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Running Title

Wnt signaling stabilizes mRNA of GLI1

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Abstract

Wnt and Hedgehog signaling pathways play central roles in embryogenesis, stem cell maintenance, and tumorigenesis. However, 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 appears to be important to several functions of Wnt, including survival and proliferation of colorectal cancer cells.

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 (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 (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 (Ptch) inhibits the activity of another transmembrane protein, Smoothened (Smo), resulting in inactivation of Hh signaling. Binding of the Hh ligand to Ptch abrogates the inhibitory effect of Ptch 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 Ptch have been shown to constitutively activate the Hh signaling pathway (reviewed in (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, yet the mechanisms of these interactions are not clear. Some studies have suggested an antagonistic role of Hh signaling towards Wnt signaling. This antagonism has been reported during patterning of the dorsal somite in chick (4), in the mouse somitic mesoderm, possibly through up-regulation of SFRP2 (5), and in colonic epithelial cell differentiation and colorectal cancers, probably via a GLI1-mediated mechanism (6, 7). Conversely, a Gli-dependent activation of Wnt signaling has been demonstrated during ventro-posterior morphogenesis in Xenopus embryos (8) and during epithelial transformation, likely *via* 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 demonstrate that this mechanism employs 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 sub-cloned into pOTB7 (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. CRD-BP shRNA was described previously (16). In brief, we used the siRNA Target Finder and Design Tool (http://www.ambion.com/techlib/misc/siRNA_finder.html) to select siRNA sequences. The annealed shRNA inserts were cloned into the p*Sinlencer* 1.0-U6 siRNA expression vector in accordance with the recommendations of Ambion. β-catenin^{S33Y} and pHR-hTcf4 (wild type) were a gift from Drs. K. Kinzler and B. Vogelstein. GLI1 shRNAs were kindly provided by Dr. F. Aberger, and 8X3'Gli BS-LucII (eight directly repeated copies of 3'Gli binding site from HNF3β floor plate enhancer cloned into pδ51LucII) was a gift of Dr. H. Sasaki. Plasmids for expression of β-galactosidase (pSV-40 β-galactosidase) were purchased from Promega (Madison, WI).

Tissue culture and transfections.

293T cells were obtained from ATCC, they were maintained in DMEM medium with 10% FBS and transfected with the calcium phosphate method (16). The cell lines HeLa, NIH3T3, HCT116, DLD1, HT-29, RKO, SW48, SW480, SW620 and CCD-841/CoTr were also obtained from ATCC and maintained in accordance with the ATCC's recommendations. The cancer cell lines DLD1D7Δ15 and LS174 T-L8 were gifts from Dr. H. Clevers. These cell lines are characterized by constitutive activation of the β-catenin/Tcf signaling. DLD1D7Δ15 cells carry a mutation in *APC* while 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

tissues culture media contained 1% penicillin and streptomycin. NIH 3T3, DLD1D7Δ15 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.

The chlorobenzothiophene-containing Hh pathway agonist (SAG) obtained from Alexis Biochemicals was diluted in DMSO and used to treat DLD1D7 Δ 15 cells at the concentration of 3nM for 30 hrs (18). Cyclopamine (Biomol) was diluted in DMSO as well and used at the concentration of 10μM (19) to treat DLD1D7 Δ15 cells for 24 hrs.

Antibodies and immunotechniques.

Antibodies against GLI1, β-actin (Santa Cruz Biotechnology) and Flag (Sigma-Aldrich) were purchased, as well as the secondary antibody conjugated with horseradish peroxidase (Chemicon). Antibody against CRD-BP was generous gift of Dr. J. Ross (20). Immunoblotting procedures were performed as described previously (21).

Whole cell extracts.

To obtain whole cell lysate for western blot analysis, the cells were lysed using a denaturing RIPA buffer containing PBS (pH 7.4), 0.5% sodium deoxycholate, 0.1% SDS, 1% (v/v) IGEPAL, 100 mM sodium orthovanadate, and proteinase inhibitors cocktail (sigma).

For UV-crosslinking reactions, protein extracts were made in non-denaturing lysis buffer containing 10 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 5% (v/v) glycerol, 0.5% (v/v) IGEPAL, and proteinase inhibitors cocktail (Sigma).

GLI1 mRNA degradation in vivo.

293T cells were transfected by calcium phosphate method with 5 μg of Tet-off plasmid (Clontech), 3 μg of pTRE-GLI1 and β-catenin^{S33Y}/Tcf4 expression vectors (3 μg each) or 4 μg of FLAG-CRD-

BP per 100-mm plate. At 48 h after transfection, the cells were treated with 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.

Luciferase Reporter Assays.

NIH 3T3 cells, DLD1D7Δ15 cells and 293T cells were transfected by lipofection with 8X3'GLI BS-LucII reporter plasmid, pSV-40 β-galactosidase (Promega) and different additional plasmids as indicated in the Figures. Luciferase activity was estimated 48 hrs 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 the supplemental table 1. 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 as well as for the fragments using SP6 DNA directed RNA polymerase to produce 5'-capped, uniformly labeled mRNAs according to the kit Riboprobe^R *in vitro* Transcription Systems (Promega). Radiolabeled probes were prepared using [α-^{32}P] UTP (800 Ci/mmol) (Amersham). Following transcription, RNase-free DNase (Promega) was added to the mixture to remove the template DNA and the probe was precipitated with 0.5 volume of 7.5 M ammonium acetate in 2.5 volumes of ethanol, and resuspended in RNase-free water.

UV cross-linking.

20 µg 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 x 10^6 cpm of each RNA probe in 96-well plate at room temperature for 20 min in 20 µl of binding buffer (10 mM HEPES [pH 7.6], 3

mM MgCl2, 40 mM KCl, 2 mM 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 Stratalinker (Stratagen) for 30 min at a distance of 5 cm from the light source. RNA not associated with protein was digested with 100 U of RNase T1 (Ambion) for 20 min at room temperature and further digested with 25 µg of RNase A (SigmaAldrich) 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). 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, in primary human tissue samples (16), in mouse tissue samples and in 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 the supplemental table 2. α-tubulin was used as a reference gene for zebrafish (25) and GAPDH as 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 et al. (27). Heterozygous *Tg(hs:Gfp∆tcf)* embryos (28) at shield stage (6 hpf) were heat shocked at 37°C for 30 or 45 minutes, then incubated at 29°C until they reached tailbud stage (10 hpf). Embryos expressing Gfp∆Tcf were identified by GFP fluorescence using a Leica MZFLIII stereoscope, then lysed for RNA extraction with Trizol (Invitrogen).

Results and Discussion

Wnt/β-catenin signaling induces expression of GLI1.

In order 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 up-regulation of GLI1 mRNA levels in HeLa cells treated with recombinant Wnt3A protein (Figure 1a). Transfection of 293T cells with β-catenin and Tcf4 resulted in elevated expression of GLI1 mRNA and protein (Figure 1b). Colorectal cancer cells DLD1D7Δ15 and LS174 T-L8, characterized by constitutive activation of the β-catenin/Tcf4 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 (Figure 1c). Similarly, blocking canonical Wnt signaling with dnTCF in zebrafish embryos resulted in reduction of *gli*1 mRNA levels (Figure 1d). These results show that Wnt/β-catenin signaling induces GLI1 expression to the extent similar to other Wnt regulated genes (*axin2* and *Tcf1*; Figure S1a-c) and this induction is evolutionary 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 over-expression of the dominant negative form of Tcf inhibited GLI1 expression in DLD1D7Δ15 and LS174 T-L8 cells, and in zebrafish embryos.

9

CRD-BP binds to the coding region of GLI1 mRNA and stabilizes it.

In order 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 Wnt transcriptional target, axin2 (29-31), this treatment did not prevent β-catenin/Tcf-dependent GLI1 induction in 293T cells (Figures S2a-b), suggesting that up-regulation of GLI1 is post-transcriptional. Further studies using doxycycline-regulated expression of GLI1 showed that Wnt signaling stabilizes GLI1 mRNA in cells (Figure 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 over-expressed (Figure 2a). Over-expression of CRD-BP also up-regulated steady state levels of GLI1 mRNA and protein (Figure 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 mRNA of GLI1 with the strongest binding observed within the first ~900 bases (41-990) of the coding region of GLI1 mRNA (Figure 2c). Overall, these results suggest that, upon Wnt/β-catenin signaling activation, CRD-BP is up-regulated, binds to the GLI1 mRNA and stabilizes it. CRD-BP did not target GLI2 and GLI3 mRNAs. These data are anticipated, since β-catenin/Tcf4 that up-regulates CRD-BP expression (16) could not induce GLI2 and GLI3 mRNA expression (Figure S2c).

Wnt/β-catenin signaling induces the expression and transcriptional activity of GLI1 in a CRD-BP dependent manner.

CRD-BP knock-down largely prevented β-catenin/Tcf-dependent GLI1 up-regulation in 293T cells, as well as in NIH3T3 cells (Figure 2d). Moreover, down-regulation of CRD-BP by shRNA prevented the induction of Gli1 transcriptional activity by the Wnt/β-catenin signaling (Figure 3a). Doxycyclin-induced inhibition of β-catenin/Tcf4 signaling resulted in down-regulation of GLI1-dependent transcription, whereas CRD-BP was able to up-regulate GLI1-dependent transcriptional activity in DLD1D7Δ15 colorectal cancer cells, regardless of the status of Wnt/β-catenin signaling (Figure 3b). Knockdown of GLI1 abrogated CRD-BP-controlled regulation of GLI-dependent transcription (Figure 3c), confirming the specificity of CRD-BP's role in GLI1 regulation. These results demonstrate 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 appears 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 (Figure 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 over-expressed 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). Since GLI1 is also up-regulated 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 up-regulation of GLI1, we tested whether GLI1 co-expression 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 (Figure 4a). Interestingly, ectopic expression of GLI1 failed to induce cyclin D1 and c-myc mRNA expression in the absence of Wnt signaling (Figure 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 cyclinD1 and c-myc. In addition, although the knock down of CRD-BP may affect the proliferation of colorectal cancer cells *via* multiple mechanisms, our data showed that over-expression 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 (Figure 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 (Figure S4b), also suggesting that other target genes are involved in partial rescue of these cell 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 (Figure S5a-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 over-expression suppressed proliferation of SW480 and HCT116 colorectal cancer cells with activated Wnt signaling (7). These cells exhibit high levels of endogenous GLI1 (Figure S5c), and, perhaps, over-expression of GLI1 does not represent relevant pathophysiological 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 (Figure S5a), further supporting the importance of GLI1 for the proliferation of colorectal cancer cells.

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) over-expressed GLI1 mRNA to different extents (Figure 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 (Figure S5c). We also analyzed Apc$^{Min+/-}$ 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 over-expressed Gli1 in their intestinal tumors but not in the normal intestine (Figures 4d and 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 up-regulation 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 et al. have recently demonstrated that Hh ligands failed to activate canonical Hh signaling in tumor epithelial cells (19), suggesting that the observed up-regulation 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 cells development in epithelia, including those of intestine (reviewed in (37)). Additionally, recent studies suggest that colorectal tumors might arise from intestinal stem cells (41). Taken together, it is likely that cross-talk between 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 employed for this cross-talk in different contexts. In this study, we identify a novel mechanism by which Wnt signaling regulates Hh signaling, and demonstrate that this mechanism employs GLI1 mRNA stabilization. To the best of our knowledge, this is the first study that demonstrates post-transcriptional regulation of the Hh signaling by the Wnt/β-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 in 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/β-catenin and Hh signaling pathways.

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FIGURE LEGENDS

Figure 1: Wnt/β-catenin signaling induces the expression of GLI1.

a. Northern blot (upper panel) analysis of GLI1 mRNA levels in HeLa cells either untreated or treated with the recombinant mouse Wnt-3A (100 ng/ml) for 15 hrs. Immunoblot (lower panel) analysis of CRD-BP expression levels in the corresponding cells.

b. Northern blot (upper panel) analysis of GLI1 mRNA levels in 293T cells transfected with pcDNA3.1 or β-catenin/Tcf4. Immunoblot (lower panel) analyses of GLI1 and CRD-BP expression levels in the corresponding cells.

c. Northern blot (upper panel) analysis of GLI1 mRNA levels in DLD1D7Δ15 and LS174 T-L8 cells untreated or treated with doxycycline (1 µg/ ml) for 24 hrs. Immunoblot (lower panel) analyses of GLI1 and CRD-BP expression levels in the corresponding cells.

7S was used as internal control for northern blot analyses and β-actin as internal control for immunoblot analyses.

Numbers in the figures a. b. and c. represent relative densitometry measurements.

d. Levels of *gli1* mRNA were determined by quantitative RT-PCR in zebrafish transgenic Tg(HS:dTCF-GFP) embryos bearing heat shock-inducible dnTCF fused to GFP. GFP-positive embryos (dTCF), which expressed dnTCF, were selected by fluorescence and analyzed separately from their GFP-negative siblings (control).

Figure 2: Wnt/β-catenin signaling modulates Hh signaling by up-regulating CRD-BP which stabilizes GLI1 mRNA.

a. 293T cells were co-transfected with Tet-off, pTRE-Tight-GLI1 and either pcDNA3.1, β-catenin/Tcf4, or Flag-CRD-BP. 48 hrs after transfection, transcription was stopped by treatment with doxycycline (1 µg/ ml) for the indicated durations, and stability of the GLI1 mRNA was analyzed by Northern blotting (upper panels) and presented graphically (lower panel).

b. Expression levels of GLI1 mRNA determined by northern blot (upper panel) analysis in 293T cells transfected with pcDNA3.1 or Flag-CRD-BP. Protein expression of GLI1 and CRD-BP were determined by immunoblot (lower panel) analyses in the corresponding cells.

c. Flag-immunoprecipitation (IP) of UV-crosslinked complexes of the 41-990, 973-1824, 1808-2730 and 2713-3600 bp fragments of GLI1 mRNA as well as the full length and proteins from 293T cells transfected with Flag-CRD-BP or pcDNA3.1 plasmid. The diagram illustrates the position of each fragment on GLI1 mRNA.

d. Northern blot (upper panel) analysis of GLI1 expression in 293T cells and immunoblot (lower panels) analyses of GLI1 and CRD-BP expression in 293T and NIH3T3 cells co-transfected with pcDNA3.1 and irrelevant shRNA, β-catenin/Tcf4 and irrelevant shRNA or β-catenin/Tcf4 and CRD-BP shRNA.

7S was used as internal control for northern blot analyses and β-actin as internal control for immunoblot analyses. Numbers in the figures b and d represent relative densitometry measurements.

Figure 3: Wnt/β-catenin signaling induces the expression of GLI1 in a CRD-BP dependent manner.

a. NIH 3T3 cells were grown in 6 well plates and co-transfected with 8X3'GLI BS-LucII reporter plasmid, pSV-40 -galactosidase and irrelevant shRNA or CRD-BP shRNA plasmid then treated with Wnt-3A (100 ng/ml) for 15 hrs as indicated. 48 hrs after transfection, the luciferase activity was estimated using luciferase reporter assay reagent (Promega). β-galactosidase was used for normalization and estimated using β-galactosidase assay reagent (Pierce).

b. DLD1D7Δ15 cells were grown in 6 well plate and co-transfected with 8X3'GLI BS-LucII reporter plasmid, pSV-40 β-galactosidase and Flag-CRD-BP or empty plasmid then treated with doxycycline (1 μg/ ml) for 24 hrs, Cyclopamine (10 μM) for 24 hours or SAG (3nM) for 30 hrs as indicated. The luciferase activity was estimated as in 3-a.

c. 293T cells grown in 6 well plate were co-transfected with 8X3'GLI BS-LucII reporter plasmid, pSV-40 β-galactosidase and Flag-CRD-BP or empty plasmid and irrelevant shRNA or GLI1 shRNA (shGLI1-C). The luciferase activity was estimated as in 3-a.

(*) The asterisks refer to $p < 0.01$ compared to controls in Student's *t*-test

Figure 4: GLI1 contributes to β-catenin and CRD-BP-dependent proliferation of colorectal cancer cells.

a. DLD1D7Δ15 and LS174 T-L8 cells were grown in 100 mm dish and co-tansfected with pTK-puro plasmid and either the empty vector or pcDNA3.1-GLI1 as indicated. 48 hrs after transfection, cells from each plate were seeded in five 100 mm plates and the cells were treated with doxicycline (1μg/ml) for 72 hrs and puromycin (8 μg /ml) for 10 days. The colonies formed were counted under the microscope.

b. DLD1D7Δ15 and LS174 T-L8 cells grown in 100 mm plates were co-transfected 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. 48 hrs after transfection, cells from each plate were seeded in five 100 mm plates and treated with puromycin (8μg/ml) for 10 days. The colonies were counted under the 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 Apc [Min -/+] mouse small intestine and colon for Gli1, CRD-BP and β-catenin. β-actin was used as internal control.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

a. -Quantitative real time RT-PCR analysis of Tcf1 and Axin2 in HeLa cells, either untreated or treated with the recombinant mouse Wnt-3A (100 ng/ml) for 15 hrs..

b. Quantitative real time RT-PCR analysis of Tcf1 and Axin2 in 293T cells transfected with pcDNA3.1 or β-catenin/Tcf4.

c. Quantitative real time RT-PCR analysis of Tcf1 and Axin2 in DLD1D7Δ15 and LS174 T-L8 cells untreated or treated with doxycycline (1 µg/ ml) for 24 hrs.

d. Quantitative real time RT-PCR analysis of GLI1 in DLD1 and HCT116 cells untreated or treated with doxycycline (1 µg/ ml) for 24 hrs.

Supplemental Figure 2

a. -Quantitative real time RT-PCR analysis of GLI1 in 293T cells transfected with pcDNA3.1, β-catenin/Tcf4 or Flag-CRD-BP as indicated and treated with actinomycin D (10µg/ml) for 4 hours.

b. -Quantitative real time RT-PCR analysis of Axin2 in 293T cells transfected as indicated in Supplemental Figure 2 a and treated with actinomycin D (10µg/ml) for 4 hours.

c. -Quantitative real time PCR for GLI2 and GLI3 in 293T cells transfected with pcDNA3.1, β-catenin/Tcf4 or Flag-CRD-BP as indicated.

Supplemental Figure 3

Quantitative real time RT-PCR (upper panel) and immunoblot (lower panel) analyses of 293T cells transfected as indicated. Note that CRD-BP shRNA-3 is only human specific, therefore mCRD-BP (mouse CRD-BP) is resistant to down-regulation by shRNA-3.

*- reported previously in (16)

Supplemental Figure 4

a. Quantitative real time RT-PCR analysis of c-myc and Cyclin D1 in DLD1D7Δ15 and LS174 T-L8 cells co-tansfected with pTK-puro plasmid and either the empty vector or pcDNA3.1-GLI1 as indicated. 48 hrs after transfection, the cells were treated with puromycin (8 µg /ml) for 5 days and then with doxicycline (1µg/ml) for 48 hrs.

b. Quantitative real time RT-PCR analysis of CRD-BP, c-myc and Cyclin D1 in DLD1D7Δ15 and LS174 T-L8 cells co-transfected 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. 48 hrs after transfection, the cells were treated with puromycin (8µg/ml) for 8 days.

Supplemental Figure 5

a. SW620, SW480, and HCT116 cells were grown in 100 mm plates and co-transfected with pTK-puro plasmid and irrelevant shRNA or GLI1 shRNAs (GLI1-A, GLI1-C). Cells from each plate were seeded in five 100 mm plates and treated with puromycin (7 μg) for 10 days. Colonies were counted under the microscope.
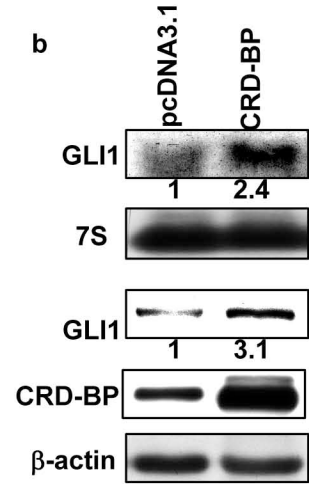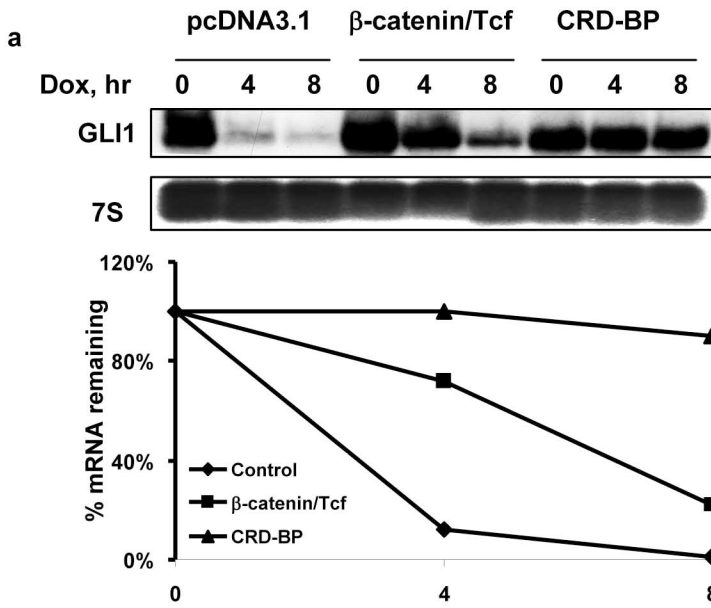
b.Quantitative real time RT-PCR for GLI1 of the cells transfected as in Supplemental Figure 5a.

c. Quantitative real time RT-PCR for GLI1 (upper panel) and immunoblot (lower panels) analyses for CRD-BP and β-catenin expression levels of colorectal cancer cell lines. β-actin was used as internal control for immunoblot analyses.

Supplemental Figure 6

Quantitative real time RT-PCR analysis for Gli1 and CRD-BP in colorectal normal and tumor samples of Apc $^{Min -/+}$ mouse intestine.
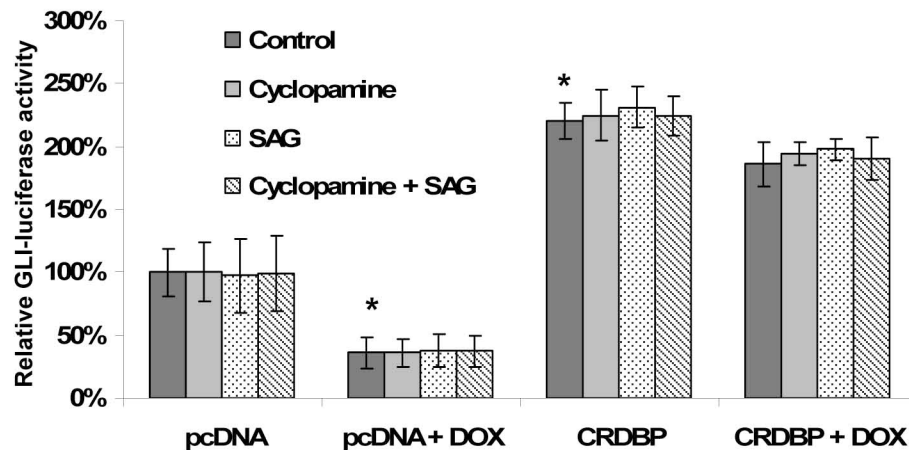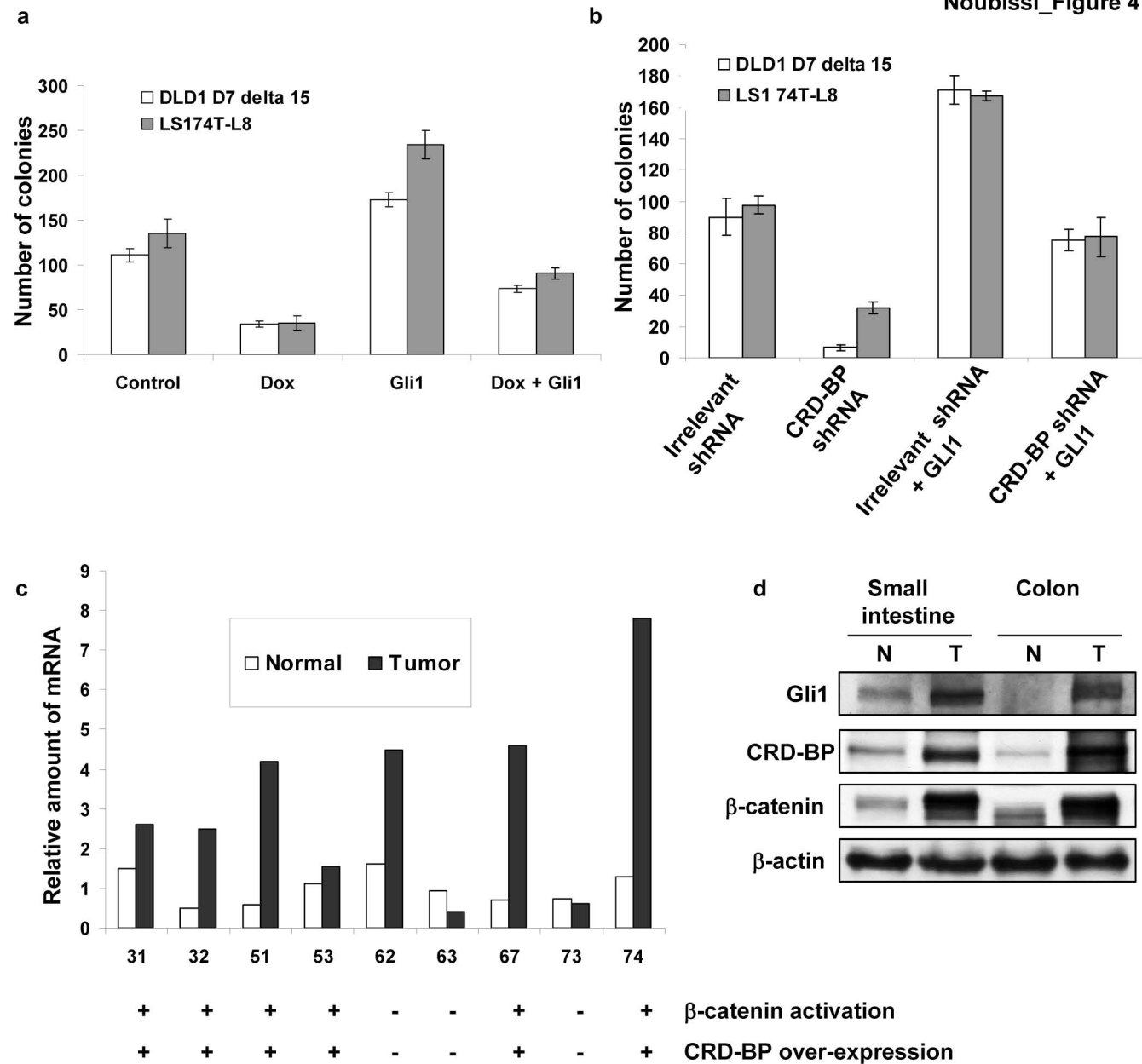
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

a. -Quantitative real time RT-PCR analysis of Tcf1 and Axin2 in HeLa cells, either untreated or treated with the recombinant mouse Wnt-3A (100 ng/ml) for 15 hrs..

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c. Quantitative real time RT-PCR analysis of Tcf1 and Axin2 in DLD1D7Δ15 and LS174 T-L8 cells untreated or treated with doxycycline (1 µg/ ml) for 24 hrs.

d. Quantitative real time RT-PCR analysis of GLI1 in DLD1 and HCT116 cells untreated or treated with doxycycline (1 µg/ ml) for 24 hrs.

Supplemental Figure 2

a. -Quantitative real time RT-PCR analysis of GLI1 in 293T cells transfected with pcDNA3.1, β-catenin/Tcf4 or Flag-CRD-BP as indicated and treated with actinomycin D (10µg/ml) for 4 hours.

b. -Quantitative real time RT-PCR analysis of Axin2 in 293T cells transfected as indicated in Supplemental Figure 2 a and treated with actinomycin D (10µg/ml) for 4 hours.

c. -Quantitative real time PCR for GLI2 and GLI3 in 293T cells transfected with pcDNA3.1, β-catenin/Tcf4 or Flag-CRD-BP as indicated.

Supplemental Figure 3

Quantitative real time RT-PCR (upper panel) and immunoblot (lower panel) analyses of 293T cells transfected as indicated. Note that CRD-BP shRNA-3 is only human specific, therefore mCRD-BP (mouse CRD-BP) is resistant to down-regulation by shRNA-3.

*- reported previously in (16)

Supplemental Figure 4

a. Quantitative real time RT-PCR analysis of c-myc and Cyclin D1 in DLD1D7Δ15 and LS174 T-L8 cells co-tansfected with pTK-puro plasmid and either the empty vector or pcDNA3.1-GLI1 as indicated. 48 hrs after transfection, the cells were treated with puromycin (8 µg /ml) for 5 days and then with doxicycline (1µg/ml) for 48 hrs.

b. Quantitative real time RT-PCR analysis of CRD-BP, c-myc and Cyclin D1 in DLD1D7Δ15 and LS174 T-L8 cells co-transfected 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. 48 hrs after transfection, the cells were treated with puromycin (8µg/ml) for 8 days.

Supplemental Figure 5

a. SW620, SW480, and HCT116 cells were grown in 100 mm plates and co-transfected with pTK-puro plasmid and irrelevant shRNA or GLI1 shRNAs (GLI1-A, GLI1-C). Cells from each plate were seeded in five 100 mm plates and treated with puromycin (7 µg) for 10 days. Colonies were counted under the microscope.

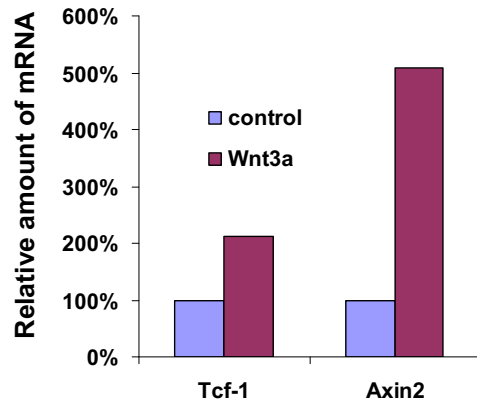**b.**Quantitative real time RT-PCR for GLI1 of the cells transfected as in Supplemental Figure 5a.

c. Quantitative real time RT-PCR for GLI1 (upper panel) and immunoblot (lower panels) analyses for CRD-BP and β-catenin expression levels of colorectal cancer cell lines. β-actin was used as internal control for immunoblot analyses.
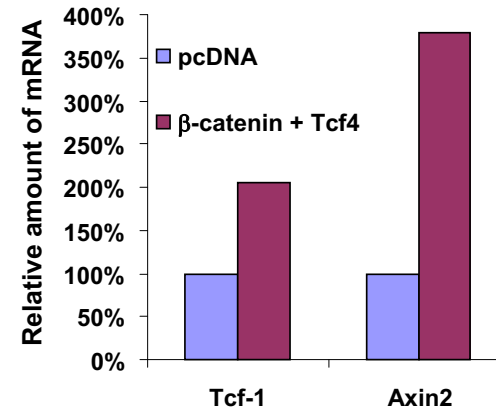
Supplemental Figure 6

Quantitative real time RT-PCR analysis for Gli1 and CRD-BP in colorectal normal and tumor samples of Apc $^{Min\ -/+}$ mouse intestine.

Relative amount
of GLI1 mRNA
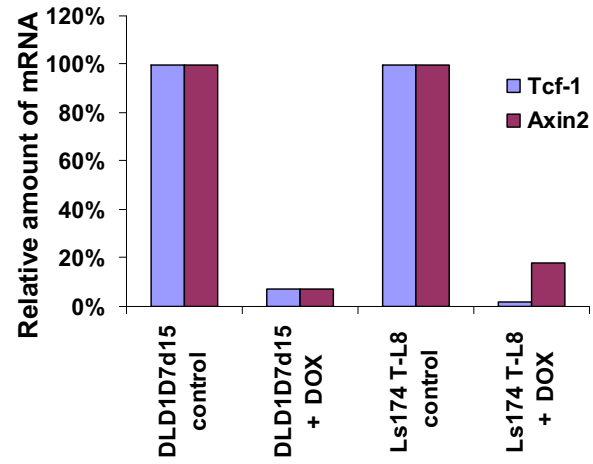
β-actin
β-catenin
CRD-BP

1 irrelevant shRNA

2 β-catenin/Tcf4 + irrelevant shRNA

3 β-catenin/Tcf4 + CRD-BP shRNA-3

4 β-catenin/Tcf4 + CRD-BP shRNA-3 +mCRD-BP

5 β-catenin/Tcf4 + CRD-BP shRNA-3 +hCRD-BP

6 β-catenin/Tcf4 + Irrelevant shRNA +mCRD-BP

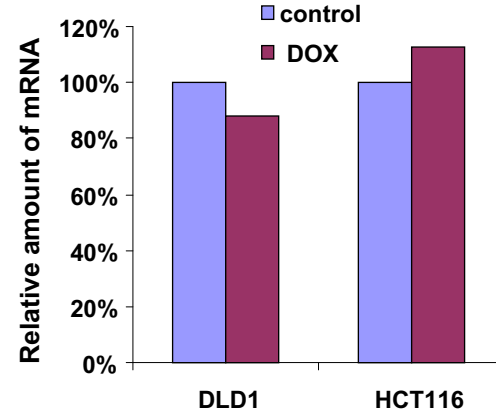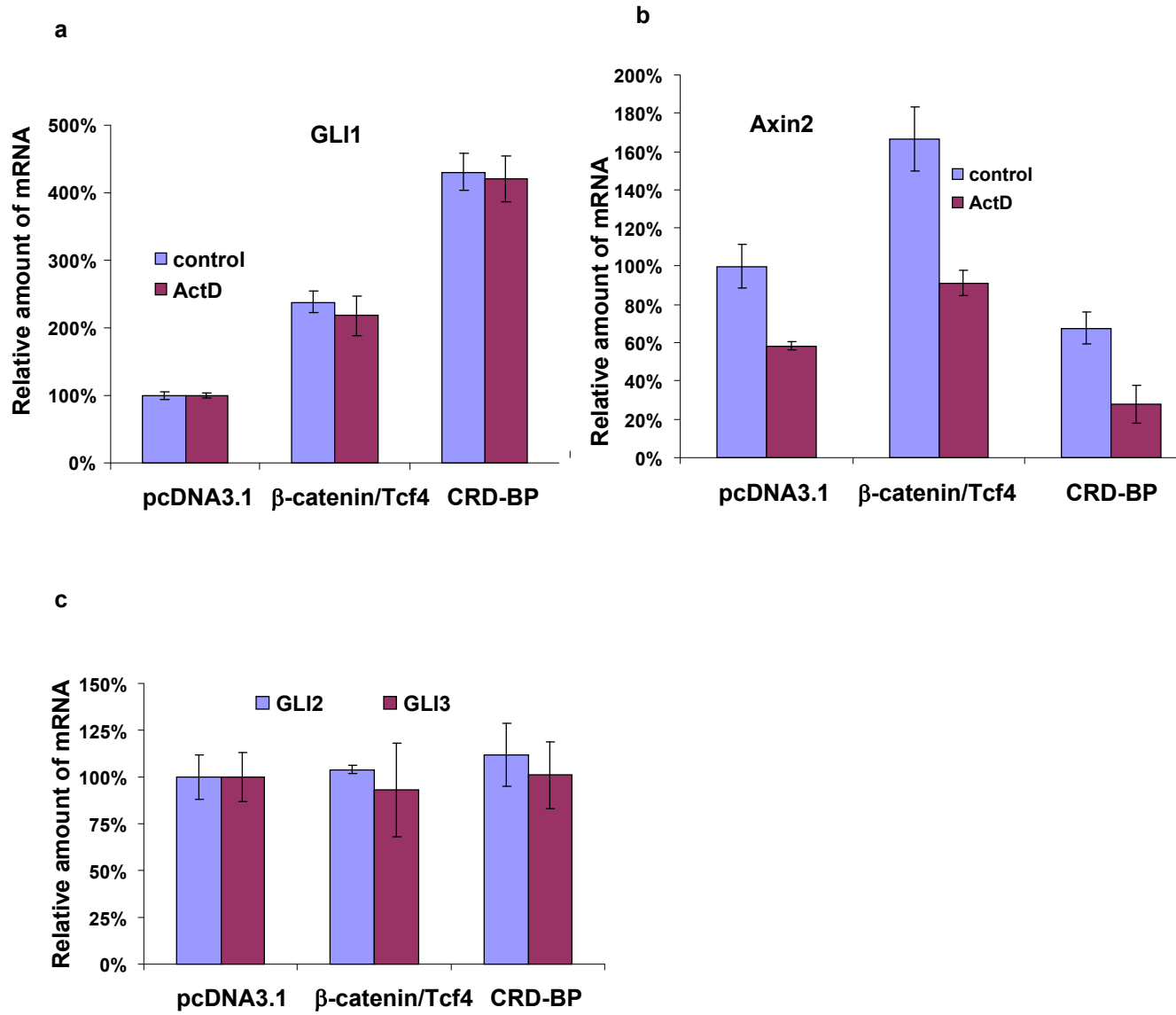7 β-catenin/Tcf4 + Irrelevant shRNA +hCRD-BP
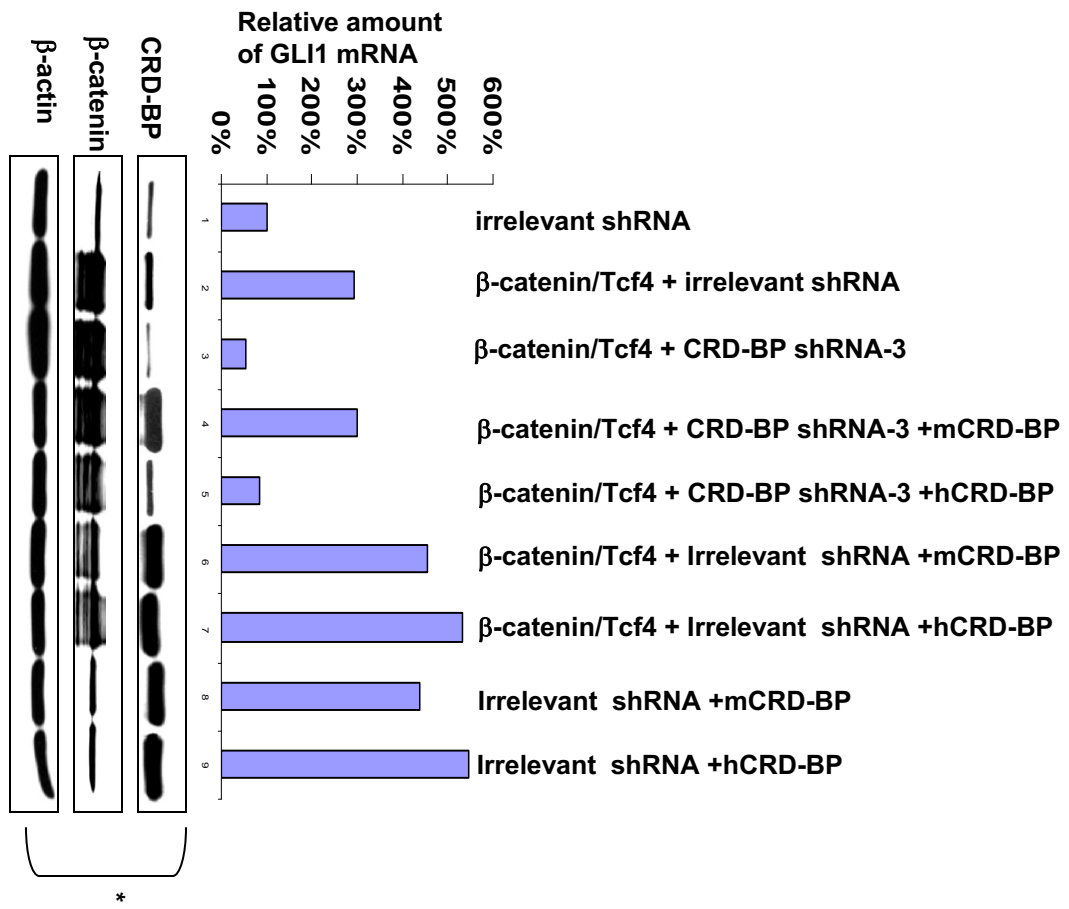
8 Irrelevant shRNA +mCRD-BP

9 Irrelevant shRNA +hCRD-BP

Noubissi_Supplemental Figure 3

Noubissi_Supplemental Figure 4

a

Relative amount of mRNA

250%
200%
150%
100%
50%
0%

Control   DOX   Gli1   DOX + Gli1

Cyclin D1 — DLD1 D7Δ15
LS174T-L8
c-myc — DLD1 D7Δ15
LS174T-L8

b

Relative amount of mRNA

250%
200%
150%
100%
50%
0%

Irrelevant shRNA   CRD-BP shRNA   Irrelevant shRNA + Gli1   CRD-BP shRNA + Gli1

Cyclin D1 — DLD1 D7D15
LS174T-L8
c-myc — DLD1 D7D15
LS174T-L8
CRD-BP — DLD1 D7D15
LS174T-L8

Supplemental Table 1: Sequences of primers used for PCR amplification of GLI1 cDNA fragments

Primers	Forward	Reverse
fragment 1	5'<u>ATTTAGGTGACACTATAGAA</u>CCCAGACAGAGTGTCCCC 3'	5' CGTGCACTTGTGTGGCTT 3'
fragment 2	5'<u>ATTTAGGTGACACTATAGAA</u>TCCCTGCCTGGCCTTATG 3'	5'ATCAAAGTCCAGGCAAGGC 3'
fragment 3	5'<u>ATTTAGGTGACACTATAGAA</u> TCCCTGCCTGGCCTTATG 3'	5'ATCAAAGTCCAGGCAAGGC 3'
fragment 4	5'<u>ATTTAGGTGACACTATAGAA</u>CCTTGCCTGGACTTTGATTC3'	5'CTGATGCAGTTCCTTTATTATCAGG3'

The underlined sequences represent SP6 promoter sequences.

Supplemental Table 2: Sequences of primers used for quantitative real-time RT-PCR

Primers	Forward	Reverse
gli1 (Zebrafish)	5'CAAGGTGGCACAAGAAGACA3'	5'TAGGTGGTCGAGGCGAATAC3'
GLI1 (human)	5'GTGCAAGTCAAGCCAGAACA3'	5'ATAGGGGCCTGACTGGAGAT3'
Gli1 (mouse)	5'ATGAAGCTAGGGGTCCAGGT3'	5'AGAAGGGAACTCACCCCAGT3'
GLI2	5'CTACAGTCCGCAAGGCCTAC3'	5'TCAGGTGCCACTTCTGTCTG3'
GLI3	5'GAGCACTTGATGCTCCACAA3'	5'GGGTGCTCTTCAGCTTTGAG3'
Cyclin D1	5'CCGTCCATGCGGAAGATC3'	5'GAAGACCTCCTCCTCGCACT3'
c-myc	5'TACCCTCTCAACGACAGCAG3'	5'TCTTGACATTCTCCTCGGTG3'
Tcf1 (TCF7)	5'TGCAGCTATACCCAGGCTGG3'	5'CCTCGACCGCCTCTTCTTC3'
Axin2	5'TCACCAAACCCATGTCTGTC3'	5'TCCAGGAAAGTTCGGAACAG3'
CRD-BP (human)	5'CTGAAGATCCTGGCCCATAA3'	5'AAGGTCTTGCAACGAGGAGA3'
CRD-BP(mouse)	5'GGCTGCTCCCTATAGCTCCT3'	5'CTGGTGGTGCAATCTTGATG3'
GAPDH (human)	5'ATGGTTGCCACTGGGGATCT3'	5'TGCCAAAGCCTAGGGGAAGA3'
GAPDH (mouse)	5'AATGTGTCCGTCGTGGATCT3'	5'CCCTGTTGCTGTAGCCGTAT3'