Wnt Signaling Stimulates Transcriptional Outcome of the Hedgehog Pathway by Stabilizing GLI1 mRNA

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Abstract
Wnt and Hedgehog signaling pathways play central roles in embryogenesis, stem cell maintenance, and tumorigenesis. However, the mechanisms by which these two pathways interact are not well understood. Here, we identified a novel mechanism by which Wnt signaling pathway stimulates the transcriptional output of Hedgehog signaling. Wnt/β-catenin signaling induces expression of an RNA-binding protein, CRD-BP, which in turn binds and stabilizes GLI1 mRNA, causing an elevation of GLI1 expression and transcriptional activity. The newly described mode of regulation of GLI1 seems to be important to several functions of Wnt, including survival and proliferation of colorectal cancer cells. [Cancer Res 2009;69(22):8572–8]

Introduction
Wnt and Hedgehog (Hh) are two major pathways that are critical in embryonic development, stem cell maintenance, and tumorigenesis. Both signaling pathways play critical roles in patterning, morphogenesis, and proliferation during embryogenesis and in tumorigenesis.

β-Catenin is a pivotal player in the canonical signaling pathway initiated by Wnt proteins. This pathway has been shown to control the establishment of the body axis at the very early stages of embryogenesis and the development of many organs and tissues, including brain, limbs, kidney, reproductive tract, teeth, and mammary glands (reviewed in ref. 1). In the absence of Wnt signaling, β-catenin (contained within a multiprotein complex of axin, APC, and GSK3β) is phosphorylated by GSK3β and subsequently degraded by ubiquitin-dependent proteolysis. Following the binding of Wnt proteins to receptors of the Frizzled and LRP families on the cell surface, GSK3β is inactivated and unphosphorylated β-catenin is released from the complex. It is subsequently translocated into the nucleus, where it forms a complex with Tcf/Lef, resulting in the activation of Wnt target genes. Mutational loss of APC, stabilizing mutations of β-catenin, or mutations in axin cause constitutive activation of the Wnt signaling pathway and lead to colorectal cancers (reviewed in ref. 2).

The Hh signaling pathway is also crucial for growth, patterning, and morphogenesis of many organs. This pathway is mediated by the Ci/GLI family of zinc finger transcription factors. In the absence of the Hh ligand, its transmembrane receptor Patched (Ptc) inhibits the activity of another transmembrane protein, Smoothened (Smo), resulting in inactivation of Hh signaling. Binding of the Hh ligand to Ptc abrogates the inhibitory effect of Ptc on Smo, thereby activating the transcription factor Ci/GLI. In vertebrates, three GLI genes have been identified, with GLI1 being predominantly a transcriptional activator and GLI2 and GLI3 acting as both activators and repressors. Aberrant regulation of the Hh pathway contributes to the development of many human cancers. Activating mutations of Smo or suppressing mutations of Ptc have been shown to constitutively activate the Hh signaling pathway (reviewed in ref. 3).

The Wnt and Hh signaling pathways, being fundamental in the coordination of developmental transitions, have been postulated to interact or cross-regulate at multiple levels; however, the mechanisms of these interactions are not clear. Some studies have suggested an antagonistic role of Hh signaling toward Wnt signaling. This antagonism has been reported during patterning of the dorsal somite in chick (4), in the mouse somitic mesoderm, possibly through upregulation of SFRP2 (5), and in colonic epithelial cell differentiation and colorectal cancers, probably through a GLI1-mediated mechanism (6, 7). Conversely, a Gli-dependent activation of Wnt signaling has been shown during ventro-posterior morphogenesis in Xenopus embryos (8) and during epithelial transformation, likely through Snail activation and E-cadherin inhibition (9). Active canonical Wnt signaling pathway has also been shown to be required for Hh pathway-driven development of basal cell carcinomas (10). Several reports have suggested that Hh signaling is controlled by Wnt signaling during embryogenesis (11, 12) and in development of colorectal cancers (13–15).

The mechanisms of cross-regulation between Wnt and Hh signaling pathways are not well understood. In this study, we identify a novel mechanism by which Wnt signaling regulates the transcriptional outcome of Hh signaling pathway. We show that this mechanism uses GLI1 mRNA stabilization by the RNA-binding protein CRD-BP, a direct target of the Wnt signaling pathway, and show its importance for colorectal tumorigenesis.

Materials and Methods
Expression vectors. The full-length GLI1 subcloned into pOTB7 [American Type Culture Collection (ATCC)] was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene) and cloned into two vectors: pTRE-Tight (Clontech) under the control of TRE promoter and pcDNA3.1 (Invitrogen) downstream of the T7 promoter.
The expression vectors for Flag-CRD-BP were a kind gift of Dr. J. Ross (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI). CRD-BP shRNA was described previously (16). In brief, we used the siRNA Target Finder and Design Tool to select siRNA sequences. The annealed shRNA inserts were cloned into the pSilencer 1.0-U6 siRNA expression vector in accordance with the recommendations of Ambion, β-Catenin\(^{331}\) and pHR-Ictcf4 (wild-type) were a gift from Drs. K. Kinzler (The Sol Goldman Pancreatic Cancer Research Center, The Ludwig Center and The Howard Hughes Medical Institute at the Johns Hopkins Kimmel Cancer Center, Baltimore, MD) and B. Vogelstein (The Sol Goldman Pancreatic Cancer Research Center, The Ludwig Center and The Howard Hughes Medical Institute at the Johns Hopkins Kimmel Cancer Center, Baltimore, MD). GLI1 shRNAs were kindly provided by Dr. F. Aberger (Department of Molecular Biology, University of Salzburg, Salzburg, Austria), and 8×3′Gli BS-LucIII (eight directly repeated copies of 3′ Gli binding site from HNF3β) floor plate enhancer cloned into pSGLucII) was a gift of Dr. H. Sasaki (Laboratory of Developmental Biology, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). Plasmids for expression of β-galactosidase (pSV-40) and β-galactosidase (Promega) were purchased from Promega.

**Tissue culture and transfections.** 293T cells were obtained from ATCC; they were maintained in DMEM medium with 10% fetal bovine serum (FBS) and transfected with the calcium phosphate method (16). The cell lines HeLa, NHEST3, HCIT116, DLD1, HT-29, RKO, SW48, SW480, SW620, and CCD-841/CoTr were also obtained from ATCC and maintained in accordance with the manufacturer’s recommendations. The cancer cell lines DLD1Δ15 and LS174 T-L8 were gifted from Dr. H. Clevers (Hubeckert Laboratory and Utrecht University, Utrecht, the Netherlands). These cell lines are characterized by constitutive activation of the β-catenin/Tcf signaling. DLD1Δ15 cells carry a mutation in APC, whereas LS174 T-L8 cells have their mutation in β-catenin. Additionally, these cells carry a doxycycline-inducible dnTcf4 (17). They were maintained in RPMI medium with 5% FBS. All our tissue culture media contained 1% penicillin and streptomycin. NIH 3T3, DLD1Δ15, and LS174 T-L8 cells were transfected with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s recommendations. The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector.

**Luciferase reporter assays.** NIH 3T3 cells, DLD1Δ15 cells, and 293T cells were transfected by lipofection with 8×3′Gli BS-LucIII reporter plasmid, pSV-40 β-galactosidase (Promega), and different additional plasmids as indicated in the figures. Luciferase activity was estimated 48 h after transfection using luciferase reporter assay reagent (Promega). β-Galactosidase used for normalization was estimated by the β-galactosidase assay reagent (Pierce).

**In vitro transcription of labeled RNA with α-\(^{32}\)P.** The fragments encompassing nucleotides 41-990, 973-1824, 1808-2730, and 2713-3600 of GLI1 cDNA were amplified by PCR downstream of the SP6 promoter using Pfu Turbo DNA polymerase (Stratagene). Primer sequences for PCR amplification are in Supplementary Table S1. The full-length GLI1 in the pOTB7 vector was linearized immediately 3′ to the target DNA insert, and *in vitro* transcription was performed for the full-length mRNA as well as for the fragments using SP6 DNA-directed RNA polymerase to produce 5′-capped, uniformly labeled mRNAs according to the kit Riboprobe *in vitro* Transcription Systems (Promega). Radiolabeled probes were prepared using [α-\(^{32}\)P]UTP (800 Ci/mmole; Amersham). Following transcription, RNase-free DNsase (Promega) was added to the mixture to remove the template DNA and the probe was precipitated with 0.5 volume of 7.5 mol/L ammonium acetate in 2.5 volumes of ethanol and resuspended in RNase-free water.

**UV cross-linking.** Twenty micrograms of protein from the whole cell extract of 293T cells transfected with either Flag-CRD-BP expression vector or pcDNA3.1 plasmid were incubated with 1.5 × 10⁶ cpm of each RNA probe in 96-well plate at room temperature for 20 min in 20 μL of binding buffer [10 mmol/L HEPES (pH 7.6), 3 mmol/L MgCl₂, 40 mmol/L KCl, 2 mmol/L DTT, 5% (v/v) glycerol, 0.5% (v/v) IGEPAL]. Heparin and yeast tRNA were added to final concentrations of 2.5 μg/μl and 50 ng/μl, respectively, for an additional 10 min. The 96-well plate was then placed on ice and irradiated at 254 nm UV light in a Stratallinker (Stratagen) for 30 min at a distance of 5 cm from the light source. RNA not associated with protein was digested with 100 units of RNase A (Sigma Aldrich) at 37°C for 15 min. The remaining RNA-protein complexes were incubated overnight with 2 μg of anti-Flag antibody and 25 μl of protein A/G-plus agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were washed six times in lysis buffer, boiled with 20 μl of sample buffer, and separated in 8% SDS-PAGE. The gel was dried and exposed to X-ray film for a week.

**Real-time PCR.** Real-time PCR for quantitative measurements in zebrafish embryos, human primary tissue samples (16), mouse tissue samples, and cell lines was done using SYBR Green PCR Core reagents (Applied Biosystems). Primer sequences for Gli1, Gli2, Gli3, Axin2, Tcf1 (22), cyclin D1 (23), c-myc (24), and CRD-BP are in Supplementary Table S2. α-Tubulin was used as a reference gene for zebrafish (25) and GAPDH as a reference gene for human (16) and mouse samples.

**Zebrafish strains and embryo culture.** Adult zebrafish were maintained according to established methods (26). Embryos were obtained from natural matings and staged according to Kimmel and colleagues (27). Heterozygous *Tg(thsGpfct)* embryos (28) at shield stage (6 hpf) were heat shocked at 37°C for 30 or 45 min, then incubated at 29°C until they reached tailbud stage (10 hpf). Embryos expressing GfpTcf were identified by green fluorescent protein (GFP) using a Leica MZFLIII stereooscope, then lysed for RNA extraction with Trizol (Invitrogen).

**Results and Discussion**

**Wnt/β-catenin signaling induces expression of GLI1.** To analyze the effect of Wnt/β-catenin signaling on the modulation of the Hh pathway, we studied the expression of the GLI family transcription factors in different cell types after Wnt/β-catenin signaling was either activated or inhibited. We observed an upregulation of GLI1 mRNA levels in HeLa cells treated with recombinant Wnt3A protein (Fig. 1A). Transfection of 293T cells with β-catenin and Tcf4 resulted in elevated expression of GLI1 mRNA and protein (Fig. 1B). Colorectal cancer cells DLD1Δ15 and LS174 T-L8, characterized by constitutive activation of the β-catenin/Tcf4 system, were treated with doxycycline (1 μg/mL). At 48 h after transfection, the cells were assayed by doxycycline (1 μg/mL) and harvested at different time points after treatment. The levels of GLI1 mRNA were analyzed by Northern blot analysis with a GLI1-specific probe.

* http://www.ambion.com/techlib/misc/siRNA_finder.html

**Wnt Signaling Stabilizes GLI1 mRNA**
signaling, carry doxycycline-inducible dominant-negative Tcf4 mutant (dnTcf4). Both of these cell lines showed a significant reduction in GLI1 expression in the presence of doxycycline at the RNA and protein levels (Fig. 1C). Similarly, blocking canonical Wnt signaling with dnTcf in zebrafish embryos resulted in reduction of gli1 mRNA levels (Fig. 1D). These results show that Wnt/β-catenin signaling induces GLI1 expression to the extent similar to other Wnt-regulated genes (axin2 and Tcf3; Supplementary Fig. S1A–C) and this induction is evolutionarily conserved and not cell type restricted. Our findings corroborate a recent study that suggested enhancement of GLI transcriptional activity by β-catenin in different human cancer cells (14). Our results also show that GLI1 induction is Tcf dependent, as overexpression of the dominant-negative form of Tcf inhibited GLI1 expression in DLD1D7Δ15 and LS174 T-L8 cells and in zebrafish embryos.

**CRD-BP binds to the coding region of GLI1 mRNA and stabilizes it.** To investigate the mechanism of GLI1 regulation by Wnt/β-catenin signaling, 293T cells transfected with β-catenin/Tcf were treated with actinomycin D to inhibit transcription, and GLI1 mRNA expression was determined. In contrast to the regulation of the Wnt transcriptional target axin2 (29–31), this treatment did not prevent β-catenin/Tcf–dependent GLI1 induction in 293T cells (Supplementary Figs. S2A and B), suggesting that upregulation of GLI1 is posttranscriptional. Further studies using doxycycline-regulated expression of GLI1 showed that Wnt signaling stabilizes GLI1 mRNA in cells (Fig. 2A). We have previously reported that Wnt/β-catenin signaling induces the expression of mRNA binding protein, CRD-BP (16). This protein was shown to bind and stabilize different mRNAs, including the mRNA of the proto-oncogene c-myc (32), the mRNA of the β-TrCP1 ubiquitin ligase receptor (16, 33), and the mRNA of MDR1, the multidrug resistance P-glycoprotein gene (34). We sought to determine whether GLI1 mRNA expression could be affected by CRD-BP as well. We found that GLI1 mRNA half-life was drastically increased when CRD-BP was overexpressed (Fig. 2A). Overexpression of CRD-BP also upregulated steady-state levels of GLI1 mRNA and protein (Fig. 2B). Because CRD-BP is an mRNA binding protein, we hypothesized that it might directly bind to GLI1 mRNA and induce its stabilization. Indeed, CRD-BP interacted directly with GLI1 mRNA, with the strongest binding observed within the first ~900 bases (41–990) of the coding region of GLI1 mRNA (Fig. 2C). Overall, these results suggest that, upon Wnt/β-catenin signaling activation, CRD-BP is upregulated and then binds to the GLI1 mRNA and stabilizes it. CRD-BP did not target GLI2 and GLI3 mRNAs. These data are anticipated because β-catenin/Tcf4 that upregulates CRD-BP expression (16) could not induce GLI2 and GLI3 mRNA expression (Supplementary Fig. S2C).

**Wnt/β-catenin signaling induces the expression and transcriptional activity of GLI1 in a CRD-BP–dependent manner.** CRD-BP knockdown largely prevented β-catenin/Tcf–dependent GLI1 upregulation in 293T cells, as well as in NIH3T3 cells (Fig. 2D and Supplementary Fig. S3). Moreover, downregulation of CRD-BP by shRNA prevented the induction of GLI1 transcriptional activity by the Wnt/β-catenin signaling (Fig. 3A). Doxycycline-induced
inhibition of β-catenin/Tcf4 signaling resulted in downregulation of GLI1-dependent transcription, whereas CRD-BP was able to upregulate GLI1-dependent transcriptional activity in DLD1Δ15 colorectal cancer cells, regardless of the status of Wnt/β-catenin signaling (Fig. 3B). Knockdown of GLI1 abrogated CRD-BP–controlled regulation of GLI-dependent transcription (Fig. 3C), confirming the specificity of the CRD-BP role in GLI1 regulation. These results show that control of GLI1 expression and activity by the Wnt/β-catenin signaling depends on CRD-BP.

Interestingly, this regulation of GLI-dependent transcriptional activity seems to be independent of upstream Hh signaling, as neither inhibitor (cyclopamine) nor activator (SAG) of SMO had any effect on either basal or Wnt- and CRD-BP–regulated GLI-luciferase (Fig. 3B). These data, whereas confirming a previous observation that upstream Hh pathway inhibitors do not affect GLI transcriptional activity in colorectal cancer cells (19), suggest a novel, SMO-independent, mode of regulation of GLI transcriptional outcome by Wnt signaling pathway.
GLI1 contributes to β-catenin and CRD-BP–dependent proliferation of colorectal cancer cells. Several reports point to the involvement of Hh signaling in the genesis of colorectal cancers (13, 15), where the role of Wnt signaling is well established. Enhancement of GLI transcriptional activity by β-catenin has been shown in different human cancer cells (14), although the mechanism of this activation is not clear. Interestingly, a recent study reported the absence of canonical Hh signaling in a variety of epithelial (including colorectal) cancer cells (19). However, GLI1 protein was also found to be overexpressed in some colorectal cancers independently of Shh signaling (35, 36). Taken together, these studies imply that Wnt signaling controls Hh signaling in colorectal cancers. In cancer cells, Hh is primarily a proliferative stimulus (37) and GLI1 is a transcriptional mediator of Hh signaling (38). Because GLI1 is also upregulated by the Wnt/β-catenin signaling, we sought to investigate whether GLI1 contributes to Wnt/β-catenin–dependent proliferation of human colorectal cancer cells. To assess this function of Wnt/β-catenin–mediated upregulation of GLI1, we tested whether GLI1 coexpression could rescue Wnt/β-catenin–dependent colony formation when this pathway is inhibited. GLI1 could indeed partially rescue the ability of DLD1D7Δ15 and LS174 T-L8 cells to form colonies when Wnt/β-catenin signaling was inhibited by doxycycline treatment–induced expression of dnTcf4 (Fig. 4A). Interestingly, ectopic expression of GLI1 failed to induce cyclin D1 and c-myc mRNA expression in the absence of Wnt signaling (Supplementary Fig. S4A), suggesting that the partial rescue of the ability of DLD1D7Δ15 and LS174 T-L8 cells to form colonies by GLI1 is probably independent of cyclin D1 and c-myc. In addition, although the knockdown of CRD-BP may affect the proliferation of colorectal cancer cells through multiple mechanisms, our data showed that overexpression of GLI1 resulted in attenuation of the inhibitory effect of CRD-BP shRNA in colony formation in colorectal cancer cells DLD1D7Δ15 and LS174 T-L8 (Fig. 4B). This indicates that GLI1 contributes to Wnt- and CRD-BP–dependent proliferation of DLD1D7Δ15 and LS174 T-L8 cells. GLI1 did not affect the expression of cyclin D1 and c-myc mRNA in these experiments either (Supplementary Fig. S4B), which also suggests that other target genes are involved in partial rescue of these cells to form colonies by GLI1. GLI1 contribution to the growth of colorectal cancer cells was not limited to DLD1D7Δ15 and LS174 T-L8 cells: Knockdown of GLI1 (using two independent GLI1-targeting shRNA constructs) drastically decreased the ability of SW480, SW620, and HCT116 cells to form colonies (Supplementary Fig. S5A and B), indicating a requirement for GLI1 in proliferation and survival of colorectal cancer cells. Our data support previous findings that showed the involvement of GLI1 in colorectal cancer cell proliferation (13, 15). In contrast, another study showed that GLI1 overexpression suppressed proliferation of SW480 and HCT116 colorectal cancer cells with activated Wnt signaling (7). These cells exhibit high levels of endogenous GLI1 (Supplementary...
overexpression of GLI1 does not represent relevant pathophysiologic conditions. On the other hand, knockdown of GLI1 expression in a variety of colorectal cancer cells, including SW480 and HCT116, resulted in dramatic inhibition of colony formation (Supplementary Fig. S5 A), further supporting the importance of GLI1 for the proliferation of colorectal cancer cells.

The Wnt/β-catenin pathway is an important player in colorectal carcinogenesis. We next assessed the expression of GLI1 in primary human colorectal tumor samples and colorectal cancer cell lines. We found that all human colorectal tumor samples previously characterized by an activation of β-catenin and high levels of CRD-BP (16, 39) overexpressed GLI1 mRNA to different extents (Fig. 4C). Similarly, we have found a positive correlation between activation of β-catenin signaling and expression of CRD-BP and GLI1 in a panel of established colorectal cancer cell lines (Supplementary Fig. S5A). We also analyzed ApcMin+/− mice, heterozygous for a nonsense mutation in the APC locus that predisposes them to the development of multiple adenomas throughout the entire intestinal tract (40), for expression of Gli1. These mice overexpressed Gli1 in their intestinal tumors but not in the normal intestine (Fig. 4D and Supplementary Fig. S6). These findings further support both a role for GLI1 in colorectal cancer formation and its regulation by Wnt/β-catenin signaling. Increased expression of GLI1 mRNA was previously reported in human colonic adenocarcinomas (13); however, its association with Wnt/β-catenin signaling was not studied. Two other studies observed that the upregulation of GLI1 expression in colorectal carcinomas was not always consistent with the expression pattern of Shh, suggesting that the Hh pathway might be activated by other regulatory mechanisms in colorectal carcinomas (35, 36). Another study reported that Indian hedgehog (Ihh) signaling stimulates colonic epithelial differentiation and inhibits proliferation by antagonizing Wnt signaling (6); however, Yauch and colleagues have recently shown that Hh ligands failed to activate canonical Hh signaling in tumor epithelial cells (19), suggesting that the observed upregulation of GLI1 in colorectal cancer cells may not be a result of activation of upstream Hh signaling. A body of evidence points to a regulatory role of Wnt and Hh pathways in stem cell development in epithelia, including those of the intestine (reviewed in ref. 37). Additionally, recent studies suggest that colorectal tumors might arise from intestinal stem cells (41). Taken together, it is likely that cross-talk between

Figure 4. GLI1 contributes to β-catenin and CRD-BP–dependent proliferation of colorectal cancer cells. A, DLD1Δ15 and LS174 T-L8 cells were grown in a 100-mm dish and cotransfected with pTK-puro plasmid and either the empty vector or pcDNA3.1-GLI1 as indicated. Forty-eight hours after transfection, cells from each plate were seeded in five 100-mm plates and the cells were treated with doxycycline (1 μg/mL) for 72 h and puromycin (8 μg/mL) for 10 d. The colonies formed were counted under a microscope. B, DLD1Δ15 and LS174 T-L8 cells grown in 100-mm plates were cotransfected with pTK-puro plasmid and irrelevant shRNA, CRD-BP shRNA, irrelevant shRNA and pcDNA3.1-GLI1, or CRD-BP shRNA and pcDNA3.1-GLI1 as indicated. Forty-eight hours after transfection, cells from each plate were seeded in five 100-mm plates and treated with puromycin (8 μg/mL) for 10 d. The colonies were counted under a light microscope. C, levels of GLI1 mRNA in primary colorectal normal and tumor human tissue samples determined by quantitative RT-PCR. D, immunoblot analysis of proteins isolated from normal (N) and tumor (T) samples of ApcMin+/− mouse small intestine and colon for Gli1, CRD-BP, and β-catenin. β-Actin was used as internal control.
the Wnt and Hh pathways exists in the development of colorectal cancers and one proposed mechanism of this interaction involves control of GLI1 expression by both pathways.

Wnt and Hh are pivotal pathways similar to each other in several respects (42, 43). These pathways interact at multiple levels in embryonic organ patterning as well as in the development of some cancers. It is likely that different mechanisms are used for this cross-talk in different contexts. In this study, we identify a novel mechanism by which Wnt signaling regulates Hh signaling and show that this mechanism uses GLI1 mRNA stabilization. To the best of our knowledge, this is the first study that shows posttranscriptional regulation of the Hh signaling by the Wnt/?-catenin signaling pathway. It is also the first study to identify CRD-BP as a mechanistic link between the two pathways. Altogether, this work sheds light on the regulatory role of Wnt signaling in controlling Hh signaling and offers significant new insight on the involvement of the two pathways in colorectal cancer development. These findings suggest CRD-BP as a valuable candidate drug target for therapy in cancers associated with deregulation of Wnt/?-catenin and Hh signaling pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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