VBP1 modulates Wnt/β-catenin signaling by mediating the stability of the transcription factors TCF/LEFs

The Wnt/β-catenin pathway is one of the major pathways that regulates embryonic development, adult homeostasis, and stem cell self-renewal. In this pathway, transcription factors T-cell factor and lymphoid enhancer factor (TCF/LEF) serve as a key switch to repress or activate Wnt target gene transcription by recruiting repressor molecules or interacting with the β-catenin effector, respectively. It has become evident that the protein stability of the TCF/LEF family members may play a critical role in controlling the activity of the Wnt/β-catenin signaling pathway. However, factors that regulate the stability of TCF/LEFs remain largely unknown. Here, we report that pVHL binds to all four TCF/LEF family members and von Hippel-Lindau tumor-suppressor protein (pVHL). Either overexpression or knockdown of VBP1 decreased Wnt/β-catenin signaling activity in both cultured cells and zebrafish embryos. Mechanistically, VBP1 directly binds to all four TCF/LEF family members and von Hippel-Lindau tumor-suppressor protein (pVHL). Either overexpression or knockdown of VBP1 increases the association between TCF/LEFs and pVHL and then decreases the protein levels of TCF/LEFs via proteasomal degradation. Together, our results provide mechanistic insights into the roles of VBP1 in controlling TCF/LEFs protein stability and regulating Wnt/β-catenin signaling pathway activity.

The evolutionarily conserved canonical Wnt/β-catenin pathway plays a pivotal role in various biological processes, such as cell proliferation and cell fate determination during embryonic development and tissue homeostasis (1–4). Misregulation of Wnt/β-catenin signaling is associated with many human diseases, such as human birth defects, cancer, and degenerative disorders (2–6). In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by a multicomponent degradation complex, consisting of the scaffolding protein Axin, the tumor-suppressor adenomatous polyposis coli, casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β), which results in proteasomal degradation of β-catenin. In the nucleus, TCF/LEF acts as a transcriptional repressor by interacting with the transcriptional-like enhancer of split (TLE)/Groucho protein (7, 8). In the presence of Wnt ligands, stabilized β-catenin accumulates and translocates to the nucleus to form a complex with TCF/LEFs, leading to the transcriptional activation of Wnt target genes (3, 4). Therefore, the outcome of Wnt/β-catenin signaling is controlled by the complex of β-catenin and TCF/LEFs. As key components of Wnt/β-catenin signaling, TCF/LEFs are conserved among different species. A single TCF/LEF gene is found in Drosophila and worm genomes, whereas vertebrate genomes harbor four TCF/LEF genes (9). It was reported that nuclear concentrations of both TCF/LEFs and β-catenin are important for establishing an optimal β-catenin:TCF ratio, which is crucial for the activity of Wnt/β-catenin signaling in Xenopus and Caenorhabditis elegans (10, 11). Thus, Wnt/β-catenin signals may regulate gene expression by dynamically controlling the concentrations of TCF/LEFs and β-catenin in the nucleus. In contrast with the well-understood posttranslational regulation of β-catenin, regulation of TCF/LEF proteins awaits further elucidation. Studies have shown that the mitogen-activated protein kinase–related protein kinase Nemo-like kinase (NLK) is involved in the suppression of Wnt target gene expression by phosphorylating TCF (in C. elegans called POP1), thereby inhibiting its DNA-binding affinity (12). Previous reports suggested that some members of TCF/LEFs are involved in the ubiquitin-proteasome pathway (13–15). For instance, TCF7L2 and LEFI are ubiquitylated and degraded by an E3 ubiquitin ligase NARF, which is the abbreviation of NLK-associated RING finger protein (15). Despite the diverse and specific functions, TCF/LEFs are often expressed in overlapping patterns and the proteins display common structural features. Therefore, the regulators of the protein stability of all four TCF/LEFs may exist and need to be explored further.

The von Hippel-Lindau protein (pVHL) is the substrate recognition component of an E3 ubiquitin ligase complex (16). Inactivation of the VHL gene occurs in most hereditary von Hippel-Lindau disease and sporadic clear-cell renal carcinomas (16, 17). Well-documented canonical targets of pVHL are the α subunits of hypoxia-inducible factors (HIF-α) (18, 19). In addition, accumulated evidence has indicated that pVHL also participates in the regulation of Wnt/β-catenin signaling. A previous study reported that pVHL regulates Wnt/β-catenin

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Haifeng Zhang1,2, Xiaozhi Rong1,2,*, Caixia Wang1,2, Yunzhang Liu1,2, Ling Lu1,2, Yun Li1,2, Chengtian Zhao3,4,†, and Jianfeng Zhou1,2,‡

From the 1Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China, the 2Laboratory for Marine Drugs and Bioproducts, Pilot National Laboratory for Marine Science and Technology, Qingdao, China, the 3Institute of Evolution and Marine Biodiversity and College of Marine Biology, Ocean University of China, Qingdao, China, and the 4Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology, Qingdao, China

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†For correspondence: Jianfeng Zhou, jfzhou@ouc.edu.cn; Xiaozhi Rong, rongxiaozhi@ouc.edu.cn.
‡These authors contributed equally to this work.
*These authors contributed equally to this work.

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signaling by reducing β-catenin protein levels through Jade-1 (20, 21). Additionally, pVHL binds to DVL and ubiquitinates it, facilitating DVL degradation via the autophagy–lysosome pathway (22). pVHL binding protein 1 (VBP1) was identified as a binding partner of pVHL by a yeast two-hybrid assay (23). Several studies have suggested that VBP1 exerts its function via pVHL. For example, VBP1 mediated the degradation of HIV-1 integrase with pVHL (24). Specifically, VBP1 bound to HIV-1 integrase and promoted its binding to the E3 ubiquitin autophagy pathways (25). Furthermore, VBP1 bound to mismatch repair protein family member, hMSH4, and promoted its degradation facilitating DVL degradation via the autophagy pathway (26, 27). Given that pVHL regulates Wnt/β-catenin signaling at multiple levels and VBP1 functions as a pVHL binding protein, however, whether VBP1 also regulates Wnt/β-catenin signaling remains unknown.

In the present study, we investigated the role of VBP1 in regulating Wnt/β-catenin signaling both in vitro and in vivo. Unexpectedly, our results demonstrated that either overexpression or knockdown of VBP1 decreased Wnt/β-catenin signaling activity in vitro and in vivo. We revealed that VBP1 directly binds to all four TCF/LEF family members and to pVHL. Intriguingly, either overexpression or knockdown of VBP1 increased the interaction between TCF/LEFs and pVHL, which then promoted TCF/LEFs protein degradation via the proteasomal pathway. Using a pVHL deficient cell line, we uncovered that VBP1 regulated TCF/LEF protein stability via pVHL. Together, our study provides insights into the mechanism of protein stability regulation of all TCF/LEF family members by VBP1.

Results

Overexpression of VBP1 inhibited Wnt/β-catenin signaling in vitro and in vivo

Because pVHL regulates the Wnt/β-catenin pathway at multiple levels and VBP1 serves as a binding protein to cooperate with pVHL in many biological processes (24–26), we assume that VBP1 may participate in the regulation of the Wnt/β-catenin pathway. To test this possibility, we examined the effect of VBP1 on Wnt/β-catenin signaling both in vitro and in vivo. We first used the TOPFlash reporter, a well-established Wnt/β-catenin signal-responsive reporter, which contains tandem repeats of the TCF/LEF response element, to examine the role of VBP1 in the Wnt/β-catenin pathway in cultured cell lines. Transfection of HEK293T cells with a plasmid encoding the FLAG-tagged VBP1 protein significantly decreased basal Wnt/β-catenin reporter activity (Fig. 1A). In addition, overexpression of VBP1 decreased the expression level of several Wnt/β-catenin direct target genes including AXIN2, CCND1, and CDK2 in HEK293T cells (Fig. 1B). Interestingly, the expression of DKK1, which is a repressor of the Wnt/β-catenin pathway was not changed by VBP1 overexpression. To determine whether Wnt/β-catenin signaling was affected by VBP1 overexpression, we conducted Western blotting analysis, and the capped mRNA of VBP1 was generated and injected into 1–2 cell stage zebrafish embryos, and then the Wnt/β-catenin reporter activity was measured at 6 hpf (hours postfertilization). As shown in Fig. 1C, forced expression of VBP1 mRNA inhibited endogenous Wnt/β-catenin activity in zebrafish embryos. Taken together, these results suggested that overexpression of VBP1 inhibited endogenous Wnt/β-catenin signaling activity in vitro and in vivo.

To further confirm whether VBP1 indeed regulated the Wnt/β-catenin signaling pathway, we investigated the genetic interaction between VBP1 and the core components of the Wnt/β-catenin signaling pathway in HEK293T cells and zebrafish embryos. Induction of Wnt/β-catenin signals by Wnt3a, β-catenin-binding site (28, 29), constitutively activated β-catenin (β-CatΔN), or constitutively activated Tcf7l1 (VP16-Tcf7l1ΔN, a β-catenin–independent VP16-Tcf7l1 fusion protein that lacks the β-catenin-binding site) in HEK293T cells resulted in dramatically increased Wnt/β-catenin reporter activity (Fig. 1D). Overexpression of VBP1 inhibited Wnt/β-catenin reporter activity induced by Wnt3a, BIO, β-CatΔN, and VP16-Tcf7l1ΔN in HEK293T cells (Fig. 1D). Similarly, overexpression of VBP1 inhibited Wnt/β-catenin reporter activity induced by Wnt3a and VP16-Tcf7l1ΔN in zebrafish embryos (Fig. 1E). Taken together, the above results implied that VBP1 is likely to inhibit Wnt/β-catenin activity at the TCF/LEF level. To further confirm the inhibitory effect of VBP1 on Wnt/β-catenin signaling in vivo, we performed epistatic analysis between VBP1 and the core components of the Wnt/β-catenin signaling pathway on the formation of the dorsoventral axis in zebrafish embryos. Because the VBP1/Vbp1 amino acid sequence is highly conserved in vertebrates from fish to mammals (Fig. S1A), we co-injected human VBP1 or zebrafish vbp1 mRNA and mRNA for wt3a, β-catΔN, or vp16-tcf7l1ΔN into zebrafish embryos. Consistent with a previous report (30), injection of wt3a, β-catΔN, or vp16-tcf7l1ΔN mRNA into zebrafish embryos resulted in dorsalized phenotypes at 12.5 hpf, which were neutralized by co-injection with either human VBP1 or zebrafish vbp1 mRNA, suggesting that the Wnt inhibitory action of VBP1/Vbp1 is evolutionary conserved and acts at the TCF/LEF level (Fig. 1, F–I). Collectively, these results suggested that overexpression of VBP1/Vbp1 inhibits Wnt/β-catenin signaling at TCF/LEF level in vitro and in vivo.

Knockdown of VBP1 reduced Wnt/β-catenin signaling and TCF-dependent transcriptional activation

To delineate the role of endogenous VBP1 in Wnt/β-catenin signaling, HEK293T and U2OS cell lines stably expressing VBP1 shRNA were established. The knockdown efficiency of VBP1 was confirmed by Western blotting analysis, and each shRNA greatly reduced the VBP1 protein levels (Fig. 2A). Using these cell lines, we tested whether VBP1 knockdown affected the expression of Wnt/β-catenin target genes. As shown in Fig. 2B, knockdown of VBP1 significantly reduced the expression
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Figure 1. Overexpression of VBP1 inhibits Wnt/β-catenin signaling. A, TOPFlash luciferase assays in HEK293T cells with 500-ng FLAG-tagged VBP1 plasmid transfection. Expression of FLAG-VBP1 was confirmed by Western blotting. Data are from four independent experiments with individual data points shown. B, relative mRNA levels of indicated Wnt target genes in control or VBP1-overexpressing HEK293T cells were analyzed by qRT-PCR. Data are from three independent experiments with individual data points shown. C, TOPFlash luciferase assays in zebrafish embryos with 600-ng VBP1 mRNA injection. Data are from three independent experiments with individual data points shown. D, TOPFlash luciferase assays in Wnt3a-, β-CatΔN-, or VP16-Tcf7l1ΔN-treated HEK293T cells with VBP1 overexpression. Control or VBP1-overexpressing HEK293T cells were cotransfected with Renilla and TOPFlash plasmids. The activation of Wnt/β-catenin signal was induced by BIO (1 μM) treatment or by indicated plasmid DNAs (15 ng of Wnt3a, 30 ng of β-CatΔN, or 50 ng of VP16-Tcf7l1ΔN) transfection. Data are from three independent experiments with individual data points shown. E, TOPFlash luciferase assays in wnt3a- or vp16-tcf7l1ΔN-injected zebrafish embryos with VBP1 overexpression. TOPFlash and Renilla plasmids were co-injected into embryos with gfp or VBP1 mRNA. The activation of Wnt/β-catenin signal was induced by injecting 100 pg of wnt3a mRNA or 50 pg of vp16-tcf7l1ΔN mRNA. Data are from three independent experiments with individual data points shown. F, overexpression of VBP1/vbp1 antagonized wnt3a, β-catΔN, or vp16-tcf7l1ΔN-induced dorsalized phenotype in zebrafish embryos. Lateral view of representative injected embryos at 5-somite stage was shown. Scale bar, 200 μm. G–I, quantitation of embryos displaying normal or dorsalized phenotype in F. Data are from three independent experiments, and the embryo number of each group is shown on the top. The values are mean ± S.D. Unpaired t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, nonsignificant.

level of Wnt/β-catenin target genes, including AXIN2, LEF1, CCND1, and CDK2 in HEK293T cells. Similar results were observed in U2OS cells (Fig. 2C). These results suggested that knockdown of VBP1 reduced endogenous Wnt/β-catenin signaling in vitro. We next determined the effect of Vbp1 knockdown on the Wnt/β-catenin signaling activity in vivo using a transgenic zebrafish Wnt/β-catenin reporter line, also known as the TOPdGFP reporter line. GFP expression in the zebrafish TOPdGFP reporter line was controlled by a promoter with multiple TCF/LEF response elements, which allowed the determination of Wnt/β-catenin activity and the identification of responsive cells through the expression of GFP protein (31). To ascertain the cell or tissue that we should observe in zebrafish embryos, we first investigated the spatiotemporal expression pattern of vbp1 during embryogenesis using RT-PCR and whole-mount in situ hybridization. We found that the vbp1 transcript was maternally deposited and ubiquitously expressed before 24 hpf (Fig. S1, B and C); however, its expression was enriched in the midbrain region at 24 hpf (Fig. S1C). Coincidentally, the midbrain region at 24 hpf was also rich in the strong fluorescence signal in the Wnt/β-catenin reporter line (31), suggesting that this region is suitable for observing the effect on the Wnt/β-catenin reporter after depletion of Vbp1. Knockdown of Vbp1 was performed using two nonoverlapping translation-blocking morpholinos (MOs) because of translation-blocking MO inhibited translation of maternally deposited mRNA. The effectiveness of MOs (MO1 and MO2) was confirmed by blocking the translation of a vbp1 5′-UTR-GFP reporter plasmid, and both MOs significantly blocked the translation of GFP mRNA in zebrafish embryos (Fig. S1D). Subsequently, control MO (8 ng), MO1 (4 ng), or MO2 (8 ng) was injected into TOPdGFP transgenic embryos at the 1–2-cell stage, respectively. In control MO–injected embryos, a strong green fluorescence signal, indicative of Wnt/β-catenin activity, was observed in the midbrain region at 24 hpf (Fig. 2D). In contrast, the GFP intensity was dramatically reduced in the Vbp1 MO-injected embryos (Fig. 2, D and E).
VBP1 modulates stability of the TCF/LEF proteins

A

HEK293T

Scramble shVBP1-1 shVBP1-2 (kDa)

VBP1

GAPDH

B

HEK293T

Scramble shVBP1-1 shVBP1-2

Relative mRNA levels

AXIN2 LEF1 CCND1 CDK2

C

U2OS

Scramble shVBP1-1 shVBP1-2

Relative mRNA levels

AXIN2 LEF1 CCND1 CDK2

D

e19p

cMO MO1 MO2

E

N= 49 59 59

Relative intensity

cMO MO1 MO2

F

U2OS

HEK293T

Relative luciferase activity

Wnt3a LiCl Vwp1 β-CatΔN Vp16-Tcf71ΔN

G

U2OS

HEK293T

Relative luciferase activity

LiCl Vwp1 β-CatΔN Vp16-Tcf71ΔN

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The decrease of fluorescence intensity was not because of morphological changes in the midbrain, which were indicated by the normal expression of midbrain marker otx2 (Fig. 2D). These results suggested that depletion of Vbp1 in zebrafish embryos decreased the endogenous Wnt/β-catenin activity in vivo. Collectively, depletion of VBP1/Vbp1 reduced endogenous Wnt/β-catenin signaling both in vitro and in vivo.

We next investigated at which level VBP1 knockdown reduced Wnt/β-catenin activity. Again, Wnt/β-catenin activity was induced by Wnt3a, LiCl (GSK3β inhibitor) (32), β-CatΔN, and VP16-Tcf7l1ΔN, at multiple levels (Fig. 2F). Knockdown of VBP1 in cultured cells dramatically reduced the Wnt/β-catenin activity induced by these molecules (Fig. 2F). These results implied that depletion of VBP1 reduced Wnt/β-catenin signaling at the TCF/LEF transcriptional level. We further tested whether the effect of knockdown of VBP1/Vbp1 on Wnt/β-catenin signaling is specific. Because the effect of VBP1/Vbp1 on Wnt/β-catenin signaling is evolutionarily conserved between zebrafish and humans, we introduced zebrafish Vbp1 into VBP1-depleted human cells to test whether zebrafish Vbp1 could rescue the reduced Wnt/β-catenin activity in cultured human cells. As expected, expression of shRNA-resistant zebrafish Vbp1 significantly rescued the reduced Wnt/β-catenin activity by VBP1 knockdown in the background of LiCl treatment and β-CatΔN overexpression (Fig. 2G). These results indicated that the reduction of Wnt signaling by VBP1 knockdown was specific and that the effect of knockdown of VBP1/Vbp1 on Wnt/β-catenin signaling was evolutionarily conserved. Taken together, our results demonstrated that depletion of VBP1/Vbp1 attenuated Wnt/β-catenin signaling at the TCF/LEF transcriptional level.

**Overexpression or knockdown of VBP1 promoted degradation of TCF/LEFs**

Given that either overexpression or knockdown of VBP1 attenuated Wnt/β-catenin signaling at the TCF/LEF level and VBP1 is a binding protein of pVHL, which is a subunit of an E3 ubiquitin ligase complex, we speculated that VBP1 might regulate TCF/LEF protein stability. To this end, we co-overexpressed VBP1 and TCF/LEFs in HEK293T cells. When cells were cotransfected with plasmids containing Myc-tagged TCF/LEFs and FLAG-tagged VBP1, exogenous TCF/LEF protein levels were reduced (Fig. 3A). In addition, overexpression of VBP1 reduced the endogenous TCF7L2 protein levels in HEK293T cells (Fig. 3B). This effect was restored after the addition of the proteasome inhibitor MG132 (Fig. 3C), indicating that TCF7L2 degradation promoted by overexpressed VBP1 depended on the proteasomal pathway.

We next examined the effect of VBP1 knockdown on TCF/LEF protein levels. Knockdown of VBP1 decreased the protein levels of endogenous TCF7L1 and TCF7L2 in HEK293T cells (Fig. 3D). In contrast, the expression of TCF7L1 and TCF7L2 mRNA was not changed upon the depletion of VBP1 when measured by qRT-PCR analysis (Fig. 3E). These results implied that TCF7L1 and TCF7L2 protein levels were regulated by VBP1 through a posttranslational mechanism. To determine whether knockdown of VBP1 accelerated degradation of TCF7L2, we measured TCF7L2 t1/2 in HEK293T cells treated with cycloheximide (CHX), a protein synthesis inhibitor. The t1/2 of TCF7L2 was significantly shortened when VBP1 was knocked down (Fig. 3, F and G). Because the HEK293T cell line was regarded as a Wnt-off cell line, we want to determine whether knockdown of VBP1 regulates TCF/LEF protein stability in Wnt-activated colon cancer cells. Knockdown of VBP1 also decreased TCF7L1 and TCF7L2 protein levels in Wnt-activated HCT116 colon cancer cells, which harbor an oncogenic mutation in β-catenin (Fig. 3H). These data suggested that knockdown of VBP1 reduced TCF/LEF protein stability in both active and inactive Wnt/β-catenin signaling status. Furthermore, the reduction of TCF7L2 protein levels by VBP1 depletion was restored by MG132 treatment in both HEK293T and HCT116 cells (Fig. 3, I and J), indicating that VBP1 depletion–mediated TCF7L2 degradation was also dependent on the proteasomal pathway. Protein degradation by the proteasomal pathway may be ubiquitin-dependent and ubiquitin-independent (33). To address the type of TCF/LEF protein degradation, we examined the ubiquitylation of Tcf7l2 under the VBP1-overexpressed condition. Myc-Tcf7l2 and FLAG–HIF-1α were cotransfected with VBP1 simultaneously into HEK293T cells, and then ubiquitylation analysis was performed after immunoprecipitation. VBP1 induced a dramatic enhancement in the ubiquitylation of HIF-1α, as previously reported (27), but failed to induce the ubiquitylation of Tcf7l2. Considering that the reduction of Tcf7l2 protein levels by overexpression or depletion of VBP1 was restored in addition of a proteasome inhibitor, our data suggested that Tcf7l2 likely underwent a ubiquitin-dependent proteasomal pathway (Fig. 3K). Taken together, these results implied that VBP1 depletion promoted degradation of the TCF7L2 in both basal and activated Wnt/β-catenin activity status via proteasomal pathway.
VBP1 modulates stability of the TCF/LEF proteins

A

HEK293T

Flag-VBP1
Myc
Histone H3
- - + (+ kDa)
- - + (+ kDa)
- - + (+ kDa)
- - + (+ kDa)

B

HEK293T

Flag-VBP1
TCF7L2
GAPDH
- + - (kDa)
- + - (kDa)
- - + (kDa)
- - + (kDa)

C

HEK293T

MG132
Flag-VBP1
Myc
Flag
GAPDH
- - + (kDa)
- - + (kDa)
- - + (kDa)
- - + (kDa)

D

HEK293T

Scramble
shVBP1-1
shVBP1-2
TCF7L1/7L2
VBP1
Histone H3
- - -
- - -
- - -
- - -

E

HEK293T

Relative mRNA levels

F

HEK293T

CHX(Hrs): 0 2 4 8
TCF7L2E
TCF7L2M/S
GAPDH
- - -
- - -
- - -
- - -

G

Relative level of TCF7L2E

H

HCT116

TCF7L1/7L2
VBP1
Histone H3
- - -
- - -
- - -
- - -

I

HEK293T

MG132
TCF7L2
VBP1
GAPDH
- - -
- - -
- - -
- - -

J

HCT116

MG132
TCF7L2
VBP1
GAPDH
- - -
- - -
- - -
- - -

K

HEK293T

Ub-HA
Flag-HIF-1α
Myc
TCF7L2
VBP1-GFP
MG132
- - +
- - +
- - +
- - +

L

HEK293T

Ub-HA
Flag-HIF-1α
Myc
TCF7L2
VBP1-GFP
MG132
- - +
- - +
- - +
- - +

IP: Myc
Flag
GFP
Input
- - -
- - -
- - -
- - -
**VB1 modulates stability of the TCF/LEF proteins**

To understand the mechanism underlying the regulation of TCF/LEF protein stability by VB1, we first tested the possible interaction between VB1 and TCF/LEFs. The co-immunoprecipitation (Co-IP) assay detected VB1 in the immunoprecipitates of four Myc-tagged TCF/LEF family members in HEK293T cells (Fig. 4A). Additionally, when FLAG-tagged VB1 was overexpressed in HEK293T cells, it formed a complex with endogenous TCF7L2 and pVHL (Fig. 4B). Furthermore, purified GST–VB1 protein pulled down all four Myc-tagged TCF/LEF members (Fig. 4C, left panel). Likewise, a direct protein-protein interaction among VB1, endogenous TCF7L2, and FLAG-tagged pVHL was confirmed by the GST pulldown assay (Fig. 4C, right panel). These results indicated that VB1 directly interacted with TCF/LEF and pVHL in HEK293T cells. We next assessed whether VB1 and Tcf7l1 interact with each other in living HeLa cells using a previously established bimolecular fluorescence complementation (BiFC) assay (34). As shown in Fig. 4D, the green BiFC signals indicated that VB1 interacted with both Tcf7l1 and Tcf7l1ΔN in the nucleus, whereas VB1 bound to Tcf7l1ΔNLS (lacking the nuclear localization signal) in the cytoplasm, suggesting that VB1 and Tcf7l1 did interact with each other in living cells. Subsequently, we generated a series of TCF7L2 domain-deleted mutants to map the region of TCF7L2 involved in the interaction with VB1 (Fig. 4E). By Co-IP assay, we found that the TLE/Groucho binding region–deleted mutant of TCF7L2 exhibited rather weak interactions with VB1 compared with WT TCF7L2, high mobility group box–deleted, or C-terminal region–deleted mutant (Fig. 4F). This suggests that the TLE/Groucho binding region in TCF7L2 may play an important role for its interaction with VB1.

Of note, pVHL was also present in the complex containing VB1 and TCF/LEF (Fig. 4B). In addition, VB1, as a pVHL binding protein, participates and regulates pVHL-related biological processes. We speculated that VB1-mediated regulation of TCF/LEF stability was related to pVHL function. We, therefore, overexpressed or knocked down VB1 protein in VHL-deficient clear-cell renal carcinoma 786-O cells. As expected, TCF7L2 protein levels were not changed in 786-O cells with either overexpression or knockdown of VB1 (Fig. 5, A and B). These data suggested that pVHL is required for VB1 to regulate the stability of TCF/LEFs. We next performed a series of Co-IP analyses to detect the association between endogenous TCF7L2 and pVHL with VB1 overexpression or knockdown in different cell lines of distinct Wnt/β-catenin activity. We observed that the association between endogenous TCF7L2 and pVHL increased under the condition of either VB1 overexpression or knockdown in HEK293T cells (Fig. 5, C and D). Similarly, knockdown of VB1 in Wnt-activated HCT116 colon cancer cells also increased the interaction between TCF7L2 and pVHL (Fig. 5E). These results indicated that the presence of pVHL is required for VB1 to regulate the stability of TCF/LEFs and that alteration of VB1 protein levels increases the association between TCF/LEF and pVHL, which may promote the degradation of TCF/LEF proteins.

Because colorectal cancers often display overactivated Wnt/β-catenin signaling and VB1 regulates the protein stability of TCF/LEFs in HCT116 colon cancer cells, we next determined whether VB1 has a physiological role in colorectal cancer cells. The alterations of Wnt/β-catenin target genes were measured in HCT116 cells after overexpression or knockdown of VB1. Similar with the effect of VB1 in HEK293T cells, either overexpression or knockdown of VB1 in HCT116 cells reduced the expression of Wnt/β-catenin target genes, including AXIN2, CCND1, and CDK2, but did not reduce the expression of Wnt/β-catenin signaling pathway negative feedback gene, DKK1 (Fig. 5, F and G). These results suggested that VB1 might have physiological roles in colorectal cancers. It is well-known that activation of Wnt/β-catenin signaling promotes proliferation of colorectal cancer cell. It is interesting to know whether alternation of VB1 decreases the proliferation of colorectal cancers. We, therefore, examined the effect of VB1 knockdown on proliferation of HCT116 cells using a colony formation assay. Our results indicated that depletion of VB1 inhibited the proliferation of HCT116 cells, which was highlighted by the significant reduction in the number of colonies (Fig. 5, H and I). These results suggest that VB1 plays a physiological role in the cell proliferation of colorectal cancers.

**Discussion**

In the present study, we show that VB1 plays an important role in regulating the Wnt/β-catenin signaling pathway, which is known as an important signaling pathway that controls developmental processes in the embryo and regulates adult homeostasis and stem cell self-renewal (35). Transcription factors TCF/LEFs and their cofactor β-catenin represent the key components of the canonical Wnt pathway (36). However, compared with our knowledge on the regulation of β-catenin,
posttranslational regulation of TCF/LEF proteins is poorly understood. Previous studies show that TCF family members LEF1, TCF7L2, and TCF7L1 are phosphorylated by HIPK2, leading to the dissociation of LEF1, TCF7L2, and TCF7L1 from their target promoter (37, 38). NLK inhibited the Wnt/β-catenin pathway via TCF7L2 and LEF1 phosphorylation, thus dissociating them from their target DNAs (12, 39, 40). A recent study showed that NARF was involved in the ubiquitylation of TCF7L2 and LEF1 as a RING finger-type E3 ubiquitin ligase (15). Here we found that VBP1 binds to all four TCF/LEF family members, and we also showed that either lower or excessive protein level of VBP1 facilitates the association between pVHL and TCF/LEFs and then promotes the degradation of TCF/LEFs via pVHL. Because it is possible that Wnt/β-catenin signals also regulate gene expression by dynamically controlling the concentration of TCF/LEFs, these findings provide
evidence that VBP1 and pVHL are involved in regulation of protein levels of TCF/LEFs, and our results suggested that the stability of TCF/LEFs controlled by VBP1 and pVHL is critical for the regulation of Wnt/b-catenin signaling in vitro and in vivo.

We observed that overexpression of VBP1 decreased the expression of Wnt target genes and Wnt/b-catenin reporter activity in vivo and in vitro. Likewise, VBP1/Vbp1 antagonized the Wnt/b-catenin–induced dorsalizing activity in zebrafish embryos. Surprisingly, depletion of VBP1 also attenuated Wnt/b-catenin activity in vivo and in vitro. Furthermore, we demonstrated that VBP1 regulated the Wnt/b-catenin signaling pathway at the TCF/LEF-dependent transcriptional level by genetic interaction analysis. These data presented here raised an interesting question regarding lower or excessive VBP1 reducing Wnt/b-catenin signaling activity. To answer this question, we showed that either overexpression or knockdown of VBP1 reduced the stability of TCF/LEFs, which might have resulted
from increased association between pVHL and TCF/LEFs when VBP1 was overexpressed or knocked down. Consequently, this reduced the activity of Wnt/β-catenin signaling. Similarly, a recent study revealed that either overexpression or knockdown of SRPK1 decrease the association between Akt and PHLPP1 and then interfere with PHLPP-mediated dephosphorylation of Akt, resulting in the promotion of cancer (41). This observation indicated that VBP1 may not be the only example that lower or excessive protein levels resulted in the same biological effect. Our findings suggest that the protein levels of VBP1 are tightly correlated with the stability of TCF/LEFs and reveal an unusual regulatory mechanism of the well-studied Wnt/β-catenin signaling pathway.

There is no evidence that VBP1 has any enzymatic activity to promote direct protein degradation. VBP1 was identified as a binding protein of pVHL, which is a component of an E3 ubiquitin ligase complex (42). VBP1 has been shown to cooperate with pVHL in regulating protein stability. For example, VBP1 regulated tubulin degradation together with pVHL (26). A similar partnership between VBP1 and pVHL was also reported. In brief, VBP1 mediated the interaction between HIV-1 integrase and pVHL, promoting integrase degradation (24). VBP1 also facilitated hMSH4 destruction partly through the pVHL-mediated proteasomal pathway (25). Based on these results, we postulated that VBP1 regulated TCF/LEF protein stability through pVHL. Indeed, our data demonstrated that VBP1 had no apparent effects on TCF/LEF protein stability in VHL-deficient 786-O cells. Moreover, our results showed that VBP1 directly interacted with TCF and pVHL. These observations suggested the possibility of VBP1 forming a complex with TCF and pVHL. Moreover, a recent study showed that VBP1 stabilizes pVHL by inhibiting the ubiquitination of pVHL and then facilitates pVHL-mediated ubiquitination of HIF-1α (27). A previous study shows that VBP1 localizes to the nucleus when it is co-overexpressed with pVHL (23). Based on these results, we suggested the following model of knockdown or overexpression of VBP1 promoting the degradation of TCF/LEFs through pVHL: at the basal level, VBP1 binds and protects TCF/LEFs from degradation. When VBP1 levels were lower in the cell and the association of pVHL with TCF/LEFs increased, proteasomal degradation of TCF/LEFs via pVHL occurred. In contrast, excessive VBP1 may stabilize pVHL and promote its translocation into the nucleus, also resulting in the increased interaction between pVHL and TCF/LEFs, and in turn promoted the degradation of TCF/LEFs. This model is based on the different binding ability of VBP1 and VHL with TCF/LEFs, which needs to be elucidated. This raises the potential of the coupled mechanism of the expression levels of VBP1, pVHL, and TCF/LEF, and the possibility needs to be systematically investigated in animal models in the future. Unexpectedly, VBP1 did not induce enhancement in the ubiquitination of Tcf7l2 but increased the ubiquitination levels of HIF-1α simultaneously. However, the addition of the proteasome inhibitor MG132 restored the protein level of TCF7L2 irrespective of VBP1 being overexpressed or depleted. This means that VBP1-mediated TCF/LEF degradation via pVHL likely undergoes the ubiquitin-independent proteasomal pathway. The exact molecular mechanism remains to be elucidated in the future.

It should be mentioned that pVHL regulates Wnt/β-catenin signaling by reducing β-catenin levels and promoting DVL degradation (21, 22). VBP1 might also regulate these components. Therefore, we cannot exclude the possibility that the output of Wnt/β-catenin activity might be compromised when VBP1 is overexpressed or knocked down.

In conclusion, our study demonstrated that VBP1 controlled the stability of all four TCF/LEFs, the important Wnt/β-catenin signaling effectors, by cooperating with pVHL. Thus, these results expanded our current knowledge on the regulation of TCF/LEF stability and modulation of the Wnt/β-catenin signaling pathway. However, further studies are still required to elucidate the precise mechanism by which pVHL and VBP1 regulate TCF/LEF stability. The TCF/LEF family in vertebrates contains four members, which are expressed in distinct but broadly overlapping patterns. Mutants of each appear as distinct phenotypes (43). In addition, dysregulation of Wnt/β-catenin signaling links various diseases. Further study on the relationship among VBP1, pVHL, TCF/LEF, and Wnt/β-catenin signaling in developmental process and diseases could be performed in the future.

**Experimental procedures**

**Antibodies and reagents**

Primary antibodies against Myc (sc-40), β-catenin (sc-7199), VBP1 (sc-390465), and pVHL (sc-135657) were purchased from Santa Cruz Biotechnology. Antibodies against TCF7L2 (2569), GFP (2555), and HA (3724) were obtained from Cell Signaling Technology. The antibody against TCF7L1/L2 (ab12065) was provided by Abcam. The antibody against GAPDH (D110016) was purchased from BBI Life Sciences (Crumlin, UK). The antibody against histone H3 (P30266) was obtained from Abmart. The FLAG M2 antibody (F1804) was provided by Sigma-Aldrich. The antibody against β-actin (abs13797S) was obtained from Absin Bioscience. Small molecules CHX (66-81-9) and BIO (667463-62-9) were purchased from Sigma-Aldrich. MG132 (HY-13259) was from MedChemExpress.

**Plasmids**

The plasmids pCS2-6×Myc-Tcf7, pCS2-6×Myc-Tcf7l1, pCS2-6×Myc-Tcf7l2, pCS2-6×Myc-Lef1, pCDNA3-Myc-VN-Tcf7l1, pCDNA3-Myc-VN-Tcf7l1ΔN, and pCDNA3-Myc-VN-Tcf7l1ΔNLS were gifts from Dr. Wei Wu (Tsinghua University, China). The plasmid pGEX-2T (28-9546-53) was obtained from GE Healthcare. pCS2-FLAG-VBP1, pCS2-FLAG-VBP1-VC, pGEX-2T-VBP1, pCDNA3.1-TCF7L2-HA, and pCS2-VP16-Tcf7l1ΔN were generated by PCR subcloning. Briefly, the human VBP1 ORF was amplified by PCR from HEK293T cell cDNA using the following primers: F, 5′-CGGGATCCGGCGGACCATGCGCGGCGTTTAAAGGACACG-3′; R, 5′-CCGGAATTCTTAATGGATGACTG-3′. The resultant product was subcloned into the pCS2-GFP, pCS2-FLAG, or pGEX-2T, respectively. pcDNA2-GFP and pcDNA2-FLAG were obtained by inserting GFP or FLAG tag into pcDNA2+ vector (a gift from Dr. Cunming Duan, University of Michigan, Ann Arbor). TCF7L2 was amplified by PCR from RD cell cDNA using the following primers: F, 5′-CGGGATCCGGCGGACCATGCGCGGCGTTTAAAGGACACG-3′; R, 5′-CCGGAATTCTTAATGGATGACTG-3′.
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AGACTTG-GTACGGAGGAG-3’

Zebrafish strains

WT (Tübingen) and TOPdGFP zebrafish strains were used in this study. The embryos were incubated in Embryo Medium at 28.5°C. The embryos were strictly staged according to standard methods (44). All experimental protocols were approved by and conducted in accordance with the Ethical Committee of Experimental Animal Care, Ocean University of China.

Cell lines and transfections

HEK293T, HeLa, HCT116, and U2OS cell lines were purchased from ATCC and cultured in DMEM (HyClone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin. The target sequence

5’-CCGG-AATTCCAGGCAATGATCC-3’

CAT-3

AGACTTG-GTACGGAGGAG-3’

quantitative RT-PCR

Total RNA was isolated using RNAise plus reagent (Takara Bio), and 2.0 μg of total RNA was used as a template for reverse transcription. The cDNA was synthesized with M-MLV reverse transcriptase (Promega). Quantitative PCR analyses were carried out with the iTaq SYBR GREEN Supermix and iCycler apparatus (Bio-Rad). Each sample was analyzed in duplicate, and qPCR primers were as follows: GADPH, forward (F): 5’-TCCGACGTCAAGGCAGATTCTCTT-3’ and reverse (R): 5’-GGCCCTCCATCTTGTCTT-3’; AXIN2, F: 5’-CTGGGTCTTTGTTGAACTGTTG-3’ and R: 5’-AGTTGTCACAGCCCAAGAC-3’; CCND1, F: 5’-CCATCGAGGAGGTGTGTC-3’ and R: 5’-AGCTGCTGAGGAGGG-3’; CDK2, F: 5’-CTGGCTTTGTTGAACTGTTG-3’ and R: 5’-AGTTGTCACAGCCCAAGAC-3’. Gene expression analysis was performed using the comparative ΔΔCT method with GADPH normalization. Template-free negative controls were included in each experiment.

Immunoblot and immunoprecipitation

Immunoblot and Co-IP were performed as described previously (46). In brief, 1 × 10^6 cells were seeded in a 35-mm dish and the cells were harvested at 24 h posttransfection; then the cells were lysed with radioimmune precipitation assay buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5) supplemented with protease and phosphatase inhibitors on ice for 15 min. Lysates were then centrifuged at 12,000 × g for 10 min at 4°C. Protein samples were mixed with 5 × Laemmli sample buffer, and proteins were separated by SDS/PAGE, transferred to PVDF membranes, and incubated with primary antibodies overnight at 4°C after blocking with 5% nonfat milk. Primary antibodies were detected with HRP-conjugated secondary antibody (Beyotime Biotechnology). For Co-IP experiments, 6 × 10^6 cells were

MO, mRNA synthesis, and microinjection

Two antisense MOs (Gene Tools, Philomath, OR, USA) were designed for targeting the translation start site of zebrafish vbp1. The sequences were MO1 (5’-ATATAAGTATGCGGCTGCTCTT-3’) and MO2 (5’-CAATGAAGCAGAGGTAGTATTCGCTCTT-3’). The sequence of control MO was: 5’-CCCTACCTACCTATTATA-3’. The target sequence
seeded in a 100-mm dish and harvested at 48 h posttransfection. The cells were then lysed in IP lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and protease and phosphatase inhibitors. Cleared cell lysates were incubated with the appropriate antibody (1–2 μg) overnight at 4 °C, followed by a 6-h incubation with protein A/G agarose beads at 4 °C. In all cases, the immune complexes bound to protein A/G beads were washed four times with IP lysis buffer and detached from the agarose using SDS loading buffer for immunoblot analysis.

The in vivo ubiquitination assay was performed with hot lysis-extracted protein lysates based on the protocol previously described (47). Briefly, HEK293T cells were cotransfected with HA-Ub, FLAG-HIF-1α, and Myc-Tcf7l2, along with empty vector or VBP1-GFP. The cells were treated with 20 μM MG132 for 8 h before harvesting, then lysed in “hot SDS” lysis buffer (2% SDS, 150 mM NaCl, and 10 mM Tris-HCl, pH 8.0) with 2 mM N-ethylmaleimide and protease inhibitors, and lysates were boiled for 10 min and then diluted with nine volumes of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% Triton). The samples were incubated at 4 °C for 30–60 min with rotation and precleared by centrifugation at 12,000 × g for 5 min before performing immunoprecipitation.

**GST pulldown assays**

*Escherichia coli* BL21 competent cells were transformed with GST or GST-VBP1 and then induced with 0.1 mM isopropyl β-d-thiogalactopyranoside at 37 °C for 5 h. The cells were collected by centrifugation, washed once with ice-cold PBS, and then lysed by sonication in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) on ice. The GST or GST-VBP1 extract was then mixed with GSH Sepharose 4B Beads (GE Healthcare) overnight on a rotator at 4 °C. These mixtures were centrifuged, and the precipitates were collected with an equal amount of indicated cell lysates of HEK293T and rotated for 2 h at 4 °C. After extensive washing with the lysis buffer, the precipitated proteins were eluted using SDS/PAGE. The bound proteins were detected by indicated antibodies or Coomassie Blue staining.

**BiFC and immunofluorescence**

BiFC assays were performed as previously described (34). Venus fluorescence protein was selected as the reporter for complementation. Human VBP1 was fused with the carboxyl (VC, amino acids 155–239) terminal of Venus and then subcloned into the pBl bidirectional vectors. *Xenopus* Tcf7l1, Tcf7l1ΔN, and Tcf7l1ΔNLS were fused, respectively, with the amino (VN, amino acids 1–173) terminal of Venus. For immunofluorescence staining, 1 × 10^5 cells were grown on coverslips inside a 6-well plate. After transfection, the cells were fixed with 4% paraformaldehyde, then permeabilized for 5 min at room temperature with PBS containing 0.25% Triton X-100 and blocked with 3% BSA in PBS. The cells were then incubated with anti-Myc antibody overnight at 4 °C. Goat anti-mouse Cy3 was used as a secondary antibody. After 4’,6-diamidino-2-phenylin-

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**VBP1 modulates stability of the TCF/LEF proteins**

Cells (6 × 10^5) were seeded in 6-well tissue culture plates and then incubated overnight. After 12–18 h of incubation, the cells were cultured with CHX at a concentration of 100 μg/ml and incubated for various hours. The cells were harvested at the indicated time points. The lysates were prepared and subjected to immunoblotting analysis. The protein stability was determined by the percentage of TCF7L2 normalized to that of GAPDH at an indicated point compared with that at the initial point.

**Plate cloning formation assay**

The colony formation assay was performed using lentivirus-infected HCT116 cells expressing either scramble shRNA or VBP1 shRNA. For each group, cells were plated at a density of 800 cells/well in a 6-well plate (Corning) and cultured in DMEM-high glucose supplemented with 10% FBS for 14 days. Culture medium was changed every 3 days. After most of the cells that had undergone plasmid integration survived at day 7 posttransfection, the surviving cells were allowed to expand in the culture plate, and when they reached a high confluence, these cells were cryo-preserved as a polyclonal line.

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**Generation of stable cell lines**

For shRNA-transduced experiments, vectors named GV248 were purchased from Genechem (Shanghai, China). VBP1 shRNA-1 sequence 5′-GGAAGACCTTGGACTTCTCGAG-3′ and VBP1 shRNA-2 sequence 5′-CAAGGATGACTCTACAAAGAACA-3′ were used. Lentivirus was produced in HEK293T cells. Briefly, 2 × 10^6 HEK293T cells were seeded in a 60-mm dish with 4 ml of DMEM containing 10% FBS without antibiotic. Next day, pCMV-dR8.2 dvpr, pCMV-VSV-G, the lentivirus vector GV248 containing the GFP reporter gene and the puromycin resistance gene, and the shRNA sequence were cotransfected into the packaging cell line by Lipofectamine 2000. After 18 h, the transfection medium was replaced with fresh DMEM + 10% FBS, and medium containing the lentivirus was harvested after 72 h. Target cells were infected with filtered lentivirus; the infection was carried out in the presence of polybrene (8 μg/ml). At 72 h postinfection, 1.0 μg/ml of puromycin was added to the infected culture plates. The medium was changed every 2–3 days. Cells without integration of transfected plasmid died, whereas cells that had undergone plasmid integration survived at day 7 posttransfection. The surviving cells were allowed to expand in the culture plate, and when they reached a high confluence, these cells were cryo-preserved as a polyclonal line.
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Statistical analysis

All graphs were generated using GraphPad Prism5 Software. Values are presented as the means ± S.D. Statistical analyses were performed using a two-tailed, unpaired Student’s t test or one-way analysis of variance followed by Tukey’s post-test. A value of \( p < 0.05 \) was considered statistically significant. Unless otherwise indicated, all experiments contained three replicates per condition.

Data availability

All the data are contained within this article and in the supporting information. All the data are to be shared upon request (Jianfeng Zhou, Ocean University of China, jfzhou@ouc.edu.cn).

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Abbreviations—The abbreviations used are: VBP, pVHL binding protein; pVHL, von Hippel-Lindau tumor suppressor protein; TCF, T-cell factor; LEF, lymphoid enhancer factor; GSK, glycogen synthase kinase; TLE, transducin-like enhancer of split; NLK, Nemo-like kinase; NARF, NLK-associated RING finger protein; HIF, hypoxia-inducible factor; hpf, hours post fertilization; MO, morpholino; CHX, cycloheximide; Co-IP, co-immunoprecipitation; BiFC, bimolecular fluorescence complementation.

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