

## Blocking Wnt Secretion Reduces Growth of Hepatocellular Carcinoma Cell Lines Mostly Independent of $\beta$ -Catenin Signaling



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### Abstract

Aberrant activation of Wnt/ $\beta$ -catenin signaling plays a key role in the onset and development of hepatocellular carcinomas (HCC), with about half of them acquiring mutations in either *CTNNB1* or *AXIN1*. However, it remains unclear whether these mutations impose sufficient  $\beta$ -catenin signaling or require upstream Wnt ligand activation for sustaining optimal growth, as previously suggested for colorectal cancers. Using a panel of nine HCC cell lines, we show that siRNA-mediated knockdown of  $\beta$ -catenin impairs growth of all these lines. Blocking Wnt secretion, by either treatment with the IWP12 porcupine inhibitor or knockdown of WLS, reduces growth of most of the lines. Unexpectedly, interfering with Wnt secretion does not clearly affect the level of  $\beta$ -catenin signaling in the majority of lines, suggesting that other mechanisms underlie the growth-suppressive effect. However, IWP12 treatment did not induce autophagy or endoplasmic reticulum (ER) stress, which may have resulted from the accumulation of Wnt ligands within the ER. Similar results were observed for colorectal cancer cell lines used for comparison in various assays. These results suggest that most colorectal and liver cancers with mutations in components of the  $\beta$ -catenin degradation complex do not strongly rely on extracellular Wnt ligand exposure to support optimal growth. In addition, our results also suggest that blocking Wnt secretion may aid in tumor suppression through alternative routes currently unappreciated.

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### Introduction

Hepatocellular carcinoma (HCC) is considered as the fifth most common cancer and the third main reason for cancer-related death with 748,000 cases and 695,000 deaths each year [1,2]. The etiology of HCC includes hepatitis B virus or hepatitis C virus infection, alcohol liver disease, nonalcoholic steatohepatitis, and aflatoxin-B1 exposure [3]. More than 80% of all HCCs occur in Eastern and Southeastern Asia where the main cause is hepatitis B virus combined with exposure to aflatoxin-B1 [4]. In Europe, Japan, and the United States, hepatitis C virus represents the dominant risk factor, together with alcohol abuse and nonalcoholic fatty liver diseases [5,6].

Wnt/ $\beta$ -catenin signaling plays an important role in a wide range of biological processes, involving embryonic patterning, cell proliferation, differentiation, angiogenesis, carcinogenesis, metastasis, and drug resistance [7–9]. Underscoring the relevance of this pathway, many tumor types including HCC exhibit enhanced Wnt/ $\beta$ -catenin signaling that strongly contributes to tumor growth [10]. Activation of Wnt/ $\beta$ -catenin signaling starts with the secretion of Wnt ligands. Wnts produced within the endoplasmic reticulum (ER) are

palmitoylated by the Wnt acyl-transferase porcupine (PORCN), which is essential for their secretion and signaling activity. Following this lipid modification, Wntless (WLS) is needed to shuttle the Wnt proteins from the Golgi to the plasma membrane where they can signal in an autocrine or paracrine manner [11]. In the absence of upstream Wnt signaling,  $\beta$ -catenin is phosphorylated at N-terminal Ser/Thr residues by a multiprotein complex consisting of the

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adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1 $\alpha$ . Phosphorylated  $\beta$ -catenin is then ubiquitinated, leading to its proteasomal degradation [12–14]. The overall effect is low  $\beta$ -catenin levels in the cytoplasm and nucleus of unstimulated cells.

Upon binding of Wnt ligands to Frizzled and LRP5/6 co-receptors, Disheveled (DVL) becomes phosphorylated, subsequently resulting in the inhibition of the  $\beta$ -catenin destruction complex [15]. As a result,  $\beta$ -catenin is stabilized and is able to translocate to the nucleus and associate with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt/ $\beta$ -catenin target genes and thereby affecting cellular decisions [4]. In addition to this classical (canonical) Wnt signaling pathway, Wnts can also signal in an alternative (noncanonical) fashion independent of  $\beta$ -catenin through associating with Frizzled and ROR1/2 receptors instead of LRP5/6. Activation of noncanonical Wnt signaling mainly affects cellular processes involved in migration and cellular polarity [8].

Hepatocarcinogenesis is a multistep process, progressing from a normal hepatocyte to a transformed phenotype as a result of the accumulation of aberrant genetic and epigenetic modifications and activation of various signaling pathways [16–18]. Increasing evidence indicates that activation of Wnt/ $\beta$ -catenin signaling is critical in hepatic oncogenesis [19,20]. About 40% to 70% of HCCs are characterized by nuclear accumulation of  $\beta$ -catenin, the hallmark of active signaling. Various molecular and genetic alterations contribute to aberrant activation of Wnt/ $\beta$ -catenin signaling. Mutations within components of the canonical Wnt/ $\beta$ -catenin signaling enhance stabilization of  $\beta$ -catenin and transcriptional activity in the nucleus. Approximately one third of all HCCs carry oncogenic  $\beta$ -catenin mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation [21]. In another subset of tumors, loss-of-function mutations of negative regulators are observed in the *APC* and *AXIN1* genes, respectively in 1% to 3% and 8% to 15% of tumors [19], both causing compromised ability to degrade  $\beta$ -catenin [8]. In addition to mutations, various other mechanisms have been suggested to promote  $\beta$ -catenin signaling, including overexpression of Wnt ligands and/or their corresponding receptors, and reduced expression of extracellular inhibitors [22]. Given the importance of  $\beta$ -catenin signaling for hepatic oncogenesis, various treatments targeting this route have been evaluated [23].

Cancers harboring mutations within intracellular components of the  $\beta$ -catenin signaling pathway, i.e., mutation of APC, AXIN or  $\beta$ -catenin itself, were often considered to become largely independent of upstream regulation by extracellular Wnt ligands. This belief has however been challenged during the last years. For example, Wnt antagonists SFRPs and DKKs are reported to attenuate Wnt signaling in colorectal cancer (CRC) [24,25]. Recently, it was demonstrated that interfering with Wnt secretion or reducing the expression of specific Wnt ligands impaired the growth of APC and  $\beta$ -catenin mutant CRC cell lines [26]. These results also indicated that interfering with Wnt secretion, for example, using the newly developed PORCN inhibitors [27,28], could be useful as an additive treatment option for tumors characterized by enhanced  $\beta$ -catenin signaling. Here, we have investigated whether this also holds truth for  $\beta$ -catenin or AXIN1 mutant liver cancer cells.

## Materials and Methods

### Cell Lines

Human HCC cell lines Hep3B, Huh6, Huh7, PLC/PRF/5, SNU182, SNU398, and SNU449, and CRC cell lines CACO2,

DLD1, HT29, SW480, HCT116, LS174T, SW48, and RKO were cultured in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Breda, the Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, UT), 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (Invitrogen-Gibco). The hepatoblastoma cell line HepG2 was cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in Williams E medium (Invitrogen-Gibco, Breda, the Netherlands) complemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine. HepaRG was cultured in William's E medium supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 5  $\mu$ g/ml insulin (Sigma-Aldrich, St. Louis, MO), and 50  $\mu$ M hydrocortisone hemisuccinate (Sigma-Aldrich, St. Louis, MO). Identity of all cell lines was confirmed by STR genotyping. *CTNNB1* mutation status was confirmed in all the nine HCC cell lines by Sanger sequencing and was consistent with those reported at the Catalogue of Somatic Mutations in Cancer (<http://cancer.sanger.ac.uk>) [29].

For the preparation of conditioned medium, L-control and L-Wnt3A cells were cultured in complete Dulbecco's modified Eagle's medium, followed by collection and filtration of medium according to standard procedures. HCC and CRC cell lines were stimulated with 25% L-control or L-Wnt3A medium.

### Reagents

IWP12 (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) with a final stock concentration of 10 mM. Antibodies specific for  $\beta$ -catenin (cat. #9561, Cell Signaling Technology), disheveled adaptor protein (DVL2) (cat. #3216, Cell Signaling Technology), WLS (cat. #MABS87, clone YJ5 Millipore), LC3I/II (cat. #4108, Cell Signaling Technology), GPR177(Wls/Evi) (cat. #MABS87, Millipore) and  $\beta$ -actin (sc-47,778, Santa Cruz), Tubulin (sc-8035, Santa Cruz) anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were used for western blot analysis.

### Gene Knockdown by Small Interfering RNA (siRNA)

Smartpool ON-TARGETplus siRNAs targeting *CTNNB1* and WLS were obtained from Dharmacon. The ON-TARGETplus nontargeting siRNA #2 was used as negative control. Cells were reverse-transfected in a 96-well plate using a total of 0.2  $\mu$ l DharmaFECT formulation 4 (Thermo Fischer Scientific) and 25 nM of each siRNA per well. Following 72 hours of incubation, the effect on knockdown was determined by Western blotting.

### Quantitative Real-Time Polymerase Chain Reaction (PCR)

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, the Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC.) and subjected to quantitative real-time PCR analyses. Analyses were performed using the StepOne Real-Time PCR System and the StepOne2.0 software (Applied Biosystem, Darmstadt, Germany). Primer sequences are provided in Supplementary Table 1. All expression levels are depicted relative to the expression of *GAPDH*.

### Western Blot Assay

Cells were lysed in Laemmli sample buffer with 0.1 M DTT and heated for 5 to 10 minutes at 95°C, followed by loading and

separation on a 8% to 15% sodium dodecyl sulfate–polyacrylamide gels. After 90 minutes of running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Invitrogen) for 1.5 hours with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 ml Odyssey Blocking Buffer and 2.5 ml PBS containing 0.05% Tween 20, followed by incubation with primary antibody (all 1:1000) overnight at 4°C. The membrane was washed 3 times with PBS containing 0.05% Tween 20 followed by incubation for 1.5 hours with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE) (1:5000) at room temperature. Blots were assayed for  $\beta$ -actin or Tubulin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE). Results were visualized and quantified with Odyssey 3.0 software.

### $\beta$ -Catenin Reporter Assays

The  $\beta$ -catenin reporter assays were basically performed as previously described [30,31]. In short, 20 hours before transfection, we plated  $10^5$  cells per well on 12-well plates. Each well was transfected with 500 ng Wnt Responsive Element (WRE) or Mutant Responsive Element (MRE) vectors and 20 ng TK-Renilla using polyethylenimine (PEI) (Sigma-Aldrich, St. Louis, MO) or Fugene HD (Promega). We measured luciferase activities in a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany) and normalized the data for the transfection efficiency by using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. Transfections were performed in triplicate, and the mean and standard error were calculated for each condition. The  $\beta$ -catenin reporter activities are shown as WRE/MRE ratios.

### MTT Assay

A total of 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates and incubated at 37°C with 5% CO<sub>2</sub> for 3 hours. The medium was removed, and 100  $\mu$ l DMSO was added to each well. The absorbance of each well was read on a microplate absorbance reader (BIO-RAD) at wavelength of 490 nm. For siRNA-mediated knockdown of genes, four independent wells were assayed for each cell line at least two times, whereas for IWP12 treatment, six independent wells were used. The mean and standard error were calculated for each condition.

### Cell Cycle Analysis

At around 60% to 80% confluency, cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4°C. The cells were washed twice with PBS and incubated with 20  $\mu$ g/ml RNase at 37°C for 30 minutes followed by incubation with 50  $\mu$ g/ml propidium iodide at 4°C for 30 minutes. Then samples were tested immediately by FACS. Cell cycle was analyzed by FlowJo\_V10 software. For each treatment, two independent wells were tested for Huh6, SNU449 and Huh7 two times. The mean and standard error were calculated for each condition.

### Statistical Analysis

All results were presented as mean  $\pm$  SD. Comparisons between groups were performed with one-sample *t* test. Differences were considered significant at a *P* value less than .05.

## Results

### $\beta$ -Catenin Signaling Activity of HCC Cell Lines

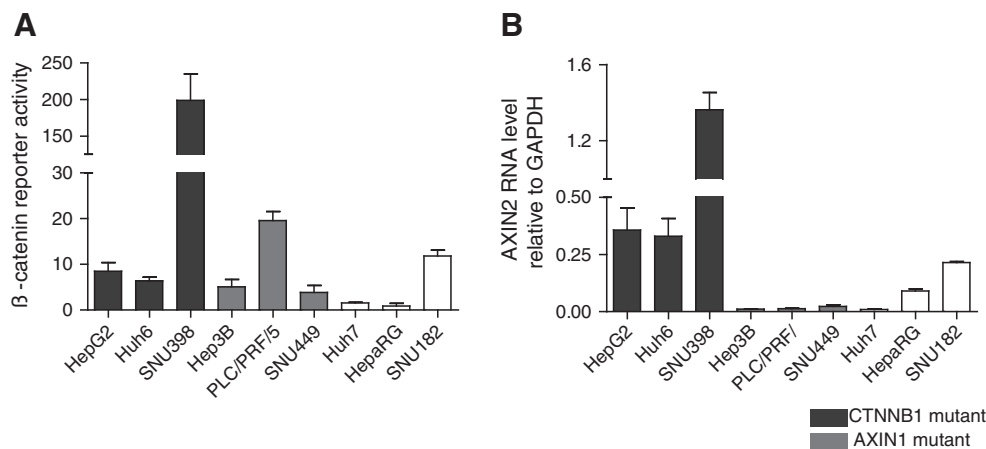
To investigate the importance of  $\beta$ -catenin signaling and Wnt secretion for sustaining cell growth, we employed nine HCC cell lines, listed in Table 1 in which gene mutations related to Wnt/ $\beta$ -catenin signaling are depicted. We also used eight CRC cell lines for comparison in various assays, known to largely depend on  $\beta$ -catenin signaling for their growth. First, we determined the baseline  $\beta$ -catenin signaling activity for all these cell lines using a  $\beta$ -catenin reporter assay and qRT-PCR of *AXIN2*, a well-established  $\beta$ -catenin target gene. As indicated in Figure 1, in line with previous publications, all  $\beta$ -catenin mutant HCC lines (SNU398, HepG2 and Huh6) showed a robust induction of both reporter activity as well as high *AXIN2* expression. The *AXIN1* mutant lines (PLC/PRF/5, Hep3B, and SNU449) also displayed enhanced reporter activity, albeit generally more modest, whereas the expression of *AXIN2* was low. Interestingly, among the HCC lines without an obvious mutation, SNU182 presented with high  $\beta$ -catenin signaling activity, both on reporter level and on *AXIN2* expression. Huh7 and HepaRG showed low reporter activity together with low *AXIN2* expression. All CRC lines, except RKO being wild type for *APC* and *CTNNB1*, showed the expected increase in reporter activity (Figure S1).

### Requirement for $\beta$ -Catenin Signaling to Sustain Efficient Cell Growth

Next, we determined the dependence on  $\beta$ -catenin signaling for supporting efficient growth by transiently transfecting smartpool siRNAs targeting *CTNNB1* or a control siRNA, followed by an MTT assay to test cell numbers after 3 days of culture. For most cell lines, we accomplished more than 80% knockdown of  $\beta$ -catenin at protein level as determined by quantitative Western blot analysis, with the exception of HepaRG in which a 65% reduction was observed (Figure 2A and Supplemental Figure S2). As indicated in Figure 2B,  $\beta$ -catenin signaling activity was clearly suppressed by siRNA-mediated

**Table 1.** Gene Mutations of Wnt/ $\beta$ -Catenin Signaling Components in HCC and CRC Cell Lines

Cell Line	Gene	AA Alteration	Zygosity
<b>HCC</b>			
HepG2	<i>CTNNB1</i>	p. W25_I140 del	Heterozygous
Huh6	<i>CTNNB1</i>	p. G34 V	Heterozygous
SNU398	<i>CTNNB1</i>	p. S37C	Heterozygous
Hep3B	<i>AXIN1</i>	p. R146*	Homozygous
PLC/PRF/5	<i>AXIN1</i>	p.(R373_M418 del)	Homozygous
SNU449	<i>AXIN1</i>	p. R712*	Homozygous
Huh7			
HepaRG			
SNU182			
<b>CRC</b>			
SW480	<i>APC</i>	p. Q1338*	Homozygous
HT29	<i>APC</i>	p. T1556 fs*3 p. E853*	Heterozygous Heterozygous
Caco2	<i>APC</i>	p. Q1367*	Homozygous
DLD1	<i>APC</i>	p. R2166*	Heterozygous
		p. I1417fs*2 p. L396 M	Heterozygous Heterozygous
	<i>AXIN1</i>	p. G659 fs*41	Heterozygous
	<i>RNF43</i>	p. S33Y	Heterozygous
SW48	<i>CTNNB1</i>	p. G659 fs*41 p. V299 fs*143	Heterozygous Heterozygous
	<i>RNF43</i>	p. S45F	Heterozygous
HCT116	<i>CTNNB1</i>	p. S45del p. R117fs*41	Heterozygous Homozygous
	<i>RNF43</i>	p. S45F	Homozygous
LS174T	<i>CTNNB1</i>	p. S45F	Homozygous
RKO	<i>RNF43</i>	p. G659 fs*41	Homozygous



**Figure 1.** Baseline  $\beta$ -catenin signaling activity in HCC cell lines. (A)  $\beta$ -Catenin luciferase reporter assay showing  $\beta$ -catenin signaling activity in HCC cell lines (mean  $\pm$  SD,  $n = 3$ ). The  $\beta$ -catenin reporter activities are depicted as WRE/MRE ratios. (B) qRT-PCR assay showing expression of the  $\beta$ -catenin target gene *AXIN2* in HCC cell lines (mean  $\pm$  SD,  $n = 3$ ). Expression levels are depicted relative to the housekeeping gene *GAPDH*.

knockdown in the three lines tested for this purpose, i.e., Huh6, SNU449 and Huh7. All HCC cell lines were inhibited significantly in their growth (Figure 2C), suggesting that  $\beta$ -catenin signaling is important for the growth of these tumor cells, even in the ones that show only low to modest levels of signaling, such as Huh7 and HepaRG. Cell cycle analysis in three lines showed that  $\beta$ -catenin knockdown provoked a dramatic G0/G1 phase arrest in Huh6 (Figure 2D). Also in Huh7, a notable increase of cells in G0/G1 is observed with a significant reduction of cells in the G2/M phase, whereas for SNU449, a trend is observed toward more cells in G0/G1 and less cells in S-phase. As expected, all five tested CRC cell lines showed a significantly reduced growth upon  $\beta$ -catenin knockdown (Figure 2E).

#### Expression Levels of Wnt Ligands in HCC Cell Lines

Taken together, the results above indicate that targeting the  $\beta$ -catenin signaling pathway represents an attractive route to suppress the growth of HCCs. This pathway has however been refractory to target in an efficient manner. More recently, inhibitors of Wnt secretion have been proposed as treatment options for malignancies dependent on Wnt-ligand secretion for their growth [26–28]. However, a prerequisite is that the tumor cells express sufficient levels of Wnt ligands capable of inducing  $\beta$ -catenin signaling. Hence, we investigated the expression profile of all 19 Wnt ligand genes in our HCC cell line panel by qRT-PCR. From the group of Wnt ligands more commonly associated with inducing  $\beta$ -catenin signaling (*WNT1*, *2*, *3*, *3A*, *8A*, *8B*, *10A*, and *10B*), only *WNT3* was clearly expressed in all HCC cell lines, followed by high *WNT10A* expression restricted to the SNU182 and HepaRG cell lines and high *WNT2* expression in SNU449 (Figure 3A). The remaining ligands of this group are barely detectable or expressed at least at 10-fold lower levels in all cell lines (Figure S3). When piling up the expression of all Wnt ligands from this group (Figure 3B), the SNU182 cell line clearly stands out as one with overall highest expression level, which may explain its high  $\beta$ -catenin signaling activity reported above. The  $\beta$ -catenin and AXIN1 mutant cell lines are among the low-to-intermediate ones.

Among the group of Wnt ligands more commonly associated with activating alternative pathways (*WNT4*, *5A*, *5B*, *6*, *7A*, *7B*, and *11*)

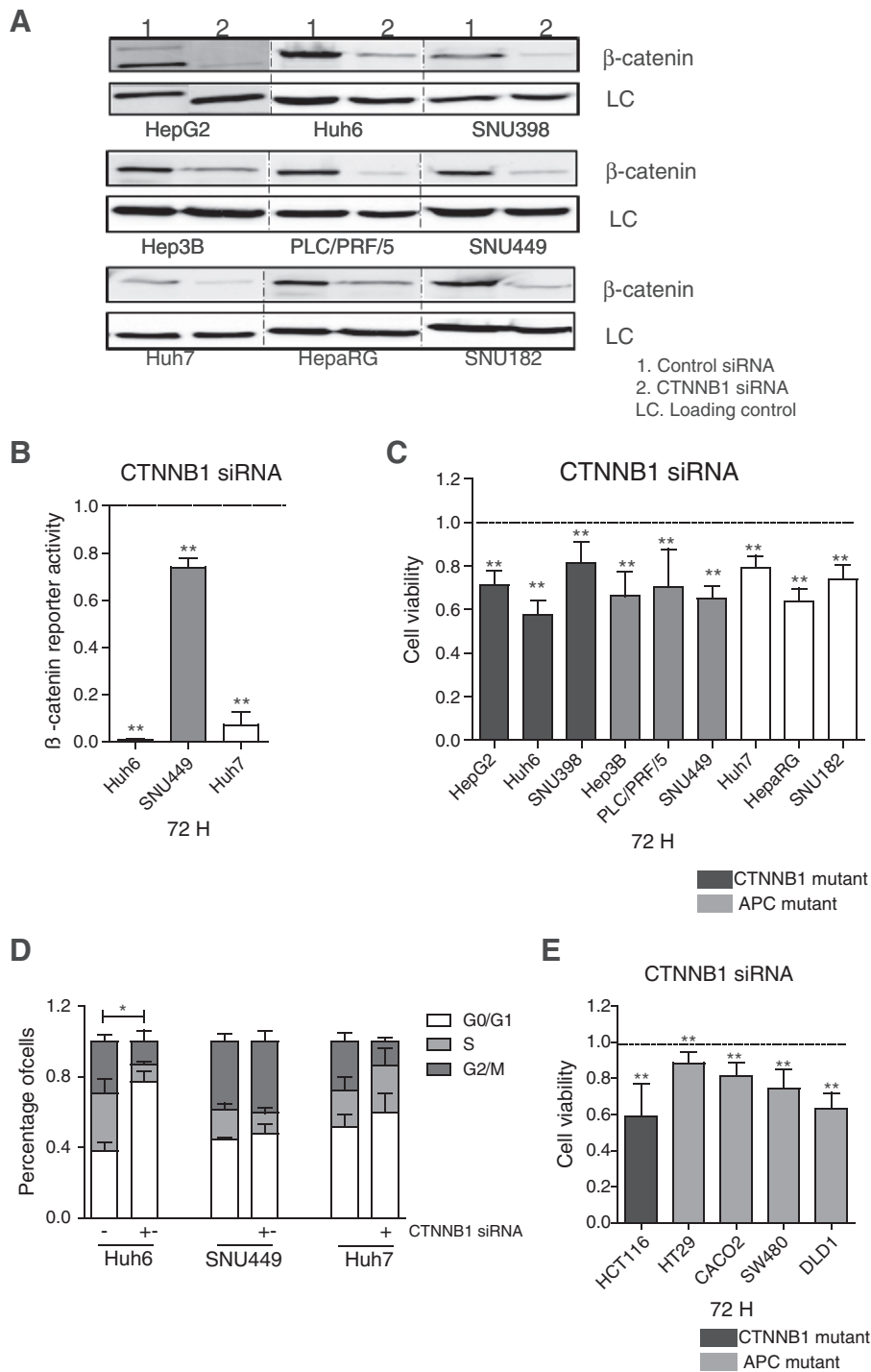
depicted in Figure S4A, *WNT5A* was most prominently expressed, being very high in HepaRG and SNU182 and readily detectable in SNU398 and SNU449. The latter cell line also shows high expression of *WNT5B* and *WNT7B*. Other Wnt ligands of this group are detectable only at low levels or absent within most cell lines. Overall, SNU182 is again the most prominent expressing cell line, whereas the  $\beta$ -catenin and AXIN1 mutant cell lines are among the low-to-intermediate expressers (Figure 3B). Of the remaining Wnt ligands (*WNT2B*, *9A*, *9B*, and *16*), *WNT2B* was highly expressed in PLC/PRF/5, SNU182, SNU398, and SNU449, whereas *WNT9B* was clearly expressed in Hep3B (Supplemental Figure S4B).

Combining the expression of all Wnt ligands shows that SNU182 has again the highest overall levels, followed by HepaRG and SNU449 (Figure 3B, right panel). As all Wnt ligands trigger the phosphorylation of DVL2 upon binding of either the FZD\_LRP5/6 or FZD\_ROR1/2 receptor complexes [32,33], we determined baseline pDVL2 levels in all cell lines. As shown in Figure 3C, the highest levels of phosphorylated DVL2 are observed in the three cell lines with the highest Wnt levels, whereas for the remaining six lines, no clear correlation can be observed.

In summary, although all HCC cell lines show a large variation in Wnt ligand expression, they all express Wnt ligands that can contribute to  $\beta$ -catenin signaling.

#### Wnt Secretion Blockage Reduces Growth of HCC Cells

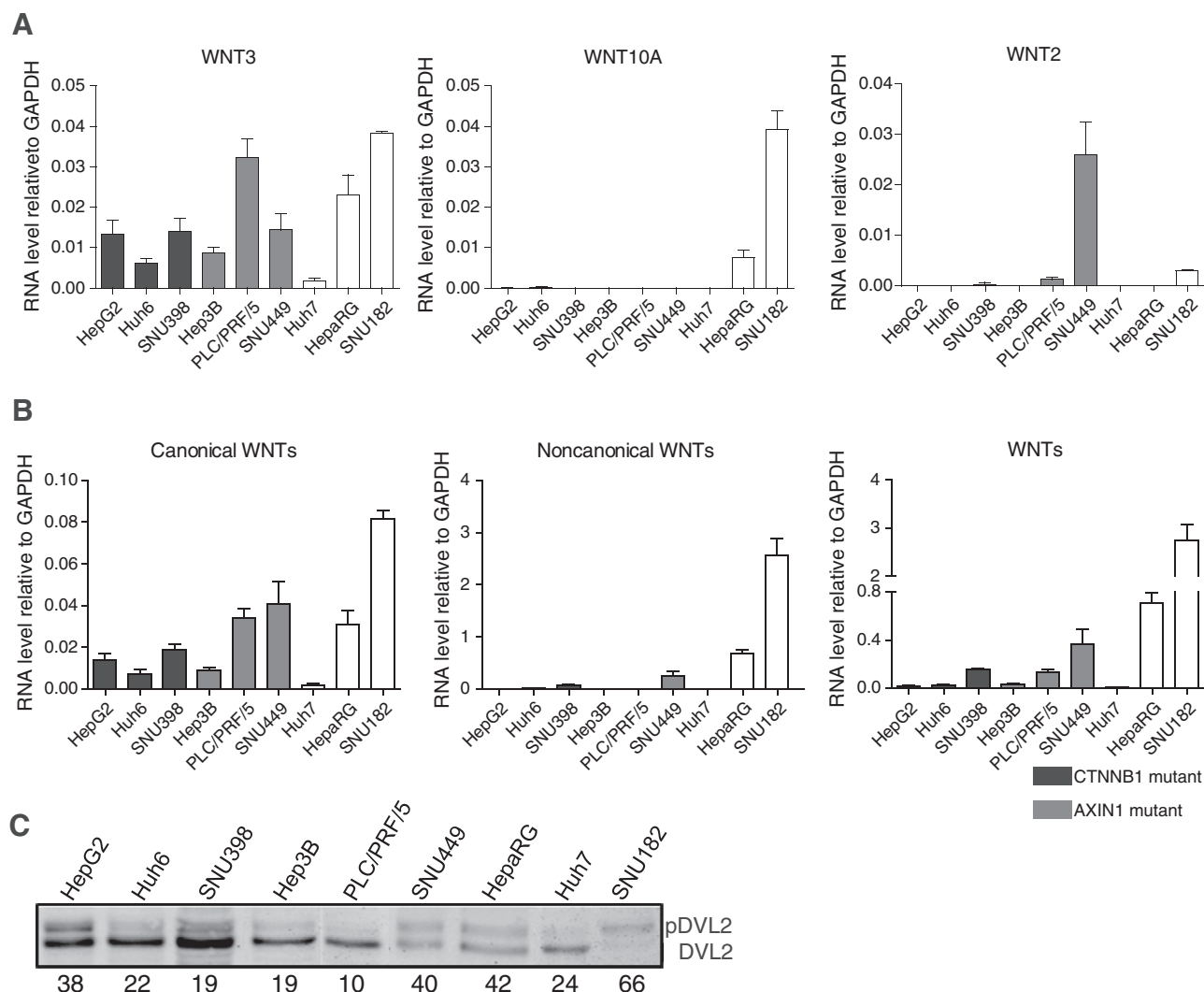
Given that most HCC cell lines show increased  $\beta$ -catenin signaling activity and expression of canonical Wnt ligands, we wished to investigate the consequences of suppressing the secretion of Wnt ligands. To this aim, we used two methods, i.e., treatment with IWP12, an effective inhibitor of PORCN required for palmitoylation of Wnt proteins [34], and knockdown of *WLS*, which shuttles the palmitoylated Wnts from the Golgi to the plasma membrane. Both treatments are expected to reduce overall levels of secreted Wnt ligands. Following 3 days of IWP12 treatment, reduced cell numbers were observed for all  $\beta$ -catenin mutant cell lines (Figure 4A), ranging from 10% reduction (Huh6) to 35% (SNU398). Among the nonmutant lines, growth of HepaRG was strongly suppressed by IWP12, whereas Huh7 and SNU182 showed more modest



**Figure 2.** Requirement of  $\beta$ -catenin signaling for sustaining HCC cell growth. (A) HCC cell lines were transiently transfected with *CTNNB1* siRNAs for 72 hours. Cell lysates were collected for Western blotting with indicated antibodies. Tubulin and  $\beta$ -actin served as loading control (LC). (B) Silencing of  $\beta$ -catenin caused the reduction of  $\beta$ -catenin signaling activity (mean  $\pm$  SD,  $n = 2$ , two times). (C) Silencing of  $\beta$ -catenin inhibited the growth of HCC cell lines as determined by MTT assay (mean  $\pm$  SD,  $n = 4$ , two times). (D) Silencing of  $\beta$ -catenin alters cell cycle progression in Huh6 (all phases significantly changed) and Huh7 cells (G2/M phase significantly reduced). SNU449 is less clearly altered, although the proportion of cells in S-phase is reduced (mean  $\pm$  SD,  $n = 2$ , two times). (E)  $\beta$ -catenin knockdown reduced cell growth in colorectal cancer cell lines. Values depicted are relative to the ones obtained with the nontargeting siRNA that are arbitrarily set to 1. \* $P < .05$ ; \*\* $P < .01$ .

reductions of their growth. The AXIN1 mutant lines were not clearly affected by IWP12 with the exception of PLC/PRF/5. Effects on growth following knockdown of *WLS* were largely in line with IWP12 treatment, with the exception of the AXIN1 mutant lines

Hep3B and SNU449 that were significantly suppressed by *WLS* knockdown and a less impressive growth reduction of HepaRG when compared with IWP12 (Figure 4B). Examples of efficient *WLS* knockdown are shown in Supplemental Figure S5.



**Figure 3.** Expression levels of Wnt family members in HCC cell lines determined by qRT-PCR. (A) Expression levels of the top three expressed “canonical” Wnt ligands (mean  $\pm$  SD,  $n = 3$ ). (B) Piled-up expression levels of canonical, noncanonical, and overall Wnt ligands, respectively. All expression levels are depicted relative to the housekeeping gene *GAPDH*. (C) Baseline levels of phosphorylated DVL2 (pDVL2) in all HCC cell lines. Values below the image represent percentage of total DVL2 that is in the phosphorylated form (upper band).

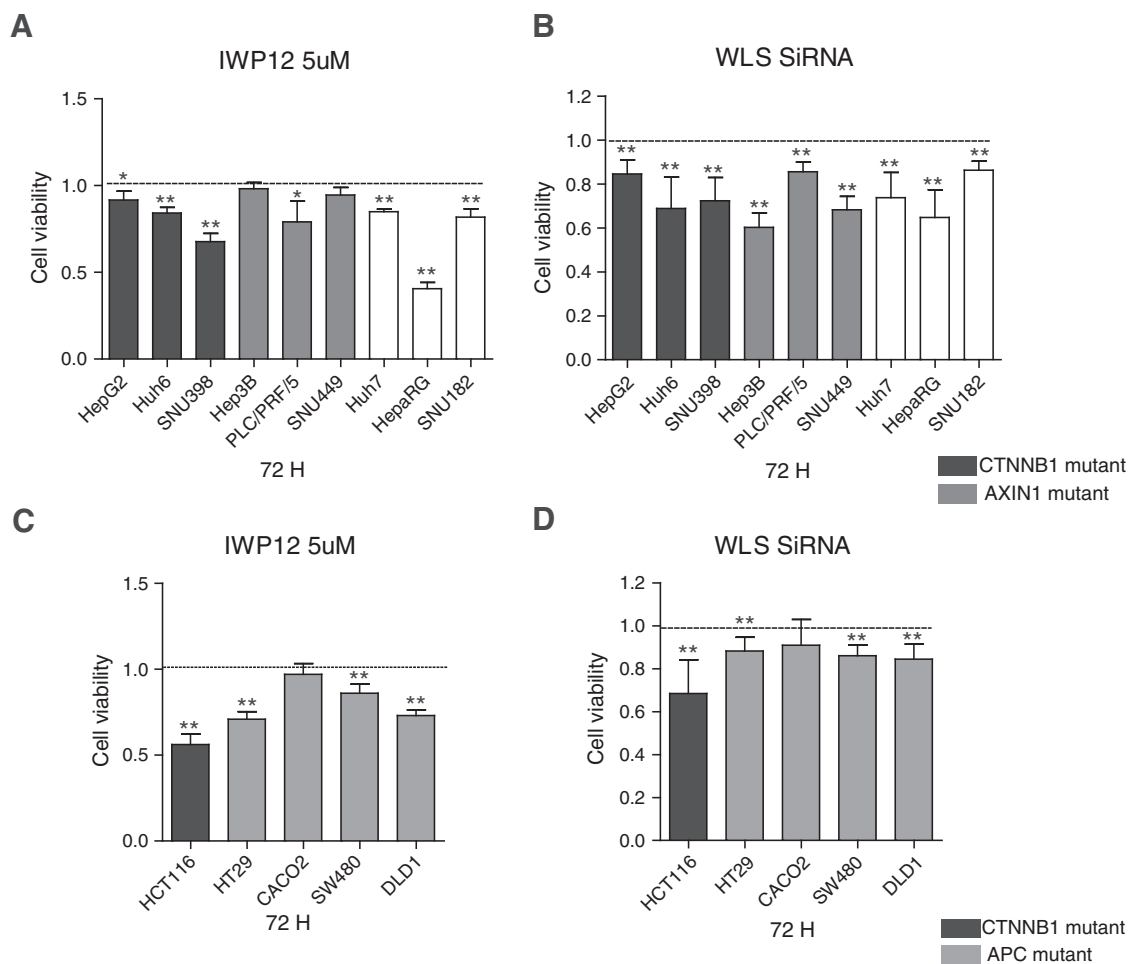
For comparison, the same assays were also performed on five CRC cell lines (Figure 4, C and D). Both treatments showed the strongest growth suppression when applied to the  $\beta$ -catenin and RNF43 mutant HCT116 cell line. Intermediate effects were observed in DLD1, HT29, and SW480, whereas CACO2 was barely affected. In conclusion, most HCC and CRC cell lines are suppressed in their growth by both IWP12 treatment as well as *WLS* knockdown.

#### Altered Exposure to Extracellular Wnt Ligands Does Not Affect $\beta$ -Catenin Signaling Activity in Most HCC Cell Lines

Previously, it was reported that Wnt secretion is required to maintain sufficiently high levels of canonical Wnt/ $\beta$ -catenin signaling activity in both APC and  $\beta$ -catenin mutant CRC cell lines [26]. Here, we asked whether the  $\beta$ -catenin and AXIN1 mutant HCC cell lines were also dependent on Wnt secretion to sustain this pathway activity. Exposure to extracellular Wnt ligands was again reduced by treating all cell lines with IWP12, after which we measured  $\beta$ -catenin signaling activity using the reporter assay and *AXIN2* qRT-PCR.

After 48 hours,  $\beta$ -catenin reporter activity was clearly suppressed only in SNU182 (Figure 5A), whereas the remaining eight HCC lines were not or only modestly inhibited in their reporter activity. Reduction of *AXIN2* expression confirmed the strong repressing effect of IWP12 in the SNU182 cell line, while no reduction was observed in the other cell lines (Figure 5B). Overall, this analysis shows that IWP12 treatment barely affects  $\beta$ -catenin signaling activity in most HCC cell lines, with the exception of SNU182.

In a true tumor setting, in addition to autocrine signaling, HCC cells are also exposed to Wnt ligands coming from the tumor microenvironment. Therefore, to determine the effects on  $\beta$ -catenin signaling of increased levels of extracellular Wnt ligands, we exposed them to L-Wnt3A conditioned medium. As shown in Figure 5C,  $\beta$ -catenin reporter activity was strongly enhanced in the Huh7 cell line and clearly activated in SNU182, both of which were confirmed by qRT-PCR for *AXIN2* (Figure 5D). Importantly, none of the  $\beta$ -catenin or AXIN1 mutant HCC lines showed enhanced  $\beta$ -catenin signaling following the addition of L-Wnt3A conditioned medium.



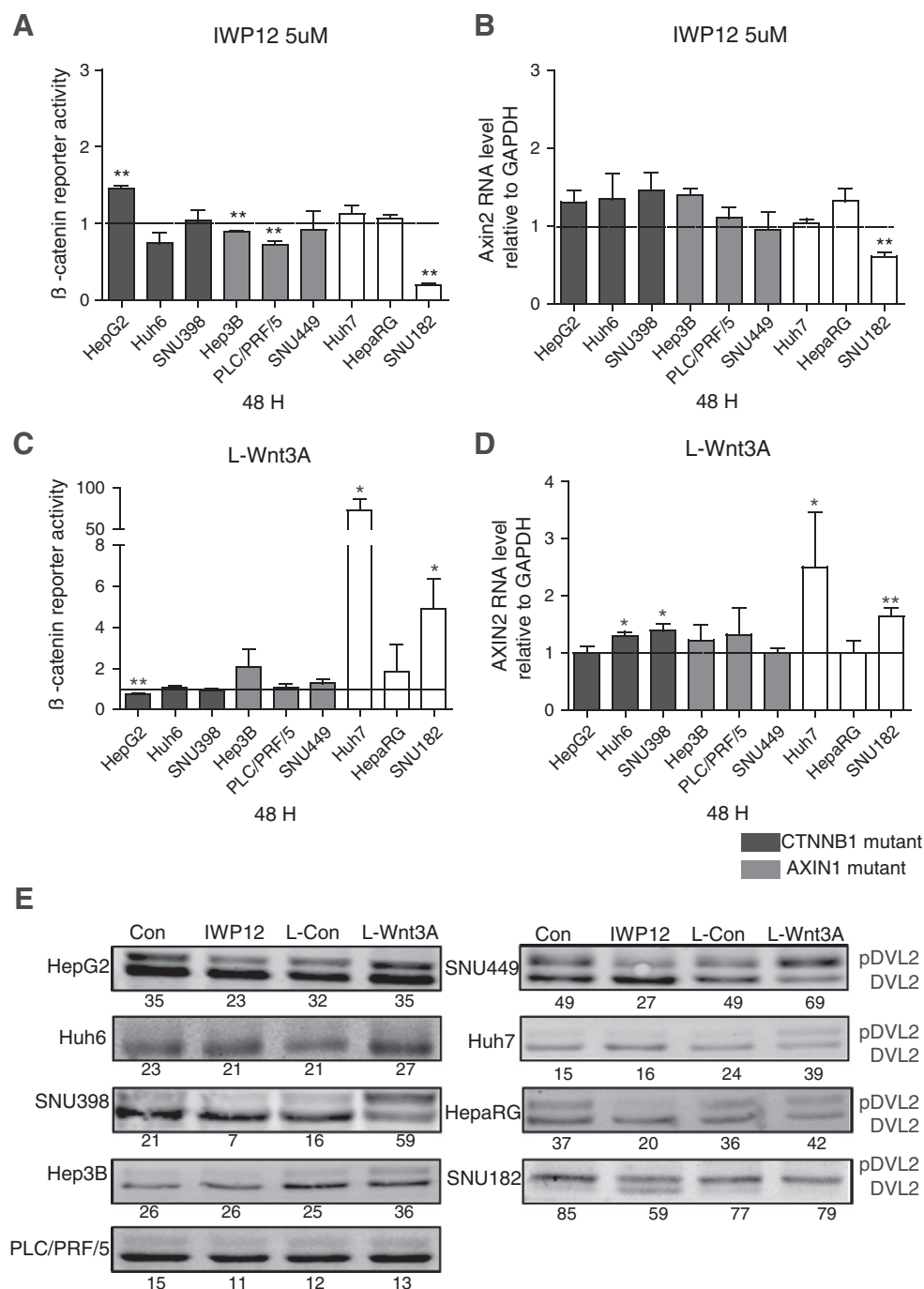
**Figure 4.** Inhibition of growth by Wnt secretion blockage. (A, C) IWP12 reduced cell growth of most HCC and CRC cell lines. Cell lines were incubated with 5  $\mu$ M IWP12 for 72 hours and tested by MTT assay (mean  $\pm$  SD,  $n = 6$ , two times). Values depicted are relative to cell numbers obtained with the control DMSO treatment that are set to 1. (B, D) Cell lines were transiently transfected with *WLS* or nontargeting siRNAs for 72 hours, followed by MTT assay (mean  $\pm$  SD,  $n = 4$ , two times). Values depicted are relative to cell numbers obtained with the nontargeting siRNA that are set to 1. \* $P < .05$ ; \*\* $P < .01$ .

These results indicate that  $\beta$ -catenin and AXIN1 mutant HCC cell lines appear largely insensitive to the level of Wnt ligand exposure for sustaining intracellular  $\beta$ -catenin signaling, which could either mean that the expressed mutant  $\beta$ -catenin or AXIN1 protein determines overall signaling levels in a dominant fashion or, alternatively, that these cells have defects in their machinery to transduce Wnt signals. To test the latter option, we determined pDVL2 levels following treatment with IWP12 or L-Wnt3A conditioned medium (Figure 5E). Phosphorylation levels were not changed in Huh6, and only a modest reduction was observed in HepG2 following IWP12 treatment. In contrast, the SNU398 cell line showed a robust response in pDVL2 levels, decreasing from 21% to 7% by IWP12 treatment and upregulating to 59% following Wnt3A treatment. Thus, both options may hold true depending on the specific cell line under investigation. High variability in pDVL2 response was also observed in the remaining AXIN1-mutant and nonmutant HCC cell lines. The PLC/PRF/5 cell line showed a low baseline pDVL2 level, which was altered neither by IWP12 nor Wnt3A. The Huh7 and Hep3B cell lines also showed low baseline levels, which can however clearly be increased by the addition of Wnt3A. On the other hand, most DVL2 was phosphorylated at baseline in SNU182, which can be inhibited by IWP12, but can hardly be further stimulated by the addition of Wnt3A. Lastly, SNU449 and

HepaRG showed intermediate pDVL2 levels at baseline that can be both reduced and activated by the respective treatments. Thus, all HCC cell lines show a large variation in both their baseline levels of pDVL2 as well as responsiveness to Wnt ligand exposure irrespective of their mutation status.

#### Response of CRC Cell Lines to Alterations in Wnt Ligand Levels

Among the eight CRC cell lines treated with IWP12, only HCT116 showed a significant reduction in  $\beta$ -catenin reporter activity and *AXIN2* expression (Figure 6, A and B). *AXIN2* expression was slightly reduced in HT29 and SW480, whereas CACO2 showed a reduced reporter activity only. Interestingly, HCT116 was also the only cell line in which both reporter activity and *AXIN2* expression could be significantly stimulated by the addition of extracellular Wnt3A (Figure 6C and D). Analysis of pDVL2 levels in a selection of 5 CRC lines showed that overall baseline levels were low with the exception of HCT116 in which 60% of DVL2 is phosphorylated (Figure 6E). IWP12 treatment shows the expected decrease in pDVL2 in HCT116, whereas none of the other cell lines showed clear alterations in pDVL2 levels following treatment with either IWP12 or Wnt3A.



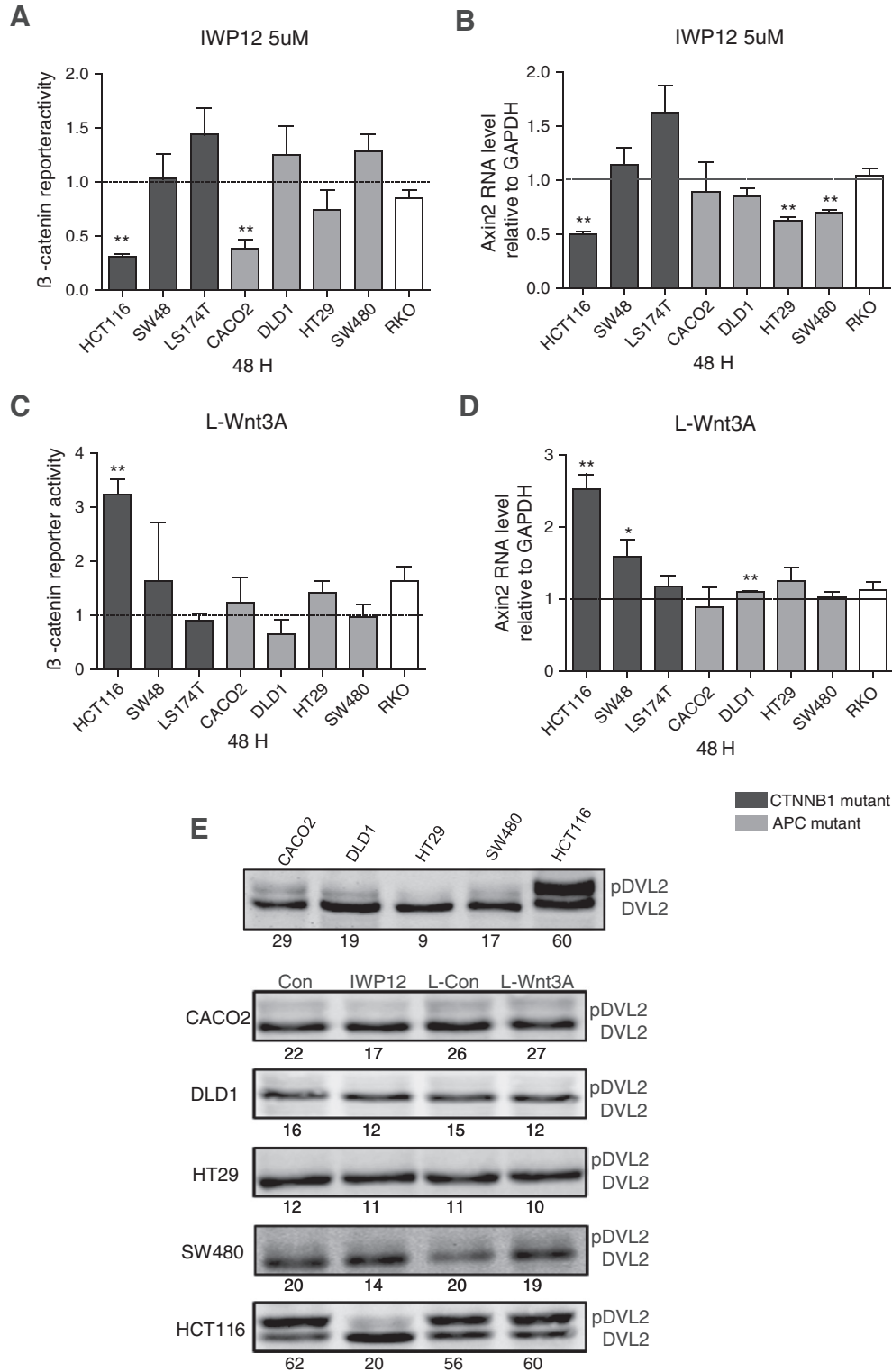
**Figure 5.** Growth reduction due to decreased Wnt secretion appears independent from  $\beta$ -catenin signaling. (A)  $\beta$ -catenin reporter activity was not clearly reduced by IWP12 in most HCC cell lines, except for SNU182 (mean  $\pm$  SD,  $n = 3$ ). (B) QRT-PCR for *AXIN2* showed that its expression following IWP12 treatment was only reduced significantly in the SNU182 cell line (mean  $\pm$  SD,  $n = 3$ , two times). (C) L-Wnt3A conditioned medium significantly promoted  $\beta$ -catenin signaling activity in Huh7 and SNU182 cell lines. A significant albeit modest reduction of reporter activity was observed in HepG2 (mean  $\pm$  SD,  $n = 3$ ). (D) Increased  $\beta$ -catenin signaling in SNU182 and Huh7 following L-Wnt3A treatment was confirmed by *AXIN2* qRT-PCR (mean  $\pm$  SD,  $n = 3$ , two times). All qRT-PCR and reporter values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 1. \* $P < .05$ ; \*\* $P < .01$ . (E) Phosphorylation level of DVL2 protein following treatment with IWP12 or L-Wnt3A conditioned medium ("Con" is DMSO only, "L-Con" is L-Control conditioned medium). Values below the images represent percentage of total DVL2 that is in the phosphorylated form (upper band).

### Blocking Wnt Secretion Does Not Lead to Increased ER Stress

Blocking Wnt secretion using IWP12 or *WLS* knockdown reduces growth of HCC cell lines, apparently largely independent of  $\beta$ -catenin signaling. These treatments however also predict the accumulation of Wnt ligands in the ER, which may lead to activation of an ER stress

response thereby reducing proliferation or inducing apoptosis. Therefore, ER stress was evaluated after IWP12 treatment in HCC cell lines using the expression of the ER-stress induced genes *CHOP* and *GRP94* as a readout. As shown in Figure 7, A and B, expression of *CHOP* was clearly increased in SNU398 (4.5-fold), whereas





**Figure 6.** Responsiveness of CRC cell lines to alterations in Wnt ligand levels. (A) β-catenin reporter activity was significantly reduced by IWP12 in CACO2 and HCT116 cell lines (mean ± SD,  $n = 3$ ). (B) QRT-PCR for *AXIN2* showed that IWP12 treatment reduced its expression in HCT116. Modest but significant reductions were observed in HT29 and SW480 cell lines (mean ± SD,  $n = 3$ , two times). (C) L-Wnt3A conditioned medium only increased β-catenin reporter activity significantly in HCT116 (mean ± SD,  $n = 3$ ). (D) *AXIN2* qRT-PCR confirmed upregulation of β-catenin signaling due to L-Wnt3A in HCT116. Significant but modest increases in *AXIN2* expression are seen in SW48 and DLD1 (mean ± SD,  $n = 3$ ). Reporter values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 1. \* $P < .05$ ; \*\* $P < .01$ . (E) Top image shows comparison of baseline pDVL2 levels within a selection of five CRC cell lines. Bottom images show pDVL2 levels following treatment with IWP12 or L-Wnt3A conditioned medium ("Con" is DMSO only, "L-Con" is L-Control conditioned medium). Values below the images represent percentage of total DVL2 that is in the phosphorylated form (upper band).

*GRP94* expression was elevated in SNU182 (2.8-fold). However, none of the other HCC cell lines displayed strong signs of induction. Overall, these results suggest that blocking Wnt secretion is not associated with the induction of a strong ER stress response.

### Wnt Secretion Inhibition Does Not Induce Autophagy

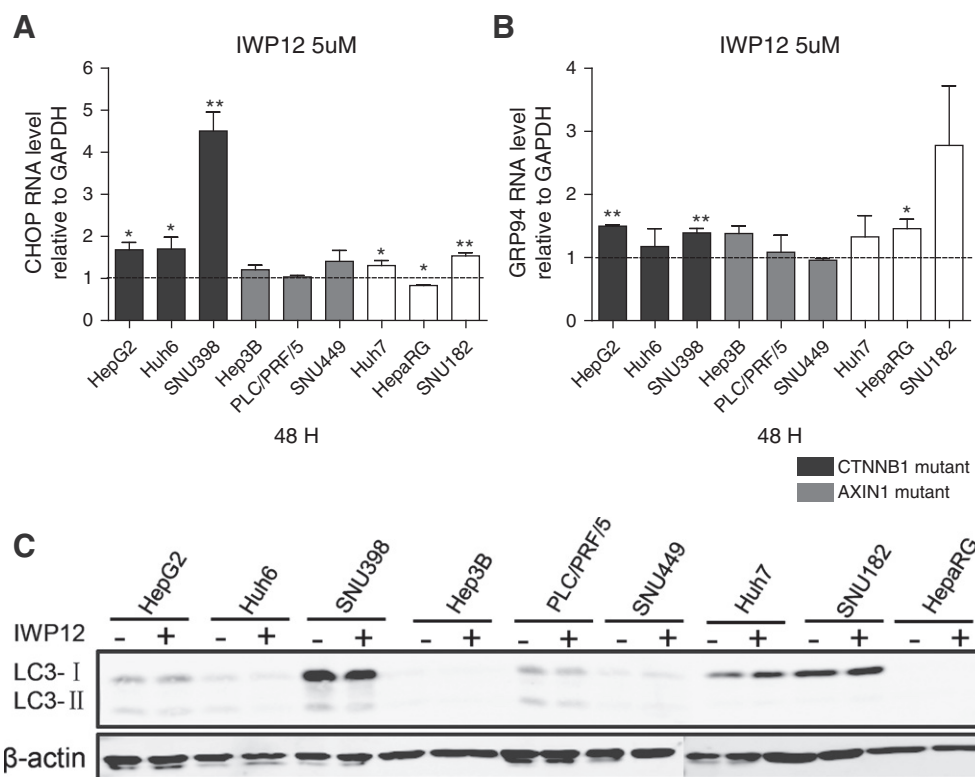
Because knockdown of  $\beta$ -catenin or suppression of  $\beta$ -catenin signaling induced autophagy and even autophagic cell death in head and neck squamous cell carcinoma cells [35] and breast cancer stem-like cells [36], we further hypothesized that blocking Wnt secretion could exert similar effects on HCC cell lines. During autophagy, the microtubule-associated protein 1A/1B-light chain 3 (LC3) is converted through lipidation into a lower migrating isoform (LC3-II) detectable by Western blot, which is used as an indicator of autophagosome formation. As indicated in Figure 7C, IWP12 treatment does not change the LC3 pattern in any of the HCC cell lines, showing that it does not induce autophagy.

### Discussion

In this article, we have investigated the importance of  $\beta$ -catenin signaling and Wnt secretion for sustaining hepatocellular carcinoma growth. Using a panel of nine HCC cell lines, we show that  $\beta$ -catenin signaling is required to support optimal growth in all of them, in line with other reports using a limited number of cell lines [37,38]. This is to be expected for cell lines carrying oncogenic  $\beta$ -catenin mutations in which the activating mutation will have provided a selective growth

advantage during tumor formation, but it also holds true for the nonmutant ones that show only low levels of baseline signaling, such as Huh7 and HepaRG, as well as the AXIN1 mutant lines. The latter observation is of relevance as it has been debated whether AXIN1 mutations lead to a significant enhancement of  $\beta$ -catenin signaling within liver cancers. This subset of tumors apparently lacks a robust nuclear  $\beta$ -catenin accumulation and shows no clear upregulation of target genes such as *AXIN2* or *GLUL* [39,40]. Also in our hands, the AXIN1 mutant lines are among the lowest expressors of *AXIN2*. Nevertheless, in these lines, *AXIN2* was readily detectable by qRT-PCR (Ct values below 28), in addition to  $\beta$ -catenin reporter activities approaching those of the  $\beta$ -catenin mutant ones. Given that also these lines are suppressed in their growth following  $\beta$ -catenin knockdown, it shows that the majority of HCCs independent of their mutational profile rely on  $\beta$ -catenin signaling for optimal growth.

Besides its role in signaling,  $\beta$ -catenin is also involved in cell-cell adhesion by directly binding to cadherins [41]. As such, the siRNA mediated knockdown that we apply here will also likely reduce the amount of  $\beta$ -catenin sequestered at these adherens junctions. However, several investigations have shown that complete loss of  $\beta$ -catenin does not automatically lead to alterations in cell adhesion, including hepatocytes and hepatocellular cancer cells [42–45]. In all cases, it was shown that  $\beta$ -catenin compensates for its loss, thereby retaining normal cell adhesion. Importantly, these studies show that it is mainly the signaling function of  $\beta$ -catenin that is affected following knockdown.



**Figure 7.** IWP12 treatment does not lead to the induction of a strong ER stress response or autophagy. Following 48 hours of IWP12 treatment, the expression of the ER stress induced response genes *CHOP* (A) and *GRP94* (B) was evaluated by qRT-PCR (mean  $\pm$  SD,  $n = 3$ ). Except for a clear induction of *CHOP* in SNU398 and *GRP94* in SNU182, IWP12 caused slight or no induction of an ER stress response in other cell lines. Values depicted are relative to those obtained for the untreated control samples that are arbitrarily set to 1. \* $P < .05$ ; \*\* $P < .01$ . (C) Wnt secretion inhibition does not enhance autophagy in HCC cell lines. After incubation with IWP12 for 48 hours, the expression of LC3-II was tested by Western blot.

Next we addressed the question to what extent does extracellular exposure to Wnt ligands contribute to the observed levels of  $\beta$ -catenin signaling. Using qRT-PCR, we tested the expression of all 19 Wnt ligands in our cell line panel. The Wnt expression profile that we observed largely corresponds with the semiquantitative analyses performed by others [22,46]. *WNT3* is the most abundantly expressed “canonical” Wnt ligand uniformly expressed in all cell lines, whereas all the others are expressed at low level or only in a subset of the cell lines. SNU182 clearly stands out as the overall highest expressor of Wnt ligands, likely explaining the high level of phosphorylated DVL2 that we observed in this cell line [33]. Among the nonmutant lines, SNU182 also showed the highest  $\beta$ -catenin reporter activity and level of *AXIN2* expression, comparable with the  $\beta$ -catenin mutant ones. As such, it is not unexpected that this cell line strongly relies on Wnt ligand secretion to retain increased  $\beta$ -catenin signaling. In fact, it is the only HCC cell line that shows a clear reduction following Wnt secretion blockage on both reporter activity as well as *AXIN2* expression level. On the other hand, the nonmutant Huh7 cell line expresses the lowest amount of Wnt ligands, explaining its low baseline signaling levels, but it is highly responsive to Wnt ligand exposure for inducing strong  $\beta$ -catenin signaling. Within a true tumor setting, it may represent a subtype of liver cancers that heavily depends on Wnt ligands expressed by cells within the tumor microenvironment, whose secretion would also be inhibited by the porcupine inhibitors employed here, whereas the SNU182 line is largely autonomous in this respect. Importantly, none of the  $\beta$ -catenin and *AXIN1*-mutant HCC cell lines are clearly affected in  $\beta$ -catenin signaling upon alterations in Wnt ligand exposure, irrespective of their source, suggesting that the expressed oncogenic  $\beta$ -catenin or mutant *AXIN1* proteins determine overall signaling levels in a dominant fashion.

In our hands, this also holds true for most of the APC and  $\beta$ -catenin mutant CRC lines that we investigated. Among eight CRC lines tested, only HCT116 shows a strongly reduced reporter activity and *AXIN2* levels following IWP12 treatment, although it is also the only one in which both  $\beta$ -catenin signaling readouts are clearly increased after Wnt3A exposure. Analysis of pDVL2 levels is largely in accordance with this lack of response, i.e., most cell lines tested show only low baseline levels that are barely changed by either treatment (DLD1, HT29, SW480), suggesting that these lines are not actively signaling through Wnt ligand receptors. These results also challenge the universal validity of the conclusions drawn by Voloshanenko et al. who proposed that colorectal cancers still strongly depend on Wnt ligand exposure for maintaining optimal  $\beta$ -catenin signaling levels [26]. Their overall well-performed study depended on a thorough analysis of the HCT116 cell line and to a lesser extent on other lines such as DLD1. Importantly, the  $\beta$ -catenin mutant HCT116 cell line is nowadays known to carry an inactivating mutation in the transmembrane E3 ubiquitin ligase RNF43, which strongly sensitizes these cells to exposure by Wnt ligands (see discussion below) [28,47–49]. As such, their study may have unknowingly overstated the importance of Wnt ligand signaling for CRC growth in general, warranting a more extensive analysis in a larger cohort of CRC samples and cell lines.

In recent years, Wnt secretion inhibitors, such as the porcupine inhibitor used in our study, have emerged as candidate drugs for treating Wnt-driven cancers. Cancers that are considered to be especially responsive to these treatments are the ones carrying somatic mutations resulting in a persistent presence of Wnt receptors at the

cell surface [28,48]. In normal cells, the Wnt/Frizzled receptors are continuously endocytosed and degraded following ubiquitination by RNF43 or its close homolog ZNRF3. Both these ubiquitin ligases are inhibited in their action by one of four secreted R-spondin proteins [50]. Consequently, mutational inactivation of RNF43/ZNRF3 and a strongly increased production of R-spondins through the generation of aberrant fusion transcripts both lead to tumor cells with high levels of Wnt/Frizzled receptor at their surface and hyperresponsiveness to Wnt ligands. These genetic aberrations have been identified in 10% to 20% of CRCs and in various other tumor types [28,51,52] but are, to the best of our knowledge, not present in HCCs, suggesting that these tumors are not prime candidates for treatment with Wnt secretion inhibitors. Nevertheless, our analysis shows that most of the HCC cell lines are reduced in their growth to varying extents following both WLS knockdown as well as IWP12 treatment.

The mechanism of the growth suppression remains more elusive at present. Except for the SNU182 cell line, we do see little evidence of  $\beta$ -catenin signaling modulation, suggesting that other mechanisms are at play. One possibility is the induction of ER stress resulting from the aberrant accumulation of Wnt ligands in the ER. However, except from increased expression of the ER-induced genes *CHOP* and *GRP94*, in resp. SNU398 and SNU182, we do not see strong evidence that Wnt secretion blockage leads to high levels of ER stress. In addition, autophagy did not contribute to the growth suppression either, as there was no visible change in the pattern of the autophagy marker LC3 following IWP12 treatment. An alternative explanation may reside in the reduced secretion of Wnt ligands more commonly signaling through  $\beta$ -catenin independent pathways. Activation of these alternative pathways has however mainly been shown to affect cellular processes involved in migration and cellular polarity and actually to counteract cell proliferation [8]. This “noncanonical” pathway has not been extensively studied in liver cancer, but the available literature does indeed support a growth suppressive effect [46,53]. Therefore, interfering with the secretion of this subset of Wnt ligands is expected to support cellular growth, which is in contrast to the growth suppression that we observed. In line with our results, Covey et al. have shown that knocking down *PORCN* in various tumor cell lines reduced their growth through a Wnt-independent pathway [54]. Also in their case, no obvious explanation could be uncovered, but both studies highlight the importance of considering alternative roles for proteins involved in Wnt secretion and their role in regulating cell growth.

In conclusion, our study shows that the majority of HCC cell lines depend on  $\beta$ -catenin signaling for maintaining optimal growth. Extracellular exposure to Wnt ligands has a minor contribution to overall  $\beta$ -catenin signaling strength in the  $\beta$ -catenin and *AXIN1*-mutant cell lines. Despite this observation, interfering with Wnt secretion through WLS knockdown or inhibition of porcupine function results in reduced growth, indicating that these proteins may have alternative roles currently unappreciated.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2016.10.004>.

### Conflicts of Interest

The authors declare no conflict of interest.

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