after transfection, GFP-positive cells were sorted out by a FAC G4 Sort Flow Cytometer (BD Biosciences) and seeded into culture plates.

For cell proliferation assays, 5×10^5 cells were seeded into 12-well plates in triplicate. Cell proliferations were counted four times by trypan blue staining at each indicated time point and are presented as total viable cell count. For MTS assays, cell viability was measured with the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. For $[^3H]$thymidine incorporation assays, 1×10^5 sorted cells were seeded into 96-well culture plates in triplicate. Forty-eight hours after seeding, $[^3H]$thymidine

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(1.0 μCi/well) was added into each well for an additional 18 h. The cells were lysed by freezing at −70°C overnight, transferred to membrane, washed with PBS thrice, and subjected to scintillation counting using the TopCount NXT (Packard Bioscience).

Chromatin immunoprecipitation assay. HeLa cells were cultured in 6-cm dishes for 24 h and then transfected with constructs encoding myc-VentX or myc-tag. Twenty-four hours after transfection, the chromatin immunoprecipitation (ChIP) assay was performed using an assay kit (Upstate Cell Signaling) following the manufacturer's instructions. Cyclin D1 promoter sequence was amplified with specific primers 5′-CGGACTACAGGGGCAACTCC-3′ (forward) and 5′-GCTGCTACTGCGCCGACAGC-3′ (reverse), and the immunoglobulin heavy chain promoter was amplified with specific primers 5′-AACCCTTTTCCCCCTCGTCT-3′ (forward) and 5′-AGCACCTGTGAGGTGGCTGC-3′ (reverse). PCR products were analyzed by electrophoresis on 1% agarose gels.

Statistical analysis. Data were analyzed using the t test. The differences with $P < 0.05$ were considered statistically significant.

Results

VentX is a xom homologue. As shown in Fig. 1*A*, the homeodomain of VentX shares 68% identical and 85% positive (similar) amino acid sequence with Xom. Using a Vector NTI protein alignment program, we found that, besides the homeodomain, VentX and Xom share strong similarities at the COOH-terminal region and the beginning portion of the NH_2-terminal region for ~10 amino acids (Fig. 1*B*). There is an unaligned portion of the NH_2-terminal region between these two molecules that is also functionally relevant (see sections below). Comparative genomic analysis shows that VentX is preserved in primates (Fig. 1*C*), but we and the others failed to find its murine homologue through expressed sequence tag (EST) database search (31).

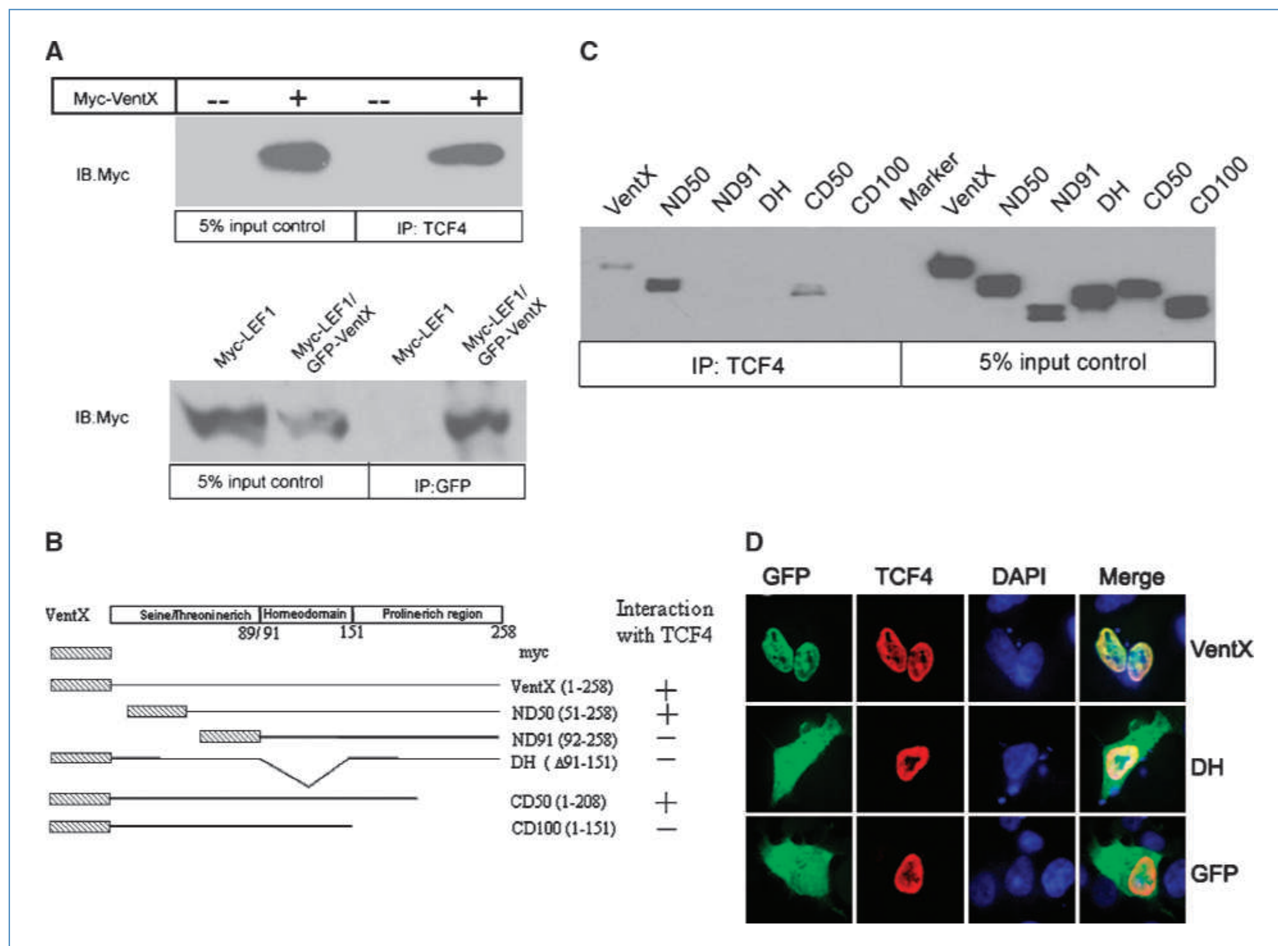

Figure 2. Physical interaction between VentX and LEF/TCF factors. *A, top, in vivo* association between VentX and TCF4 in HCT116 cells, determined by immunoprecipitation-Western blot analysis; *bottom,* interaction between VentX and LEF1 in 293T cells. *B,* schematic presentation of VentX and its deletion mutants. *C, in vivo* interaction of VentX and its deletion mutants with TCF4, determined by immunoprecipitation-Western blot analysis. *D,* colocalization of VentX and TCF4 in HCT116 cells, determined by immunostaining assay and confocal microscopy. The nuclei were labeled with DAPI.

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To identify potential functional similarity between VentX and Xom, we injected mRNA encoding VentX or Xom together with mRNA encoding activin and the Gsc-luciferase reporter construct into one of the two blastomeres of *Xenopus* embryos at the two-cell stage. As shown in Fig. 1*D*, expression of VentX inhibits activin-induced expression of the Gsc promoter, similar to the inhibition of Gsc promoter by Xom. These results are consistent with the prior finding that expression of VentX inhibits dorsalization in zebrafish (27). Nevertheless, Xom transactivates LEF/TCF-mediated transcription through its NH_2-terminal domain, which distinguishes Xom from VentX (see below; ref. 26). Thus, we conclude that VentX is a Xom homologue rather than a Xom ortholog.

VentX forms a complex with LEF/TCF transcriptional factors. To test the hypothesis that VentX interacts with LEF1/TCFs, a plasmid encoding myc-VentX was transiently transfected in colon cancer HCT116 cells, which express endogenous TCF4. The potential interaction between VentX and TCF4 was characterized by coimmunoprecipitation assay. When anti–TCF4-coated beads were applied to HCT116 cell extracts, myc-VentX was readily coimmunoprecipitated with TCF4 (Fig. 2*A, top*). To determine the specificity of the interaction between VentX and members of the LEF/TCF family, we sought to determine whether VentX binds to LEF1. As shown in Fig. 2*A* (*bottom*), 293 cells were transiently transfected with plasmid encoding GFP-VentX, either alone or together with plasmid encoding myc-LEF1. Cell lysates were obtained 48 hours after transfection and subjected to immunoprecipitation-Western blot analysis. We found that VentX forms a complex with LEF1. We next tried to determine the critical domains of VentX involved in the interaction with LEF/TCFs through deletion analysis (Fig. 2*B* and *C*). We found that when the homeodomain and its surrounding region of 50 amino acids were deleted from VentX, the resultant mutants failed to interact with anti–TCF4-coated beads by coimmunoprecipitation, suggesting that the VentX homeodomain and its surrounding region play critical roles in the interaction between VentX and LEF1/TCF factors. Consistently, we found that whereas VentX colocalized with TCF4 in a punctate manner in the nuclei of HCT116 cells (Fig. 2*D, top*), the homeobox-deleted VentX mutant was distributed throughout the cytoplasm of transfected cells (Fig. 2*D, middle*).

VentX inhibits β-catenin transactivation of LEF/TCF-mediated transcription. Our finding that VentX interacts with LEF/TCFs prompted us to examine the effects of VentX on β-catenin transactivation of LEF/TCFs. Using the LEF/TCF reporter TOPflash assay, we found that VentX alone does not activate LEF1/TCF-mediated transcription in 293T cells. Instead, we found that VentX blocks β-catenin transactivation of LEF/TCF-mediated transcription in a concentration-dependent manner (Fig. 3*A, left*). To further confirm the inhibitory effects of VentX on β-catenin signaling, we examined the effects of VentX on LEF/TCF-mediated transcription in HCT116 cells, which show elevated endogenous β-catenin activity (32). Our data show that VentX inhibits LEF1/TCF-mediated transcription in a concentration-dependent manner in HCT116 cells (Fig. 3*A, right*). To explore the mechanism underlying VentX inhibition of β-catenin/LEF/TCF signaling, myc-VentX was transiently expressed in HCT116 cells, and the effects of VentX on the levels of β-catenin expression and the formation of the β-catenin and TCF4 complex were determined. We found that VentX does not change the level of β-catenin mRNA or protein (data not shown; Fig. 3*B*); rather, our data showed that VentX disrupts the formation of a β-catenin and TCF4 complex, which is required for β-catenin transactivation of LEF/TCF target genes (33). As shown in Fig. 3*B*, when the HCT116 cell lysates were subjected to immunoprecipitation with anti-TCF4 beads, with increasing levels of myc-VentX in the immunocomplex, there was a corresponding decrease in the levels of β-catenin in the immunocomplex. These results suggest that disruption of the formation of β-catenin and LEF/TCF complex serves as a mechanism of VentX inhibition of β-catenin/LEF/TCF signaling.

VentX modulates the expression of β-catenin/LEF1/TCF downstream genes. We next examined the effects of VentX on the expression of endogenous LEF/TCF target genes, such as cyclin D1 (18, 34). First, we explored the potential interaction between VentX and the cyclin D1 promoter using a ChIP assay. A plasmid encoding myc-VentX and the control myc-tag were transiently transfected into HeLa cells, which were later subjected to immunoprecipitation with antibodies against myc-tag. The cyclin D1 promoter or IgG heavy chain Cmμ region, which was used as a negative control, was amplified with specific primers. As shown in Fig. 3*C*, VentX binds specifically to the cyclin D1 promoter but not the Cmμ region. The effect of VentX on the expression of cyclin D1 was further tested with the cyclin D1 promoter-luciferase assay. Consistent with VentX inhibition of LEF/TCF-mediated transcription, we found that VentX blocks transactivation of the cyclin D1 promoter-luciferase construct in HCT116 cells, similar to its effect on the TOPflash reporter construct (Fig. 3*D, left*). Consistent with an inhibitory effect of VentX on the expression of cyclin D1, Western blot analysis showed that VentX causes a concentration-dependent decrease in levels of the intracellular cyclin D1 (Fig. 3*D, right*).

Expression profile of VentX in normal tissues and cancer cell lines. National Cancer Institute EST data[4] suggest that VentX is rarely expressed in adult tissues. To explore the physiologic role of VentX, we screened 30 human adult tissues for VentX expression using a cDNA array. We found that VentX is predominately expressed in peripheral blood leukocytes (PBL; Fig. 4*A*). Lineage analysis of PBLs showed that VentX is expressed in both myeloid and lymphoid lineages, including monocytes, B cells, T cells, and neutrophils (Fig. 4*B*). Given the function of LEF/TCF factors in B-cell proliferation and development, we further characterized VentX expression during B-cell development. Our data showed that VentX expression is regulated during B-cell development, with the most abundant expression in

[4] http://cgap.nci.nih.gov/Tissues/VirtualNorthern

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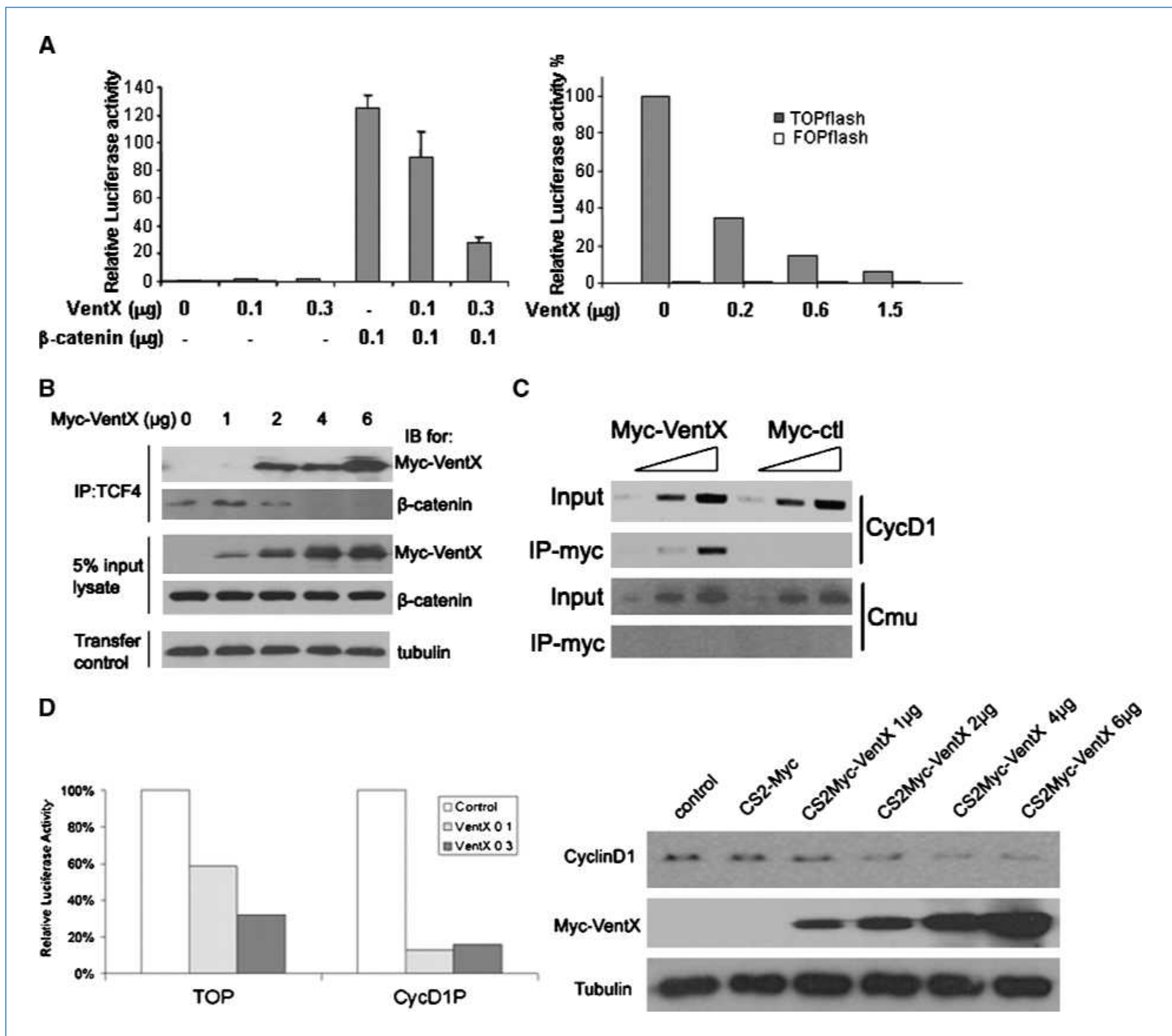

Figure 3. VentX inhibits β-catenin transactivation of LEF/TCF-mediated transcription. *A,* VentX inhibits β-catenin activation of TOPflash. *Left,* 293T cells were transfected with TOPflash reporter construct (0.1 μg) and the indicated expression vectors, and luciferase activity was measured. Luciferase activity in cells transfected with TOPflash alone was designated 1 unit. *Right,* VentX inhibits TOPflash activation in HCT116 cells. HCT116 cells were transfected with TOPflash or control FOPflash reporter constructs together with a construct encoding VentX (or normalized equal amount of empty vector control). Luciferase activity in cells transfected with TOPflash alone was designated 100% activity. *B,* VentX expression disrupted the formation of the β-catenin/TCF4 complex. *C,* ChIP assay showing the formation of a complex between VentX and cyclin D1 promoter. HeLa cell lysates transfected with plasmids encoding myc-VentX or myc-tag were immunoprecipitated with anti-myc antibody. Cyclin D1 promoter or the control immunoglobulin heavy chain (Cmμ) was amplified with specific primers. Note that only cyclin D1 primers amplified a PCR product of expected size. *D,* VentX inhibits activation of cyclin D1 promoter in HCT116 cells. *Left,* HCT116 cells were transfected with cyclin D1 promoter-luciferase construct or TOPflash reporter constructs together with increasing amounts of plasmid encoding VentX as indicated. Luciferase activity in cells transfected with the TOPflash reporter constructs alone was designated as 100%. *Right,* HCT116 cell lysates transfected with increasing amounts of plasmid encoding VentX were subjected to immunoblot with anti–cyclin D1 antibody. Tubulin was used as a loading control. *Columns,* mean; *bars,* SD.

peripheral circulating B cells (Fig. 4*C*). We have further characterized VentX expression in cancer cell lines derived from both solid and hematopoietic malignancies. Of the tested solid cancer cell lines, including colon cancer cell lines HCT116, HT29, Caco2, and SW480; hepatocellular carcinoma HepG2; breast cancer cell line MDA-231 and MCF7; neuroblastoma cell line SK-N-AS; prostate cancer cell lines PC3 and LnCap; cervical cancer cell line HeLa[5]; and eight hematopoietic cancer cell lines (Fig. 5*D*), VentX is rarely expressed except in the Nalm16 cell, a lymphoblastic acute leukemia cell line.

[5] Unpublished data.

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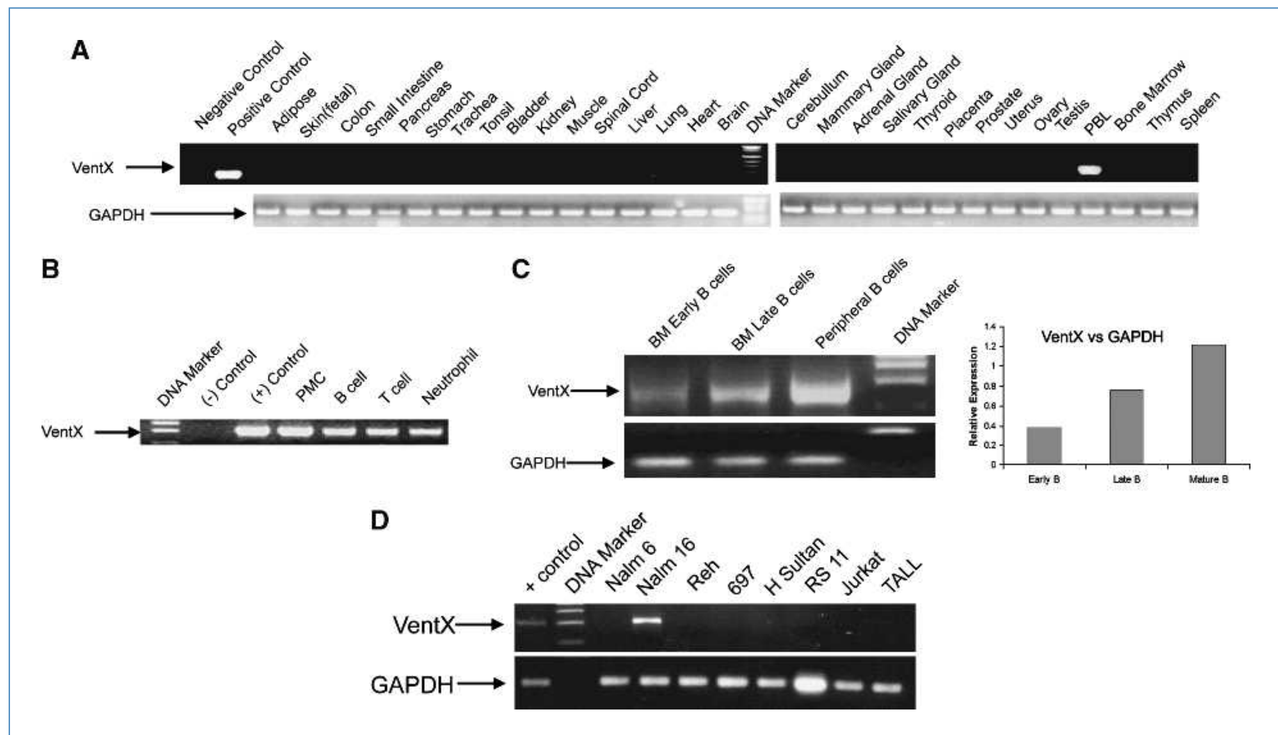

Figure 4. VentX expression is highly regulated and predominant in hematopoietic cells. *A,* tissue expression profiling of VentX expression in 30 adult tissues using a cDNA array and 30 cycles of PCR. GAPDH was used as internal control. Note that VentX is predominantly expressed in PBLs. *B* to *D,* RT-PCR analysis of VentX expression in indicated primary and cancer cells. *B,* lineage analysis of VentX expression in peripheral hematopoietic cells of myeloid and lymphoid lineages, including monocytes, T cells, B cells, and neutrophils. *C,* VentX expression is regulated during B-cell development. *Left,* VentX expression at indicated B-cell development stages; *right,* quantitative measurement of VentX expression with densitometry. GAPDH was used as internal control. *D,* VentX expression in indicated hematopoietic cancer cell lines, including B acute lymphoblastic leukemia cell lines (Nalm6, Nalm16, Reh, 697, and RS11), T acute lymphoblastic leukemia cell lines (Jurkat and TALL), and Burkitt lymphoma cell line (HS Sultan).

VentX is a negative regulator of cell proliferation. Our finding that VentX disrupts β-catenin/LEF/TCF signaling and inhibits the expression of its downstream cell cycle regulator (Fig. 3) prompted us to assess the potential role of VentX in cell proliferation. Given the physiologically restricted VentX expression in hematologic cells and the results of screening tests on cancer cell lines, we determined to use the lymphoblastic leukemia cell lines Reh and Nalm16 as the models in which to examine the role of VentX on cell proliferation through gain-of-function and loss-of-function approaches. First, we tested the effect of VentX on the proliferation of Reh, which does not express endogenous VentX. Plasmid encoding GFP-VentX was transiently transfected into Reh cells, the positively transfected cells were sorted with FACS, and the effects of VentX on cell proliferation were determined by viable cell count (Fig. 5*A, left*), MTS assay (Fig. 5*A, middle*), and [^{3}H]thymidine incorporation assay (Fig. 5*A, right*). Our data show that VentX expression strongly inhibits the proliferation of Reh cells. Similar results were also obtained with VentX on the proliferation of other cancer cells.[5]

To further evaluate the effect of VentX on cell proliferation, we tested the effects of downregulation of VentX expression in Nalm16 cells, which express endogenous VentX (Fig. 5*B* and *C*), using a shRNA approach. Four constructs of VentX shRNA were transfected into the Nalm16 cells. The effectiveness of these constructs in downregulating VentX expression was determined by RT-PCR (Fig. 5*B, top*). The effectiveness of the VentX shRNA was further determined by Western blot analysis using a specific VentX antibody raised in the laboratory (Fig. 5*B, middle* and *bottom*). As shown in Fig. 5*B*, whereas construct 3 had high specific activity against VentX expression, construct 1 exerted little effect. Subsequently, the constructs were used to transfect Nalm16 cells, and positively transfected cells were selected by puromycin resistance. The effects of VentX on cell proliferation were determined by viable cell count and MTS assay. As shown in Fig. 5*C*, whereas downregulation of VentX with construct 3 is associated with hyperproliferation of Nalm16, neither the control construct nor construct 1 had any effect on the proliferation of Nalm16 cells. To determine whether the effects of VentX on cell proliferation relate to the expression of LEF/TCF target genes, we examined the effects of downregulating VentX on the expression of cyclin D1. In contrast to the effect of overexpression (Fig. 3*D*), downregulation of VentX is associated with increased cellular levels of cyclin D1 (Fig. 5*D*). Thus, VentX downregulates cell proliferation at least partly through inhibiting β-catenin/LEF/TCF signaling

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and the expression of its downstream cell cycle regulators such as cyclin D1.

VentX expression is downregulated in peripheral blood of CLL patients. The abundant VentX expression in peripheral circulating B lymphocytes and its role in regulating Wnt signaling prompted us to further examine VentX expression in primary blood samples from newly diagnosed and untreated CLL patients. All CLL samples contained a minimum of 95% CD19$^+$ leukemia cells. RT-PCR of the total RNA level showed a significant reduction of VentX expression in all 10 CLL samples (Fig. 6*A*). The CD5$^+$ subset of normal B cells represents the normal counterparts of CLL cells (35). Therefore, as another control, we further verified the expression of VentX in CD5$^+$ subset of normal B cells (Fig. 6*A, right*). The downregulation of VentX expression in chronic leukemia patients is associated with an elevation of cyclin D1 expression (Fig. 6*B*), which is consistent with previous findings by Lu and colleagues (7). These results suggest a role of VentX in regulating Wnt/LEF/TCF signaling and the pathogenesis of CLL.

Discussion

Blocking LEF/TCF activation by Wnt/β-catenin has been considered a potential target for cancer prevention and treatment. In this study, we identified VentX, a human homologue of Xom, as a LEF/TCF-associated inhibitor of cell

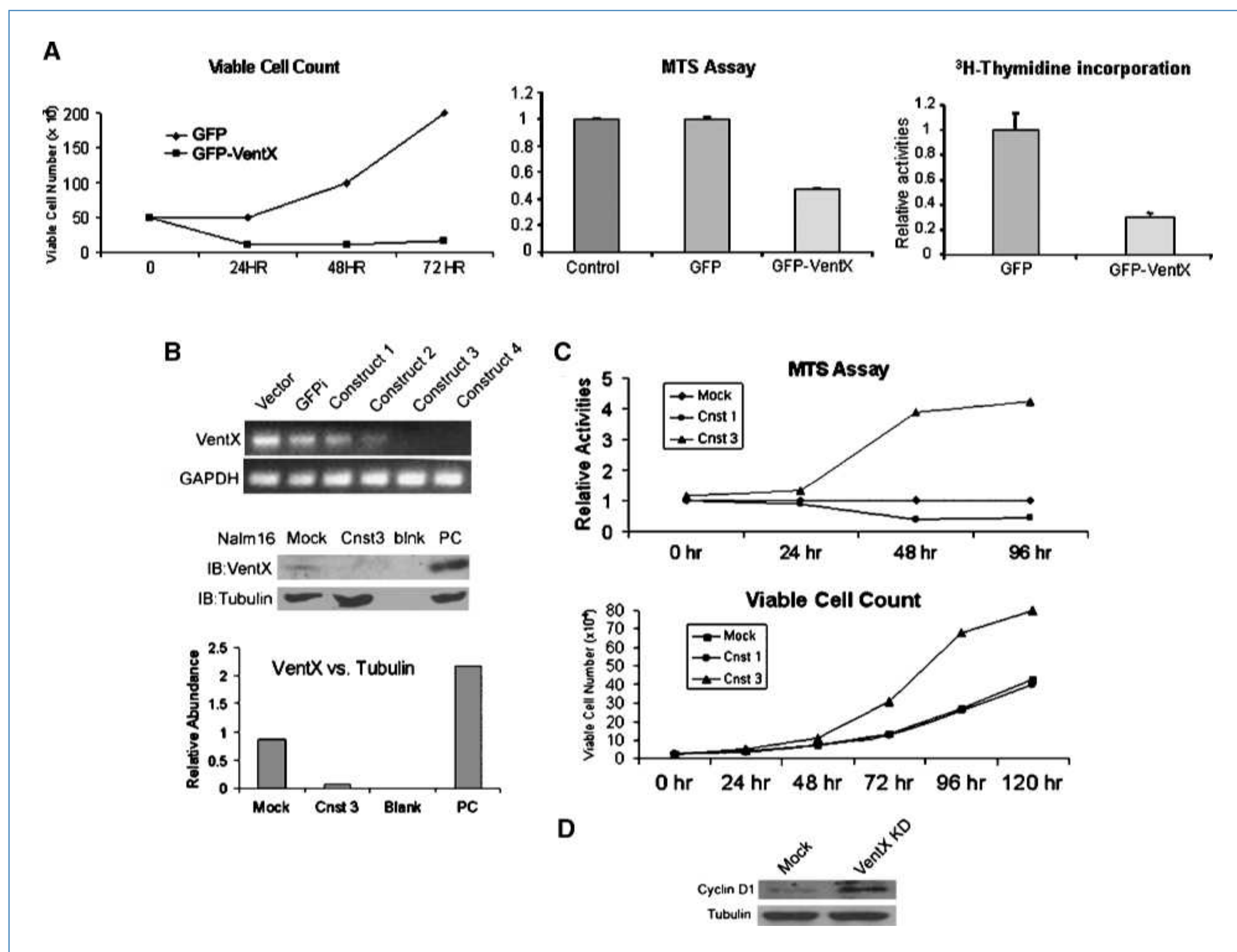

Figure 5. VentX is a negative regulator of cell proliferation. *A,* overexpression of VentX inhibits cell proliferation. Reh cells were transfected with plasmids encoding GFP or GFP-VentX. Cell proliferation was determined by counting viable cells at indicated time points using trypan blue staining (*left*), and cell viability was further measured by MTS assay (*middle*) and [^{3}H]thymidine incorporation assay (*right*). *B* to *D,* downregulation of VentX promotes cell proliferation. *B,* downregulation of VentX expression with VentX shRNA. Nalm16 cells were transfected with constructs encoding VentX shRNA as indicated. *Top,* the effect of these VentX shRNA on the expression of endogenous VentX was determined by RT-PCR. Expression of VentX in control-transfected and VentX shRNA–transfected cells was further determined by immunoblot using VentX-specific antibody (*middle*) and quantified by densitometry (*bottom*). Note that construct 3 but not construct 1 effectively knocks down the expression of VentX in Nalm16 cells. *C,* effects of downregulation of VentX on the viability and proliferation of Nalm16 as measured by MTS assay (*top*) and viable cell count (*bottom*). *D,* downregulation of VentX leads to elevated expression of cyclin D1. All experiments were repeated. *Columns* and *points,* mean of representative experiments done in triplicate; *bars,* SD.

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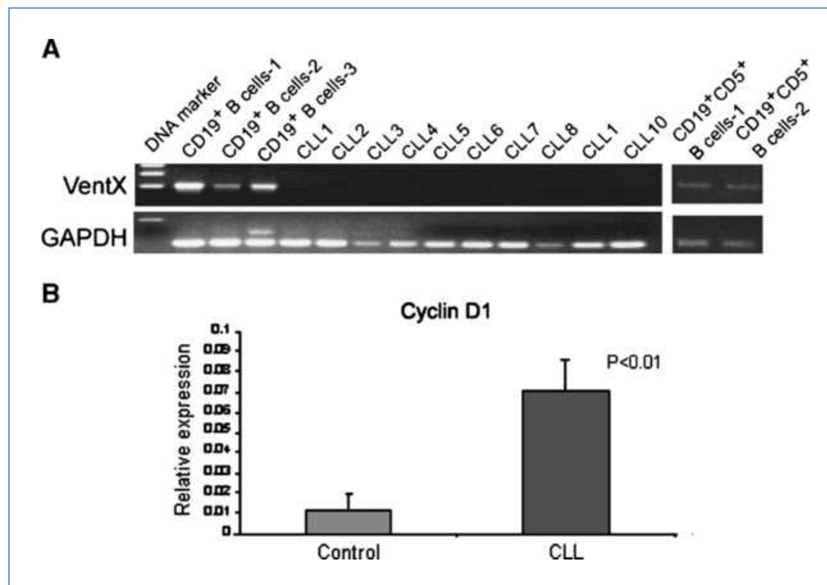

Figure 6. VentX is implicated in leukemogenesis of lymphocytic leukemia. *A,* VentX expression was markedly reduced in peripheral blood samples from newly diagnosed untreated CLL patients. mRNA was extracted from blood samples of 10 CLL patients as well as peripheral circulating $CD19^+$ and $CD19^+$ $CD5^+$ B cells from healthy adults, and VentX expression was measured using RT-PCR. Note that VentX expression is readily detectable in peripheral circulating B cells from healthy individuals but markedly decreased in blood samples (>95% B cells) from CLL patients. *B,* downregulation of VentX in lymphocytic leukemia is associated with corresponding elevation in the expression of cyclin D1. GAPDH was used as internal control. All experiments were repeated. *Columns,* mean of representative experiments done in triplicate; *bars,* SD.

proliferation, functioning at least in part by blocking β-catenin transactivation of LEF/TCF-mediated transcription. Our findings that VentX expression is significantly downregulated in CLL suggest a potential role of VentX in the pathogenesis of hematologic malignancies.

VentX was initially cloned from a human BM cDNA library and was identified as the first mammalian homologue of the *Xenopus laevis Vent* (*Xvent*) class of diverged homeobox genes. Moretti and colleagues (27) found that VentX expression is increased in BM mononuclear cells in recovery patients after chemotherapy. Consistent with a potential role in the development of hematopoietic cells, we found that VentX expression is regulated during B-cell development, with the most abundant expression in the mature circulating B cells. Besides B cells, VentX is also expressed in peripheral T cells, mononuclear cells, and neutrophils. Thus, detailed analysis is needed to further characterize the expression and function of VentX in hematopoietic organs, such as BM and spleen, as well as other tissues and organs.

Several mechanisms have been offered to account for the modification of β-catenin transactivation of LEF/TCF-dependent gene expression. For example, Chibby was identified as a β-catenin–associated inhibitor of β-catenin/LEF1/TCF signaling, functioning through disruption of a complex formed between β-catenin and LEF/TCF factors (33). In comparison, hypermethylated in cancer 1, a tumor suppressor, was found to inhibit the activation of LEF/TCF target oncogenes by sequestering the β-catenin/TCF complex from entering the nucleus (36). Here, we present biochemical and immunocytochemistry data, suggesting that VentX functions as a LEF/TCF-associated negative regulator of canonical Wnt/β-catenin/LEF/TCF signaling by disruption of the complex formation between β-catenin and LEF/TCF factors. A potential physiologic role of VentX in modulating LEF/TCF-dependent gene transcription was further indicated by ChIP analysis, showing the interaction between VentX and the cyclin D1 promoter, which contains LEF/TCF binding sites (18, 34).

Abnormal activation of the Wnt signaling pathway, which leads to elevated expression of LEF/TCF downstream oncogenes, has been implicated in the pathogenesis of lymphocytic leukemia. In CLL, the expression of *cyclin D1*, the LEF/TCF downstream gene, is significantly elevated. Here, we find that the expression of VentX is significantly downregulated in CLL, with concomitant elevation of cyclin D1 expression. A potential role of VentX in regulating B-cell proliferation is also indicated by our finding that overexpression of VentX inhibits the proliferation of Reh cells, whereas downregulation of VentX expression in Nalm16 cells accelerates Nalm16 proliferation (Fig. 5). The findings that VentX is downregulated in hematopoietic malignancies and that expression of VentX inhibits the proliferation of malignant hematopoietic cells suggest that VentX is a putative tumor suppressor, functioning at least partly through inhibiting the expression of LEF/TCF downstream oncogenes such as cyclin D1. Questions remain as to the potential application of VentX in predicting clinical behavior of lymphocytic leukemia and the role of VentX as a novel target of exploitation for identifying therapeutic drugs for cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843–50.
2. Clevers H. Wnt/β-catenin signaling in development and disease. Cell 2006;127:469–80.
3. Reya T, O'Riordan M, Okamura R, et al. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity 2000;13:15–24.
4. Zhao C, Blum J, Chen A, et al. Loss of β-catenin impairs the renewal of normal and CML stem cells *in vivo*. Cancer Cell 2007;12:528–41.
5. Simon M, Grandage VL, Linch DC, Khwaja A. Constitutive activation of the Wnt/β-catenin signalling pathway in acute myeloid leukaemia. Oncogene 2005;24:2410–20.
6. Ysebaert L, Chicanne G, Demur C, et al. Expression of β-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. Leukemia 2006;20:1211–6.
7. Lu D, Zhao Y, Tawatao R, et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2004;101:3118–23.
8. Behrens J, von Kries JP, Kuhl M, et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996;382:638–42.
9. Molenaar M, van de Wetering M, Oosterwegel M, et al. XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. Cell 1996;86:391–9.
10. Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, Kemler R. Nuclear localization of β-catenin by interaction with transcription factor LEF-1. Mech Dev 1996;59:3–10.
11. Brantjes H, Barker N, van Es J, Clevers H. TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. Biol Chem 2002; 383:255–61.
12. Hurlstone A, Clevers H. T-cell factors: turn-ons and turn-offs. EMBO J 2002;21:2303–11.
13. Roose J, Molenaar M, Peterson J, et al. The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 1998;395:608–12.
14. Brantjes H, Roose J, van De Wetering M, Clevers H. All Tcf HMG box transcription factors interact with Groucho-related co-repressors. Nucleic Acids Res 2001;29:1410–9.
15. Cavallo RA, Cox RT, Moline MM, et al. *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. Nature 1998;395: 604–8.
16. Waltzer L, Bienz M. *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature 1998;395:521–5.
17. Brannon M, Brown JD, Bates R, Kimelman D, Moon RT. XCtBP is a XTcf-3 co-repressor with roles throughout *Xenopus* development. Development 1999;126:3159–70.
18. Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
19. Behrens J. Control of β-catenin signaling in tumor development. Ann N Y Acad Sci 2000;910:21–33, discussion 33–5.
20. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–50.
21. Koide T, Hayata T, Cho KW. *Xenopus* as a model system to study transcriptional regulatory networks. Proc Natl Acad Sci U S A 2005; 102:4943–8.
22. Messenger NJ, Kabitschke C, Andrews R, et al. Functional specificity of the *Xenopus* T-domain protein Brachyury is conferred by its ability to interact with Smad1. Dev Cell 2005;8:599–610.
23. Schmidt JE, von Dassow G, Kimelman D. Regulation of dorsal-ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene Vox. Development 1996;122:1711–21.
24. Onichtchouk D, Gawantka V, Dosch R, et al. The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of *Xenopus* mesoderm. Development 1996;122:3045–53.
25. De Robertis EM, Kuroda H. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu Rev Cell Dev Biol 2004;20: 285–308.
26. Gao H, Wu B, Giese R, Zhu Z. Xom interacts with and stimulates transcriptional activity of LEF1/TCFs: implications for ventral cell fate determination during vertebrate embryogenesis. Cell Res 2007;17: 345–56.
27. Moretti PA, Davidson AJ, Baker E, Lilley B, Zon LI, D'Andrea RJ. Molecular cloning of a human Vent-like homeobox gene. Genomics 2001;76:21–9.
28. Holland PW, Booth HA, Bruford EA. Classification and nomenclature of all human homeobox genes. BMC Biol 2007;5:47.
29. Zhu Z, Kirschner M. Regulated proteolysis of Xom mediates dorsoventral pattern formation during early *Xenopus* development. Dev Cell 2002;3:557–68.
30. Le Y, Zhu BM, Harley B, et al. SOCS3 protein developmentally regulates the chemokine receptor CXCR4-FAK signaling pathway during B lymphopoiesis. Immunity 2007;27:811–23.
31. Ku M, Howard S, Ni W, Lagna G, Hata A. OAZ regulates bone morphogenetic protein signaling through Smad6 activation. J Biol Chem 2006;281:5277–87.
32. Morin PJ, Sparks AB, Korinek V, et al. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 1997;275:1787–90.
33. Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, Moon RT. Chibby, a nuclear β-catenin-associated antagonist of the Wnt/ Wingless pathway. Nature 2003;422:905–9.
34. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 1999;96:5522–7.
35. Gale RP, Foon KA. Chronic lymphocytic leukemia. Recent advances in biology and treatment. Ann Intern Med 1985;103:101–20.
36. Valenta T, Lukas J, Doubravska L, Fafilek B, Korinek V. HIC1 attenuates Wnt signaling by recruitment of TCF-4 and β-catenin to the nuclear bodies. EMBO J 2006;25:2326–37.

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VentX, a Novel Lymphoid-Enhancing Factor/T-Cell Factor–Associated Transcription Repressor, Is a Putative Tumor Suppressor

Hong Gao, Yi Le, Xiaoming Wu, et al.

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