Dapper1 Is a Nucleocytoplasmic Shuttling Protein That Negatively Modulates Wnt Signaling in the Nucleus*

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Wnt signaling, via the activation of the canonical β-catenin and lymphoid enhancer factor (LEF)/T-cell factor pathway, plays an important role in embryogenesis and cancer development by regulating the expression of genes involved in cell proliferation, differentiation, and survival. Dapper (Dpr), as a Dishevelled interactor, has been suggested to modulate Wnt signaling by promoting Dishevelled degradation. Here, we provide evidence that Dpr1 shuttles between the cytoplasm and the nucleus. Although overexpressed Dpr1 was mainly found in the cytoplasm, endogenous Dpr1 was localized over the cell, and Wnt1 induced its nuclear export. Treatment with leptomycin B suggested that Dpr1 negatively modulates the basal activity of Wnt/β-catenin signaling in the nucleus by keeping LEF1 in the repressive state. Thus, Dpr1 controls Wnt/β-catenin signaling in both the cytoplasm and the nucleus.

The secreted growth factors of the Wnt family play key roles in early embryogenesis and tissue homeostasis in adults by modulating cell proliferation, differentiation, morphology, and migration. Appropriate control of their signaling activity is essential for normal physiological activity as dysregulation of their signaling is associated with various types of human diseases such as cancer (1–5). In the canonical Wnt signaling pathway, the soluble form of the core factor β-catenin is mainly targeted for ubiquitination and proteasomal degradation, which is promoted by the destruction complex of Axin, adenomatous polyposis coli (APC)2, and glycogen synthase kinase 3β (6–9). The binding of Wnt to its cell surface receptors, Frizzled (Fz) and low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) leads to the recruitment of Dishevelled (Dvl) to Fz and Axin to LRP5/6, resulting in the disruption of the destruction complex and consequently the accumulation of β-catenin. The accumulated β-catenin moves into the nucleus and activates the transcription of target genes through interacting with T-cell factor (TCF)/lymphoid enhancer binding factor (LEF) transcription factors (10–13).

Dapper (Dpr), which was first identified as an interacting protein of Dvl, a central mediator in Wnt signaling, controls Xenopus embryogenesis by functioning as a general antagonist of Dvl to modulate the β-catenin-dependent and JNK-dependent Wnt signaling (14, 15). Several Dpr orthologs, including Dpr1, Dpr2, and Dpr3, have been identified in zebrafish, mouse, and human (16–20). The function of Dpr has been shown to be evolutionally conserved from fish to human (20, 21). However, recent studies suggested that the Dpr family members may also positively regulate Wnt signaling through distinct mechanisms. Zebrafish Dpr1 and Dpr2 were reported to positively modulate the canonical β-catenin or noncanonical calcium-planar cell polarity pathways of Wnt signaling during fish embryo development, respectively (18). Frodo, a Dpr homolog that shares 90% identity at the amino acid level to Xenopus Dpr, has been suggested to positively synergize with Dvl to induce secondary axis and be required for normal eye and neural tissue development (15). In addition, we have demonstrated that zebrafish and mouse Dpr genes specifically inhibited transforming growth factor β/Nodal signaling during mesoderm induction by promoting lysosomal degradation of its type I receptors (19, 21).

Dpr has been also implicated to be associated with tumorigenesis. Human Dpr1 was down-regulated in hepatocellular carcinoma, and this down-regulation was correlated with the cytoplasm accumulation of β-catenin (22).

Our recent work demonstrated that human Dpr1 promotes Dvl2 degradation by a lysosome inhibitor-sensitive mechanism in the cytoplasm (20), whereas Frodo, in addition to interact with Dvl, has been shown to associate with other multiple proteins in both the cytoplasm and the nucleus; that is, TCF3 (23), cell cycle and DNA replication-related protein Dbf4 (24), and the catenin protein family member p120-catenin (25). The subcellular localization of Dpr proteins seems complex. Endoge-

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2 The abbreviations used are: APC, adenomatous polyposis coli; Dvl, Dishevelled; siRNA, small interfering RNA; PBS, phosphate-buffered saline; ChlP,

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ous Dpr1 was showed to be found throughout the cytoplasm as punctate spots and diffusely in the nucleus in both cultured cells and Xenopus animal cap (14, 20). Although ectopic Dpr1 and Dpr2 were predominantly present in the cytoplasm, they were also visible in the nucleus of some cells (14, 18). The observation that some of the C-terminal fragments of Dpr1 are localized almost exclusively in the nucleus further suggested that Dpr1 might have a role in the nucleus (20). However, the physiological significance of Dpr1 in the nucleus is still unclear.

In this study we provide evidence that human Dpr1 shuttles between the nucleus and the cytoplasm. We have further identified a classical nuclear localization signal (NLS) and a nuclear export signal (NES) within Dpr1. In addition, we found that mutation of the NES rendered the exclusive nuclear localization of Dpr1, and this Dpr1 mutant still retained its ability of inhibiting Wnt signaling. Finally we showed that Dpr1 could disrupt the LEF1-β-catenin complex and recruit co-repressor HDAC1 to LEF1.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Human Dpr1 cDNA and siRNA against Dpr1 were described previously (20). Myc-tagged Dpr1 mutants and deletions were generated by restriction digestions and PCR and subcloned into PCMV-Myc. All of the sequences were verified by DNA sequencing. HDAC constructs were kindly provided by Dr. Xin-Hua Feng (Baylor College of Medicine). Dvl construct was provided by Dr. Xi He (Children’s Hospital, Harvard Medical School) (26). c-Myc promoter luciferase reporter was provided by Drs. Bert Vogelstein and Kenneth W. Kinzler (Johns Hopkins University School of Medicine) (27), and LEF1, β-catenin, LEF-luciferase and Topflash-luciferase were provided by Dr. Zhijie Chang (Tsinghua University).

Cell Culture and Establishment of Stable Cell Lines—HEK293T, HeLa, and SW480 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), nonessential amino acids, L-glutamine, and penicillin/streptomycin in a 5% CO2-containing atmosphere at 37 °C. To generate stable cells expressing Dpr1-specific siRNA construct, HeLa cells were transfected with siRNA constructs in pSUPER with specific anti-Dpr1 sequence or anti-nonspecific sequence (20) using Lipofectamine (Invitrogen), and stable transfectants were selected with 0.5 μg/ml puromycin (Invitrogen) for 14 days. Individual clones were then obtained after confirmation of Dpr1 expression by immunoblotting and reverse transcription (RT)-polymerase chain reaction.

Transfection, Immunoprecipitation, and Immunoblotting—HEK293T or HeLa cells were transiently transfected using the calcium phosphate method or Lipofectamine. At 36 h post-transfection the cells were lysed with 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Roche Applied Science) for 30 min at 4 °C. After 12,000 × g centrifugation for 15 min, the lysates were immunoprecipitated with specific antibody and protein A-Sepharose (Zymed Laboratories Inc.) for 3 h at 4 °C. Thereafter, the precipitants were washed 3 times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95 °C and analyzed by SDS-PAGE. Immunoblotting was performed with primary antibodies and then secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were visualized by chemiluminescence. Goat anti-LEF1, mouse anti-β-catenin, and mouse anti-HDAC1 antibodies were purchased from Santa Cruz Biotechnology. Anti-Dpr1 antibody was described previously (20).

Subcellular Fractionation—HEK293T cells were harvested by scraping and resuspended in hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol) containing protease inhibitors (Roche Applied Science) on ice for 15 min. Then Nonidet P-40 was added to 0.001% (for HEK293T) or 0.005% (for HeLa), and cells were vortexed for 30 s and centrifuged at 1000 × g at 4 °C for 5 min. The low speed centrifugation was washed with the ice-cold phosphate-buffered saline (PBS) three times and then resuspended in an equivalent volume of hypotonic lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and lysed by sonification. After centrifugation at 15,000 g for 15 min at 4 °C, the supernatant (nuclear extracts) was collected for analysis.

Immunofluorescence—HeLa cells grown on glass coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% bovine serum albumin in PBS for 60 min. The cells were then incubated with primary antibodies diluted in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 3 h, washed twice with PBS, and then incubated with rhodamine-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) for an additional 40 min. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma). Images were obtained with confocal Olympus Fluoview 500 microscope.

Luciferase Reporter Assays—HEK293T cells were transfected with various plasmids as indicated in the figures. 36 h after transfection, the cells were harvested, and luciferase activities were measured by aluminometer (Berthold Technologies). Reporter activity was normalized to the co-transfected Renilla. Experiments were repeated in triplicate, and the data represent the mean ± S.D. of three independent experiments.

In Vitro Synthesis of mRNA and Microinjection of Zebrafish Embryos—Capped mRNAs were in vitro synthesized with the Cap-Scribe kit (Roche Applied Science). The synthesized mRNA was purified using the RNAeasy Mini kit (Qiagen) and dissolved in nuclease-free water. 20 pmol of synthetic mRNA was injected into 1-cell embryos using a gas-driven microinjector (Sutter Instruments). Injection dose was an estimated amount received by a single embryo. For the mRNA injection experiment, the control embryos were injected with green fluorescence protein (GFP) mRNA.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was carried out essentially as described previously (28). The cell lysates were subjected to immunoprecipitation. Precipitated genomic DNA pellets were subjected to PCR. The primers used to amplify the human c-Myc promoter harboring the third LEF/TCF binding elements, as described in Sierra et
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FIGURE 1. Dpr1 shuttles between the cytoplasm and the nucleus. A and D, LMB treatment elicits redistribution of exogenously expressed Dpr1 from the cytoplasm into the nucleus. HeLa cells transfected with Myc-tagged Dpr1 (0.5 μg) for 36 h were treated with (D) or without (A) 100 pM LMB for another 30 min and then processed for anti-Myc immunofluorescence. C–F, Wnt-1 induces translocation of endogenous Dpr1 from the nucleus to the cytoplasm. HeLa cells transfected with (C and F) or without (B and E) Wnt-1 were treated with (E and F) or without (B and C) Wnt-1 for 8 h and then harvested for detecting endogenous Dpr1 localization by indirect immunofluorescence using anti-Dpr1 antibody followed by rhodamine-conjugated secondary antibody and visualized under a fluorescence microscope. Nuclei were counterstained blue with diamidino-2-phenylindole (DAPI). Scale bar, 10 μm.

RESULTS

Dpr1 Shuttles between the Cytoplasm and the Nucleus—Previous studies have shown that endogenous Xenopus and human Dpr proteins are distributed in both the nucleus and cytoplasm, whereas ectopic Dpr1 stays mainly in the cytoplasm as punctate dots (14, 18, 20). To further investigate the cellular distribution of Dpr1, Myc-tagged full-length human Dpr1 was expressed in HeLa cells and analyzed via immunofluorescent confocal studies. Consistent with previous reports, full-length Dpr1 displayed a punctate pattern in the cytoplasm (Fig. 1A). We then examined the distribution of endogenous Dpr1 in HeLa cells using a specific polyclonal antibody against Dpr1 and found that endogenous Dpr1 was distributed in the punctate pattern throughout the cytoplasm and also apparently stained in the nucleus (Fig. 1B). This observation coincides with the localization pattern of endogenous Xenopus Dpr (14).

Interestingly, upon expression of Wnt-1 in the cells, Dpr1 tended to shift mostly into the cytoplasm instead (Fig. 1C). These data raise the possibility that Dpr1 may be capable of shuttling between the cytoplasm and the nucleus.

To validate the above possibility, we asked whether leptomycin B (LMB), an inhibitor of the nuclear export mediated by export receptor chromosome maintenance region 1 (31), has any effect on the localization of Dpr1. When HeLa cells transfected with full-length Dpr1 were treated with LMB for 30 min, almost all the Dpr1 was accumulated in the nucleus (Fig. 1D). In addition, endogenous Dpr1 accumulated around and in the nucleus upon LMB treatment regardless of Wnt-1 stimulation (Fig. 1, E and F).

The Functional NES and NLS Mediate the Nucleocytoplasmic Shuttling of Dpr1—The translocation of proteins larger than 40–60 kDa in and out of the nucleus is usually dependent on the active transport mediated by nuclear transport receptors, which recognize certain specific NLSs and NESs (32). Dpr1 is about 92 kDa and seems too large to diffuse freely through nuclear pores. We, thus, reasoned that Dpr1 may have functional NES and NLS signals to mediate its nucleocytoplasmic shuttling.

To map the region of Dpr1 responsible for its shuttling between the cytoplasm and the nucleus, we generated several truncation mutants of Dpr1 (Fig. 2A) and then examined their subcellular localization in HeLa cells. All deletion mutants were correctly expressed, as indicated by immunoblotting analysis (data not shown). As shown in Fig. 2B, the N-terminal deletions Dpr1(N1) and Dpr1(N2) were predominantly localized in the cytoplasm, and LMB treatment induced a partial nuclear location of Dpr1(N1) and the complete nuclear accumulation of Dpr1(N2), indicating that the putative NES element was localized in the N-terminal region of amino acids 1–311. The C-terminal deletions Dpr1(C2) and Dpr1(C3) exclusively localized in the nucleus (Fig. 2B), whereas Dpr1 Dpr1(C1), which only missed the N-terminal 96 amino acids, showed similar punctate distribution in the cytoplasm similar to that of wild-type Dpr1. LMB treatment resulted in the nuclear accumulation of Dpr1(C1). As to the central region truncations, Dpr1(C1), which only missed the N-terminal 96 amino acids, showed similar punctate distribution in the cytoplasm similar to that of wild-type Dpr1. LMB treatment resulted in the nuclear accumulation of Dpr1(C1). As to the central region truncations, Dpr1(C1), which only missed the N-terminal 96 amino acids, showed similar punctate distribution in the cytoplasm similar to that of wild-type Dpr1. LMB treatment resulted in the nuclear accumulation of Dpr1(C1). As to the central region truncations, Dpr1(C1), which only missed the N-terminal 96 amino acids, showed similar punctate distribution in the cytoplasm similar to that of wild-type Dpr1. LMB treatment resulted in the nuclear accumulation of Dpr1(C1).
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By inspecting the amino acid sequence of human Dpr1, we found one potential leucine-rich NES at positions amino acids 132–141 (Fig. 2C) and one potential bipartite NLS at amino acids 610–623 (Fig. 2E), and both the potential NES and NLS sequences were highly conserved from zebrafish to human. To examine the importance of these potential NES and NLS, point mutations within the putative motifs were generated with two NES mutants, Dpr1(NESm) and Dpr1(NESm2), and two NLS mutants, Dpr1(NLSm) and Dpr1(NLSm2). Strikingly, we found that replacement of the anterior two residues (L132A, I136A) in the NES (Dpr1(NESm)) led to complete nuclear accumulation of the mutant Dpr1 protein as indicated by immunostaining in HeLa cells (Fig. 2D), indicating that these two residues are essential for the nuclear export of Dpr1. In contrast, the localization of Dpr1(NESm2) was relatively unaffected when Leu-139 and Leu-141 were substituted with Ala.

We did similar manipulation on the potential NLS by point mutations, yielding two mutants, Dpr1(NLSm), with the Ala substitution at Lys-622 and Lys-623, and Dpr1(NLSm2), with the Ala substitution at Lys-610 and Lys-611 (Fig. 2E). Both mutants exhibited similar cytoplasmic punctate distribution as wild-type protein in HeLa cells (Fig. 2F). However, LMB treatment did not alter the cytoplasmic distribution of Dpr1(NLSm), although it caused partial nuclear accumulation of Dpr1(NLSm2) (Fig. 2F). These data suggest that the bipartite NLS is important for the nuclear transport of Dpr1 and imply that the residues Lys-622 and Lys-623 play a key role in the nuclear localization of Dpr1. Localization of these mutants was also examined in HEK293T cells, and similar results were obtained. Furthermore, Wnt-1 stimulation showed no detectable influence on that of these mutants (data not shown). Together these results validate that Dpr1 protein shuttles between the cytoplasm and the nucleus, and this shuttling is mediated by the functional NES and NLS sequences.

Dpr1 Antagonizes Wnt Signaling in the Nucleus—To explore the functional significance of Dpr1 localization in the nucleus, the effect of Dpr1(NESm) and Dpr1(NLSm) on Wnt signaling was examined. HEK293T cells were transiently transfected with the Wnt-responsive reporter LEF-luciferase or Topflash-luciferase, Wnt1 together with wild-type or mutant Dpr1. As shown in Fig. 3A and B, wild-type Dpr1 and its two mutants interfered with the Wnt1-induced expression of the reporters LEF-luciferase and Topflash-luciferase in a dose-dependent manner, although the nuclear-localized Dpr1(NESm) is less effective, whereas none of them had effect on the control reporter Fopflash-luciferase (data not shown). Similar results were obtained in HeLa cells (data not shown).

To further confirm the inhibitory effect of Dpr1 in the nucleus, we examined the effect of different Dpr1 mutants on the β-catenin-stimulated expression of LEF-luciferase and found that all three forms of Dpr1 reduced LEF-luciferase expression induced by β-catenin in a dose-dependent manner (Fig. 3C). β-Catenin(SA) is a mutant unable to undergo phosphorylation-dependent degradation and has been shown to strongly induce the expression of target genes (33). Dpr1 was also able to inhibit β-catenin(SA)-activated reporter expression (Fig. 3D). Consistent with these data, as shown in Fig. 3E and F, all three forms of Dpr1 repressed the basal expression of LEF-luciferase in HEK293T cells as well as in SW480, a colon cancer cell line that harbors APC mutations, and endogenous β-catenin is abnormally accumulated and active (33). These results indicate that Dpr1 can function in the nucleus to down-regulate Wnt signaling.

Our previous work demonstrated that Dpr1 can promote Dvl degradation (20). To examine whether Dpr1 variants with different intracellular localizations retain this function, we compared the abilities of different mutants of Dpr1 to induce Dvl2 degradation. HEK293T cells were transfected with FLAG-
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The above results strongly suggest that albeit less robustly, Dpr1 can inhibit Wnt signaling in the nucleus. And this inhibition did not interfere with the stability of Dvl nor block the nuclear translocation of β-catenin. To elucidate the underlying mechanism, we examined whether Dpr1 associates with the nuclear components of the Wnt pathway. The TCF/LEF family members, high mobility group box transcription factors, play an essential role in the Wnt pathway by consecutively binding to the promoter of many downstream target genes. Frodo, a Dpr1 homolog, was reported to bind to Xenopus TCF3 (23). We, therefore, investigated whether Dpr1 could interact with members of this protein family. As shown in Fig. 4A, when tagged Dvl2 and Myc-tagged wild-type or mutant Dpr1 along with GFP as a control. When expressed at the same level, wild-type Dpr1 (WT) and the cytoplasm-localized Dpr1(NLSm) could effectively reduce the protein level of Dvl2, whereas the nuclear Dpr1(NESm) had nearly no such effect, as judged by immunoblotting (Fig. 3G). No change of GFP level was observed, which confirmed the specificity of Dpr1-promoted Dvl2 degradation. This indicated that Dpr1-mediated degradation of Dvl mainly occurred in the cytoplasm, whereas the inhibitory effect of Dpr1(NESm) on Wnt signaling should be because of other mechanisms.

We then explored whether these Dpr1 mutants have any effect on the nuclear translocation of β-catenin. HEK293T cells were transfected with Wnt-1 and Myc-tagged Dpr1 variants, and the nuclear fractions were extracted for immunoblotting. As shown in Fig. 3H, the expression of the cytoplasm-localized Dpr1(NLSm) resulted in less β-catenin in the nucleus, whereas the nuclear Dpr1(NESm) had no such effect. The nuclear distribution of Dpr1 mutants confirmed our immunofluorescence results.

We further confirmed the function of the nuclear mutant of Dpr1 in zebrafish embryos by injecting mRNA of wild-type Dpr1, Dpr1(NESm), and Dpr1(NLSm) into zebrafish embryos at a one-cell stage. The expression of Dpr1-induced embryonic abnormalities such as enlarged telencephalons, eyes, and reduced trunk and tail at 24 h post-fertilization embryos (Fig. 3I), reminiscent of the defects found in Wnt8 signaling-defective embryos (18, 34). Statistical analysis showed that when 20 pmol of mRNA was injected, Dpr1(WT) caused posterior defects in 43% of the injected embryos, and similarly, Dpr1(NLSm) caused an abnormality rate of 49%. Nuclear Dpr1(NESm) showed a less pronounced influence, resulting in only 35% aberrant embryos with modest abnormalities (Fig. 3I). Nevertheless, these in vivo results further demonstrate that all three forms of Dpr1 can antagonize Wnt signaling, although the nuclear Dpr1(NESm) works more modestly.

Dpr1 Disrupts the LEF1-β-Catenin Complex in the Nucleus—The above results strongly suggest that albeit less robustly, Dpr1 can inhibit Wnt signaling in the nucleus. And this inhibition did not interfere with the stability of Dvl nor block the nuclear translocation of β-catenin. To elucidate the underlying mechanism, we examined whether Dpr1 associates with the nuclear components of the Wnt pathway. The TCF/LEF family members, high mobility group box transcription factors, play an essential role in the Wnt pathway by consecutively binding to the promoter of many downstream target genes. Frodo, a Dpr1 homolog, was reported to bind to Xenopus TCF3 (23). We, therefore, investigated whether Dpr1 could interact with members of this protein family. As shown in Fig. 4A, when

β-catenin. HEK293T cells were transfected with Wnt-1 (0.2 μg) and Myc-Dpr1 variants (0.5 μg each) as indicated. At 36 h post-transfection, the cells were harvested, and total cell lysates (TL) and nuclear fractions were extracted for immunoblotting. I, overexpression of Dpr1(NESm) interferes with normal development of zebrafish embryos. Embryos were injected with 20 pmol of mRNA encoding GFP, Dpr1(WT), Dpr1(NESm), or Dpr1(NLSm) at the single-cell stage. The embryo phenotype was observed 24 h after fertilization. Representative embryos with phenotypes induced by the expression of different Dpr1 were shown, with GFP-injected embryos as control. Embryo deficiency was summarized in the table (n indicates the number of embryos injected).
coexpressed, HA-tagged LEF1 could be detected in the Myc-tagged Dpr1 immunoprecipitation complex. This interaction was mediated by the C-terminal fragment (amino acids 631–836) of Dpr1, Dpr1(C3) (Fig. 4B).

-β-Catenin, a core transcription activator of the canonical Wnt signaling pathway, shuttles between the cytoplasm and the nucleus and interacts with TCF/LEF to activate target genes. *Xenopus* Dpr1 and Frzd1 were reported to form complex with β-catenin (14) and p120-catenin (25), respectively, and the binding was indicated to occur mainly in the cytoplasm. We, therefore, asked whether human Dpr1 could also bind to β-catenin. For this purpose we transfected HEK293T cells with Myc-tagged Dpr1 and performed immunoprecipitation using anti-Myc antibody and found that endogenous β-catenin was in the precipitated complex (Fig. 4C). Interestingly, all three forms of Dpr1 associated with endogenous β-catenin (data not shown), indicating that Dpr1 could bind to β-catenin in both the cytoplasm and the nucleus. To further confirm this interaction, we carried out immunoprecipitation assays at endogenous protein levels using specific β-catenin antibody and found that endogenous Dpr1 could be detected in the immunoprecipitated complex (Fig. 4D). Domain mapping showed that the C-terminal fragment (amino acids 631–836) of Dpr1, Dpr1(C3), accounted for the binding to β-catenin (Fig. 4E). Conversely, all the fragments of β-catenin except C3 were able to interact with Dpr1, albeit C4 and N4 interacted weakly, suggesting that the amino acids 266–408 of β-catenin, which covers the armadillo repeat 4–6, was essential for their binding (Fig. 4F and G).

These results suggested that Dpr1 is in the LEF1-β-catenin complex in the nucleus. Previous studies have shown that the armadillo repeats 2–9 of β-catenin are the binding sites for LEF1/TCF and its antagonists, Tob1 and ICAT (10, 35, 36). As LEF1 and β-catenin bind to the same region of Dpr1, Dpr1 and Dpr1 associate with β-catenin in the overlapped region, it is possible that Dpr1 competes with β-catenin to bind to LEF1. To test this possibility, we transfected FLAG-tagged β-catenin(SA) and HA-LEF1 in HEK293T cells with or without Myc-tagged Dpr1 variants. The nuclear fraction was extracted for anti-FLAG immunoprecipitation and subsequent immunoblotting analysis. In accordance with previous studies (10), β-catenin(SA) interacted with LEF1 (Fig. 4H, upper panel). Furthermore, this interaction was greatly impaired in the presence of Dpr1(NESm) despite that the total levels of β-catenin(SA) and LEF1 in the lysates remained constant (Fig. 4H, lower panels). Dpr1(NLSm) also disrupted the formation of the β-catenin(SA) and LEF1 complex. It could be because Dpr1(NLSm) reduced the entry of β-catenin(SA) into the nucleus (Fig. 4H, lower panels).

It is known that β-catenin associates with the promoter of its target genes through TCF/LEF and c-Myc is one of the β-catenin-LEF targets (27). To further confirm that Dpr1 disrupts the LEF1-β-catenin complex associated with the genomic DNA, we carried out the ChIP assay. After anti-FLAG or anti-Myc immunoprecipitation to pull down protein-DNA complex from the transfected HEK293T cells, PCR was carried out to detect the c-Myc promoter region (nucleotides −1413 to −1108). As shown in Fig. 4I, both β-catenin(SA) and Dpr1(NESm) were found to associate with the TCF binding element region of the c-Myc promoter, and Dpr1(NESm) decreased the binding to DNA of β-catenin(SA). Taken together, our data indicate that Dpr1 can interfere with the
interaction of β-catenin and LEF1 and, thus, antagonize Wnt signaling in the nucleus.

Dpr1 Recruits HDAC1 to LEF1 in the Nucleus—TCF/LEF transcription factor family members were reported to act as repressors of Wnt target genes in the absence of Wnt signal, mainly by binding with various nuclear co-repressors such as Groucho, CtBP, and HDACs (37–41). We, thus, attempted to address whether HDAC proteins might also contribute to the inhibitory effect of Dpr1 in the nucleus. HEK293T cells were transfected with various constructs as indicated in Fig. 5A. The co-immunoprecipitation assay showed that Dpr1 formed a complex with HDAC1 but not highly conserved HDAC2, defining the specificity of their interaction. Dpr1 was also detected in the anti-HDAC1 immunoprecipitated complex at endogenous protein levels (Fig. 5B), which confirmed that the interaction could take place at the physiological condition. We further mapped the regions of Dpr1 responsible for this interaction, and found that the C-terminal Dpr1(C3) strongly interacted with HDAC1, and the N-terminal Dpr1(N1) also has a weak binding activity (Fig. 5C).

To determine whether HDAC1 contributes to the inhibitory function of Dpr1 in the nucleus, we investigated the effect of HDAC1 on LEF1-luciferase expression. Consistent with previous report (42), HDAC1 did not repress Wnt-1 induced LEF1 reporter transcription when transfected alone (Fig. 5D), which might be due to a high level of endogenous HDAC1 protein in the cells. However, co-expression of HDAC1 enhanced the inhibitory effect of wild-type and nuclear Dpr1 (NESm) but not cytoplasmic Dpr1 (NLSm) (Fig. 5D). All three Dpr1 forms were expressed at similar levels as judged by immunoblotting (data not shown). This suggested that HDAC1 might contribute to Dpr1 inhibition on Wnt signaling in the nucleus. To further confirm the role of HDAC1 in mediating the inhibitory effect of Dpr1 in Wnt-1-induced transcriptional activation, we tested whether trichostatin A, a HDAC inhibitor, could reverse this inhibition. Although trichostatin A treatment did not influence the expression level of Dpr1 (data not shown), it could counteract the inhibitory ability of Dpr1(NESm) on Wnt-1-stimulated reporter expression but had virtually no effect on that of Dpr1 (NLSm) (Fig. 5E). Together these results suggest that HDAC1 cooperates with Dpr1 to antagonize Wnt signaling in the nucleus.

As HDAC1 can bind to Dpr1 and collaborate with nuclear Dpr1 to decrease LEF-luciferase reporter expression, we then explored whether nuclear Dpr1 could influence the LEF1 and HDAC1 complex. We transfected into HEK293T cells with FLAG-tagged HDAC1 and HA-tagged LEF1 in HEK293T cells with or without Myc-tagged Dpr1 variants and performed anti-FLAG co-immunoprecipitation assay of the nuclear fractions. As shown in Fig. 5F, HDAC1 could associate with LEF1, which is in accordance with the previous report. More importantly, both wild type (WT) Dpr1 and Dpr1(NESm) enhanced the interaction between HDAC1 and LEF1 without interfering the protein expression levels, whereas the cytoplasmic Dpr1(NLSm) exhibited no such effect as it was not found in the nucleus.

The above data suggest that Dpr1 might enhance the LEF1 recruitment of HDAC1 to repress gene expression. To further test this hypothesis, we carried out anti-HDAC1 ChIP assay. In agreement with an early report (29), HDAC1 bound to the c-Myc promoter (Fig. 5G). Consistent with the above result that nuclear Dpr1 enhanced the interaction between LEF1 and HDAC1, Dpr1(NESm) increased the association of HDAC1 with the c-Myc promoter, suggesting that Dpr1 might affect the nuclear localization of HDAC1.
with the promoter (Fig. 5G). Together, the above data indicate that Dpr1 can enhance the interaction of HDAC1 with LEF1 to the target gene promoter and repress transcription. Therefore, Dpr1 could inhibit Wnt signaling in the nucleus by virtue of two mechanisms; disruption of the β-catenin-LEF1 interaction and facilitation of co-repressor HDAC1 recruitment to gene promoters.

**Knockdown of Dpr1 Expression Increases the Wnt-1-mediated Expression of Wnt Targets c-Myc and Cyclin D1 and Promotes Cell Cycle Progression**—To evaluate the physiological function of endogenous Dpr1, we generated Dpr1 knockdown stable cell line by transfecting HeLa cells with the siRNA construct targeted against Dpr1, which is expressed endogenously in this cell line (20). Immunoblotting and RT-PCR confirmed Dpr1 knockdown in Dpr1 siRNA cells but not in control cells (nonspecific siRNA) (data not shown). In contrast to control, Dpr1 knockdown remarkably induced the expression of the Wnt target genes c-Myc and cyclin D1, as shown by RT-PCR (Fig. 6A). Consistently, the Wnt-1-induced expression of reporters driven by their promoters was up-regulated in Dpr1 knockdown cells (Fig. 6, B and C). In addition, both Dpr1(NESm) and Dpr1(NLSm) could counteract the effect of Dpr1 siRNA with Dpr1(NLSm) working more efficiently (Fig. 6, B and C). As both c-Myc and cyclin D1 are important factors promoting cell cycle progress and, thus, cell proliferation, we investigated the function of Dpr1 in the nucleus to interfere with Wnt3a-induced cell cycle progression. As shown in Fig. 6D, like Dpr1(WT), overexpression of Dpr1(NESm) resulted in accumulation of more cells in the G1 phase. In contrast, Dpr1 siRNA cells escaped from the G1 phase more quickly than the control nonspecific siRNA cells (Fig. 6E), indicating that knockdown of Dpr1 could lead to growth advantage to overcome G1 arrest. Taken together, these data suggest that endogenous Dpr1 as well as exogenously expressed nuclear Dpr1 form can repress the expression of c-Myc and cyclin D1 and, thus, inhibit cell cycle progression.

**DISCUSSION**

Dvl and β-catenin are key players in the canonical Wnt signaling pathway. In the absence of Wnt, β-catenin is degraded via the proteasomal pathway (1, 2, 5). β-Catenin degradation is initiated by phosphorylation and ubiquitination, both of which are promoted by a complex containing Axin, glycogen synthase kinase 3β, casein kinase 1, β-transducin repeat-containing protein and others. The binding of Wnt to its cell surface receptors results in the membrane recruitment of Dvl and, thus, disassembly of the β-catenin degradation complex. Dpr was first identified as an interacting partner of Dvl protein (14). Because Dvl is genetically and biochemically placed upstream of the β-catenin degradation complex, Dpr has been mainly proposed to function in the cytoplasm via increasing glycogen synthase kinase 3β and Axin in the β-catenin degradation complex and, thus, resulting in a decrease of soluble β-catenin (14) or promoting the degradation of Dvl protein (20). In this study we demonstrated that human Dpr1 shuttles between the cytoplasm and the nucleus. We have identified the nuclear localization signal and the nuclear export signal and found that disruption of its NLS or NES results in the constant cytoplasmic or nuclear localization of Dpr1, respectively. Interestingly, the forced localization in the nucleus of Dpr1 by NES mutations still renders the inhibitory effect of Dpr1 on Wnt/β-catenin signaling as shown by reporter assay. We further showed that Dpr1 interferes with the canonical Wnt signaling via two non-competing mechanisms; that is, disrupting β-catenin-LEF1 complex formation and enhancing LEF1-HDAC1 complex formation. Finally, we found that the nuclear localization of Dpr1 may contribute to its function in controlling cell cycle progression.

**Shuttling of Dpr1 between the Cytoplasm and the Nucleus**—Many components of the Wnt signaling pathway, such as APC, Axin, and glycogen synthase kinase 3β, have been initially reported to function in the cytoplasm and then found to shuttle into the nucleus (43–48). The cytoplasmic shuttling of both
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APC and Axin was shown to promote the export of β-catenin from the nucleus (43, 44, 46). APC can also function in the nucleus as an adaptor to bring β-catenin to CtxBP, thus resulting in the formation of a complex that sequesters free nuclear β-catenin available for interaction with TCF (49). Furthermore, a recent study suggested that APC and β-transducin repeat-containing protein, a ubiquitin E3 ligase component involving in β-catenin degradation, can repress c-Myc expression by their direct binding to the c-Myc promoter (29). In addition, Dvl protein, which remains in the cytoplasm as punctate dots and are colocalized with Dpr1 when they are co-expressed (20), was also reported to be able to undergo nucleocytoplasmic shuttling, and its presence in the nucleus was thought to be critical for its promoting function of Wnt signaling (50, 51). All these observations indicate that the shuttling activity and the nucleus localization of these components may have important roles in mediating and/or regulating Wnt signaling.

In this study our immunofluorescence analysis revealed both cytoplasmic and nuclear distribution of endogenous Dpr1, although the exogenously expressed Dpr1 were mainly as punctate dots in the cytoplasm. LMB treatment resulted in its accumulation in the nucleus, indicating that Dpr1 constantly shuttled in and out of the nucleus. The nuclear accumulation of Dpr1 upon LMB treatment also suggests that the nuclear export of Dpr1 is dependent on the nuclear export receptor nucleoplasmin (53). The NLS of homolog Frodo has also been found to function in the nucleus; it could prevent the formation of the β-catenin-LEF1 complex and recruit co-repressor HDAC1 to LEF1 to help maintain LEF1 in a repressive state in the nucleus. Wnt1 treatment led to the nuclear export and the cytoplasmic accumulation of Dpr1. In the cytoplasm, Dpr1 promotes the degradation of Dvl or β-catenin (14, 20). In addition, Dpr1 can bind to β-catenin (Fig. 4, C and D), and the expression of cytoplasmic Dpr1(NLSm) decreases the nuclear β-catenin level, whereas the nuclear Dpr1(NESm) has no such effect, strongly suggesting that Dpr1 might sequester β-catenin in the cytoplasm through direct interaction. Less β-catenin(SA) detected in the nuclear fraction in the presence of Dpr1(NLSm) (Fig. 4H) further supported this hypothesis. Consistent to the nuclear function of Dpr1, its homolog Frodo has been also found to function in the nucleus; it associates with TCF3 and influences TCF3-dependent transcription (23). Furthermore, Frodo can interact with and stabilize the p120-catenin protein (25).

Therefore, Dpr1 can function as an antagonist of Wnt signaling in both the cytoplasm and the nucleus. In the absence of Wnt signaling, Dpr1 is mainly localized in the nucleus, where it could prevent the formation of the β-catenin-LEF1 complex and recruit co-repressor HDAC1 to LEF1 to help maintain LEF1 in a repressive state. In the presence of Wnt ligands, Dpr1 undergoes the nuclear export, where Dpr1 promotes the degradation of Dvl and β-catenin. In addition, Dpr1 can also sequester β-catenin in the cytoplasm through direct interaction.

In summary, our findings extend our understanding of the molecular mechanisms underlying the negative regulation of Wnt signaling by Dpr1. In parallel to its function of promoting Dvl protein for degradation in the cytoplasm, Dpr1 can exert its inhibitory effect in the nucleus by keeping LEF1 in the repressive state. The nuclear function of Dpr1 might be very important in a tight control of the basal activity of β-catenin in the absence of Wnt. Depending on cell type or state, the two possible mechanisms of Dpr1 repressing function may work jointly or independently. In addition, the nucleocytoplasmic shuttling
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Of Dpr1 may also influence the nuclear and cytoplasmic distribution of its partners such as Dvl and β-catenin, which needs further investigation. Because Dpr1 has been shown to regulate JNK pathway (14), it remains to be tested whether Dpr1 also regulates noncanonical Wnt signaling in the nucleus.

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REFERENCES

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