Targeting Wnt/β-catenin Signaling in Liver Cancers

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Chapter 1

General Introduction and Outline of This Thesis

Liver cancers

Liver cancer is one of the leading global health care issues. Primary liver cancers include hepatocellular carcinoma (HCC), cholangiocarcinoma and hepatoblastoma, the latter which is mainly observed in children. Focal nodular hyperplasia and hepatocellular adenoma are benign hepatocellular tumors that develop most frequently in women without cirrhosis (1). Other tumors observed in the liver, such as fibrosarcoma, angiosarcoma, leiomyosarcoma and lymphoma, are rare but have malignant potential.

Around 90% of liver cancer patients are diagnosed with HCC, the fifth most frequent cancer and highly relevant to cancer related deaths worldwide (2, 3). Hepatitis B (HBV) and hepatitis C (HCV) viruses together with alcohol abuse, obesity-induced non-alcoholic steatohepatitis (NASH) and aflatoxin-B1 exposure are considered as the major causes for HCC, which show regional preferences. In parts of Asia and Africa, high incidence of HCC is linked to elevated HBV and aflatoxin-B1 prevalence (4). In western countries, the main causes are HCV, alcohol abuse and NASH (5, 6).

Hepatocarcinogenesis is a complex pathological process that can take decades from tumor cell initiation to the final malignant tumor. Etiological factors mentioned above lead to chronic hepatitis, which gives rise to fibrosis and progression to cirrhosis around 10 years later (7). Cirrhosis chronically alters the liver microenvironment which potentiates the initiation and progression of HCC. During this process, the accumulation of aberrant genetic and epigenetic modifications elicits the dysregulation of signaling pathways. This in turn facilitates the transformation from the precancerous dysplastic hepatocytes into early HCC that ultimately progresses to malignant phenotypes.

Wnt/β-catenin signaling

The evolutionarily conserved Wnt signaling pathway is involved in both physiological and pathophysiological processes (8-10). Wnt signaling is triggered by Wnt ligands. These ligands are generated within the endoplasmic reticulum (ER), modified by palmitoylation by the Wnt acyl-transferase porcupine (PORCN) and shuttled by Wntless (WLS) from the Golgi to the plasma membrane where they can signal in an autocrine or paracrine manner (Figure.1) (11, 12). Nineteen Wnt ligands have been identified in the human genome. Based on the

dependency of β-catenin to transduce the signal, Wnt signaling is subdivided into canonical Wnt/β-catenin pathway, the noncanonical planar cell polarity pathway, or the noncanonical Wnt/calcium pathway. In this thesis, we mainly focus on the canonical Wnt/β-catenin pathway, largely relevant to HCC.

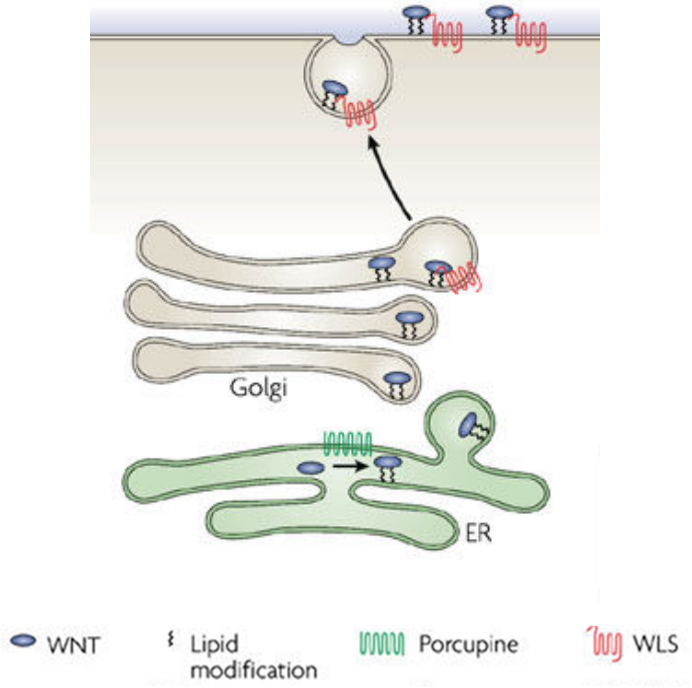

Figure.1 Wnts are lipid modified by PORCN in the ER and escorted by WLS from the Golgi to the plasma membrane for secretion. Figure adapted from reference Hausmann G, et al (11).

Canonical Wnt/β-catenin signaling is normally turned off in tissues of adults with the exception of part of stem cell niches (13). Regulation of Wnt/β-catenin signaling is fine-tuned at both extracellular and intracellular levels. Extracellularly, Wnt ligands are captured by Wnt antagonists, such as secreted Frizzled-related proteins (SFRPs), dickkopf (DKKs) and the Wnt inhibitory factor (WIF) (14). Intracellularly, the cytosolic transcription factor β-catenin is tightly regulated by a multiprotein complex composed of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1α. Kinase CK1α triggers the priming site at Ser45 of β-catenin allowing the subsequent phosphorylation at Thr41, Ser37 and Ser33 by GSK3. Then the β-transducin repeat containing protein (βTRCP) recognizes the phosphorylated β-catenin for subsequent proteolysis (14, 15). The overall effect is to maintain minimal levels of free cytosolic β-catenin (Figure.2).

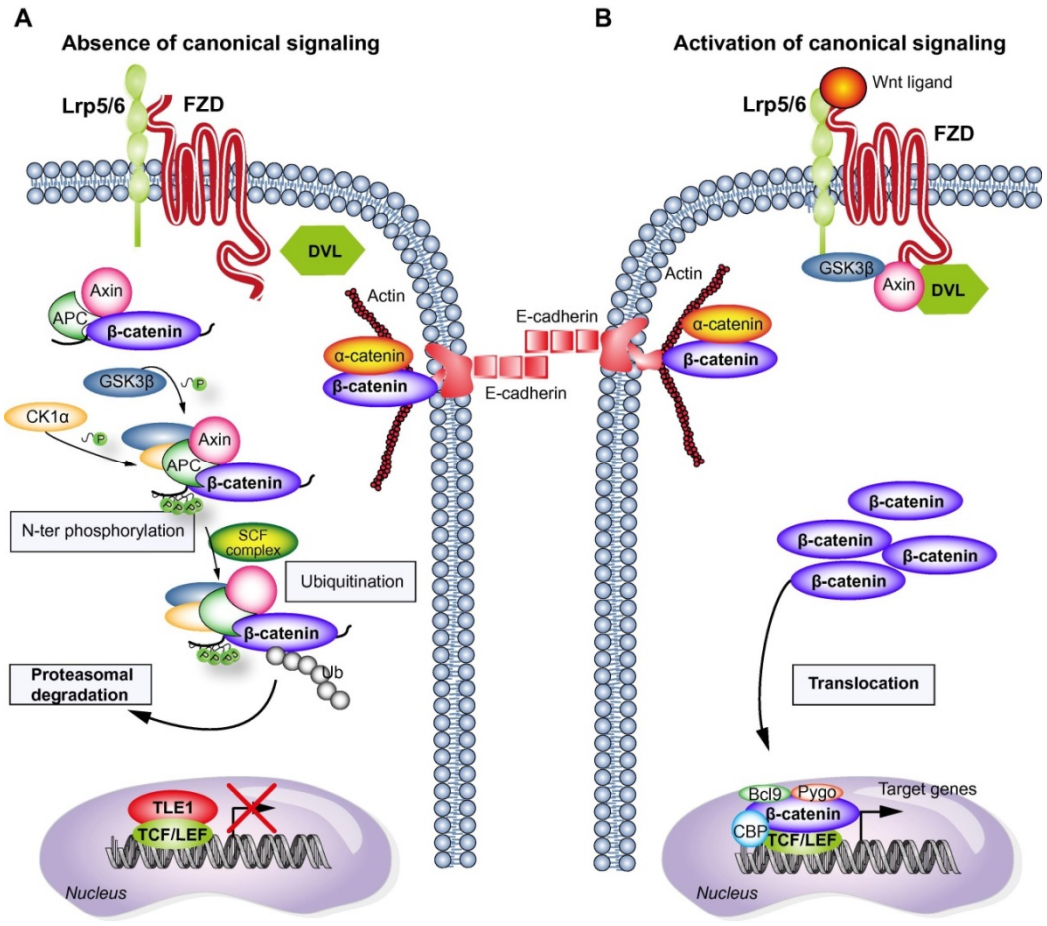

Figure.2 The canonical Wnt/β-catenin signaling pathway. (A) In the absence of Wnt ligands, β-catenin is phosphorylated by a degradation complex consisting of GSK3β, CK1α, APC and AXIN1/AXIN2. Phosphorylated β-catenin is targeted for proteasomal degradation after ubiquitination by the SCF protein complex. In the nucleus, the TCF/LEF transcription factor activity is repressed by TLE-1. (B) Activation of canonical Wnt/β-catenin signaling leads to the dissociation of the degradation complex. As a result, β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it promotes the expression of target genes *via* interaction with TCF/LEF transcription factors and other proteins such as CBP, Bcl9, and Pygo. Both figure and text adapted from reference Pez F, et al (16).

Wnt ligands initiate the signaling by binding with a member of the frizzled receptor (FZD) family and one of the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors. Then the scaffolding proteins disheveled (DVL) and AXIN are recruited to the membrane, leading to the disassembly of the multiprotein β-catenin destruction complex (17) and subsequent accumulation of unphosphorylated β-catenin (active β-catenin) in the cytoplasm. The active β-catenin translocates to the nucleus (18) and binds transcription factors of the T-cell factor (TCF7, TCF7L1 and TCF7L2)/lymphoid enhancer-binding factor (LEF) family, triggering the transcription of downstream Wnt/β-catenin target genes (16, 19) (Figure.2). Thus, the mechanism underlying the regulation of canonical Wnt/β-catenin

signaling is complicated. Functional mutations of these related proteins could lead to the dysregulation of target gene transcription.

Aberrant activation of Wnt/β-catenin signaling in hepatocellular carcinoma

Inappropriate activation of Wnt/β-catenin signaling is critical in HCC (14, 16, 20-22). The central component transcription factor β-catenin shows nuclear accumulation in around 40%-70% of HCC patients. Distinctive molecular or genetic alterations have been identified to stabilize β-catenin and to aberrantly trigger Wnt/β-catenin signaling, such as elevated level of upstream Wnt ligands or cell surface receptors and decrease of extracellular inhibitors [26]. In addition, frequently gain-of-function mutations are observed in exon3 of the CTNNB1 gene (encoding β-catenin) (15-25%) at the phosphorylation residues (23), which result in the expression of mutant β-catenin resistant to proteolytic degradation. Loss-of-function mutations of negative regulators are reported in AXIN1 (10.4%), AXIN2 (3.3%) and APC (1.4%) (24), evidently contrasting with the situation in colorectal cancer (CRC) where up to 80% of cancers display mutated APC (25, 26). Frameshift mutations or genomic deletions in these genes elicit the compromised function of the multiprotein complex to degrade β-catenin and is thus also related to enhanced Wnt/β-catenin signaling.

The relative mutation frequencies of these various Wnt/β-catenin signaling elements are different in HCC as compared to other cancers, e.g. sporadic CRC. The reason underlying these disparities could be due to different etiology of HCC, such as HBV or HCV, which shows preference on the different type of mutations that arise in liver genomes as compared to other sites in the body (24, 27). It may also derive from the fact that in different organs, optimal cancer-driving Wnt/β-catenin signaling mutations may be substantially different, resulting in selection pressure for different types of mutations (25).

Target Wnt/β-catenin signaling

In light of the importance of aberrant activation of Wnt/β-catenin signaling in HCC, components involved in this pathway could be promising therapeutic targets for HCC therapy. After decades of extensive research to identify these Wnt/β-catenin signaling inhibitors, there have been numerous small molecules discovered that may possess this potential. In table 1,

we provide a summary of the reported Wnt/β-catenin signaling inhibitors tested in different tumor types. Some of these inhibitors target the upstream components of Wnt/β-catenin signaling including the porcupine protein, Wnt ligands, FZD receptors and co-receptors. Others aim at interfering with the intracellular signal transduction process by targeting DVL, AXIN, GSK3, CK1α or β-catenin itself, or affect its interaction with co-activators or transcriptional factors. Some of these inhibitors are currently undergoing clinical trials.

Table.1 Wnt/β-catenin signaling inhibitors undergoing preclinical and clinical evaluation

Targets	Compounds	Diseases	Stage	References
Porcupine	LGK974	Pancreatic adenocarcinoma, BRAF mutant colorectal cancer head and neck squamous cell carcinoma	Phase 1	(28)
	IWPs	Colon cancer	Preclinical	
	Wnt-C59	Mammary tumor Pancreatic cancer Colorectal cancer	Preclinical	(29, 30)
	ETC-1922159	Mammary tumor Teratocarcinomas	Phase 1	(31)
FZD1/2/5/7/8	OMP-18R5 (vantictumab)	Breast cancer Lung cancer Pancreas cancer Colon cancer	Phase 1	(32)
FZD7	sFZD7	**HCC**	Preclinical	(33)
FZD8	OMP-54F28	**Liver cancers (HCC)** Ovarian Cancer Pancreas cancer Solid tumor	Phase 1	(21)
LRP6	Niclosamide	Prostate; Breast	Preclinical	(34)
	Silibinin	Prostate; Breast	Preclinical	(35)
LRP5/6	Salinomycin	breast, prostate, lung, gastric, osteosarcoma, **HCC**	Preclinical	(36-41)
Wnt1	Anti-Wnt1	**HCC**, CRC, Lung cancer, sarcoma, breast cancer, head-neck squamous cell carcinoma	Preclinical	(42-46)
Wnt2	Anti-Wnt2	Melanoma, mesothelioma, lung caner	Preclinical	(47-49)
Wnt10b	Anti-Wnt10b	head-neck squamous cell carcinoma	Preclinical	(46)
Wnt ligands	WIF-Fc/ SFRP-Fc	**HCC**	Preclinical	(50)
DVL	NSC668036			(51)
	3289-8625	Prostate cancer		(52)
	FJ9	Melanoma, lung cancer		(53)
Tankyrase/AXIN	XAV939	CRC, neuroblastoma, breast cancer	Preclinical	(54-56)
	IWR-1	CRC, prostate	Preclinical	(57)
	JW55	CRC	Preclinical	(58)
GSK3β	DIFs	Leukemia	Preclinical	(59-63)

		HeLa		
CK1α	Pyrivinium	CRC	Preclinical	(64, 65)
β-catenin phosphorylation	CGK062	CRC, **HCC**, prostate cancer	Preclinical	(66)
β-catenin ubiquitination	Hexachlorophene	CRC	Preclinical	(67)
	Isoreserpine	CRC	Preclinical	(68)
β-catenin	β-catenin siRNA	**HCC**	Preclinical	(69)
	BBI608	Glioblastoma, CRC, **HCC**, gastric cancer, pancreas cancer, lung cancer	Phase 1/2	(70)
β-catenin/CBP	ICG-001	CRC, breast cancer, pancreatic cancer, head-neck squamous cell carcinoma	Preclinical	(71-74)
	PRI-724	Pancreatic adenocarcinoma, leukemia, CRC, HCV-induced cirrhosis, solid tumor	Phase 1	
β-catenin/TCF	PKF115-548 PKF222-815 CGP049090 FH535	**HCC**, CRC, lung cancer	Preclinical	(75-78)
cyclooxygenases	NSAIDS	CRC	Preclinical	(79-82)
β-catenin/E-cadherin	Vitamin derivatives	CRC	Preclinical	(83)
β-catenin nuclear export	Peg-IFN	**HCC**	Preclinical	(84)

Aim of the thesis

During last decades, a tremendous progression of knowledge and understanding about liver cancer development has been witnessed. The progression in clinical technology has been instrumental for early detection. In addition, the Barcelona clinic liver cancer (BCLC) staging and treatment model that also takes into account remaining liver functionality and general health of the patient, has been broadly endorsed worldwide. These achievements facilitate the early diagnosis and enable more efficient treatment strategies to improve the outcome of HCC patients. Nevertheless, HCC related mortality is still high worldwide. Given that HCC individuals show extensive phenotypic and molecular heterogeneity, it is important to identify the molecular features and genomic traits of HCC patients, thus aiding reasonable stratification and optimal personal treatment decisions.

As one of the critical contributing factors to HCC growth, aberrant activation of Wnt/β-catenin signaling derives from a variety of molecular alterations involved in this pathway, especially mutations of β-catenin and AXIN1. As illustrated in Table 1, a variety of drugs targeting Wnt/β-catenin signaling have been developed, but most of them have not been

thoroughly tested for liver cancer. In the current thesis, our first aim is to explore the effectiveness of two classes of drugs in a panel of HCC cell lines, i.e. an extracellular Wnt secretion inhibitor and cytosolic tankyrase inhibitor. The second aim is to compare the response and sensitivity for these inhibitors depending on the specific β-catenin signaling defect present in these HCC cell lines, i.e. *CTNNB1* or *AXIN1* mutations.

Our third aim is to achieve a better understanding the mechanism through which *AXIN1* mutations contribute to HCC cell growth. As a critical negative regulator of Wnt/β-catenin signaling, mutation or deletion of AXIN1 is expected to support tumor growth by enhancing β-catenin signaling. However, *AXIN1* mutations were shown to not cause a robust induction of β-catenin target genes in liver cancers (3, 85). Hence, the specific role of *AXIN1* mutation in reprogramming Wnt/β-catenin signaling in liver cancers remains under debate. Lastly, we also intend to find new potential molecular targets regulating Wnt/β-catenin signaling in HCC.

Outline of this thesis

In **chapter 1**, we have provided a general introduction of Wnt/β-catenin signaling and its role in the initiation and progression of liver cancers. In **chapter 2**, we review how aberrant activated Wnt/β-catenin signaling interacts with the HCV viral components and potentiates the progression from hepatitis C to HCC. In **chapter 3**, we investigate the dependency of extracellular Wnt secretion to support growth in 9 HCC cell lines characterized by mutations in either *CTNNB1*, *AXIN1* or no obvious mutation in a β-catenin signaling related component, also in comparison with CRC cell lines. In **chapter 4**, we use the same cell line panel to explore whether the inhibition of tankyrase, by endorsed inhibitors XAV939 as well as IWR-1, stabilizes AXIN1/2 and attenuates Wnt/β-catenin signaling in HCC cells. We further compare the contribution of AXIN1 and AXIN2 in this process. **In chapter 5**, we test the expression levels of serine-threonine kinase receptor-associated protein (STRAP) in patient HCC tissues and investigate its function in regulating Wnt/β-catenin signaling activity in our panel of HCC cell lines. In a related project (**chapter 6)**, we describe a novel approach to support the identification of Familial Adenomatous Polyposis carriers among children with hepatoblastoma tumors employing β-catenin immunohistochemical staining and mutation analysis.

The novel insights derived from this thesis will be summarized and discussed in **chapter 7** and **chapter 8**, which will provide a better understanding of Wnt/β-catenin signaling in liver cancers and experimental evidence for future stratification and optimal treatment decision of patients carrying Wnt-driven liver cancers.

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Chapter 2

Action and function of Wnt/β-catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma

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Abstract

Hepatitis C virus (HCV) infection is one of the leading causes for hepatocellular carcinoma (HCC) worldwide, but the mechanistic basis as to how chronic HCV infection furthers the HCC process remains only poorly understood. Intriguingly, accumulating evidence indicates that HCV core and nonstructural proteins provoke activation of the Wnt/β-catenin signaling pathway, whereas the evidence supporting a role of Wnt/β-catenin signaling in the onset and progression of HCC is compelling. Convincing molecular explanations as how expression of viral effectors translate into increased activity of the Wnt/β-catenin signaling machinery are still largely lacking, hampering design of rational strategies aimed at preventing HCC. Furthermore, how such increased signaling is especially associated with HCC oncogenesis in the context of HCV infection remains obscure as well. Here we review the body of contemporary biomedical knowledge on the role of Wnt/β-catenin pathway in the progression from chronic hepatitis C, to cirrhosis and HCC and explore potential hypotheses as to the mechanisms involved.

Keywords: Hepatocellular carcinoma; Wnt/β-catenin signaling; hepatitis C virus

Introduction

HCV is estimated to infect up to 2% of the global population (around 180 million people worldwide) [1] with approximately 3-4 million new infections each year [2, 3]. Following infection, 60%-80% of affected individuals eventually develop chronic hepatitis [4]. After around 10 years of infection, 5%-10% of these chronically infected patients progress to cirrhosis [5]. In addition to the high mortality associated with advanced cirrhosis *per se*, annually another 2.0%-6.6% of cirrhotic patients with HCV infection progress to HCC [6, 7]. Understanding the details as to how HCV infection can promote the HCC process is thus of critical importance for the rational design of novel avenues aimed at the prevention and treatment of HCC.

Distinct from hepatitis B virus (HBV), a DNA virus that can integrate into the human genome and thus directly provoke genomic alterations potentially leading to cancer [8], HCV is a RNA virus lacking a DNA intermediate phase in its life cycle and therefore its infection of liver cells is not associated with damage to the host genetic material *per se* [9]. Hence, the tumor promoting potential of HCV derives from indirect interaction with the hepatocyte genome. However, other pathogens with a similar infection route are less clearly associated with progression towards HCC, compare for instance hepatitis E virus infection. It thus appears that HCV has specific properties that promote further hepatocyte transformation.

The Wnt/β-catenin pathway is an attractive candidate to mediate the HCV-specific effects leading to hepatocyte oncogenic transformation. Activation of this pathway is clearly contributing to hepatocarcinogenesis as indicated by the detection of recurrent genetic mutations of Wnt/β-catenin signaling pathway components in HCC that appear especially frequent in HCV-related tumors. Intriguingly, HCV-derived viral proteins appear to be capable of autonomous activation of Wnt/β-catenin signaling, although the underlying molecular mechanisms remain poorly understood. Here we explore potential hypotheses explaining these effects and summarize documented interactions of Wnt/β-catenin signaling components in HCC patients with HCV infection. We propose that the Wnt/β-catenin signaling pathway constitutes a rational target for the prevention and treatment of HCV-associated HCC.

Wnt/β-catenin signaling

Wnt/β-catenin signaling is a pivotal morphogenetic pathway and accordingly associated with a host of physiological and pathophysiological processes, including embryonic patterning, cell proliferation, differentiation, angiogenesis and especially cancer [10-12]. Wnt signalling is initiated by binding of Wnt ligands to their cognate receptors. These Wnt ligands are 40kDa cysteine-rich glycoproteins [13], which following synthesis and primary glycosylation on the endoplasmic reticulum are palmitoylated by Wnt acyl-transferase porcupine protein in the Golgi apparatus. Secretion of Wnts then requires the evenness interrupted/wntless/G protein-coupled receptor 177 (Evi/Wls/GPR177), which shuttles palmitoylated Wnts to the plasma membrane, where they are released by the cell and initiate autocrine or paracrine signaling. Hitherto, nineteen Wnts have been identified in the human genome [14], and because annotation of *Homo sapiens* DNA is now quite complete it is unlikely further Wnt paralogues will be discovered. Wnts can provoke different modes of cellular signaling, either mediated by β-catenin or independent of this protein. According to the dependence on β-catenin for provoking cellular effects, Wnts are classified into canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) subgroups [15, 16]. In this review we shall focus on the canonical Wnts, as these are most associated with HCC in general and HCV-infection associated HCC in particular.

Except for several stem cell niches, canonical Wnt/β-catenin signaling is typically not active in tissues of adult individuals [17], despite constitutive production of Wnt ligands. This is a result of the action of a range of Wnt antagonists, such as secreted Frizzled-related proteins (SFRPs), dickkopf (DKKs) and the wnt inhibitory factor (WIF) [18]. In this non-signaling state, cytosolic β-catenin is continuously phosphorylated at Ser33, Ser37, Thr41 and Ser45 residues located in exon3 by a multiprotein complex consisting of adenomatous polyposis coli (APC), AXIN, glycogen synthase kinase-3β (GSK3β) and casein kinase 1 (CK1). These phosphorylations cause β-catenin to be recognized and poly-ubiquitinated by the β-transducin repeat containing protein (βTrCP) followed by β-catenin degradation in the proteasome [18, 19]. The overall effect is that minimal free cytosolic β-catenin is available for nuclear signaling and thus Wnt-mediated gene transcription is absent under normal conditions.

Upon binding of Wnt ligands to a complex consisting of the frizzled receptor (FZD) and co-receptors, which include the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), the scaffolding protein disheveled (DVL) is recruited to the membrane, an event that in turn

causes the disassembly of the multiprotein β-catenin destruction complex. This results in rescue of β-catenin from proteasomal degradation and thus the accumulation of β-catenin in the cytoplasm, eventually causing β-catenin translocation to the nucleus [20]. In the nucleus, β-catenin binds transcription factors of the T-cell factor (TCF7, TCF7L1 and TCF7L2) 4/lymphoid enhancer-binding factor (LEF) family, triggering transcription of downstream Wnt target genes, including *CYCLIND1*, *AXIN2*, *C-MYC*, *RING FINGER PROTEIN 43* (*RNF43*) and *ZINC/RING FINGER PROTEIN 3* (*ZNRF3*) [21, 22]. RNF43 and ZNRF3 are two closely related transmembrane E3 ligases, which remove surface FZD receptors by promoting their endocytosis [23]. This E3 ligase activity is in turn negatively modulated by R-spondins (RSPO) and the leucine-rich repeat-containing G-protein coupled receptor 4/5/6 (LGR4/5/6) that sequestrate RNF43 and ZNRF3 from FZD receptors by forming a tripartite complex [24]. Hence regulation of Wnt target gene transcription is complex allowing for extensive regulation but also for mechanisms leading to deregulation of target gene transcription in pathophysiology.

Further complexity is added by the role of β-catenin in cell-cell adhesion where it acts, independent of its transcriptional activity, by forming a complex with cadherins and facilitating the formation of cellular junctions between adjacent hepatocytes. The β-catenin captured in these cell-adhesion complexes represents a dynamic pool of β-catenin capable of nuclear signaling following several stimuli. One of these stimuli is β-catenin tyrosine phosphorylation by receptor tyrosine kinases activated by growth factors produced by epithelial as well as stromal cells. In particular, phosphorylation of β-catenin residue Tyr654 results in its release from cadherins and an increase in TCF-mediated transcriptional activity [25-28]. Furthermore, the adherence pool of β-catenin also appears to be under indirect control of Wnt signaling itself. Upon activation of canonical Wnt/β-catenin signaling, the suppression of GSK3β leads to the upregulation of SNAIL [29]. As SNAIL is a repressor of the *CDH1* gene encoding E-cadherin [30, 31], this will lead to reduced E-cadherin production. Diminished E-cadherin causes the dissociation of the complex and subsequent internalization of β-catenin and accumulation of β-catenin in the perinuclear endocytic recycling compartment which promotes translocation to the nucleus to activate Wnt/β-catenin signaling [32, 33]. Hence pathogens can also provoke β-catenin signaling by disrupting intercellular junctions, in addition to direct effects on elements of the Wnt signaling cascade involved in regulating β-catenin-mediated transcription.

Aberrant activation of Wnt/β-catenin signaling during HCC

Important in the context of potential modulation by HCV infection in relation to HCC is that aberrant signal transduction in general and β-catenin signaling in particular, is one of the key characteristics of hepatocarcinogenesis [34]. Functional deregulation of Wnt/β-catenin signaling is reported frequently in HCC strongly suggesting that this pathway is important in this tumor type. Various genetic and molecular alterations have been identified to be pro-oncogenic in a variety of settings, and have as a common denominator that they stabilize β-catenin thus provoking enhanced transcriptional activity of Wnt target genes. Table 1 summarizes the relative mutation frequency of Wnt/β-catenin signaling elements in HCC patients. Employing HCC cohorts from different countries, the most prevalent are activating mutations in *CTNNB1* (encoding β-catenin) followed by loss-of-function mutations in *AXIN1, AXIN2 and APC.* The relative mutation frequencies of these various Wnt/β-catenin signaling elements are different in HCC as compared to other cancers, *e.g.* sporadic colorectal cancer. The reason why these differences emerge may result from different etiology of HCC and thus the type of mutations induced in liver genomes as compared to other sites in the body, but may also derive from the fact that in different organs, optimal cancer-driving Wnt/β-catenin signaling mutations may be substantially different, resulting in selection pressure for different types of mutations [35, 36]. As indicated in Table 1, in HCCs around 22.1% harbor specific gain-of-function mutations of *CTNNB1*. Missense, insertion or partial deletions within *CTNNB1* exon 3 lead to the generation of a mutant β-catenin protein preventing the proper phosphorylation of amino acids Ser33, Ser37, Thr41 and Ser45 resulting in compromised degradation and thus stabilization of β-catenin in the cytoplasm. Less frequently, loss-of-function mutation of *AXIN1*, *AXIN2* or *APC* is found in 10.4%, 3.3% and 1.4% of HCCs respectively, evidently contrasting with the situation in colorectal cancer where up to 80 % of cancers display mutated APC [36, 37]. Frameshift or deletion in these genes yields impaired ability of the destruction complex to degrade β-catenin and is thus also associated with enhanced Wnt/β-catenin signaling. Overexpression of upstream ligands or cell surface receptors and reduction of extracellular inhibitors have been reported to stimulate activation of this pathway in HCC as well [38]. Thus evidently, at some stage in the progression towards full-blown HCC, acquisition of increased Wnt/β-catenin signaling provides liver cancer cells a relative advantage over cells not having

such mutations. Here we shall argue that especially HCV infections create the conditions which allow pre-carcinogenic cancer cells to exhibit such enhanced Wnt/β-catenin signaling.

High frequency of *CTNNB1* mutation in HCV related HCC

HCV infection presents a substantial clinical challenge, for which only direct anti-viral medication appears a suitable solution [75]. If left untreated or not timely-recognized, persistent HCV infection causes immune-mediated chronic liver damage and compensatory hepatic regeneration by inducing cell proliferation and thus creates a microenvironment permissive for the induction of genetic alterations to the hepatocyte genome [76]. Following HCV infections, genetic abnormalities accumulate relatively slowly during the sequence of chronic hepatitis and increased cirrhosis that finally progresses to HCC. Consequently, the selective growth advantage provided to hepatocytes with a malignant phenotype eventually facilitates the development of phenotypically and genetically heterogeneous HCC [77]. As one of the principal proto-oncogenes in HCC development, the relatively high frequency of *CTNNB1* mutations in HCV-related HCC is especially striking, in the view of the relative absence of such mutations in HBV-related liver cancers but also in the view of their paucity in not-virally associated HCC (Table 2). Indeed, around 26.7% of HCV-related HCC harbor a *CTNNB1* mutation, which is much higher than that observed in HBV-associated HCC (11.6%) or that observed in total non-virally-associated HCC (21.2%). Furthermore, we noticed that, different from colorectal cancers which mainly show Thr41 and Ser45 mutations [36], HCV-related HCC shows a preference for *CTNNB1* mutations from Asp32 to Ser37 residues [45, 47, 49, 59, 68, 70, 71] (Fig.1). Recently, a genotype-phenotype correlation was shown for *CTNNB1* mutations, suggesting that activating mutations occurring at the Asp32 to Ser37 residues lead to higher signaling levels than mutations at Thr41 and Ser45 [39]. This may partially explain the preference. It also could be attributable to the mutagenic dose demanded to induce HCC.

Table 1. Genetic mutation in components of Wnt/β-catenin pathway in HCC

Reference	Patient N	Mutant samples N(%)				Region
		CTNNB1	AXIN1	AXIN2	APC	
Rebouissou et al.[34]	373	146(39)	NA	NA	NA	France, Spain, Italy
Hirotsu et al.[35]	9	2(22.2)	NA	NA	NA	Japan
Schulze et al.[36]	243	95(37.4)	27(11.1)	3(1.2)	4(1.6)	France, Italy, Spain
Kan et al.[37]	88	14(15.9)	4(4.5)	2(2.3)	2(2.3)	China
Kitao et al.[38]	134	27(20.1)	NA	NA	NA	Japan
Ding et al.[39]	156	15(9.6)	NA	NA	NA	China
Tornesello et al.[40]	67	10(14.9)	NA	NA	NA	Southern Italy
Cleary et al.[41]	87	20(22.9)	NA	NA	NA	Canada, NC
Guichard et al.[42]	125	41(32.8)	19(15.2)	NA	2(1.6)	France
Lachenmayer et al.[43]	90	29(32.2)	NA	NA	NA	USA, Netherlands, Italy, Spain, Germany
Li et al.[44]	139	28(20.1)	NA	NA	NA	USA, Netherlands, China
Cieply et al.[45]	32	9(28.1)	NA	NA	NA	USA
Bengochea et al.[46]	62	16(25.8)	NA	NA	NA	Thailand, France
Austinat et al.[47]	40	10(25)	2(5)	NA	NA	Germany
Kim et al.[48]	36	1(2.8)	9(25)	NA	NA	Korea
Zucman-Rossi et al.[49]	45	18(40)	5(11.1)	NA	NA	France
Boyault et al.[50]	120	34(28.3)	13(10.8)	NA	NA	France
Zucman-Rossi et al.[51]	96	12(12.5)	NA	NA	NA	France
Park et al.[52]	81	13(16)	5(6.2)	NA	NA	Korea
Ishizaki et al.[53]	89	10(11.2)	13(14.6)	9(10.1)	NA	Japan
Cui et al.[54]	34	15(44.1)	NA	NA	NA	China
Edamoto et al.[55]	100	24(24)	NA	NA	0	Japan, Switzerland
Taniguchi et al.[56]	73	14(19.2)	7(9.6)	2(2.7)	NA	UK
Wong et al.[57]	60	7(11.7)	NA	NA	NA	China
Mao et al.[58]	262	37(14.1)	NA	NA	NA	Taiwan
Cui et al.[59]	34	15(44.1)	NA	NA	NA	China
Laurent–Puig et al.[60]	137	26(19)	12(8.8)	NA	NA	France
Devereux et al.[61]	62	5(8.1)	NA	NA	NA	China
Hsu et al.[62]	434	57(13.1)	NA	NA	NA	Taiwan
Satoh et al.[63]	87	0(0)	5(5.7)	NA	NA	Japan
Huang et al.[64]	22	9(41)	NA	NA	NA	Japan, Switzerland
Legoix et al.[65]	119	21(17.6)	NA	NA	NA	France
Terris et al.[66]	73	14(19.2)	NA	NA	NA	France
Kondo et al.[67]	38	9(24)	NA	NA	NA	Japan
Nhieu et al.[68]	35	12(34.3)	NA	NA	NA	France
Miyoshi et al.[69]	75	14(18.7)	NA	NA	NA	Japan
de La Coste et al.[70]	31	8(25.8)	NA	NA	NA	France
Total	3788	837(22.1)	121(10.4)	16(3.3)	8(1.4)	

Mutations at Ser45 require the selective duplication of the mutated allele as second activating hit, whereas only one activating hit for mutations at the Asp32 to Ser37.Although *CTNNB1* mutation appear a late stage event in the progression to HCC [56], the high rate of *CTNNB1* mutations observed may be directly and causally related to the HCV infectious process as *in vitro* studies show that both acute and chronic HCV infections provoke specifically *CTNNB1* mutations, in hematological model systems and HCCs [78]. Evidently, clarifying the relationship between infection with a non-integrating virus and subsequent *CTNNB1* mutations may prove exceedingly useful for designing strategies aimed at preventing HCV-associated HCC.

Table 2. Comparison of *CTNNB1* mutation in subtypes of HCC

References	CTNNB1 mutant samples N(%)			Mutation type	Amino acid	Region
	HCV	HBV	NV			
Hirotsu et al.[35]	2/5(40)	0/1(0)	0/3(0)	Missense	Gly34, His36	Japan
Kitao et al.[38]	12/55(21.8)	4/34(11.8)	11/44(25)	NA	NA	Japan
Ding et al.[39]	NA	12/110(10.9)	3/46(6.5)	Missense	Asp32,Gly34, Ser37, Thr41,Ser45	China
Tornesello et al.[40]	10/57(17.5)	0/10(0)	NA	Missense	Asp32, Ser33,Gly34 Ile35, Ser37, Ser45	Southern Italy
Kan et al. [37]	NA	12/81(14.8)	NA	Missense	Asp32, Ser33,Gly34 Ile35, Ser37, Thr41, Ser45	China
Guichard et al.[42]	8/24(33.3)	4/35(11.4)	30/80(37.5)	Missense Insertion Deletion	Asp32, Ser33, Ser37, Thr41,Thr42 Ser45	France
Li et al.[44]	14/45(31.1)	6/52(11.5)	9/44(20.5)	Missense Deletion	Asp32, Ser33,Gly34, His36, Ser37, Thr41, Ser45 ,Asn387	USA, Netherlands, China
Bengochea et al.[46]	8/20(40)	3/18(16.7)	5/24(20.8)	Missense Insertion	Asp32, Ser33, Ser37, Thr41 Ser45	Thailand, France
Kim et al.[48]	0/4(0)	0/21(0)	1/14(7.1)	Missense	Ser33	China
Park et al.[52]	0/6(0)	13/78(16.7)	NA	Missense Deletion	Asp32, Ser33,Gly34 Ile35, His36, Ser37, Thr41, Ser45	Korea
Edamoto et al.[55]	16/51(31.4)	5/26(19.2)	3/23(13)	Missense	Asp32, Ser33, His36, Ser37, Thr41, Ser45	Japan, Switzerland
Wong et al.[57]	0/2(0)	5/48(10.4)	2/10(20)	Missense Deletion	Asp32, Ser33,Gly34 Ile35, Ser37, Thr41, Ser45	China
Hsu et al.[62]	23/92(25)	30/323(9.3)	4/19(21.1)	Missense Deletion	Asp32, Gly34, Thr41, Ser45	Taiwan
Huang et al.[64]	9/22(41)	NA	NA	Missense	Asp32, Ser33,Ser37, Thr41, Ser45	Japan, Switzerland
Legoix et al.[65]	7/30(23.3)	5/26(19.2)	13/64(20.3)	Missense Deletion	Asp32, Ser33,Gly34, Ser37, Thr41, Ser45	France
Terris et al.[66]	2/7(28.6)	3/14(21.4)	9/52(17.3)	Missense Deletion	Asp32, Ser33,Gly34, Ser37, Ser45	France
Kondo et al.[67]	7/22(31.8)	1/8(12.5)	1/9(11.1)	Missense Deletion	Asp32, Ser33,Gly34 Ile35, His36, Ser37, Thr41, Ser45	Japan
Total	118/442(26.7)	103/885(11.6)	91/432(21.1)			

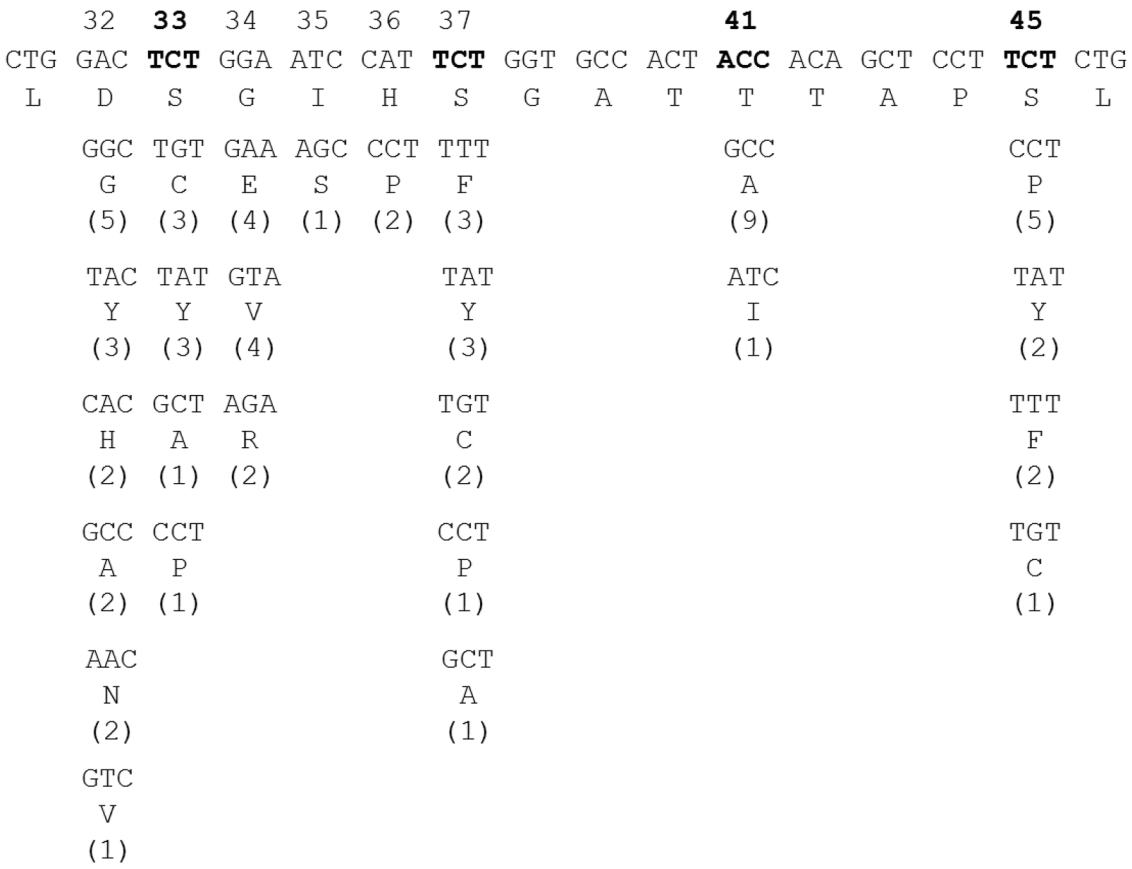

Figure 1. Summary of CTNNB1 exon 3 mutations in HCV-related HCC. Illustrated are the locations of the CTNNB1 mutations reported in 68 tumors from 65 HCC patients (one tumor with p.D32_G48del, not shown). N-terminal serine and threonine phosphorylation residues are indicated bold. Numbers in brackets are absolute number of tumors tested with given mutation.

HCV structural proteins activate Wnt/β-catenin signaling

The HCV genome is a single-stranded positive sense 9.6kb RNA molecule, which includes a single open reading frame encoding a polyprotein of ≈ 3,000 amino acids that following translation is cleaved into 10 mature proteins by both host and viral proteases. These proteins are the structural proteins (core, E1 and E2), the viroporin p7 and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The pro-oncogenic pathogenesis of HCV appears mainly mediated by the core protein and two of the non-structural proteins NS3 and NS5A [79]. These pro-oncogenic effects appear to depend largely on the potential of these proteins to mediate activation of Wnt/β-catenin signaling.

Core protein

The 21kDa core protein is the major component of HCV. Despite lacking obvious organelle localization signals in the primary sequence, it is not only detected in the cytosol, but also in th Golgi apparatus, in lipid droplets and in the nucleus [80, 81]. Remarkably, in the latter

organelle it serves as a regulator of hepatocyte transcription, facilitating Wnt/β-catenin signaling. This is brought about by upregulation of canonical Wnts, FZD and LRP5/6 receptors [82, 83] while concomitantly inhibiting transcription of Wnt antagonists SFRP2 and DKK1 [84]. The latter effect is mediated by epigenetic silencing of the promoters involved by core protein-mediated recruitment of DNA methyltransferase-1 (DNMT1) and histone deacetylase-1 (HDAC1) to the transcription start site, an effect already detected early in hepatitis infection [84, 85]. In addition, the HCV core protein mediates hypermethylation of the *CDH1* (E-cadherin) gene promoter [86]. Reduced production of E-cadherin results in diminished sequestering of β-catenin in β-catenin/E-caherin complexes and thus enhanced activation of Wnt/β-catenin signaling (Fig.2). Hence, the core protein mediates a plethora of molecular events leading to increased Wnt/β-catenin signaling and thus apparently HCV is under substantial selection pressure to provoke Wnt/β-catenin signaling. Potential sources for this selection pressure are a necessity to counteract hepatocyte apoptosis, whereas Wnt/β-catenin signaling-driven expansion of the HCV-infected compartment may be involved as well.

NS5A

The notion that HCV is under selection pressure to counteract apoptosis is further reinforced by observations that NS5A not only functions as a component of the HCV RNA replication complex [87], but also binds to the p85 regulatory subunit of phosphoinositide 3 kinase (PI3K) thus activating the downstream effector serine/threonine kinase Akt [88, 89]. Akt activation provides powerful anti-apoptotic signal and also mediates the inactivation of GSK3β, stabilization of β-catenin and subsequent stimulation of β-catenin dependent transcription [90]. In addition, the NS5A protein binds and stabilizes β-catenin directly [91], apparently independent of its effects on Akt and GSK3β [92] (Fig.2). Thus the multiple stimulatory effects of NS5A on Wnt/β-catenin signaling are also testimony of the selection pressure of HCV to increase hepatocyte Wnt/β-catenin signaling.

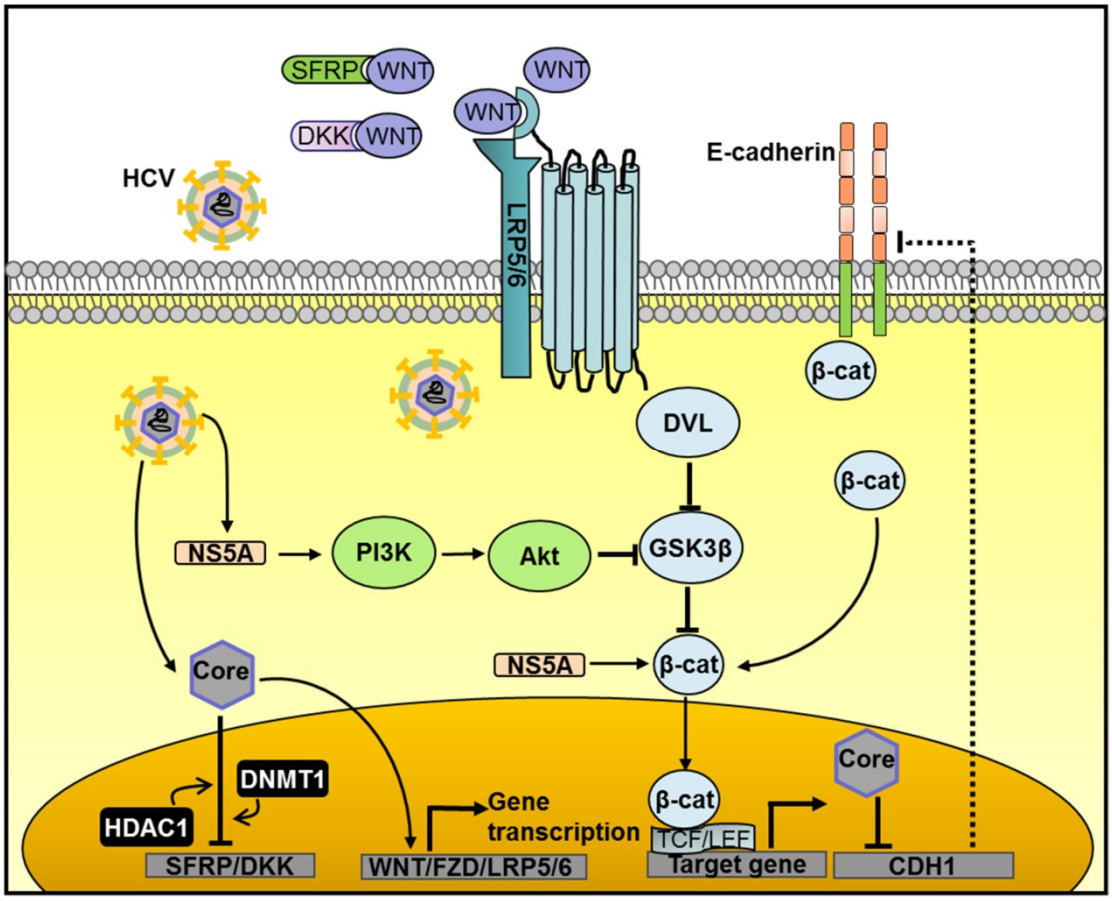

Figure 2. Wnt/β-catenin signaling is activated by HCV virus proteins. HCV core protein elevates gene expression of Wnt ligands, FZD and LRP5/6 receptors but decreases the expression of Wnt antagonists DKK and SFRP by recruiting DNMT1 and HDAC1 to their transcription start sites. In addition, HCV core protein releases β-catenin from the β-catenin/E-cadherin complexes by suppression of the *CDH1* gene promoter encoding E-cadherin. NS5A protein activates PI3K/Akt signaling leading to the inactivation of GSK3β and subsequent reduced breakdown of β-catenin, or directly stabilizes β-catenin protein. The overall effect is the cytoplasmic accumulation of β-catenin and stimulation of downstream transcription.

More Wnt/β-catenin signaling-stimulating effects

The hypotheses that successful HCV-infection critically depends on its potential to stimulate Wnt/β-catenin signaling is further supported by observations that, in addition to direct activation, HCV infection leads to elevation of microRNA-155 (miR-155) [93] and microRNA-199a-5p [94], in turn triggering Wnt/β-catenin signaling. MiR-155 acts as an oncomiR by targeting the suppressor of the cytokine signaling 1 (SOCS1) gene [95] that directly inhibits APC expression, one of the major negative regulators of Wnt/β-catenin signaling [93]. Moreover, both direct and indirect activation by HCV viral proteins may explain the notable dysregulation of Wnt/β-catenin signaling in hepatitis C and related HCC subclass. Moreover, HCV core, NS3 and NS5A proteins may facilitate further oncogenic transformation of infected hepatocytes [79] by suppression of DNA repair mechanisms, potentially causing *CTNNB1*

mutations. Support for this idea could be found in the observation that in experimental animals hepatocarcinogenic nitrosamine diethylnitrosamine (DEN) provokes cancer by inducing *CTNNB1* mutations [96, 97] and thus increased mutagenic pressure through corrupting DNA repair may be preferentially associated with this mutation. Hence effects on the DNA repair machinery exerted by HCV core, NS3 and NS5A may link increased Wnt/β-catenin signaling mediated by direct effects of these proteins early in infection to mutation-mediated activation of Wnt/β-catenin signaling later in the progression to HCC.

Wnt/β-catenin signaling paves the way for chronic hepatitis C to HCC

Inflammation

The HCV virus battles with the immune system. Thus negative modulation of inflammatory responses through enhanced Wnt/β-catenin signaling could conceivably provide further selection pressure of HCV to acquire Wnt/β-catenin signaling-activating properties. The effect of Wnt/β-catenin signaling, however, on hepatocyte immune responses remains controversial. On one hand, Wnt/β-catenin signaling could suppress the immune response by blunting T cell activation [98, 99], reducing TNF release [100] or stimulating the production of the chemokine-like chemotactic factor leukocyte cell-derived chemotaxin 2 (LECT2) and invariant NKT cells (iNKT) responses, both of which relay antiinflammatory response [101]. On the other hand, Wnt/β-catenin signaling triggers inflammatory responses by activating the pro-inflammatory NF-κB pathway, as evident from experimentation in a hepatocyte-specific APC and LECT2 knockout ($APC^{-/-}LECT2^{-/-}$) mouse model [101]. In potential agreement, germline genetic variations in Wnt/β-catenin signaling elements were significantly associated with the risk for inflammation in HCV-infected males [102]. Thus the issue as to how HCV-elicited Wnt/β-catenin signaling relates to HCV-provoked inflammation warrants further experimentation.

Fibrosis to cirrhosis and HCC development

Chronic inflammation evoked by HCV infection may culminate in liver fibrosis. Such fibrosis progresses gradually and disrupts liver physical structure and function over the course of several decades, finally resulting in fatal diseases such as cirrhosis and HCC [103]. Given HCV-stimulation of Wnt/β-catenin signaling probably evolved to support the early phases of viral infection, emerging data suggest that activated Wnt/β-catenin signaling by HCV participates

in the pathogenesis of liver fibrosis as well [102, 103], mainly by enhancing hepatic stellate cell (HSC) activation and survival [104]. The subsequent progression toward full-blown HCC is a complex process involving many various signaling pathways, but especially crosstalk between epidermal growth factor receptor (EGFR) signaling and fibroblast growth factor (FGF) receptor signaling and aberrant activation of Wnt/β-catenin signaling appears important here.

The EGFR pathway controls a variety of signals ranging from cell proliferation, cell motility, apoptosis decrease, to epithelial mesenchymal transition, upregulation of matrix metalloproteinases (MMP), and even stem cell maintenance [105]. EGFR is highly expressed in the adult liver [106] and plays an essential role in the G1/S phase transition for hepatocyte proliferation [107]. EGFR pathway dysregulation has been reported in 60% to 80% of HCC patients [108], and associated with the late stages and the degree of tumor differentiation [109, 110]. EGFR favors HCV entry through co-internalization of a HCV-CD81-EGFR complex following binding of EGFR ligands to the receptor and subsequent endocytosis [111, 112]. Following clathrin-mediated endocytosis of the EGFR, the receptor is routed for eventual intracellular degradation [113]. The viral NS5A protein, however, perturbs EGFR trafficking and degradation, increasing EGFR signaling and contributing to HCV-mediated HCC development [114]. Binding of Wnt1 and Wnt5a to FZD transactivates EGFR signaling by MMP-mediated release of soluble EGFR ligands, such as TGFα [115]. Activated β-catenin might form heterodimers with EGFR to enhance EGFR pathway activation [116]. Conversely, EGFR signaling contributes to Wnt/β-catenin signaling in various ways. Firstly, EGFR can directly induce tyrosine phosphorylation of β-catenin at residue Y654, thereby decreasing the binding with cell-adhesion complexes and releasing it for nuclear signaling [104, 117]. In fact, this phenomenon has been observed for a large number of growth factors signaling through receptor tyrosine kinases, such as HGF and FGFs that are produced in excess by the cirrhotic tissue adjacent to tumor tissue [28, 118-120]. Secondly, EGFR stimulates the PI3K/Akt and Ras/Raf/MEK/ERK cascades that both can promote β-catenin signaling through inhibiting GSK3β activity [121-125] (Fig.3). Thus HCV-mediated activation of Wnt/β-catenin signaling may initiate a vicious interaction between EGFR and Wnt signaling, promoting potentially pro-oncogenic hepatocyte proliferation.

Similar to the EGFR pathway, FGF-initiated signaling is a cardinal regulator of hepatocyte proliferation, differentiation, embryonic development and organogenesis as well as hepatic

tumorigenesis [126, 127]. Especially in chronic hepatitis C-associated HCC, activation of FGF signaling is observed [128, 129] and increased FGF levels are associated with enhanced HCV replication and release of infectious particles [130]. Crosstalk of Wnt and FGF pathways in HCV-related HCC is supported by observations that FGF signaling leads to the release of β-catenin from the β-catenin/E-cadherin complexes due to the phosphorylation of Tyr654 as described above. Furthermore, FGF2 increases expression of β-catenin mRNA, upregulates β-catenin nuclear translocation and inactivates GSK3β [131], probably mediated through activation of PI3K/Akt and Ras/Raf/MEK/ERK pathways. Conversely, Wnt/β-catenin signaling is able to activate FGF signaling by increasing *FGF18* and *FGF20* expression [132] (Fig.3). Thus again, vicious interaction between Wnt/β-catenin signaling and FGF signaling appears to occur.

Of interest, it has been reported that the Src homology region 2 domain-containing phosphatase-2 (SHP-2) can be activated by HCV structural E2 protein [133]. Thus conceivably SHP-2 may be an effector on EGFR and FGF signaling in HCV related HCC. Overexpression of SHP-2 promotes liver tumor cell growth and metastasis by coordinately activating not only PI3K/Akt and Ras/Raf/MEK/ERK pathways [121] but also Wnt/β-catenin signaling [134]. The latter effect is due to tyrosine dephosphorylation of parafibromin/Cdc73, acting as a tumor suppressor inhibiting *CYCLIND1* and *C-MYC*, together with SUV39H1. As a result, parafibromin acquires the ability to bind β-catenin stably, overriding the repression effect and inducing the expression of Wnt target genes [134] (Fig.3). Together, these results suggest that SHP-2 is one of the critical molecules enhanced during early HCV infection and contributes to the later progression to final HCC, which needs further investigation.

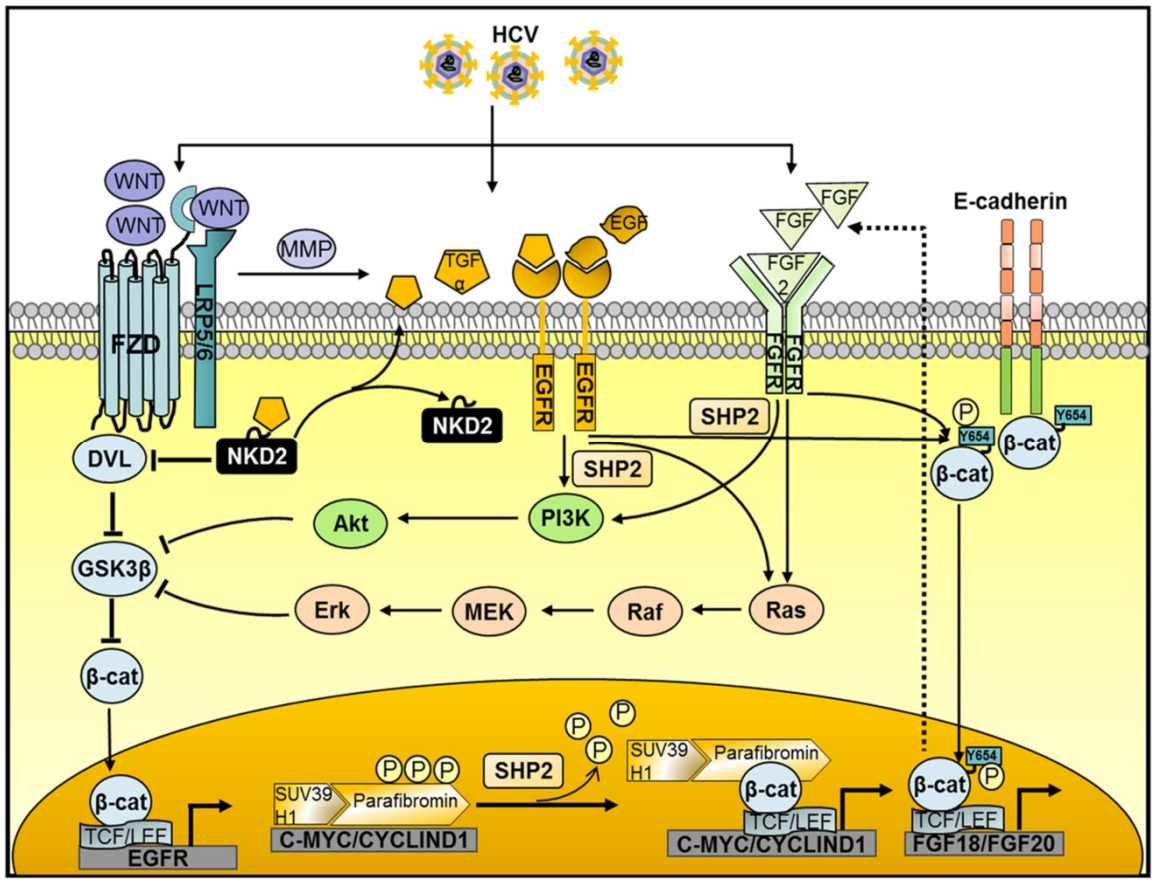

Figure 3. Crosstalk of Wnt/β-catenin pathway with EGFR and FGF pathways in HCV related HCC. HCV promotes Wnt signaling as well as EGFR and FGF pathways. The Wnt/β-catenin and EGFR pathways activate each other. Binding of Wnt ligands with FZD receptors transactivates EGFR signaling by MMP-mediated release of soluble EGFR ligands. EGFR signaling transactivates Wnt/β-catenin signaling through PI3K/Akt and Ras/Raf/MEK/Erk pathways but also by releasing β-catenin from β-catenin/E-cadherin complexes due to residue Tyr654 phosphorylation. Activated β-catenin forms heterodimers with EGFR and in turn promote EGFR pathway. On the other hand, Wnt signaling stimulates FGF signaling by inducing FGF18 and FGF20 ligand expression. In turn, the association of FGF19 to FGFR leads to the release of β-catenin from the β-catenin/E-cadherin complexes. FGF2 signaling inhibits GSK3β activity through PI3K/Akt and Ras/Raf/MEK/Erk pathways. Activated SHP-2 in both PI3K/Akt and Ras/Raf/MEK/Erk pathways dephosphorylates parafibromin which acquires the ability to bind β-catenin stably, overriding the repression effect on the *CYCLIND1* and *C-MYC* expression and triggering downstream signaling.

Conclusion

As one of the important cascades involved in HCV-related HCC initiation and development, Wnt/β-catenin signaling is aberrantly activated by HCV viral core and NS5A proteins. In turn, stimulated Wnt/β-catenin signaling promotes progression of hepatitis C during inflammation and fibrosis eventually promoting cirrhosis and HCC. This interaction is further aggravated by a vicious circle involving the EGFR and FGF pathways.

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Chapter 3

Blocking Wnt secretion reduces growth of hepatocellular carcinoma cell lines mostly independent of β-catenin signaling

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Abstract

Aberrant activation of Wnt/β-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 β-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 knock-down of β-catenin impairs growth of all these lines. Blocking Wnt secretion, either by 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 β-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 β-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.

Keywords: Hepatocellular carcinoma; Wnt/β-catenin signaling

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 (HBV) or Hepatitis C virus (HCV) infection, alcohol liver disease, non-alcoholic steatohepatitis (NASH) and aflatoxin-B1 exposure [3]. More than 80% of all HCCs occur in Eastern and Southeastern Asia where the main cause is HBV combined with exposure to aflatoxin-B1 [4]. In Europe, Japan and the United States, HCV represents the dominant risk factor, together with alcohol abuse and nonalcoholic fatty liver diseases [5, 6].

Wnt/β-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/β-catenin signaling that strongly contributes to tumor growth [10]. Activation of Wnt/β-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, β-catenin is phosphorylated at N-terminal Ser/Thr residues by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1α. Phosphorylated β-catenin is then ubiquitinated, leading to its proteasomal degradation [12-14]. The overall effect is low β-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 β-catenin destruction complex [15]. As a result, β-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/β-catenin target genes thereby affecting cellular decisions [4]. In addition to this classical (canonical) Wnt signaling pathway, Wnts can also signal in an alternative (non-canonical) fashion independent of β-catenin through associating with Frizzled and ROR1/2 receptors instead of

LRP5/6. Activation of non-canonical 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/β-catenin signaling is critical in hepatic oncogenesis [19, 20]. About 40%-70% of HCCs are characterized by nuclear accumulation of β-catenin, the hallmark of active signaling. Various molecular and genetic alterations contribute to aberrant activation of Wnt/β-catenin signaling. Mutations within components of the canonical Wnt/β-catenin signaling enhance stabilization of β-catenin and transcriptional activity in the nucleus. Approximately one third of all HCCs carry oncogenic β-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 1-3% and 8-15% of tumors [19], both causing compromised ability to degrade β-catenin [8]. In addition to mutations, various other mechanisms have been suggested to promote β-catenin signaling, including overexpression of Wnt ligands and/or their corresponding receptors, and reduced expression of extracellular inhibitors [22]. Given the importance of β-catenin signaling for hepatic oncogenesis, various treatments targeting this route have been evaluated [23].

Cancers harboring mutations within intracellular components of the β-catenin signaling pathway, i.e., mutation of APC, AXIN or β-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 β-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 β-catenin signaling. Here, we have investigated whether this also holds truth for β-catenin or AXIN1 mutant liver cancer cells.

Materials and methods

Cell lines

Human HCC cell lines Hep3B, Huh6, Huh7, PLC/PRF/5, SNU182, SNU398, SNU449 and CRC cell lines CACO2, DLD1, HT29, SW480, HCT116, LS174T, SW48 and RKO were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 µg/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 µg/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 µg/ml streptomycin, 5 µg/ml insulin (Sigma-Aldrich, St Louis, MO), and 50 µM 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 COSMIC, 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 DMEM 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 10mM. Antibodies specific for β-catenin (Cat. #9561, Cell Signaling Technology), dishevelled adaptor protein (DVL2) (Cat. #3216, Cell Signaling Technology), WLS (Cat.#MABS87**,** clone YJ5 Millipore), LC3 Ⅰ/Ⅱ (Cat. #4108, Cell Signaling Technology), GPR177(Wls/Evi) (Cat.#MABS87, Millipore) and β-actin (sc-47778, 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.

Table1. Gene mutations of Wnt/β-catenin signaling components in HCC and CRC cell lines

Cell line	Gene	AA alteration	Zygosity
HCC			
HepG2	*CTNNB1*	p.W25_I140 del	Heterozygous
Huh6	*CTNNB1*	p.G34V	Heterozygous
SNU398	*CTNNB1*	p.S37C	Heterozygous
Hep3B	*AXIN1*	p.R146*	Homozygous
PLC/PRF/5	*AXIN1*	p.(R373_M418 del)	Homozygous
SNU449	*AXIN1*	p.R712*	Homozygous
Huh7			
HepaRG			
SNU182			
CRC			
SW480	*APC*	p.Q1338*	Homozygous
HT29	*APC*	p.T1556fs*3	Heterozygous
		p.E853*	Heterozygous
Caco2	*APC*	p.Q1367*	Homozygous
DLD1	*APC*	p.R2166*	Heterozygous
		p.I1417fs*2	Heterozygous
	AXIN1	p.L396M	Heterozygous
	RNF43	p.G659fs*41	Heterozygous
SW48	*CTNNB1*	p.S33Y	Heterozygous
	RNF43	p.G659fs*41	Heterozygous
		p.V299fs*143	Heterozygous
HCT116	*CTNNB1*	p.S45del	Heterozygous
	RNF43	p.R117fs*41	Heterozygous
LS174T	*CTNNB1*	p.S45F	Homozygous
RKO	*RNF43*	p.G659fs*41	Heterozygous

Gene knockdown by small interfering RNA (siRNA)

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

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). 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

StepOnev2.0 software (Applied Biosystem, Darmstadt, Germany). Primer sequences are provided in supplementary Table1. 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 5-10 minutes at 95 ℃, followed by loading and separation on a 8-15% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h 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 (PBS-T), followed by incubation with primary antibody (all 1:1000) overnight at 4 °C. The membrane was washed 3 times with PBS-T followed by incubation for 1.5 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for β-actin or Tubulin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantified with Odyssey 3.0 software.

β-catenin reporter assays

The β-catenin reporter assays were basically performed as previously described [30, 31]. In short, twenty 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 β-catenin reporter activities are shown as WRE/MRE ratios.

MTT assay

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% CO2 for 3 h. The medium

was removed and 100 µl 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, 4 independent wells were assayed for each cell line at least two times, whereas for IWP12 treatment 6 independent wells were used. The mean and standard error were calculated for each condition.

Cell cycle analysis

Around 60%-80% confluency, cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4℃. The cells were washed twice with PBS and incubated with 20ug/ml RNase at 37°C for 30 min followed by incubation with 50ug/ml Propidium Iodide (PI) at 4°C for 30min. 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 ± SD. Comparisons between groups were performed with one sample t test. Differences were considered significant at a *p* value less than 0.05.

Results

β-catenin signaling activity of HCC cell lines

To investigate the importance of β-catenin signaling and Wnt secretion for sustaining cell growth, we employed 9 HCC cell lines, listed in Table 1 in which gene mutations related to Wnt/β-catenin signaling are depicted. We also used 8 CRC cell lines for comparison in various assays, known to largely depend on β-catenin signaling for their growth. First, we determined the baseline β-catenin signaling activity for all these cell lines using a β-catenin reporter assay and qRT-PCR of *AXIN2*, a well-established β-catenin target gene. As indicated in Figure 1, in line with previous publications, all β-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 β-catenin signaling activity, both on reporter level and *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).

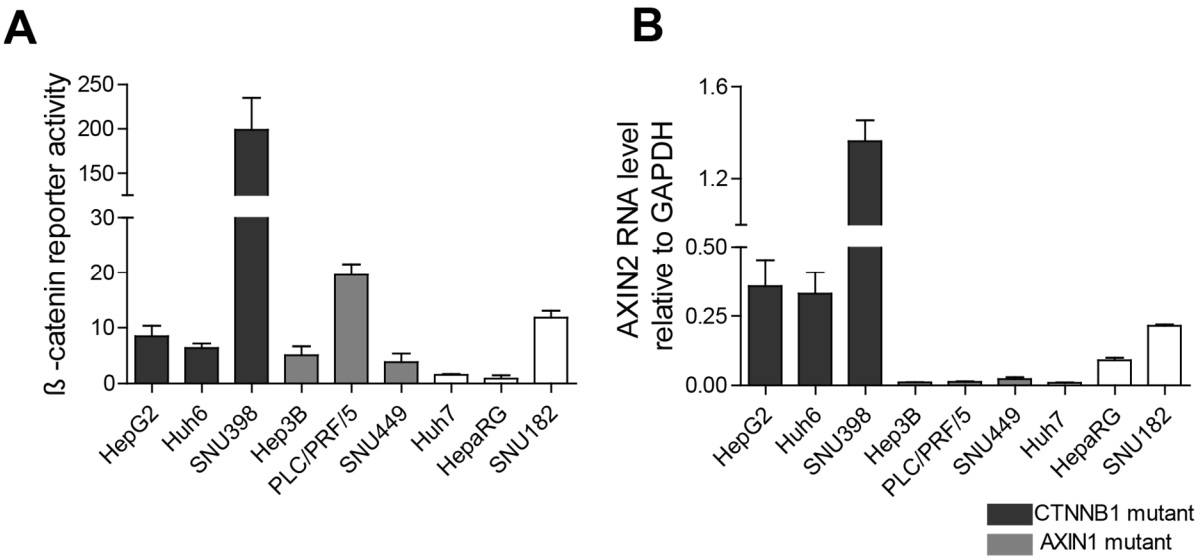

Fig.1. Baseline β-catenin signaling activity in HCC cell lines. (A) β-catenin luciferase reporter assay showing β-catenin signaling activity in HCC cell lines (mean ± SD, n = 3). The β-catenin reporter activities are depicted as WRE/MRE ratios. (B) QRT-PCR assay showing expression of the β-catenin target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

Requirement for β-catenin signaling to sustain efficient cell growth

Next, we determined the dependence on β-catenin signaling for supporting efficient growth by transiently transfecting smartpool siRNAs targeting *CTNNB1* or a control siRNA, followed by a MTT assay to test cell numbers after 3 days of culture. For most cell lines we accomplished more than 80% knock-down of β-catenin at protein level as determined by quantitative western blot analysis, with the exception of HepaRG in which a 65% reduction was observed (see Figures 2A and S2). As indicated in Figure 2B, β-catenin signaling activity was clearly suppressed by siRNA mediated 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 β-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 β-catenin knockdown provoked a dramatic G0/G1 phase arrest in Huh6 (Fig 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 towards 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 β-catenin knockdown (Figure 2E).

Expression levels of WNT ligands in HCC cell lines

Taken together, the results above indicate that targeting the β-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 β-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 β-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 β-catenin signaling activity reported above. The β-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

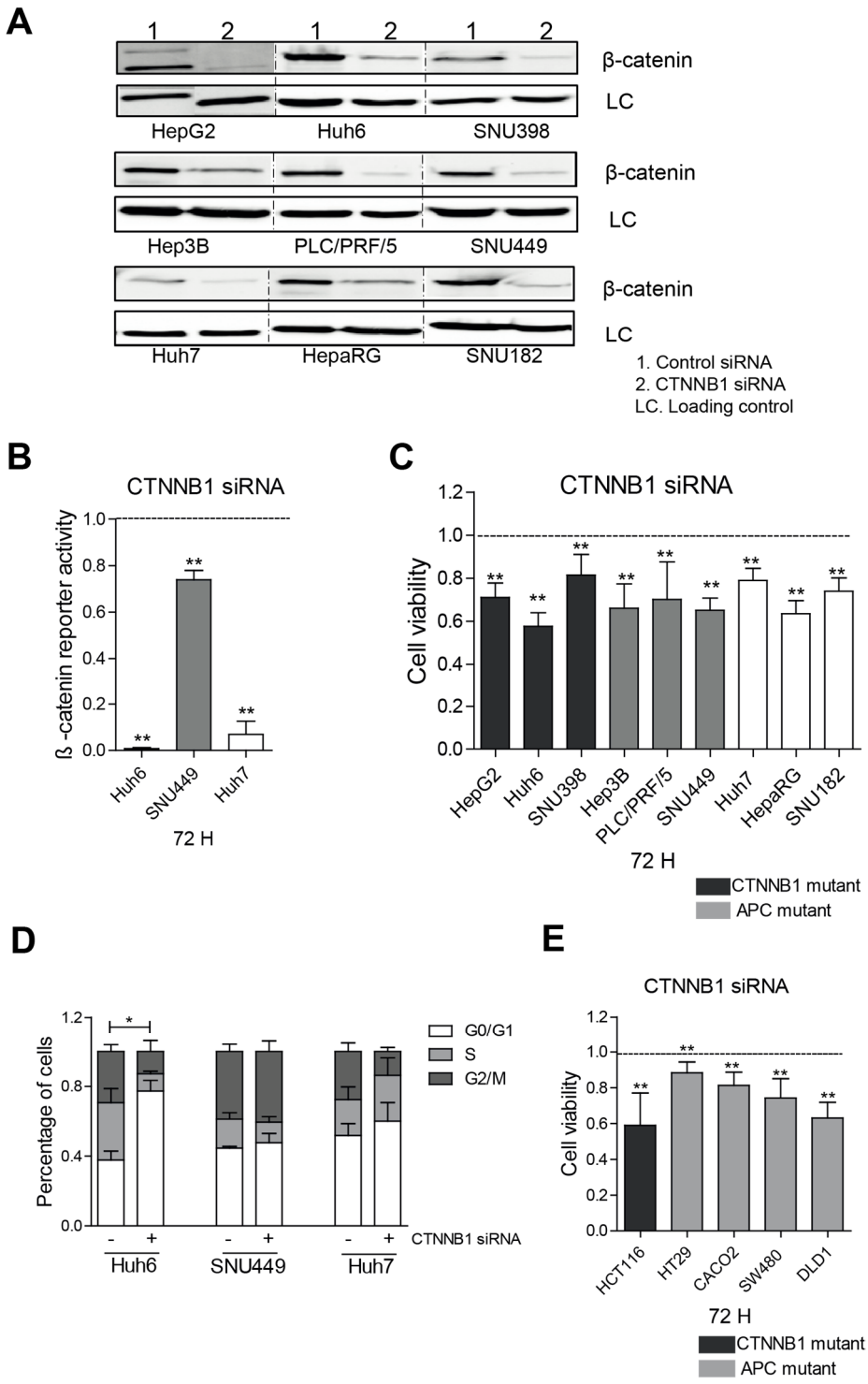

Fig.2. Requirement of β-catenin signaling for sustaining HCC cell growth. (A) HCC cell lines were transiently transfected with *CTNNB1* siRNAs for 72 hours. Cell lysates were collected for western blotting with indicated antibodies. Tubulin and β-actin served as loading control (LC). (B) Silencing of β-catenin caused the reduction of β-catenin signaling activity (mean ± SD, n=2, two times). (C) Silencing of β-catenin inhibited the growth of HCC cell lines as determined by MTT assay (mean ± SD, n=4, two times). (D) Silencing of β-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 ± SD, n=2, two times). (E) β-catenin knockdown reduced cell growth in colorectal cancer cell lines. Values depicted are relative to the ones obtained with the non-targeting siRNA that are arbitrarily set to 1. *p<0.05; **p<0.01.

most cell lines. Overall, SNU182 is again the most prominent expressing cell line, whereas the β-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, while *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, highest levels of phosphorylated DVL2 are observed in the three cell lines with 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 β-catenin signaling.

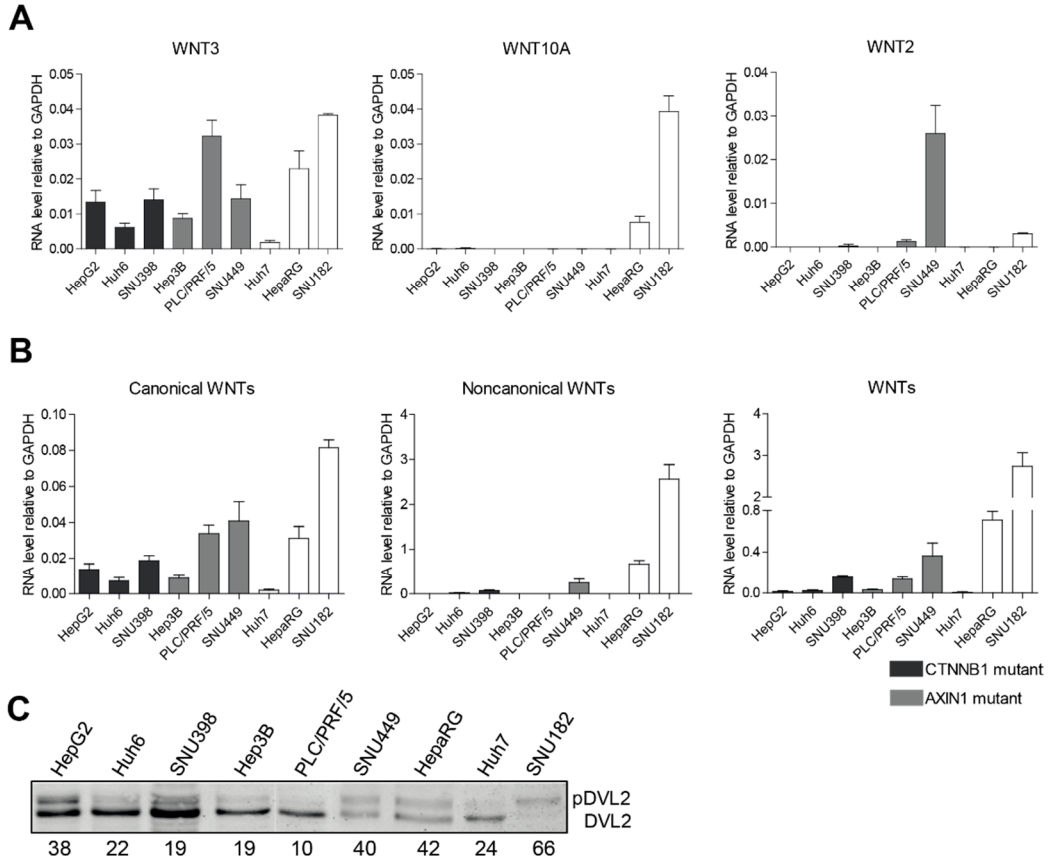

Fig.3. Expression levels of Wnt family members in HCC cell lines determined by qRT-PCR. (A) Expression levels of the top three expressed "canonical" Wnt ligands (mean ± SD, n=3). (B) Piled-up expression levels of canonical, non-canonical and overall Wnt ligands, respectively. All expression levels are 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).

Wnt secretion blockage reduces growth of HCC cells

Given that most HCC cell lines show increased β-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 three days of IWP12 treatment, reduced cell numbers were observed for all β-catenin mutant cell lines (Figure 4A), ranging from 10% reduction (Huh6) to 35% (SNU398). Among the non-mutant lines, growth of HepaRG was strongly suppressed by IWP12, whereas Huh7 and SNU182 showed more modest 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.

For comparison, the same assays were also performed on five CRC cell lines (Figure 4C,D). Both treatments showed the strongest growth suppression when applied to the β-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.

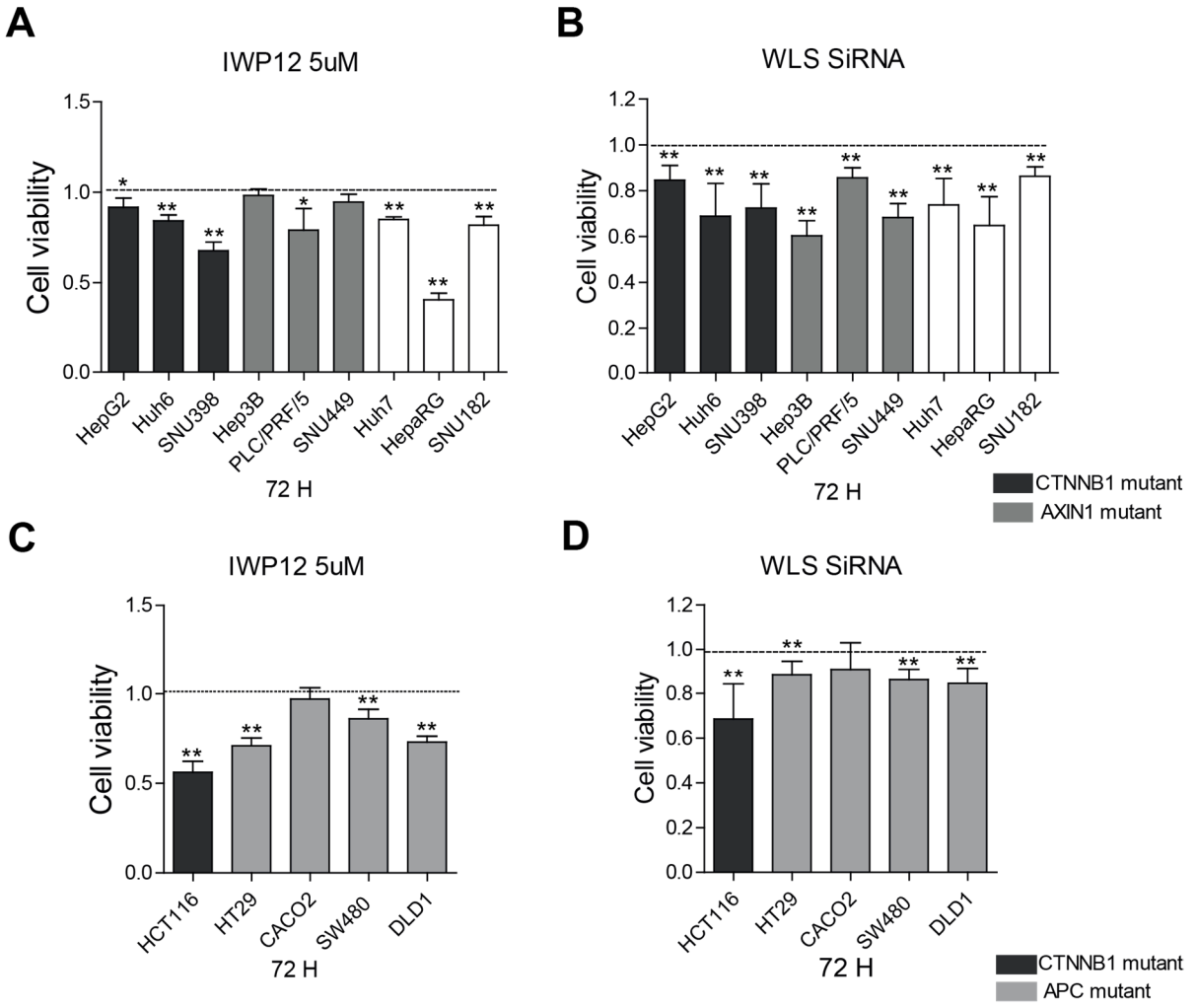

Fig.4. Inhibition of growth by Wnt secretion blockage. (A, C) IWP12 reduced cell growth of most HCC and CRC cell lines. Cell lines were incubated with 5 µM IWP12 for 72 hours and tested by MTT assay (mean ± SD, n=6, two times). Values depicted are relative to cell numbers obtained with the control DMSO treatment that are set to 1. (B, D) Cell lines were transiently transfected with *WLS* or non-targeting siRNAs for 72 hours, followed by MTT assay (mean ± SD, n = 4, two times). Values depicted are relative to cell numbers obtained with the non-targeting siRNA that are set to 1. **p*<0.05; ***p*<0.01.

Altered exposure to extracellular Wnt ligands does not affect β-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/β-catenin signaling activity in both APC and β-catenin mutant CRC cell lines [26]. Here, we asked whether the β-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 β-catenin signaling activity using the reporter assay and *AXIN2* qRT-PCR. After 48 hours, β-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 β-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 β-catenin signaling of increased levels of extracellular Wnt ligands, we exposed them to L-Wnt3A conditioned medium. As shown in Figure 5C, β-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 β-catenin or AXIN1 mutant HCC lines showed enhanced β-catenin signaling following the addition of L-Wnt3A conditioned medium.

These results indicate that β-catenin and AXIN1 mutant HCC cell lines appear largely insensitive to the level of Wnt ligand exposure for sustaining intracellular β-catenin signaling, which could either mean that the expressed mutant β-catenin or AXIN1 protein determine 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 upregulation 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 non-mutant 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 both be 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.

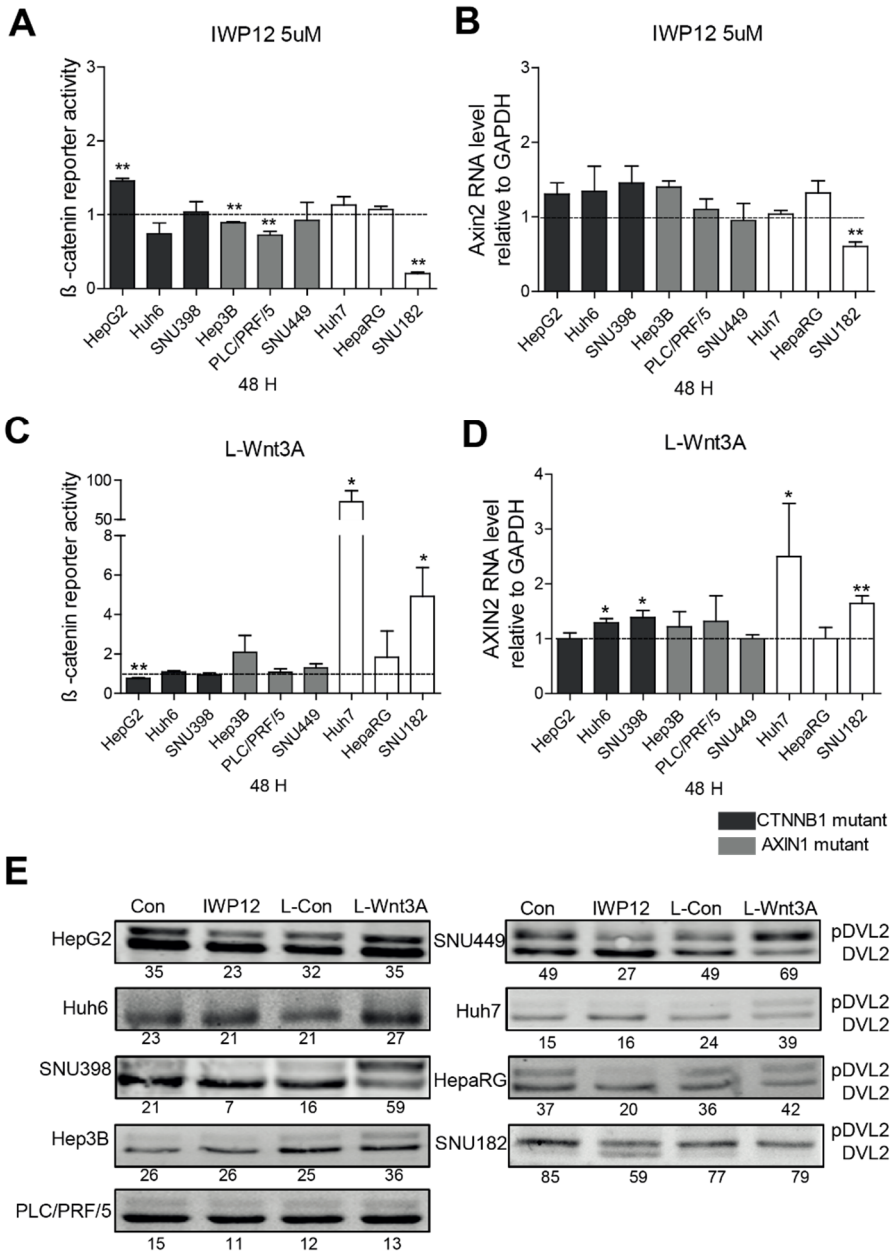

Fig.5. Growth reduction due to decreased Wnt secretion appears independent from β-catenin signaling. (A) β-catenin reporter activity was not clearly reduced by IWP12 in most HCC cell lines, except for SNU182 (mean ± SD, n=3). (B) QRT-PCR for *AXIN2* showed that its expression following IWP12 treatment was only reduced significantly in the SNU182 cell line (mean ± SD, n=3, two times). (C) L-Wnt3A conditioned medium significantly promoted β-catenin signaling activity in Huh7 and SNU182 cell lines. A significant albeit modest reduction of reporter activity was observed in HepG2. (mean ± SD, n=3). (D) Increased β-catenin signaling in SNU182 and Huh7 following L-Wnt3A treatment was confirmed by *AXIN2* qRT-PCR (mean ± 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*<0.05; ***p*<0.01. (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).

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 β-catenin reporter activity as well as *AXIN2* expression (Figure 6A,B). *AXIN2* expression was slightly reduced in HT29 and SW480, while 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,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.

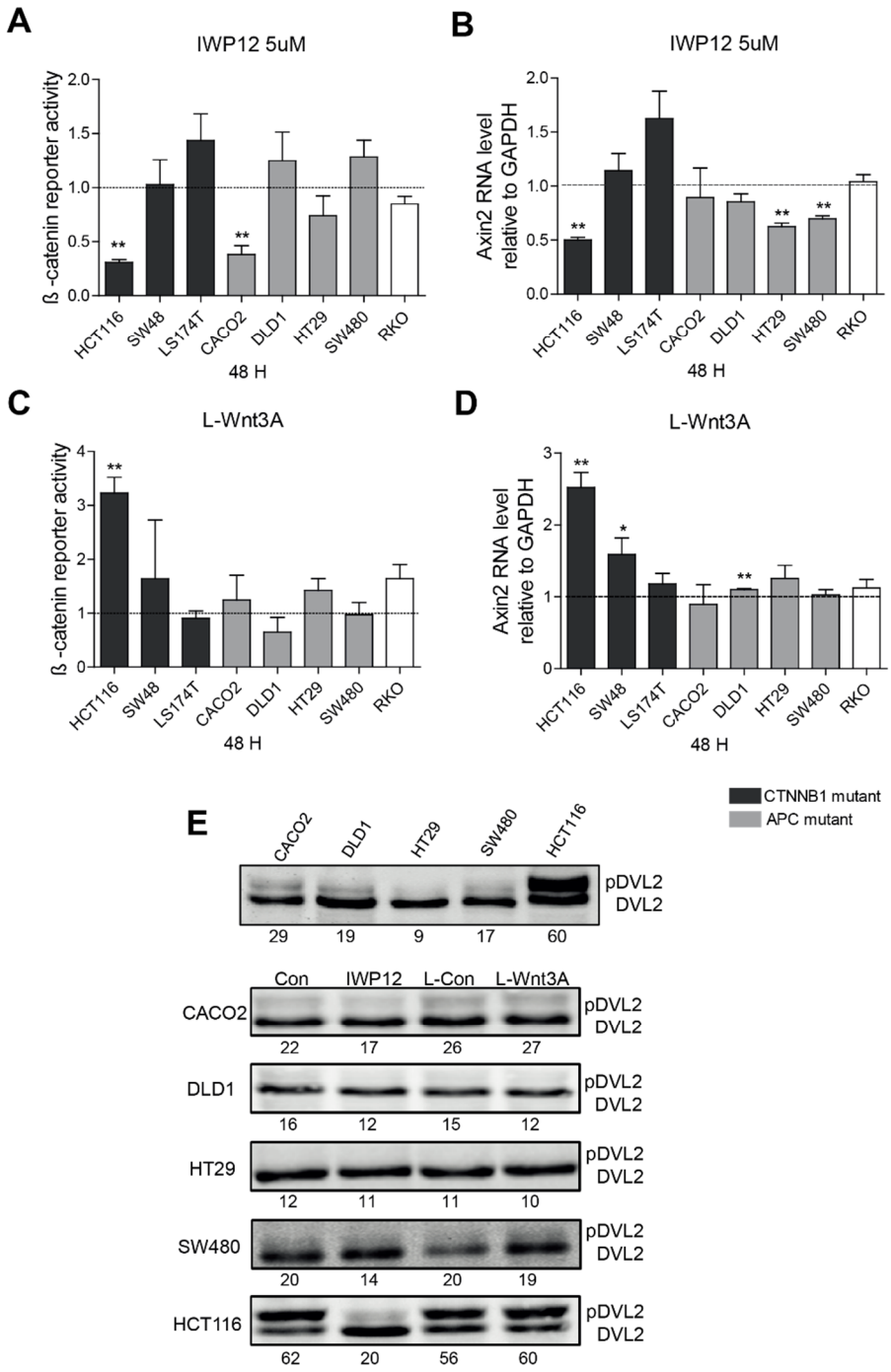

Fig.6. Responsiveness of CRC cell lines to alterations in Wnt ligand levels. (A) β-catenin reporter activity was significantly reduced by IWP12 in CACO2 and HCT116 cell lines (mean ± SD, n=3). (B) QRT-PCR for *AXIN2* showed that IWP12 treatment reduced its expression in HCT116. Modest but significant 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-Wnta3A 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*<0.05; ***p*<0.01. (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).

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 β-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 read-out. As shown in Figure 7A and 7B, expression of *CHOP* was clearly increased in SNU398 (4.5-fold), whereas *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.

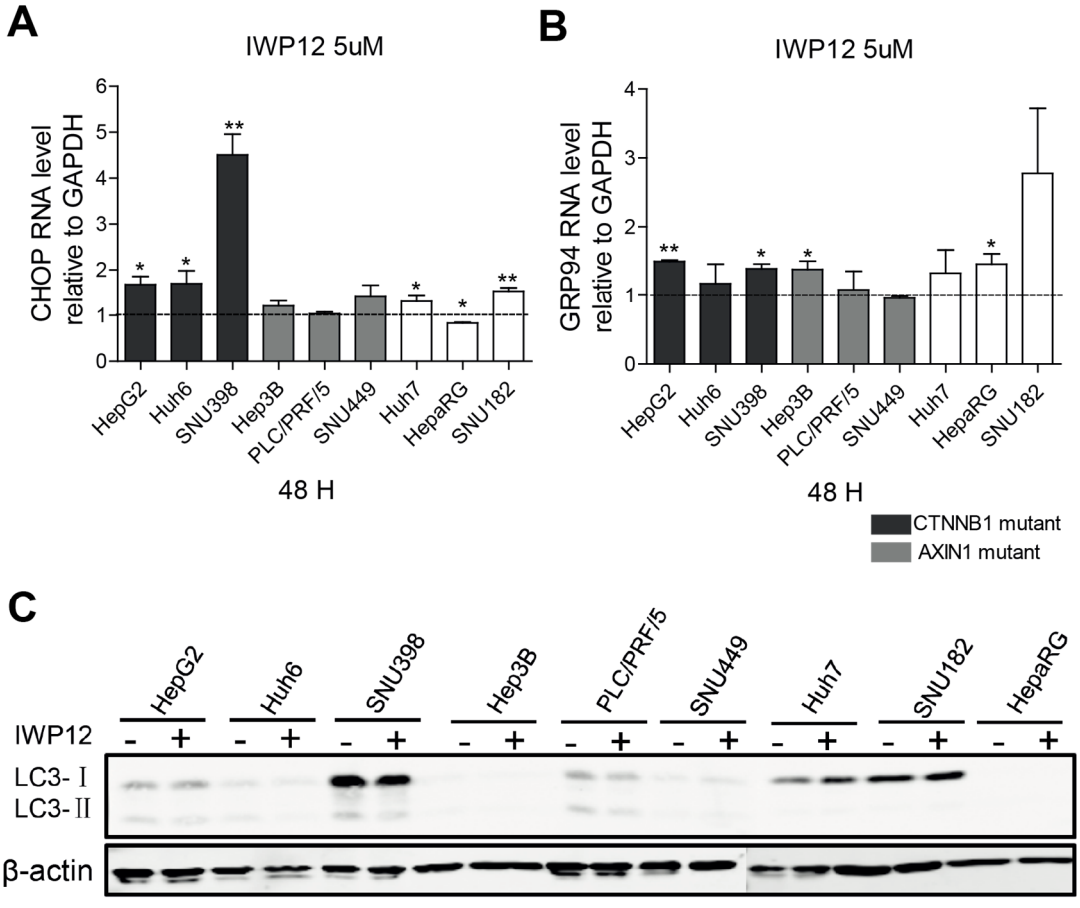

Fig.7. IWP12 treatment does not lead to the induction of a strong ER stress response or autophagy. Following 48 hours of IWP12 treatment, the expression of the ER stress induced response genes *CHOP* (A) and *GRP94* (B) was evaluated by qRT-PCR (mean ± SD, n=3). Except for a clear induction of *CHOP* 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*<0.05; ***p*<0.01. (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.

Wnt secretion inhibition does not induce autophagy

Since knockdown of β-catenin or suppression of β-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 β-catenin signaling and Wnt secretion for sustaining hepatocellular carcinoma growth. Using a panel of 9 HCC cell lines we show that β-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 β-catenin mutations in which the activating mutation will have provided a selective growth advantage during tumor formation, but it also holds true for the non-mutant 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 β-catenin signaling within liver cancers. This subset of tumors apparently lacks a robust nuclear β-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 β-catenin reporter activities approaching those of the β-catenin mutant ones. Given that also these lines are suppressed in their growth following β-catenin knockdown, it shows that the majority of HCCs independent of their mutational profile, rely on β-catenin signaling for optimal growth.

Besides its role in signaling, β-catenin is also involved in cell-cell adhesion by directly binding to cadherins [41]. As such, the siRNA mediated knock-down that we apply here, will also likely reduce the amount of β-catenin sequestered at these adherens junctions. However, several investigations have shown that complete loss of β-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 ɣ-catenin compensates for its loss, thereby retaining normal cell adhesion. Importantly, these studies show that it is mainly the signaling function of β-catenin that is affected following knock-down.

Next we addressed the question to what extent extracellular exposure to Wnt ligands contributes to the observed levels of β-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 semi-quantitative 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 non-mutant lines, SNU182 also showed the highest β-catenin reporter activity and level of *AXIN2* expression, comparable with the β-catenin mutant ones. As such, it is not unexpected that this cell line strongly relies on Wnt ligand secretion to retain increased β-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 non-mutant 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 β-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 β-catenin and AXIN1-mutant HCC cell lines are clearly affected in β-catenin signaling upon alterations in Wnt ligand exposure, irrespective of their source, suggesting that the expressed oncogenic β-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 β-catenin mutant CRC lines that we investigated. Among 8 CRC lines tested, only HCT116 shows a strongly reduced reporter activity and *AXIN2* levels following IWP12 treatment, while it is also the only one in which both β-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 β-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 β-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 or 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 hyper-responsiveness to Wnt ligands. These genetic aberrations have been identified in 10-20% 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 β-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 β-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 "non-canonical" 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 observe. 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 β-catenin signaling for maintaining optimal growth. Extracellular exposure to Wnt ligands has a minor contribution to overall β-catenin signaling strength in the β-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 Figures and Tables

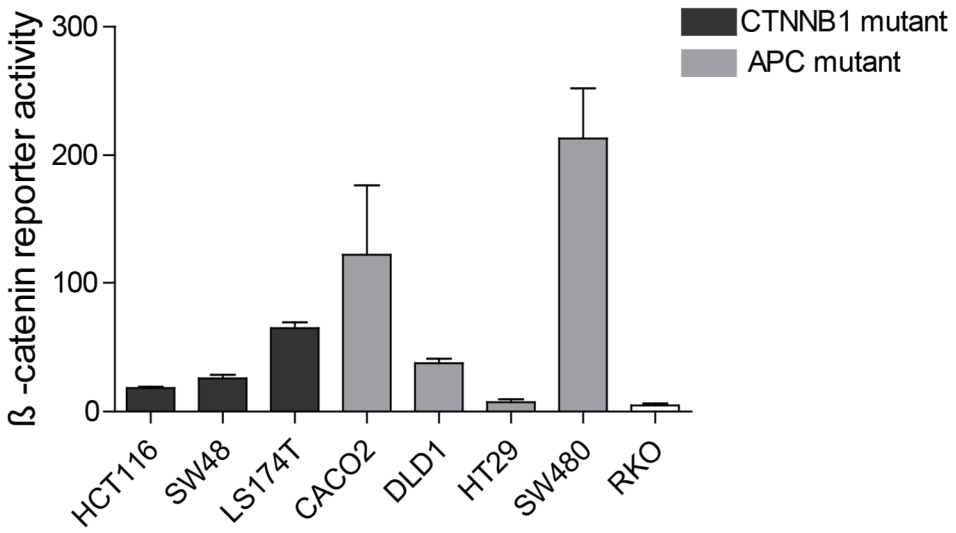

Fig.S1. Wnt/β-catenin signaling activity in CRC cell lines determined by a β-catenin luciferase reporter assay (mean ± SD, n=3).

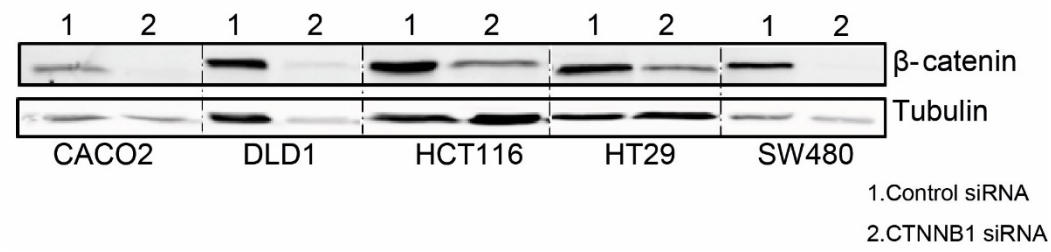

Fig.S2. SiRNA mediated silencing of β-catenin in CRC cell lines confirmed by western blotting.

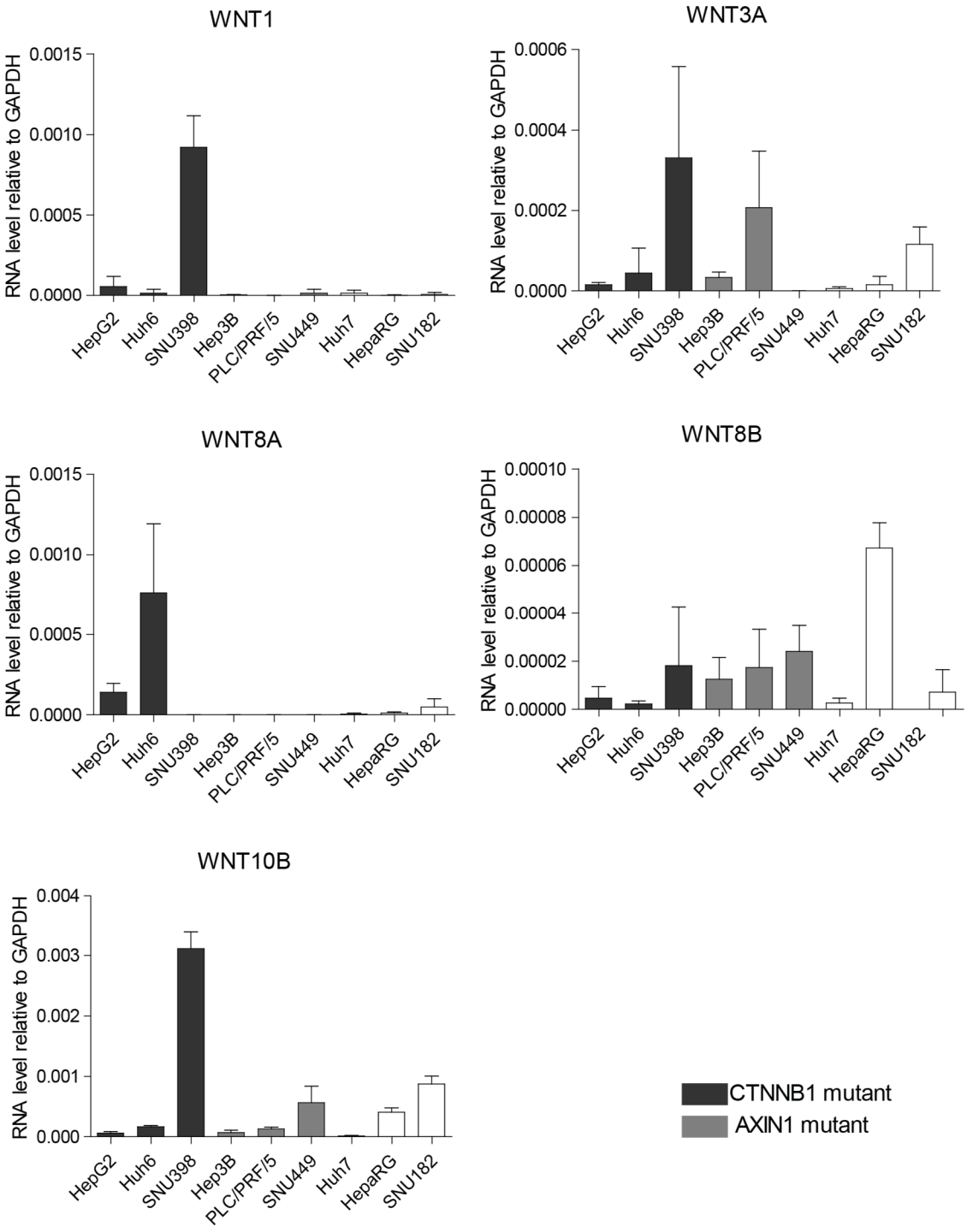

Fig.S3. Expression levels of remaining "canonical" Wnt ligands in HCC cell lines tested by qRT-PCR (mean ± SD, n =3).

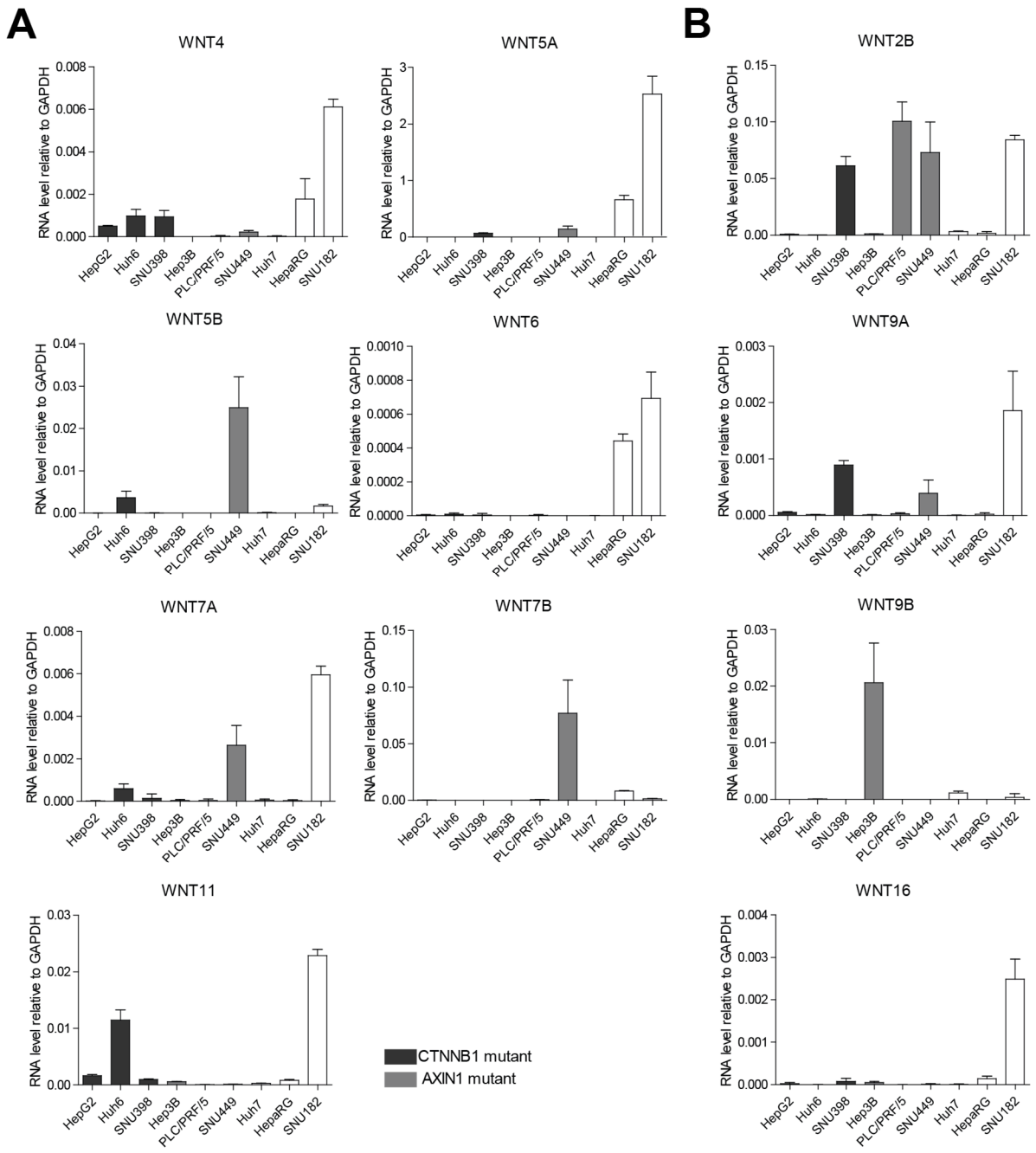

Fig.S4. Expression levels of "non-canonical" (A) and unclassified (B) Wnt ligands in HCC cell lines tested by qRT-PCR (mean ± SD, n =3).

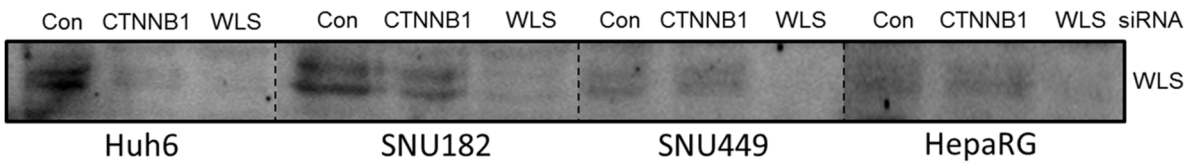

Fig.S5. SiRNA mediated silencing of *WLS* in HCC cell lines tested by western blotting.

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Chapter 4

Evaluation of AXIN1 and AXIN2 as targets of tankyrase inhibition in hepatocellular carcinoma cells

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In preparation

Abstract

Aberrantly activated Wnt/β-catenin signaling plays an important role in the initiation and progression of hepatocellular carcinoma (HCC). The ADP-ribose polymerase tankyrase enzymes promote AXIN turnover, an important negative regulator of β-catenin signaling, thereby enhancing its signaling activity. Tankyrase inhibitors destabilize β-catenin and are considered promising therapeutics for β-catenin-driven cancers, which have been largely investigated in colorectal cancers. However, the knowledge regarding the efficacy of tankyrase inhibitors in HCC subgroups carrying distinct genetic mutations in components of the β-catenin signaling pathway, remains limited. Here we first show that the tankyrase inhibitor XAV939 does not clearly stabilize AXIN1 or AXIN2 protein in our panel of 9 HCC cell lines, with the exception of AXIN2 in the *CTNNB1* mutant ones. RNA analysis revealed low expression levels for *AXIN2* and especially for AXIN1, providing an explanation for the minimal protein accumulation that we observe. Nevertheless, tankyrase inhibition diminished β-catenin signaling in most non-*CTNNB1* mutant HCC cell lines. The reduced signaling activity was however not accompanied with a clear growth suppression in these lines. Next, using siRNA mediated knockdown of AXIN1, AXIN2 or combinations thereof, we investigate their contribution to β-catenin signaling regulation. Recently, several reports have suggested that *AXIN1* mutation does not lead to a significant induction of β-catenin signaling. However, our analyses show that in basically all non-*CTNNB1* mutant lines both AXIN1 and AXIN2 contribute to β-catenin regulation to varying levels depending on the cell line investigated, questioning the strong statements that have been made in this regard. Overall, our analyses show that both AXIN1 and AXIN2 contribute to the regulation of β-catenin signaling in HCC. Enhancing their activity by tankyrase monotherapy provides however no effective treatment to affect their growth.

Keywords: Hepatocellular carcinoma; Wnt/β-catenin signaling, tankyrase, AXIN1 and AXIN2

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third leading cause for cancer related deaths worldwide with around 500,000 new cases diagnosed each year (1, 2). Hepatocarcinogenesis initiates with the accumulation of aberrant genetic and epigenetic modifications leading to the dysregulation of signaling pathways, which transform the normal hepatocytes towards malignant phenotypes (3).

Inappropriate activation of Wnt/β-catenin signaling has been reported frequently in HCC (4). As the central component of Wnt/β-catenin signaling, the transcription factor β-catenin is tightly regulated by a multiprotein complex composed of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and the kinases GSK3 and CK1α (4, 5). In the absence of Wnt ligands, β-catenin is constitutively phosphorylated and degraded to maintain a minimal level in the cytoplasm. Upon Wnt stimulation, the multiprotein complex dissociates causing the accumulation of cytosolic and nuclear β-catenin, which in turn triggers the transcription of specific target genes. Aberrant activation of Wnt/β-catenin signaling in HCC has been mainly attributed to activating somatic mutations in the *CTNNB1* gene coding for β-catenin (20-25%) (4, 6-8). These mutations result in single amino-acid alterations or small in-frame deletions at N-terminal phosphorylation residues that make the protein more resistant to proteolytic degradation. For these activating mutations it is well-accepted that they support tumor growth by enhancing β-catenin signaling in a dominant fashion.

Another component of the Wnt/β-catenin signaling pathway regularly inactivated in HCC is *AXIN1* (10%), while inactivating mutations of the *AXIN2* (3-4%) and *APC* (1%~2%) genes are observed less frequently (4, 6, 7). Originally, given its prominent role in the β-catenin destruction complex, mutational inactivation of AXIN1 was considered to support HCC development by aberrantly enhancing β-catenin signaling. This view has however been challenged in the last decade by several reports showing neither a clear nuclear β-catenin accumulation nor enhanced expression of β-catenin target genes in *AXIN1*-mutant HCCs (9-11). In support, AXIN1 has also been shown to regulate the activity of other proteins relevant for tumorigenesis, such as MYC, P53 and SMAD3 (12-15). Other reports, including our own work, have provided some evidence of increased β-catenin signaling in *AXIN1* mutant HCC

cells, albeit modest (10, 16, 17). Hence, the extent of β-catenin signaling following AXIN1 mutation and its relevance for supporting HCC growth is still under debate.

Besides their frequent mutational inactivation in liver cancer, the AXIN proteins have gained substantial interest in cancer research for a second reason. Their activity in the β-catenin destruction complex can be increased by so-called tankyrase inhibitors, which thus may serve as a therapeutic option to reduce the growth of β-catenin-dependent cancers. The AXIN proteins, like β-catenin itself, are under tight proteolytic control. The poly-ADP-ribosyltransferases tankyrase-1 and -2 (encoded by *TNKS* and *TNKS2*) associate with the N-terminus of AXIN proteins, resulting in their PARsylation and subsequent RNF146 mediated protein ubiquitylation and degradation (18-21), thereby limiting the activity of the destruction complex. Blocking the catalytic activity of the tankyrases, first results in accumulation of the tankyrases themselves by inhibition of their auto-PARsylation, followed by AXIN accumulation. Next, so-called degradasomes are assembled in which all components of the β-catenin destruction complex aggregate to form large multiprotein complexes leading to an efficient β-catenin turnover. The formation of these degradasomes can be visualized as discrete cytoplasmic puncta within tankyrase inhibitor treated cells. A recent boom of interest focuses on the application of these tankyrase inhibitors for the treatment of breast (22), lung (23) and especially colorectal cancer (24-28), with some successful initial results for a subset of tumors. Investigating their potential to treat HCC has been limited to a single study in which high inhibitor levels blocked the growth of some liver cancer cell lines (29).

In this study, we employed *CTNNB1*, *AXIN1* and non-mutant HCC cell lines to investigate the impact of tankyrase inhibition on Wnt/β-catenin signaling as well as cell growth, and to further explore the function of AXIN1 and AXIN2 in regulating Wnt/β-catenin signaling in HCC cells.

Materials and methods

Cell lines

CTNNB1 mutant HepG2, Huh6, SNU398, *AXIN1* mutant Hep3B, PLC/PRF/5, SNU449 and non-mutant HepaRG, Huh7, SNU182 HCC as well as CRC (DLD1 and SW480) cell lines were cultured

as reported previously [Wang, 2016 #6]. The term "non-mutant" is used throughout the paper to indicate that these lines do not contain mutations in genes known to be linked to β-catenin signaling. Identity of all cell lines was confirmed by STR genotyping. Mutation status depicted in Table 1 was confirmed in all the nine HCC cell lines by Sanger sequencing and was consistent with those reported at COSMIC, the Catalogue Of Somatic Mutations In Cancer (http://cancer.sanger.ac.uk) (30).

Table.1 Gene mutations of Wnt/β-catenin signaling components in HCC cell lines

Cell line	Gene	AA alteration	Zygosity
HepG2	*CTNNB1*	p.W25_I140 del	Heterozygous
Huh6	*CTNNB1*	p.G34V	Heterozygous
SNU398	*CTNNB1*	p.S37C	Heterozygous
Hep3B	*AXIN1*	p.R146*	Homozygous
PLC/PRF/5	*AXIN1*	p.(R373_M418 del)	Homozygous
SNU449	*AXIN1*	p.R712*	Homozygous
Huh7			
HepaRG			
SNU182			

Reagents

XAV939 and IWR-1 were purchased from Sigma-Aldrich. Antibodies specific for β-catenin (610154, BD Transduction Laboratories™), phospho-β-catenin (Ser33/37) (#2009, Cell Signaling Technology), AXIN1 ((#2087 and #3323 Cell Signaling Technology), AXIN2 ((#2151, Cell Signaling Technology), Tankyrase-1/2 (sc-365897, Santa Cruz), β-actin (sc-47778, Santa Cruz) and anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) were used for western blot analysis.

β-catenin reporter assays

The β-catenin reporter assays were performed as previously described [van Veelen, 2011 #13]. In short, we plated 5×10^4 cells per well on 24-well plates, which were transfected with 250 ng Wnt Responsive Element (WRE) or Mutant Responsive Element (MRE) vectors and 10 ng CMV-Renilla using FuGENE® HD Transfection Reagent. We measured luciferase activities and

normalized the data for the transfection efficiency by using the Dual Luciferase Reporter Assay system.

MTT assay

After incubation with XAV939 for 72 hours, cells were analyzed by MTT assay as previously reported [Wang, 2016 #6]. The mean and standard error were calculated for each condition.

Western blotting

Cells were treated with XAV939 for 16 hours and then lysed for western blotting analysis as previously reported [Wang, 2016 #6]. Results were visualized with Odyssey 3.0 software.

Immunocytochemistry

Cells were seeded on glass coverslips. After 16 hours treatment with XAV939, cells were washed with PBS, fixed in PBS-buffered 4% paraformaldehyde for 10 mins and blocked with PBS solution containing 0.05% tween-20, 5g/L skim milk and 1.5g/L glycine for 30 mins. Samples were incubated with primary antibodies (1:200) overnight at 4°C. Subsequently, samples were incubated with 1:1000 dilutions of the anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate) or anti-rabbit IgG(H+L), F(ab') 2 Fragment (Alexa Fluor 488 conjugate) secondary antibodies. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Images were detected using a Zeiss LSM510META confocal electroscope.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). CDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). Quantitative PCR was performed using Sensimix SYBRGreen (Applied Biosystems) or TaqMan (AXIN1 and AXIN2) Gene Expression Assays (Applied Biosystems). Analyses were performed using the StepOne Real-Time PCR System and the StepOnev2.0 software (Applied Biosystems, Darmstadt, Germany). All expression levels are depicted relative to the expression of *GAPDH*. Primer sequences are provided in Table S1

Gene knockdown by small interfering RNA (siRNA)

Smartpool ON-TARGETplus siRNAs targeting *AXIN1*, *AXIN2* or *APC* were purchased from Dharmacon. The ON-TARGETplus Non-targeting siRNA #2 was used as negative control. Cells were reverse-transfected in a 24-well plate using a total of 0.8 µl DharmaFECT formulation 4 (Thermo-Fisher Scientific) and 25nM of each siRNA per well. Following 72h incubation, the effect of knockdown was tested by qRT-PCR (Figure S5). Alternatively, 48h after siRNA transfection, the cells were transfected with WRE or MRE vectors and CMV-Renilla for a β-catenin reporter assay.

Colony formation assay

After trypsinization, 1000 cells for each cell line were seeded in 6-well plates and were cultured in complete DMEM medium containing 1uM XAV939 or DMSO as control. Medium was changed every three days. Two weeks later, the cells were washed with PBS, fixed in 4% PBS-buffered paraformaldehyde for 10 min and stained with crystal violet solution. Tests were performed at least in triplicates.

Statistical analysis

All results were presented as mean ± SD. Comparisons between groups were performed with Mann Whitney test. Differences were considered significant at a *P* value less than 0.05 (**P*<0.05, ***P*<0.01, ****P*<0.001).

Results

Tankyrase inhibition stabilizes AXIN2 exclusively in *CTNNB1* mutant HCC cell lines

To investigate the effect of tankyrase inhibition on liver cancer cells, we employed 9 HCC cell lines listed in Table 1, in which gene mutations related to Wnt/β-catenin signaling are depicted. At baseline all HCC cell lines showed readily detectable RNA expression of both *TNKS* and *TNKS2* genes, while by western blotting predominantly the larger tankyrase-1 variant was visible (Figure 1). Both AXIN1 and AXIN2 proteins were noticeably expressed in β-catenin mutant lines, but only weakly detectable in the remaining lines, with the exception of PLC/PRF/5 showing a clear expression of mutant AXIN1 (lacks exon 4 encoding GSK3 interaction domain).

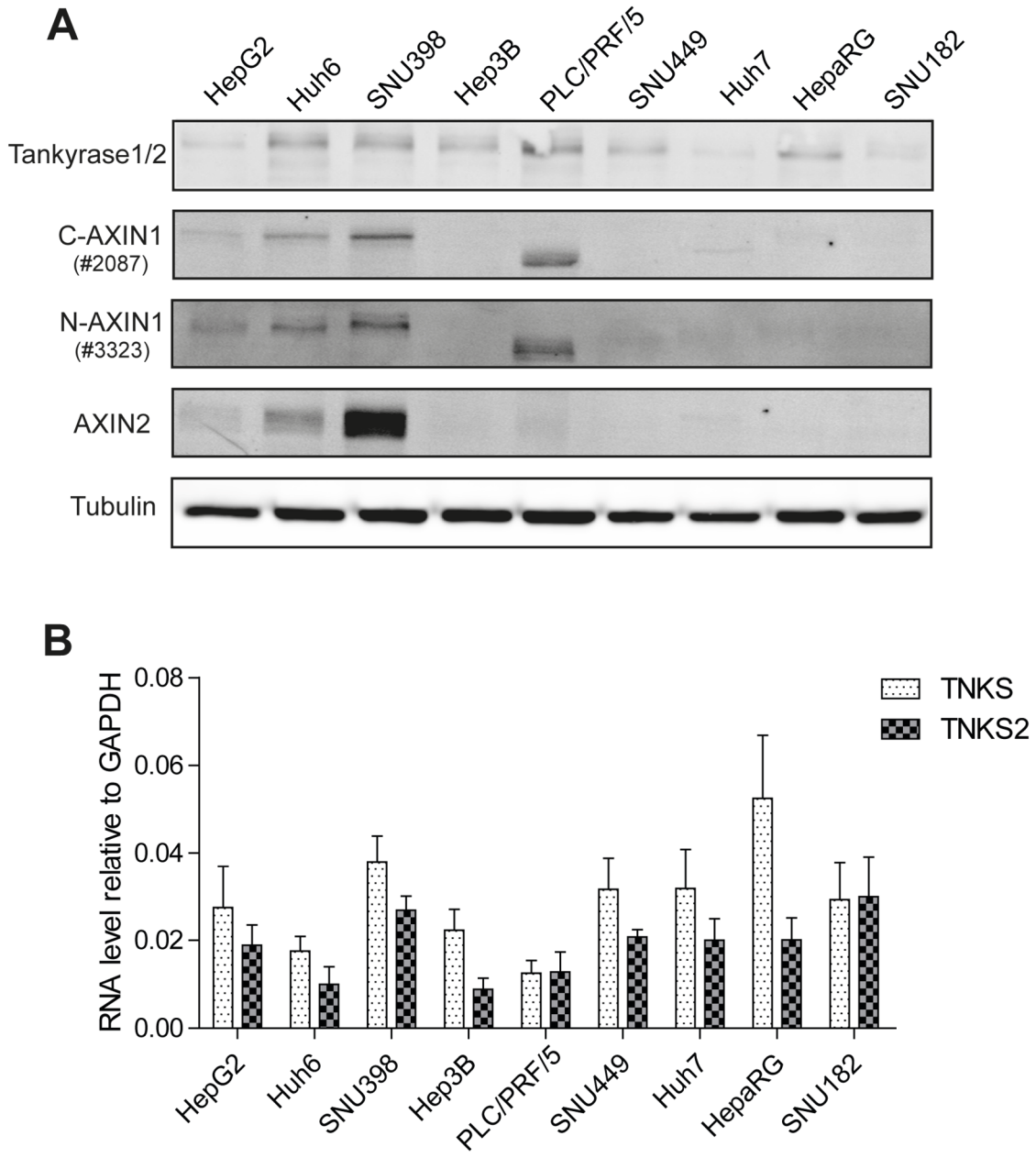

Figure 1. Baseline levels of tankyrase-1/2 and AXIN1/2 in HCC cell lines. (A) Western blotting assay showing the basal protein levels of tankyrase1/2, AXIN1 and AXIN2. (B) QRT-PCR assay showing expression of *TNKS* and *TNKS2* in HCC cell lines (mean ± SD, n=2, twice). Expression levels are depicted relative to the housekeeping gene *GAPDH*.

The tankyrase enzymes have been shown to antagonize the activity of the β-catenin destruction complex by PARsylation of AXINs (18-21). In order to test whether this also holds true for HCC, we treated HCC cell lines with tankyrase inhibitors XAV939 or IWR-1, using the CRC cell line SW480 as positive control. In accordance with previous studies on SW480 cells, XAV939 stabilized tankyrase-1/2, AXIN1 and AXIN2, increased phospho-β-catenin (p-β-catenin) and diminished total β-catenin levels (Figure S1A). In all HCC cell lines, XAV939 treatment at two different concentrations led to a robust accumulation of both tankyrase variants by blocking its auto-PARsylation (Figure 2A). Strikingly, we did not observe a clear stabilization of

either AXIN1 or AXIN2 protein in most HCC cell lines. Solely in the *CTNNB1* mutant lines HepG2, Huh6 and especially SNU398, the AXIN2 signal was noticeably enhanced. With respect to β-catenin, only in Huh6 an increase in pS33/37 phosphorylation was observed accompanied with a slight reduction in total β-catenin levels, but only clearly following treatment with 5µM XAV939. Basically identical results were obtained with a second tankyrase inhibitor (IWR-1) in the Huh6, PLC/PRF/5 and SW480 cell lines (Figure S1B). Owing to the clear stabilization of tankyrase-1/2 even at low dosage of XAV939, we used a 1µM concentration in the remainder of our study.

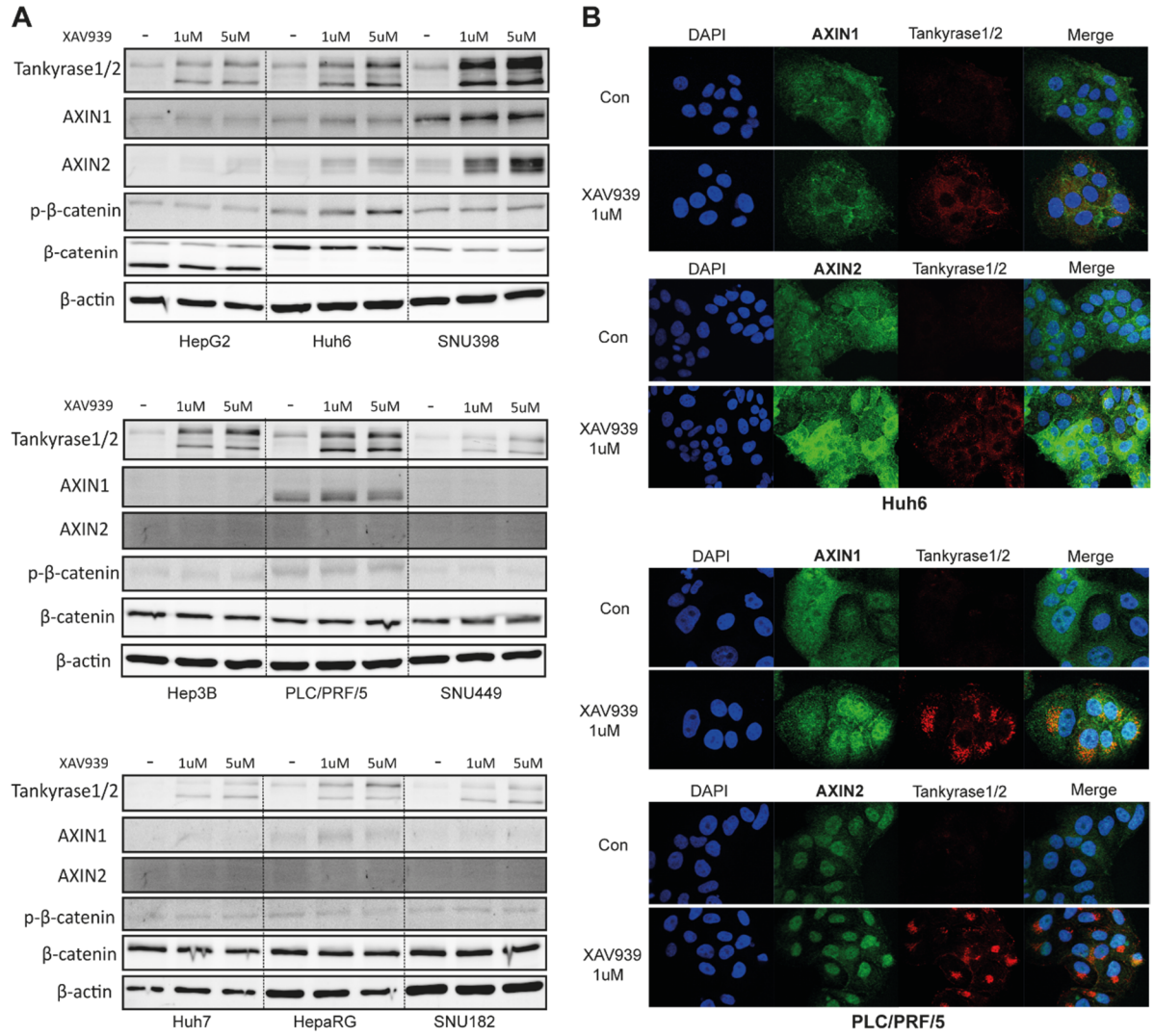

Figure 2. Tankyrase inhibition stabilizes AXIN2 exclusively in *CTNNB1* mutant HCC cell lines. (A) Western blotting assay showing levels of trankyrase-1/2, AXIN1/2, p-β-catenin and total β-catenin after XAV939 treatment (1or 5µM, 16 h). (B) Immunofluorescence staining indicating the levels and location of tankyrases and AXINs in Huh6 and PLC/PRF/5 after XAV939 treatment (1µM, 16 h).

In various cell lines tankyrase inhibition has been shown to lead to the formation of so-called β-catenin degradasomes, consisting of higher-order structures in which all components required for β-catenin degradation are present (31). These degradasomes can be visualized as AXIN- and tankyrase-positive cytoplasmic puncta (32), which were readily visible in XAV939 treated SW480 cells (Figure S2). In PLC/PRF/5 treated cells, accumulation of tankyrase in puncta was clearly discerned, but no obvious change in abundance and subcellular localization of AXIN1 and AXIN2 was visible (Figure 2B). In Huh6 cells both tankyrase and AXIN2 accumulated, while in accordance with the western blot analysis no change was observed for AXIN1.

Thus, both the western blot and immunocytochemistry analysis suggest that tankyrase inhibition leads to an efficient stabilization of AXIN2 and the formation of β-catenin degradasomes exclusively in β-catenin mutant HCC cell lines, while AXIN1 remains unaltered. In all other lines no obvious change in AXIN1 or AXIN2 is observed.

Baseline RNA levels of *AXIN2* are higher than *AXIN1* in all HCC cell lines

We wished to identify potential mechanisms underlying the lack of a clear AXIN accumulation that we fail to observe in most HCC cell lines following tankyrase inhibition. Recently, Thorvaldsen et al. showed that sustained protein translation is required for AXIN accumulation (31). Indirectly this also implies that sufficient RNA must be available to generate new AXIN protein. Hence, we compared *AXIN1* and *AXIN2* RNA expression levels both by qRT-PCR (Figure 3A, S3) as well as TaqMan analysis (Figure 3B). Uniformly *AXIN2* was expressed at considerably higher levels than *AXIN1* in all HCC cell lines, independent of their β-catenin related mutation status. In accordance with *AXIN2* being a β-catenin target gene, the expression differences were largest in the *CTNNB1* mutant lines.

Comparing the *AXIN1/2* RNA expression with their corresponding protein levels following tankyrase inhibition, a clear correlation emerges in the extent of accumulation observed. *AXIN1* RNA levels in HCC lines are apparently inadequate to generate sufficient new AXIN1 protein to visibly accumulate, whereas for AXIN2 this is only the case in the high expressing *CTNNB1*-mutant cell lines.

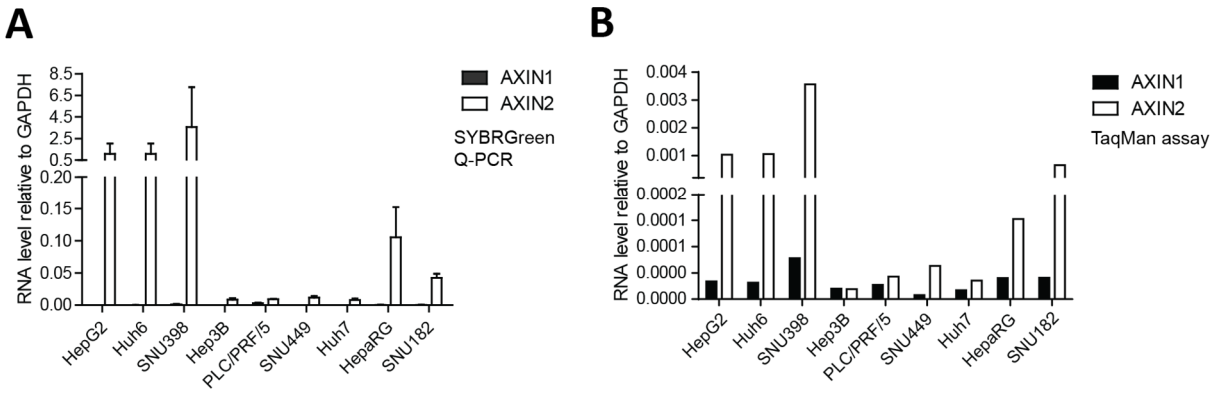

Figure 3. Baseline RNA level of *AXIN2* is higher than *AXIN1* in HCC cell lines. (A) RNA levels tested by qRT-PCR (mean ± SD, n=2, twice). (B) RNA levels tested by TaqMan Gene Expression Assay.

Tankyrase inhibition diminishes Wnt/β-catenin signaling activity in most non-*CTNNB1* mutant HCC cells

Despite that we observed no clear visual AXIN accumulation in most HCC cell lines, we set out to determine the impact of tankyrase inhibition on Wnt/β-catenin signaling activity. In line with our previous work, in untreated samples we observed a clear induction of β-catenin reporter activity in both the *CTNNB1*- and *AXIN1*-mutant lines (17). Following XAV939 treatment, *CTNNB1* mutant cells still gave a high β-catenin reporter activity, comparable to the untreated cells (Figure 4), in line with the supposed dominant activity of mutant β-catenin. Reporter activity was however clearly suppressed in both the *AXIN1* mutant as well as non-mutant lines, with the exception of HepaRG. The most prominent decreases were observed in PLC/PRF/5 and Huh7, which also displayed a minor but noticeable decrease of *AXIN2* at RNA level, a well-established β-catenin target gene (Figure S4). Thus, this analysis shows that tankyrase inhibition can reduce β-catenin signaling in *AXIN1*-mutant and non-mutant lines, despite that no obvious AXIN accumulation is observed.

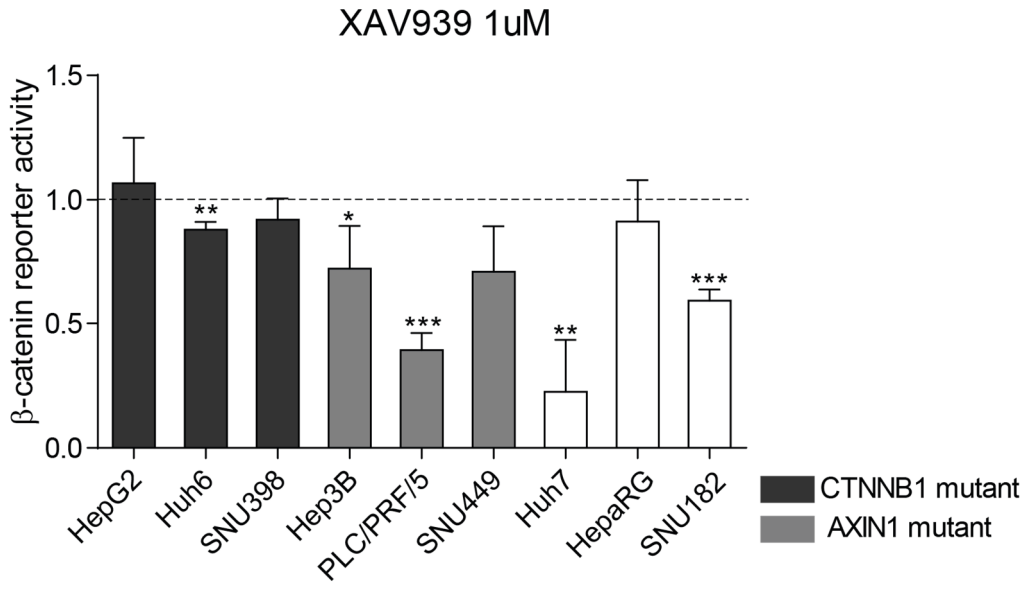

Figure 4. Tankyrase inhibitor diminishes Wnt/β-catenin signaling activity in most non-*CTNNB1* mutant HCC cells. HCC cell lines were incubated with 1μM XAV939 for 24 h followed by a β-catenin reporter assay (mean ± SD, n=2, twice). Reporter values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 1. (**P*<0.05, ***P*<0.01, ****P*<0.001).

Both AXIN1 and AXIN2 contribute to β-catenin signaling regulation in HCC cell lines

To evaluate the specific contribution of either AXIN1 or AXIN2 to the regulation of β-catenin signaling in each cell line, we applied siRNA mediated knockdown. We focused our analysis on the *AXIN1*-mutant and non-mutant lines, using SNU398 as a *CTNNB1* mutant control. *APC* knockdown was used as a positive control for activation of β-catenin signaling. Analysis by qRT-PCR confirmed the efficient knockdown of all targeted genes (Figure S5A). As expected, *APC* knockdown resulted in a strong increase of β-catenin reporter activity and *AXIN2* expression in all cell lines, with exception of the *CTNNB1* mutant SNU398 (Figrue 5 and S5B). In all three *AXIN1*-mutant lines, a comparable increase in reporter activity was observed as a consequence of *AXIN2* knockdown. Among the non-mutant cell lines a variable response was noted. In SNU182 cells reporter activity was increased to levels approaching *APC* knockdown. In Huh7 cells a clear 4-fold increase was observed, but far less-prominent as by *APC* knockdown, while HepaRG cells were barely affected in their β-catenin signaling activity (Figure 5A). QRT-PCR analyses for *AXIN2* were also performed in all *AXIN2* knock-down samples (Figure S5B). However, a meaningful interpretation is complicated by the fact that total *AXIN2* RNA levels are simultaneously downregulated by siRNA as well as upregulated by

enhanced β-catenin signaling. Hence, for the interpretation of AXIN2 knockdown we restricted ourselves to the reporter assay.

The incomplete increase in reporter activity observed in the non-mutant lines following *AXIN2* knockdown, suggests that AXIN1 still contributes to β-catenin turnover. Therefore, we knocked down its expression, using the *AXIN1*-mutant PLC/PRF/5 as negative control. We also evaluated simultaneous knockdown with *AXIN2*. As expected, the PLC/PRF/5 line was not affected by *AXIN1* knockdown, confirming the defective status of the mutant protein (Figure 5B). HepaRG and SNU182 did not show clear change in reporter activity either. In contrast, Huh7 shows a clear increase after *AXIN1* knockdown, even exceeding the value observed for *AXIN2* knockdown alone. QRT-PCR analysis of *AXIN2* showed increased expression levels in all *AXIN1* siRNA treated non-mutant lines, while it was not affected in PLC/PRF/5 (Figure S5C). Simultaneous knockdown of *AXIN1* and *AXIN2* led to a robust induction of β-catenin reporter activity in all tested lines (Figure 5B).

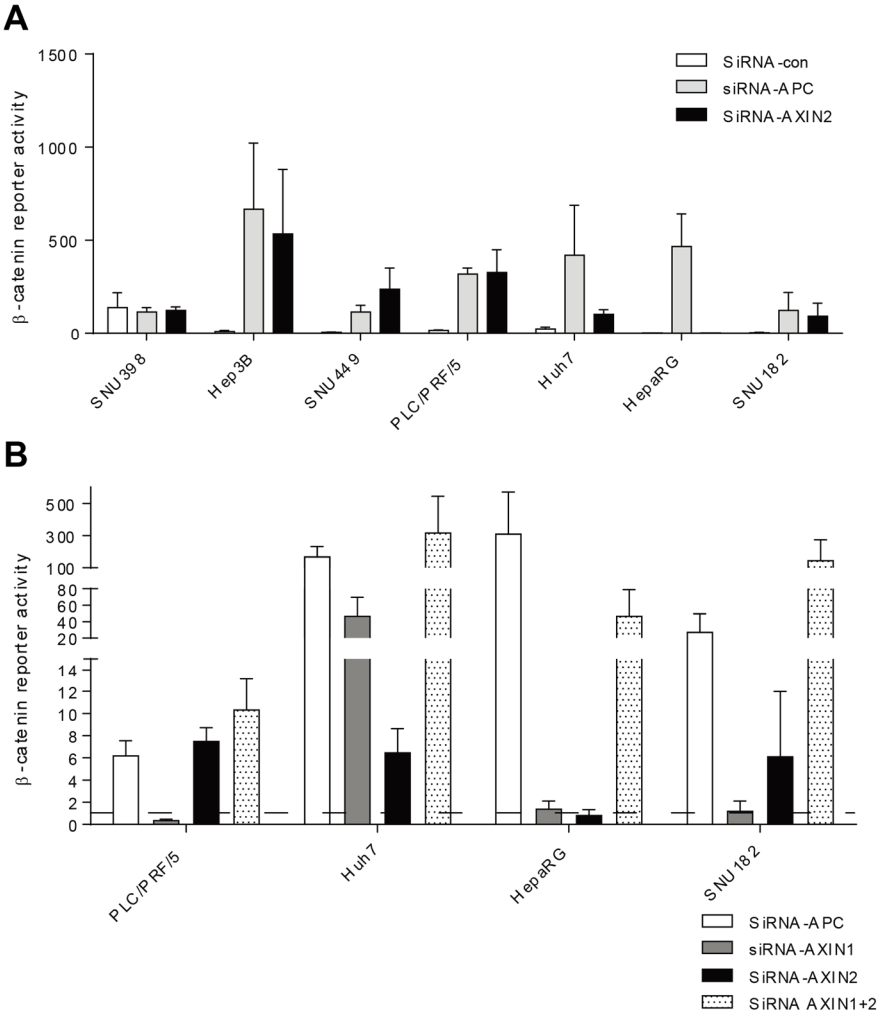

Figure 5. Both AXIN1 and AXIN2 contribute to β-catenin signaling regulation in HCC cell lines. Indicated cell lines were subjected to a β-catenin reporter assay after siRNA-mediated knockdown of *AXIN1*, *AXIN2*, a combination thereof or *APC*. (A) Both *APC* and *AXIN2* knockdown are equally effective in enhancing signaling in *AXIN1* mutant cells (mean ± SD, n=2, twice). *AXIN2* knockdown in the non-mutant lines results in an incomplete increase in reporter activity when compared with *APC* knockdown. (B) Dependency on both AXIN1 and AXIN2 to regulate Wnt/β-catenin signaling activity in non-mutant HCC cells lines (mean ± SD, n=2, twice).

Taken together, these analyses show that (i) in *CTNNB1*-mutant HCC lines β-catenin signaling is dominantly regulated by the mutant β-catenin protein and cannot be effectively modulated by alterations in APC or AXIN1/2 levels; (ii) in *AXIN1*-mutant lines both *AXIN2* and *APC* knockdown are equally effective in enhancing signaling; (iii) in non-mutant lines both AXIN1 and AXIN2 contribute to β-catenin regulation to varying levels depending on the cell lines. Overall, these analyses also show that *AXIN1* mutation or knockdown contributes to enhanced β-catenin signaling in all non-*CTNNB1* mutant lines.

Tankyrase inhibitor XAV939 does not inhibit cell growth of HCC lines effectively

XAV939 was reported to inhibit the proliferation of a subset of APC-mutant CRC cells (24). As a significant reduction in β-catenin signaling was observed in a subset of our HCC cell line panel, we examined whether this compound also exerts an inhibitory effect on HCC cells. Following three days of XAV939 treatment, we noticed a reduced cell viability in a dose-dependent manner in the CRC DLD1 cell line, which was used as positive control. However, HCC cell growth was unaltered, even at higher concentration (Figure S6). To test the effect of long-term treatment, we performed a colony formation assay treating HCC cells with 1µM XAV939 for two weeks. As indicated in Figure 6, no obvious reduction in colony number and size was observed for most cell lines (HepG2 failed to form colonies) with the exception of SNU398 and HepaRG. As these two lines did not show any evidence of reduced β-catenin signaling by XAV939 (see Figure 4), this is most likely the consequence of other cellular processes regulated by tankyrases, such as. telomere maintenance, mitosis or DNA strand break repair (33-35). Another explanation could be the potential repressive effect of XAV939 on poly(ADP-ribose) polymerases PARP1and PARP2 (24), Collectively, these findings suggest that XAV939 cannot or at most modestly affect the growth of HCC cell lines at concentrations that block tankyrase activity.

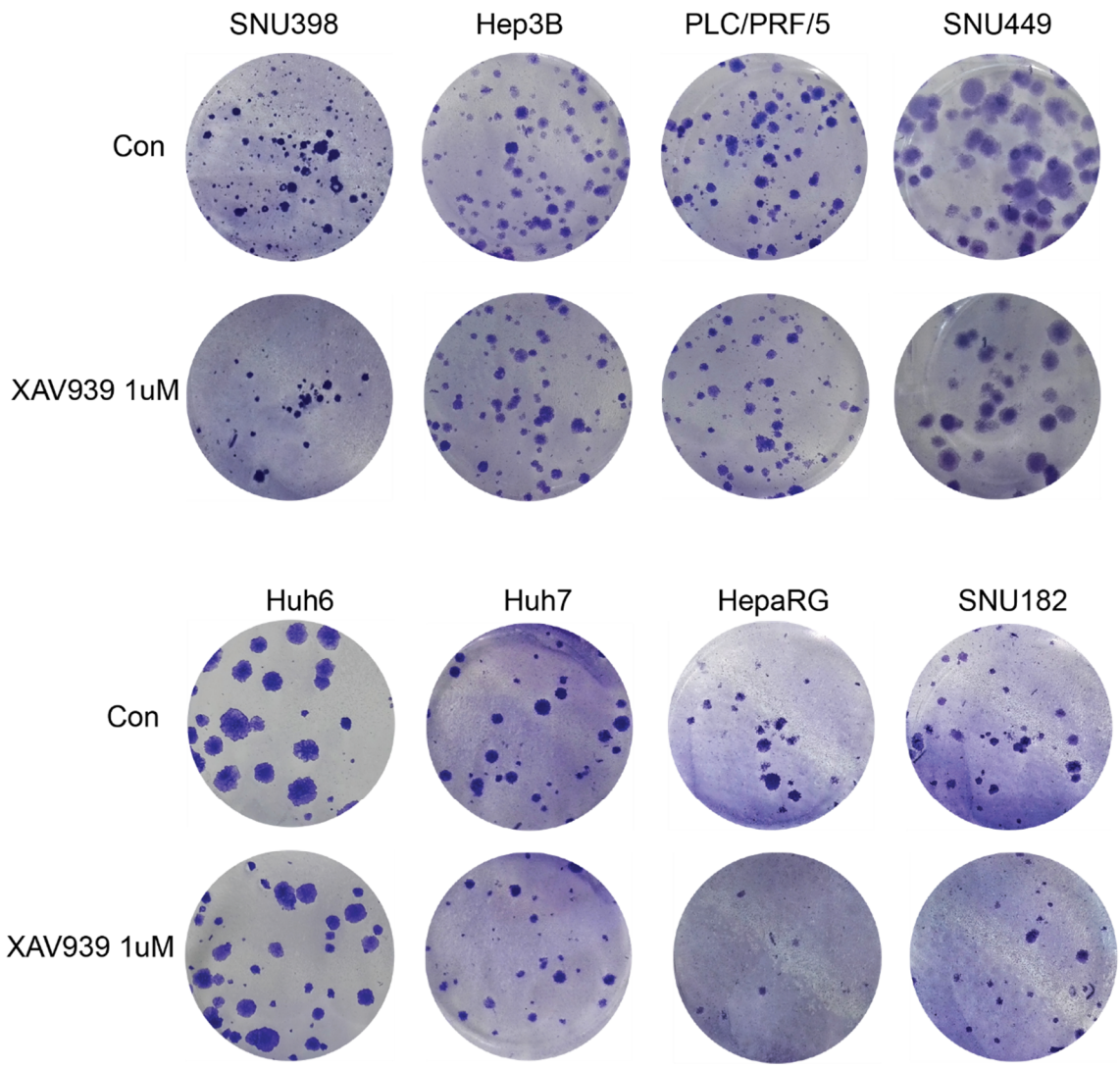

Figure 6. TNKS inhibitor XAV939 does not or at most modestly affect the colony formation capacity of HCC lines. Cells were treated with 1µM XAV939 for two weeks. Medium was changed every three days. Tests were performed at least in triplicates. HepG2 did not form colonies using this assay.

Discussion

Inappropriate activation of Wnt/β-catenin signaling has been reported frequently in HCC (4). This has been mainly attributed to somatic mutations in the *CTNNB1* gene (20-25%) (4, 6-8). A second common mechanism originally considered to lead to enhanced β-catenin signaling, is mutational inactivation of AXIN1 (10%) (6, 7). Given its activity in the β-catenin destruction complex this was a logic assumption, however several more recent reports have suggested that AXIN1 mutation in liver cancer leads to no or at most a modest increase in β-catenin signaling (9, 10, 16). Hence, the exact mechanism through which AXIN1 mutation supports HCC growth is still under debate. In addition, the AXIN proteins have gained substantial interest in cancer research because their activity in the β-catenin destruction complex can be increased by tankyrase inhibitors, which thus may serve as a therapeutic option to reduce the

growth of β-catenin-dependent cancers. Here, using a panel of 9 HCC cell lines with specific mutations in components of the β-catenin signaling pathway, we have investigated both aspects of AXIN biology.

To our initial surprise, tankyrase inhibition did not lead to a clear visual accumulation of both AXIN1 and AXIN2 protein in most HCC cell lines, with the exception of AXIN2 in the *CTNNB1*-mutant ones. This is in apparent contrast with studies of breast (22), lung (23)and especially colorectal cancer, where its application leads to an efficient accumulation of either one or both AXIN proteins (24-28). As a potential explanation we identified low RNA expression levels, especially of *AXIN1.* Recently, it was shown that sustained protein translation is required for AXIN accumulation to occur (20). Apparently, the low RNA levels are inadequate to generate sufficient new AXIN1 protein to visibly accumulate, whereas for AXIN2 this is only the case in the high *AXIN2* expressing *CTNNB1*-mutant cell lines. In contrast, most colorectal cancers are characterized by APC mutations leading to a strongly enhanced β-catenin signaling, which in turn leads to hyper-activation of *AXIN2*, explaining the efficient AXIN2 accumulation observed in this cancer type following tankyrase inhibition (24-26). Thus, our investigation suggests that *AXIN1* and *AXIN2* RNA levels could be a good predictor for the level of their tankyrase-inhibitor induced accumulation.

Despite that we observed no clear visual AXIN accumulation in the non-*CTNNB1*-mutant HCC cell lines, we notice the suppressing effect of XAV939 on β-catenin signaling activity in most of them. In addition to their enzymatic activity, the tankyrase enzymes are increasingly being recognized as scaffolding proteins that catalyze the formation of the β-catenin destruction complex (21). Most likely the XAV939 induced tankyrase stabilization allows a more efficient formation of these AXIN-containing destruction complexes leading to more β-catenin breakdown, even though thus far we fail to show the AXIN-puncta microscopically. In accordance with previous reports investigating β-catenin mutant CRC lines, the HCC cell lines expressing constitutively active mutant β-catenin variants are not affected in their signaling activity (24-26).

The reduction in β-catenin signaling accomplished with tankyrase inhibition in a subset of our lines, is however not sufficient to significantly affect their growth. This seems to contradict our previous study where all HCC cell lines were inhibited in their growth after

siRNA-mediated β-catenin knockdown (17). In this latter study we reached more than 80% reduction in total β-catenin protein levels, which is in strong contrast to basically unaltered levels in our current study. XAV939 has also been evaluated in another study involving three HCC cell lines (HepG2, Huh7 and Hep40) (29). In this report growth inhibitory effects were observed at drug concentrations (IC50s of 25-80 μM) far-exceeding the levels required for selective tankyrase inhibition, which will most likely also have inhibited the activity of other PARP enzymes (36). Taken together, these studies suggest that tankyrase inhibition at physiologically relevant concentrations is unlikely to contribute to HCC treatment as monotherapy. A potential exception could be the rare subset of HCCs carrying APC mutations leading to high level signaling of wild-type β-catenin (37-39).

As mentioned above, the exact mechanism through which *AXIN1* mutation contributes to HCC development is still under debate. The original assumption that it drives tumorigenesis by enhancing β-catenin signaling has been questioned for the following reasons: (i) AXIN1 and AXIN2 show high similarity to one another in most domains responsible for binding to other proteins (40). In accordance, AXIN2 can at least partially compensate for AXIN1-loss in the β-catenin destruction complex (41). (ii) AXIN1 has also been shown to regulate the activity of other proteins relevant for tumorigenesis, such as MYC, P53 and SMAD3 (12-15). (iii) Immunohistochemical analysis for β-catenin fails to identify an efficient nuclear accumulation in AXIN1-mutant tumors (9). Although nuclear accumulation of β-catenin is a reliable predictor of active signaling, its absence does however not fully exclude that a low level of biologically relevant signaling is active. (iv) Deletion of *AXIN1* in the mouse liver led to enlarged livers, a feature that has also been associated with increased β-catenin signaling, and a weak induction of some β-catenin target genes (10). Only late-onset hepatocellular cancers with no or at most a few cells staining positive for nuclear β-catenin were observed in these mice (10, 11). (v) Expression profile analysis using a gene signature representative of β-catenin target genes, clustered most *AXIN1*-mutated HCCs in a group with no evident β-catenin program activation, and about 20% in groups with weak or strong activation (11). Overall, these reports make a strong point that β-catenin signaling is not prominent in *AXIN1*-mutant HCCs, but they also hint to some low level activation that might be biologically relevant. In this respect, several examples have been presented in the literature showing that minor alterations in the level of β-catenin signaling can have profound biological effects (5, 42-44). In case of hepatocellular

cancer, Buchert et al. have shown that late-onset hepatocellular tumors were present in all mice carrying a hypomorphic *APC* mutation associated with just a modest increase in β-catenin signaling, while tumor formation was absent or largely prevented with slightly increased or decreased signaling (42). This narrow window of signaling effective in liver cancer formation highlights the importance of low level signaling for some cancer types and shows that it is difficult to fully exclude a role for β-catenin signaling. Our analysis shows that all three *AXIN1*-mutant HCC cell lines have increased β-catenin reporter activity, and that *AXIN1* knockdown in non-mutant lines leads to enhanced signaling, also supported by a recent investigation of *AXIN1* knockdown in Huh7 cells (11). Previously, we have also shown that all *AXIN1*-mutant lines depend on sufficiently high β-catenin levels for an optimal growth (17). Taken together, this suggests that *AXIN1* mutation leads to a modest increase of β-catenin signaling that may be relevant for hepatocellular tumorigenesis.

AXIN1-mutant tumors depend to a large extent on AXIN2 to counterbalance signals that induce β-catenin signaling. Hence, they are expected to be more prone to signal in conditions that normally activate the pathway, such as commonly encountered local tissue injury or inflammation within the tumor micro-environment. At these specific locations, *AXIN1*-mutant cells may carry a selective advantage through β-catenin mediated induction of for example tumor stem cell features. Such local effects are likely missed when expression profiles are obtained from tumors as a whole. As such, we feel that a role for β-catenin signaling in *AXIN1*-mutant HCCs cannot be ruled out completely, and will require additional experimentation such as combined *AXIN1* and *CTNNB1* deletion in mouse livers, to determine if *AXIN1*-mutant tumors can arise without β-catenin signaling. Nevertheless, it is clear that this specific subset of HCCs follows a different route to tumorigenesis and may more heavily depend on the activation of other signaling pathways such as the recently described involvement of YAP/TAZ and Notch signaling (11).

In conclusion, we show that both AXIN1 and AXIN2 contribute to the regulation of β-catenin signaling in HCC. Enhancing their activity by tankyrase monotherapy provides however no effective treatment to affect their growth.

Supplementary Tables and Figures

Supplementary table.1 Primer sequences used for qRT-PCR

Gene	Forward Sequence(5′~3′)	Reverse Sequence(5’~3’)
AXIN1	AACGACAGCGAGCAGCAGAG	AGCTTGTGACACGGCCCTGG
AXIN1[a]	CAAGAGCAGGGTTTCCCCTT	GCCGTCGAAGTCTCACCTTT
AXIN1[b]	GAACTGGTGTCCACAGACCC	CCCATCTTGGTCATCCAGCA
AXIN2	TATCCAGTGATGCGCTGACG	TTACTGCCCACACGATAAGG
TNKS1	CCTGGCAGATCCTTCAGCAA	TTGTAGCCCGCTGCTAGATG
TNKS2	TGCCAGGAGTGGCAATGAAG	TTTCTGCCATCACTTGCGTG
APC	GCGCTTACTGTGAAACCTGT	GAACACACACAGCAGGACAG
GAPDH	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT

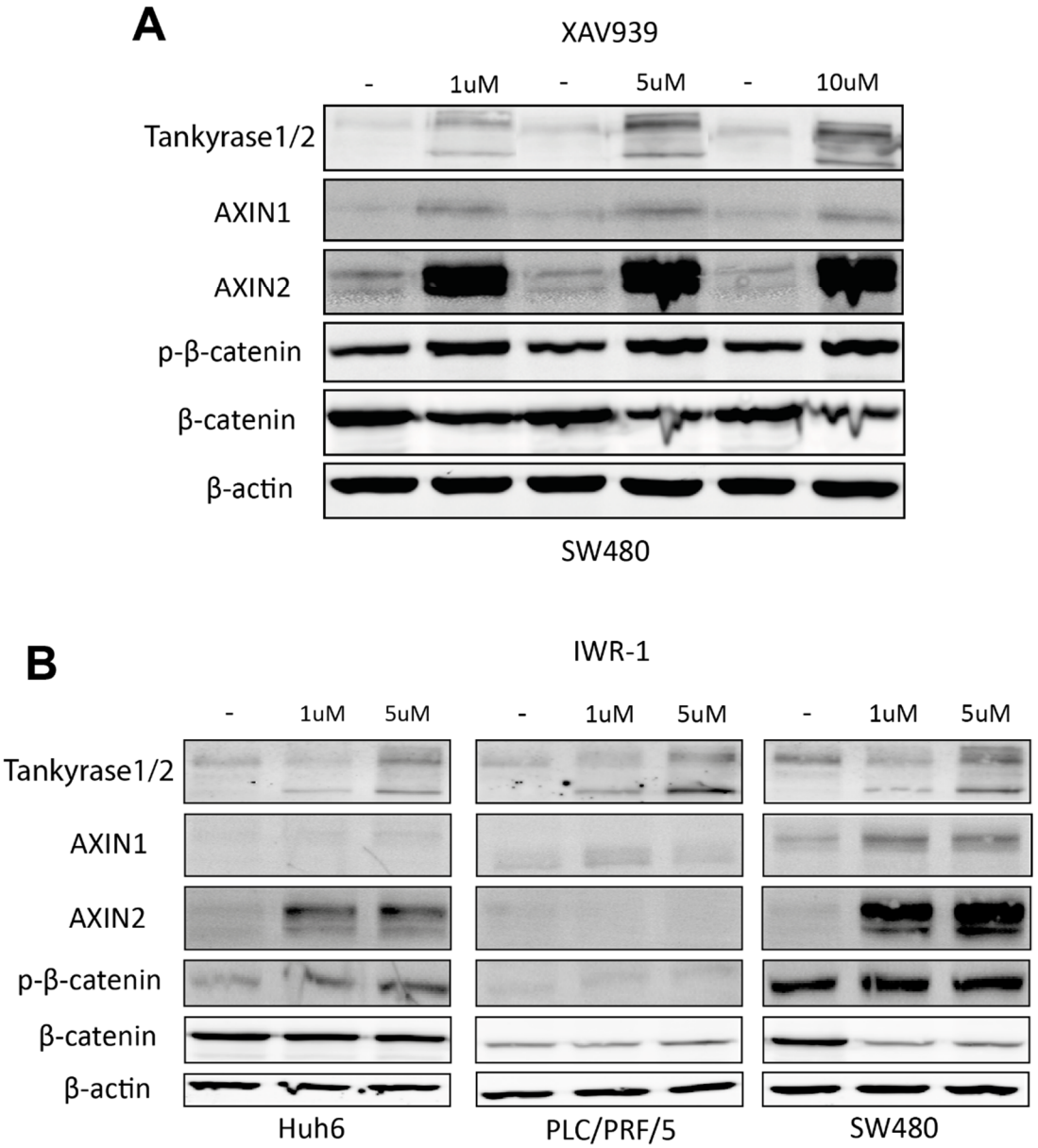

Figure S1. Effect of tankyrase inhibitors validated by western blotting. (A) In SW480 cells XAV939 stabilizes tankyrase-1/2 and AXIN1/2 protein levels, increases phospho-β-catenin (p-β-catenin) while simultaneously reducing total β-catenin. (B) Related proteins tested in SW480 and two HCC cell lines (Huh6 and PLC/PRF/5) using a second tankyrase inhibitor, i.e. IWR-1, showing basically identical results to XAV939. All these cells were treated with indicated tankyrase inhibitors for 16 h.

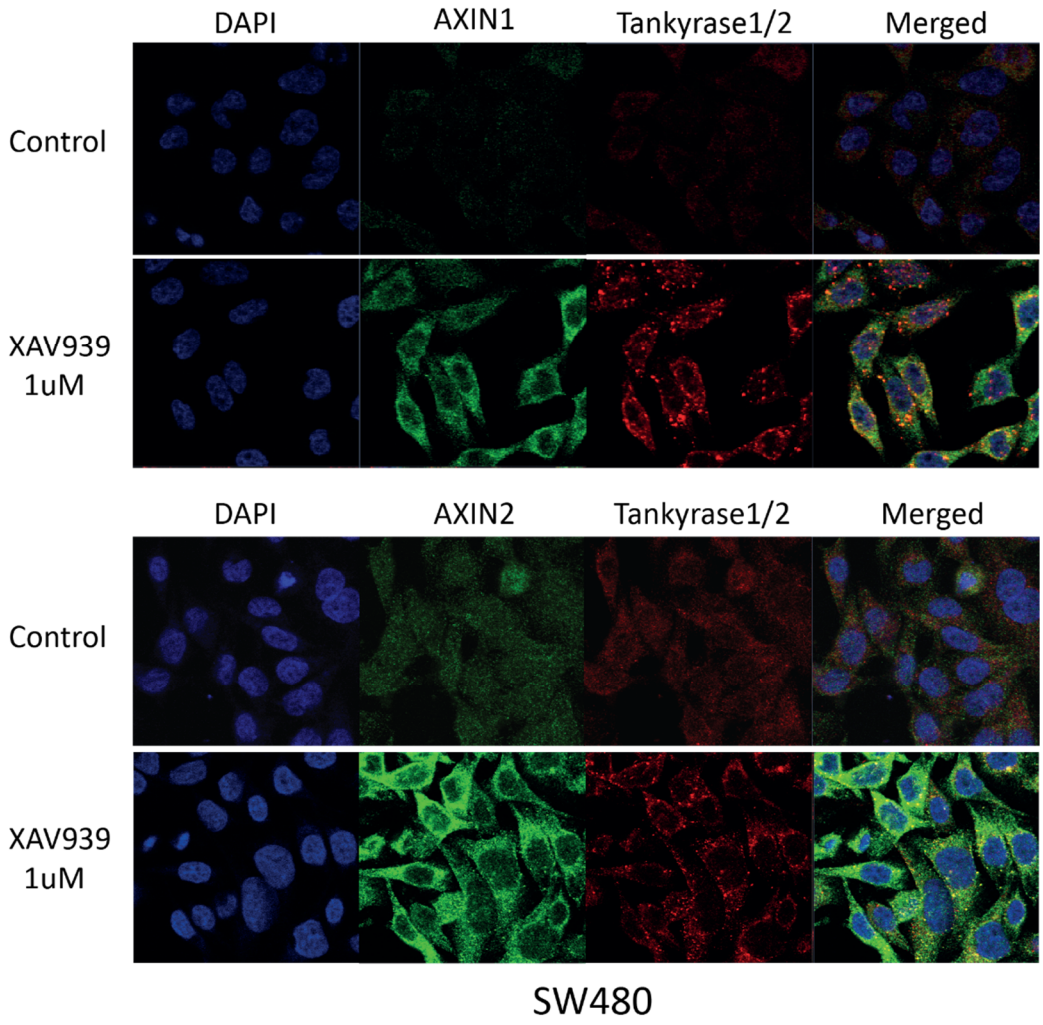

Figure S2. Immunofluorescence staining showing the abundance and subcellular localization of tankyrase-1/2 and AXIN1/2 in SW480 treated with XAV939 (1μM for 16 h).

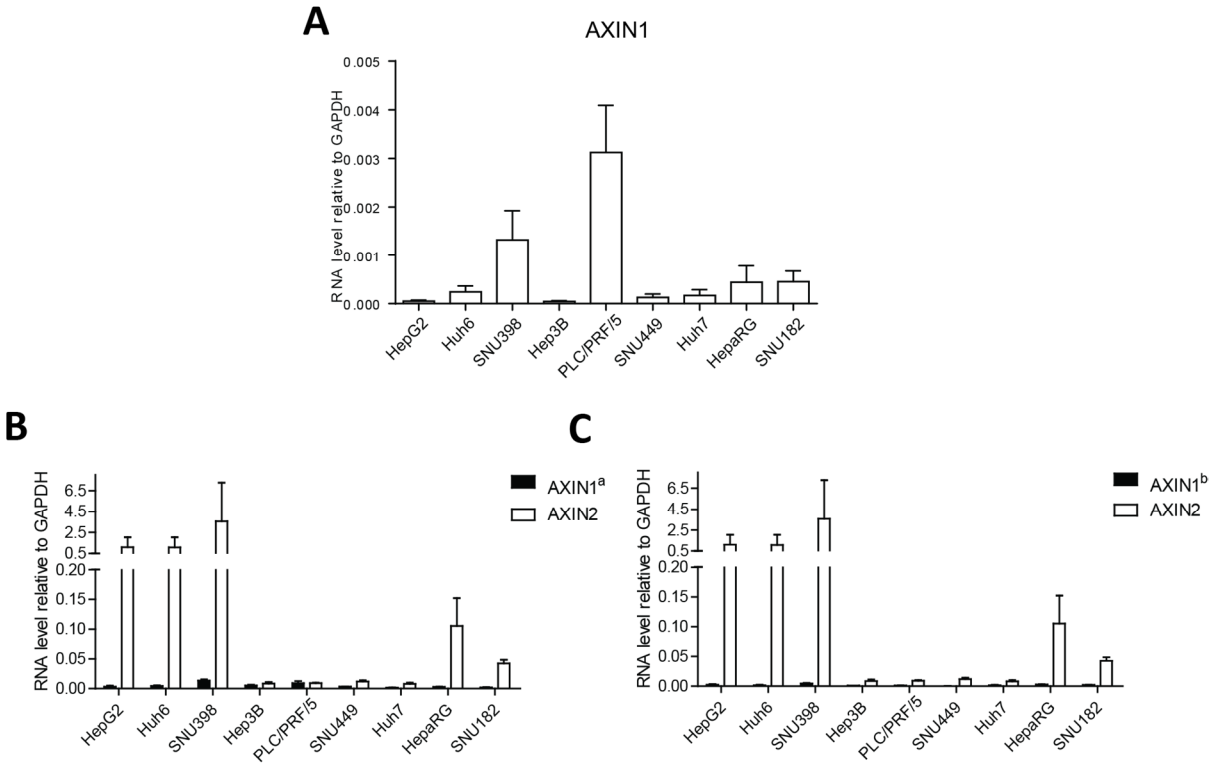

Figure S3. (A) Baseline *AXIN1* expression in HCC cell lines. (B,C) Baseline RNA level of *AXIN2* is higher than *AXIN1* in HCC cell lines as validated by two additional sets of *AXIN1* oligos (a and b) tested by qRT-PCR (mean ± SD, n=2, twice). All expression levels are depicted relative to the housekeeping gene *GAPDH*.

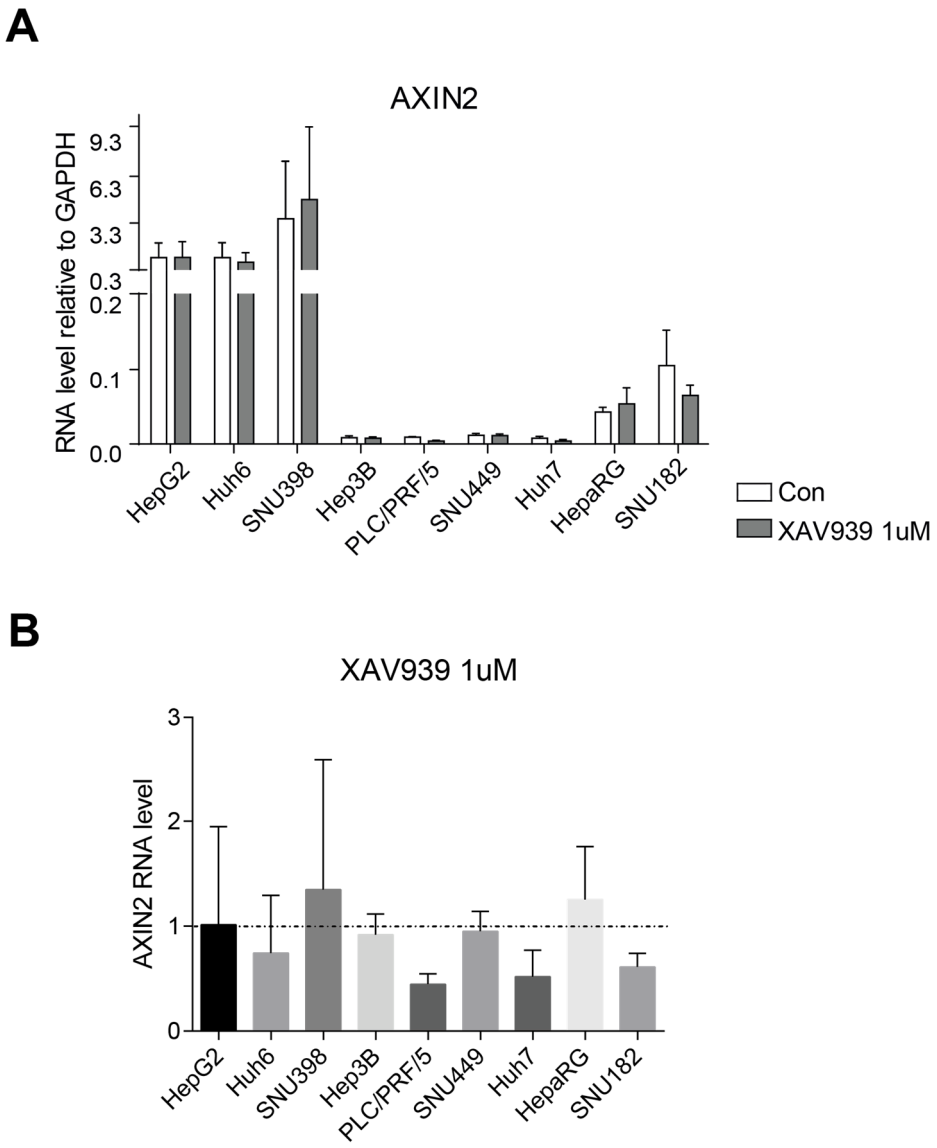

Figure S4. QRT-PCR of *AXIN2* in HCC cell lines treated with XAV939 (1µM, 24 h) (mean ± SD, n=2, twice). (A) Expression levels of *AXIN2* relative to *GAPDH*. (B) *AXIN2* levels relative to the untreated samples arbitrarily set to 1.

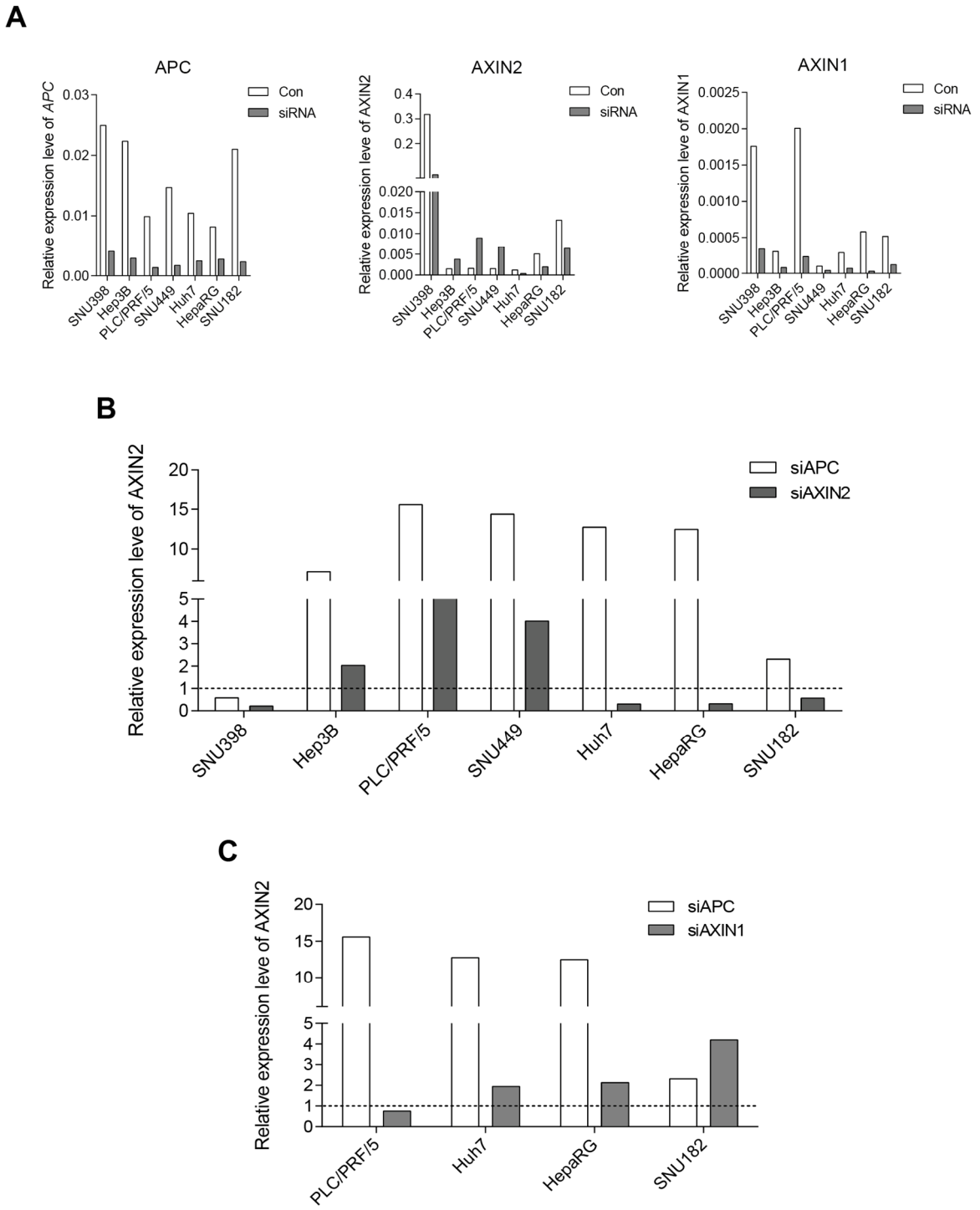

Figure S5. (A) SiRNA mediated knockdown of *APC*, *AXIN1* and *AXIN2* tested by qRT-PCR. A caveat should be mentioned for *AXIN2* knock-down in the *AXIN1*-mutant lines. Following its knockdown, β-catenin signaling is strongly enhanced in these lines, in turn resulting in the activation of *AXIN2* expression. As a result, the qRT-PCR analysis suggests an increase of *AXIN2* levels, while functionally overall AXIN2 activity is clearly reduced. (B) RNA levels of *AXIN2* tested by qRT-PCR in HCC cells after siRNA-mediated *APC* or *AXIN2* knockdown. (C) RNA levels of *AXIN2* tested by qRT-PCR in *AXIN1* siRNA treated non-mutant lines.

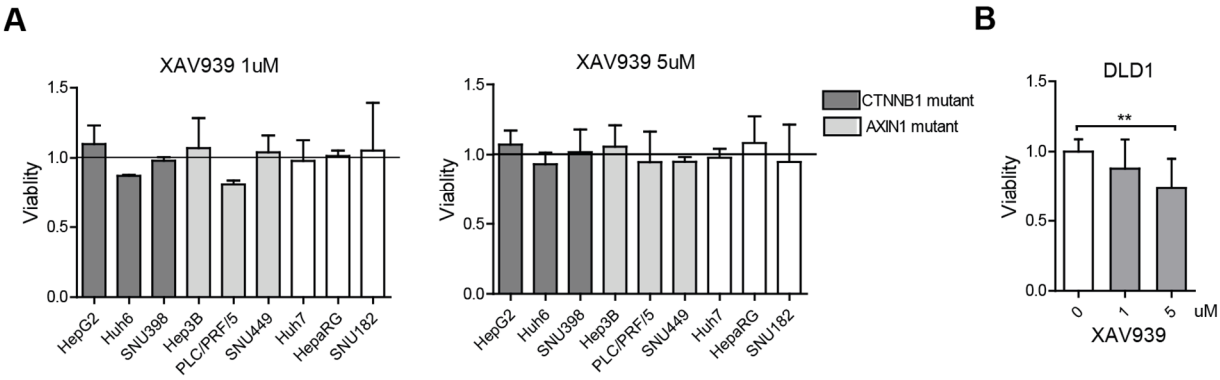

Figure S6. XAV939 does not inhibit cell viability of HCC cells in short term. (A) HCC cell lines were treated with XAV939 at 1µM or 5µM for three days (mean ± SD, n=4). (B). CRC DLD1 cells were treated with XAV939 at 1µM or 5µM for three days, showing a dose-dependent reduction (mean ± SD, n=4).

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Chapter 5

Oncogenic STRAP supports hepatocellular carcinoma cell growth through enhancing Wnt/β-catenin signaling

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Abstract

Aberrant activation of Wnt/β-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*. Recently, the scaffold protein STRAP was shown to facilitate the aberrant activation of Wnt/β-catenin signaling in colorectal cancers. However, the function of STRAP in HCC remains completely unknown. Here we show that in most HCCs increased levels of STRAP can be observed. STRAP knock-out clones generated by gene editing of Huh6, Huh7 and PLC/PRF/5 HCC cell lines, are strongly impaired in cell-cycle progression and the formation of colonies from single cells. RNA sequencing revealed that many signaling pathways and metabolic processes are affected following STRAP loss. Importantly, Wnt/β-catenin signaling was clearly impaired in all STRAP knock-out/down cell lines tested, regardless of the underlying *CTNNB1* or *AXIN1* mutation. In accordance with β-catenin's role in (cancer) stem cell maintenance, we observed reduced expression of various stem cell markers, such as *AXIN2* and *LGR5*, and concomitantly increased expression of differentiation-associated genes. Together, these results show that the increased STRAP levels observed in hepatocellular cancers, provide growth advantage among others by enhancing Wnt/β-catenin signaling. These observations also identify STRAP as a new player in regulating β-catenin signaling in HCC.

Keywords: Hepatocellular carcinoma, STRAP, Wnt/β-catenin signaling, tissue microarray, RNA sequencing

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third leading cause for cancer related deaths worldwide with around 500,000 new cases diagnosed each year (1, 2). Hepatocarcinogenesis initiates with the accumulation of aberrant genetic and epigenetic modifications leading to the dysregulation of signaling pathways, which transform the normal hepatocytes towards malignant phenotypes (3).

Inappropriate activation of Wnt/β-catenin signaling has been reported in HCC (4). As the central component of Wnt/β-catenin signaling, the transcription factor β-catenin is tightly regulated by a multiprotein complex composed of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and the kinases GSK3 and CK1α (4, 5). In the absence of Wnt ligands, β-catenin is constitutively phosphorylated and degraded to maintain a minimal level in the cytoplasm. On Wnt stimulation, the multiprotein complex dissociates causing the accumulation of cytosolic and nuclear β-catenin. The latter triggers the transcription of specific target genes. Aberrant activation of Wnt/β-catenin signaling in HCC has been attributed to activating mutations in *CTNNB1* (20-25%) or loss of function mutations in *AXIN1* (10%), AXIN2 (3-4%) and *APC* (1%~2%) (4, 6, 7).

The serine-threonine kinase receptor-associated protein (STRAP) encoded by the *STRAP* gene, harbors seven WD40-repeat domains (8). It is considered to be a scaffolding protein without enzymatic function that exerts regulatory functions on a variety of cellular processes ranging from signal transduction, transcriptional regulation, RNA processing, vesicular trafficking to cell cycle progression (9). STRAP was shown to be overexpressed and exert oncogenic properties in breast cancer, colorectal cancer (CRC) and lung carcinomas (10-12). Originally, STRAP was shown to inhibit canonical transforming growth factor-beta (TGF-β) signaling (13). Later, it became apparent that STRAP modulates various other cellular processes and signaling pathways such as signaling through ASK1, P53, PI3K/PDK1, and $P21^{Cip1}$ (9, 14, 15). More recently, Wnt/β-catenin signaling was demonstrated to be stimulated by increased STRAP in CRC through binding with GSK-3β around the catalytic domain, which diminished subsequent ubiquitin-dependent degradation of β-catenin (16). However, the function of STRAP in HCC progression remains elusive.

In this study, we investigated the expression level of STRAP in HCC tumor tissues and used clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated gene editing to knockout *STRAP* in HCC cell lines carrying distinctive mutations related to Wnt/β-catenin signaling in order to investigate the function of STRAP. Our results suggest that upregulation of STRAP protein provides growth advantage to HCC cells via enhancing Wnt/β-catenin signaling. These observations identify STRAP as a new player in regulating Wnt/β-catenin signaling in HCC.

Materials and methods

1. Cell lines

Human HCC cell lines Hep3B, HepG2, HepaRG, Huh6, Huh7, PLC/PRF/5, SNU398, SNU182 and SNU449 were cultured as reported previously (7). Identity of all cell lines was confirmed by STR genotyping. *CTNNB1* and *AXIN1* mutations were confirmed in these HCC cell lines by Sanger sequencing and were in accordance with those reported at COSMIC, the Catalogue Of Somatic Mutations In Cancer (http://cancer.sanger.ac.uk) (17). For the preparation of Huh7 conditioned medium, cells were cultured in complete DMEM medium for 3 days, followed by collection and filtration of medium according to standard procedures.

2. Tissue microarray (TMA)

TMA construction was described previously (18). Briefly, archived formalin fixed paraffin-embedded tissue samples from 141 patients who underwent hepatic resection for HCC at Erasmus MC-University Medical center, between 2004 and 2013 were collected. Three or four 0.6mm cores from the tumor area as well as two 0.6mm cores from the corresponding tumor free liver (TFL) area of these patients were taken. The TMAs were made using an automated tissue-arrayer ATA-27 (Beecher Instruments, Silver Spring MD, USA) or a manual tissue arrayer MTA-1 (Beecher Instruments).

3. DEN induction of liver tumors in mice

Mice of C57BL/6J background or mixed with C3H/HeOuJ or CD1 (all 3-4 weeks of age) were administrated weekly with Diethylnitrosamine (DEN) (intraperitoneal injection; 100 mg/kg) for 6-17 weeks to induce liver tumor formation. Mice were sacrificed 3-16 months after the last DEN injection, after which livers were fixed in PBS-buffered formalin and embedded in

paraffin according to routine procedures. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Erasmus Medical Center.

4. Reagents

The following antibodies were used for western blot analysis or immunohistochemistry staining. STRAP (611346, BD Transduction Laboratories™ and HPA027320, Atlas antibodies), β-catenin (610154, BD Transduction Laboratories™), Non-phospho (Active) β-catenin (Ser33/37/Thr41) (#8814, Cell Signaling Technology), Tubulin (sc-8035, Santa Cruz), β-actin (sc-47778, Santa Cruz) and anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA), HRP-conjugated anti-mouse polymer secondary antibody (Envision™, DAKO, Glostrup, Denmark). Propidium iodide solution, diaminobenzidine (DAB) and crystal violet solution were purchased from Sigma (St. Louis, MO).

5. Immunohistochemistry

Paraffin embedded tumor slides were deparaffinized in xylene, rehydrated in graded alcohols and then rinsed in PBS with 0.025%Trition. Antigen retrieval was performed in a microwave in Tris/EDTA(pH 8) for 10 min. Endogenous peroxidase activity was blocked by incubation in 1.5% H2O2 at room temperature for 15 min. After blocking by 5% nonfat dry milk in PBS, the sections were incubated with STRAP antibody (611346, BD Transduction Laboratories™) (1:100) at 4°C overnight. HRP-conjugated anti-mouse polymer secondary antibody was then applied for 1 h. Then reaction products were visualized using DAB and counterstained with hematoxylin. STRAP staining was scored by two independent observers. The intensity of STRAP staining was classified in three categories: 0, 1, 2, respectively correlating with weak, moderate or strong staining. In our study, we generated STRAP knock-out HCC cell lines that were used to test the specificity of the antibody (Supplemental Figure S1).

6. β-catenin reporter assays

The β-catenin reporter assays were basically performed as previously described (19, 20). In short, twenty hours before transfection, we plated 0.5×105 cells per well on 24-well plates. Each well was transfected with 250 ng Wnt Responsive Element (WRE) or Mutant Responsive Element (MRE) vectors and 10 ng CMV-Renilla using FuGENE® HD Transfection Reagent (E2311, 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 (E1980, Promega) according to the manufacturer's instruction. Transfections were performed twice in duplicate and the mean and standard error were calculated for each condition. The β-catenin reporter activities are shown as WRE/MRE ratios.

7. Western blotting

Cells were lysed in Laemmli sample buffer with 0.1 M DTT and heated for 10 minutes at 95°C, followed by loading and separation on a 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. The membrane was blocked with Odyssey Blocking Buffer followed by incubation with primary antibody (1:1000) overnight at 4°C. Anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000) were applied for 1 hour at room temperature. Blots were assayed for Tubulin or β-actin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantified with Odyssey 3.0 software.

8. Gene knockdown by small interfering RNA (siRNA)

Smartpool ON-TARGETplus siRNAs targeting *STRAP* were purchased from Dharmacon. The ON-TARGETplus Non-targeting siRNA #2 was used as negative control. Cells were reverse-transfected in a 24-well plate using a total of 0.8 µl DharmaFECT formulation 4 (Thermo Fischer Scientific) and 25nM of each siRNA per well. Following 72 h incubation, the effect of knock-down was tested by western blotting or β-catenin reporter assay. Alternatively, 48 h after reverse transfection, the cells were transfected with WRE or MRE vectors and CMV-Renilla for β-catenin reporter assay.

9. Construction of CRISPR/Cas9 *STRAP*-targeting vectors

Single guide RNAs (sgRNAs) targeting exon 1 or 2 of human STRAP were designed using the following CRISPR design tool (http://crispr.mit.edu/). Supplemental table S1 depicts the three selected sgRNAs, chosen because of lowest predicted potential exonic off-target sites. Oligos were dissolved at 100 pmol/µl and annealed by combining 10 µl of each with 2 µl of NEB buffer 3, heated in a PCR machine to 94°C for 4 minutes, removed and allowed to cool down to room temperature. Annealed oligos were diluted 1000x in water of which 1 µl was combined with

100 ng of BbsI-digested and purified pX330 in a total ligation volume of 20 µl using 1.5 units T4 DNA ligase. Next, ligated plasmids were electroporated into DH10B E. coli. After plating, correct plasmids were identified and sequence-verified using standard procedures.

10. Generation of *STRAP* knock-out HCC cell lines

Huh6, Huh7 and PLC/PRF/5 cell lines were transfected in 6-well plates using 7.5 µl FuGENE® HD Transfection Reagent (E2311, Promega) and 2 µg of each pX330 plasmid per well together with 0.2 µg GFP expression construct. GFP expression was used to select the cells that received high levels of the pX330 CRISPR/Cas9 constructs. After incubation at 37°C for 24 h, single cells were prepared for fluorescence activated cell sorting to a 96-well plate. After single cell sorting, Huh7 cells were maintained in DMEM supplemented with either 20% FCS or 25% Huh7-conditioned medium. Huh6 and PLC/PRF/5 were cultured in complete DMEM medium.

Clones grown successfully from single cells were first subjected to western blotting with anti-STRAP antibody (611346, BD Transduction Laboratories) (Supplemental Figure S2). For each cell line, apparently successful STRAP knock-out and control clones were selected for DNA sequence verification using oligos shown in Supplemental Table S2. For clones with complicated chromatograms, we also employed next-generation sequencing (NGS) on an Ion-Torrent device using the fusion method for amplicon library preparation. This method uses oligos designed to directly include barcodes and adaptors required for processing on the Ion-Torrent device (see Supplemental Table S3). PCR products were generated using Q5 proofreading polymerase (NEB) according to manufacturer's instructions, followed by purification and NGS according to routine protocols. All selected clones were re-tested for STRAP-loss using an additional STRAP antibody (HPA027320, Atlas antibodies).

11. Analysis of cell cycle

At approximately 60%-80% confluency in 12-well plates, cells were trypsinized, 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 µg/ml RNase at 37°C for 30 min followed by incubation with 50 µg/ml Propidium Iodide (PI) at 4°C for 30 min. Then samples were tested immediately by FACS. Cell cycle was analyzed by FlowJo_V10 software. Independent STRAP knock-out clones were tested for Huh6, Huh7 and PLC/PRF/5 cell lines. Tests were performed twice in duplicate. The mean and standard error were calculated for each condition.

12. Colony formation assay

After trypsinization, 1000 cells for each clone were seeded in 6-well plates and were cultured in 2ml complete DMEM medium per well. Two weeks later, the cells were washed with PBS, fixed in 4% PBS-buffered paraformaldehyde for 10 min and stained with crystal violet solution. The number of colonies were counted under a microscope. Tests were performed at least in triplicates. The mean and standard error were calculated for each condition.

13. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated with the Machery-NucleoSpin RNA II kit (BIOKE, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). CDNA was prepared from total RNA using a random-primed 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 StepOnev2.0 software (Applied Biosystem, Darmstadt, Germany). All expression levels are depicted relative to the expression of *GAPDH*. Primer sequences are provided in Supplemental Table S4.

14. RNA extraction, Illumina library preparation and sequencing

Total RNA was isolated with the Machery-NucleoSpin RNA II kit (BIOKE, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). RNA quality was checked using a RNA Pico chip on the Agilent Bioanalyzer. Library was constructed and sequenced with an Illumina HiSeqTM2000 (GATC Biotech, Konstanz, Germany). Briefly, the mRNA was enriched using oligo-dT magnetic beads, followed by fragmentation (about 200 bp). Then the first strand of cDNA was synthesized using random hexamer-primer and the second strand was further synthesized in a reaction buffer including dNTPs, RNase H and DNA polymerase I. Double stranded cDNA was purified with magnetic beads. Then, the 3'-end single nucleotide A (adenine) was added and adapters were ligated to the fragments which were enriched by PCR amplification.

15. RNA-seq analysis

The Illumina single-end reads were trimmed to remove the TrueSeq adapter sequences using Trimmomatic (v.0.33). Subsequently, the reads were mapped to the human reference genome build hg38 with the RNA-seq aligner STAR (v2.4.2a) and the Homo sapiens GENCODE v23 annotation. Raw counts were measured with summarizeOverlaps function from the

Bioconductor GenomicAlignments package (v1.12.1) using the setting mode union. The differentially expressed genes were called with a generalized linear model using a negative binomial distribution and accounting for the different cell lines (Huh6 and Huh7). The calculations were performed by the DESeq2 package (v1.16.1). We applied a Wald-test to identify statistical significant differently expressed genes with a False Discover Rate (FDR) that was calculated using Benjamini Hochberg correction and set a threshold value of 0.01. After blind variance stabilizing $\log_2$ transformation of the counts, the differentially expressed genes were used to calculate scaled gene-wise values (Z-score). The scaled values were, clustered hierarchically with complete linkage using Euclidean distances and subsequently plotted in a heat map with pheatmap package(v1.0.8). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene enrichment analyses were carried out as described previously (21). We used R(v 3.4.0) for statistics and visualization of the data.

16. Statistical analysis

All results were presented as mean ± SD or mean ± SEM as described in the figure legends. Comparison of STRAP protein staining between HCC tumor group and adjacent normal groups were performed with test of proportion. Differences were considered significant at a *P* value less than 0.05.

17. Data accessibility

The RNA-sequencing data from this study have been submitted to the Gene Expression Omnibus (GEO) (22) database under the accession number GSE101061.

Results

1. STRAP is up-regulated in patient HCC tumor tissues

In order to assess the expression level of STRAP protein in HCC lesions, we stained a tissue microarray (TMA) containing cores of 141 HCC tumors and patient matched adjacent normal tissues. The intensity of STRAP staining was classified in three categories and scored by two independent investigators resulting in a Kappa test of 0.609 (for STRAP in HCC tumors), which was deemed acceptable (Supplemental Table S5). In most normal samples STRAP protein was expressed at low to moderate levels, while it was significantly elevated in the majority of HCC tumors (Figure 1). Within the tumor cells, STRAP showed a predominant cytoplasmic location

(Figure 1D). Similar results were observed in Diethylnitrosamine (DEN) induced liver tumors in mice, in which 22 out of 28 tumor nodules showed increased STRAP expression relative to flanking normal liver tissue (Supplemental Figure S3).

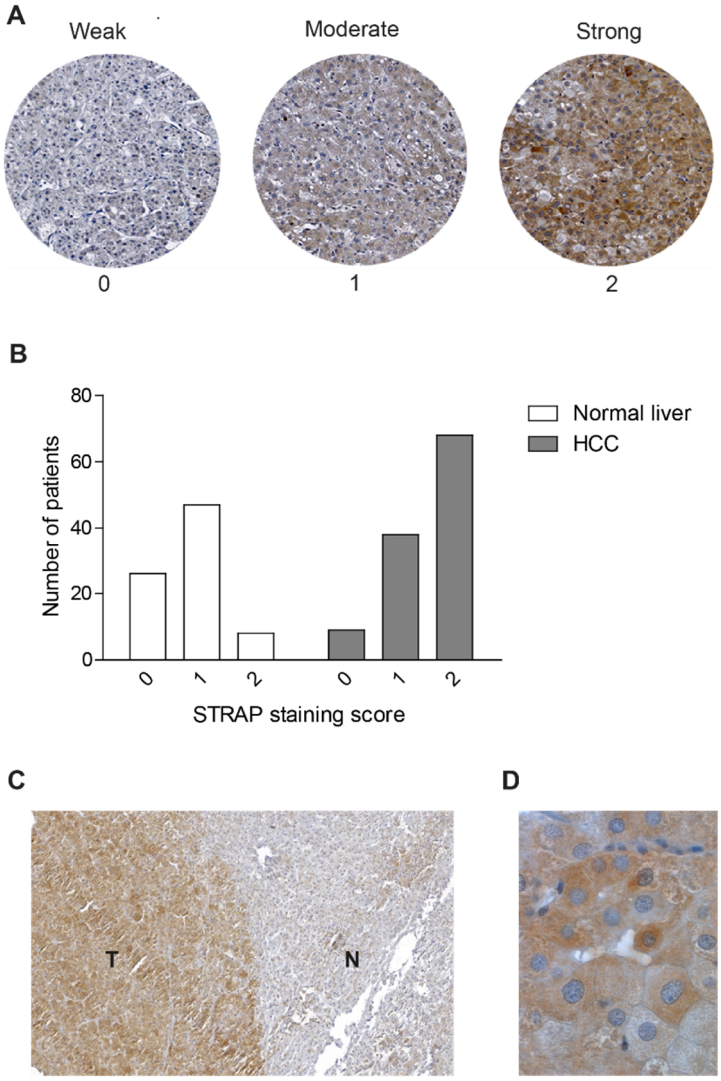

Figure 1. Elevated expression of STRAP in patient HCC tumor tissues. (A) The levels of STRAP protein positivity were scored from weak (0), moderate (1) to strong (2), both in the adjacent normal liver and HCC tumor tissue. (B) Distribution of normal liver and HCC tissues classified by above categories. Differences in the number of patients between normal liver and HCC tumors are highly significant (P<0.001, test of proportion). (C) Representative STRAP staining of a HCC tumor (T) with adjacent normal liver tissue (N); original magnification 100x. (D) STRAP shows a predominant cytoplasmic location in hepatocellular carcinoma cells; original magnification 630x.

2. Knockout of the *STRAP* gene by CRISPR/Cas9 technology

We tested the baseline expression of STRAP in our panel of 9 cell lines. All lines showed readily detectable STRAP protein and RNA, with little variation between cell lines (Supplemental Figure S4). In order to determine the function of STRAP protein for supporting cell growth and Wnt/β-catenin signaling, we used the CRISPR/Cas9 technology to disrupt *STRAP* gene

expression in the *CTNNB1* mutant Huh6, non-mutant Huh7 and *AXIN1* mutant PLC/PRF/5 cell lines. As shown in Figure 2, the STRAP protein was completely lost in the selected knock-out clones of all three cell lines. The frameshift mutations observed by Sanger and next-generation sequencing are depicted in Supplemental Figure S5.

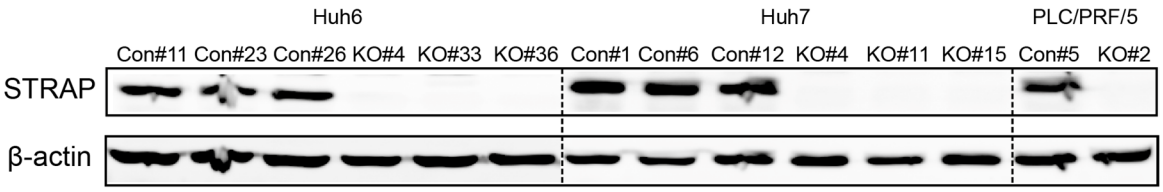

Figure 2. Knockout of the *STRAP* gene by CRISPR/Cas9 technology in HCC cell lines. Huh6, Huh7 and PLC/PRF/5 were transfected with *STRAP*-targeting vectors and single-cell sorted by FACS. The surviving clones were subjected to western blot. Depicted are STRAP protein levels of independent control and knock-out clones.

3. Transcriptome analysis of STRAP knock-out clones by RNA sequencing

To investigate the genome-wide effects of STRAP in regulating gene expression in HCC cell lines, total RNA of selected STRAP knock-out and control clones was subjected to RNA sequencing. We restricted this analysis to Huh6 and Huh7, as for these lines a minimum of 3 independent clones were available. The hierarchical clustering results successfully distinguished the Huh6 from the Huh7 cell line. Importantly, the STRAP KO Huh6 clones preferentially clustered with Huh7 KO ones. Likewise, control Huh6 and Huh7 clones were clustered. According to STRAP genotype, 5605 differentially expressed genes (threshold FDR<0.01) were clustered in both Huh6 and Huh7 (Figure 3A).

For validation of the differentially expressed genes identified from RNA sequencing, a total of eight genes were selected for qRT-PCR in Huh6 and Huh7 cell lines, which were among the top genes either up- or down-regulated. As shown in Figure 3B, $\log_2$ fold change of these genes tested by qRT-PCR significantly correlated with those from RNA sequencing (R=0.998 in Huh6 and R=0.913 in Huh7). In addition, we tested these genes in PLC/PRF/5 clones by qRT-PCR (Figure 3C). With the exception of *NTHL1* and *ABHD13*, all six other genes showed similar trends in direction and magnitude of change as observed in the Huh6 and Huh7 clones. Taken together, these data indicate that STRAP plays an important role, directly or indirectly, in the transcriptional regulation of many genes in HCC cell lines.

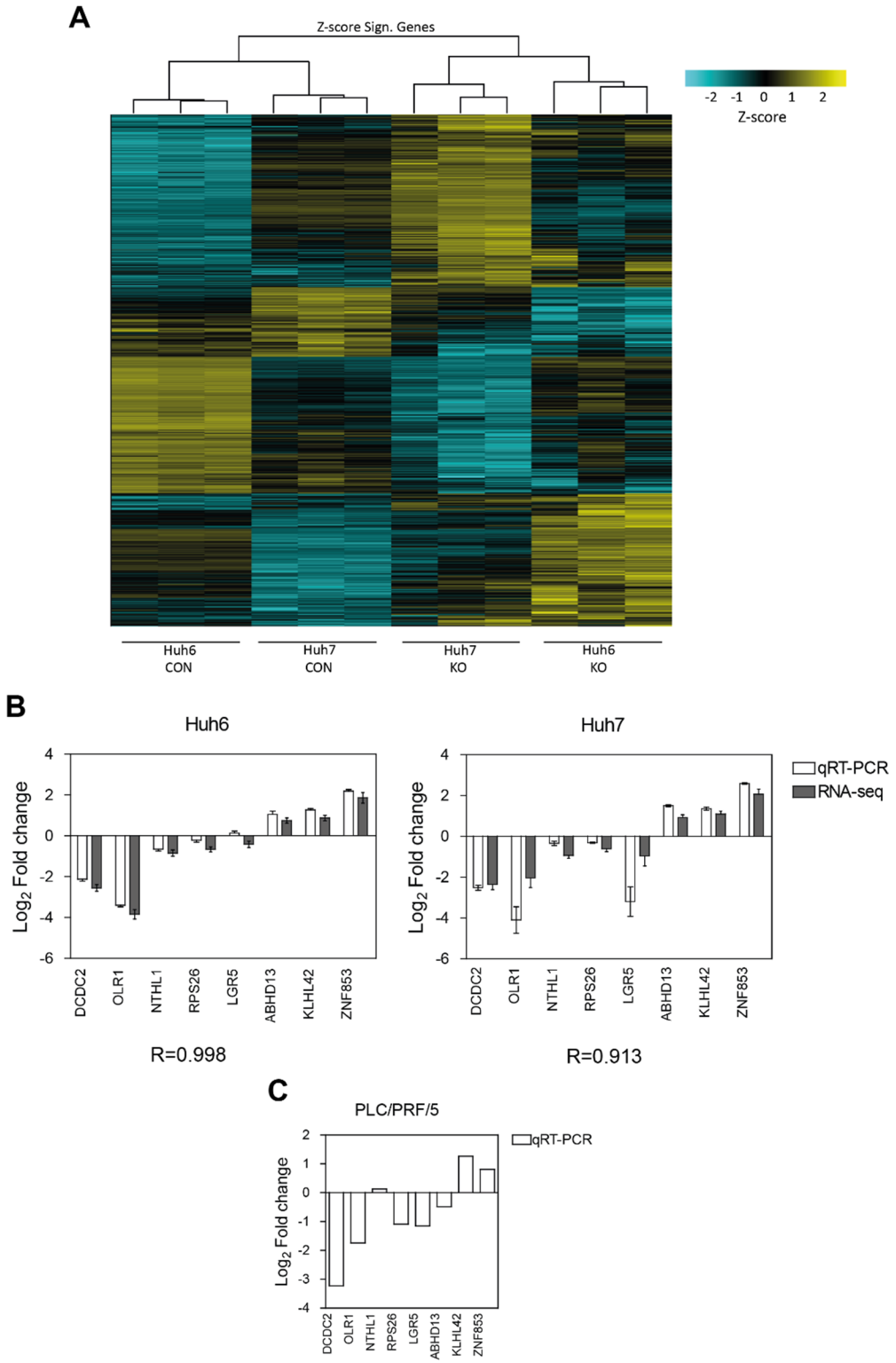

Figure 3. Differential expression profiles in STRAP knock-out Huh6 and Huh7 clones. (A) Hierarchically clustered heat map using complete linkage showing scaled Z-score color key of normalized counts of 5605 differentially expressed genes in 3 control (CON) and 3 STRAP knockout (KO) clones of Huh6 and Huh7. Columns represent clones and rows show the differentially expressed genes. (B) Comparison of relative log_2 fold changes of selected genes tested by RNA-seq and qRT-PCR. The results are presented as log_2 fold change ± Standard Error, n=3. (C) Log_2 fold change of the selected genes tested in PLC/PRF/5 control and STRAP knock-out clones. Values are depicted relative to the numbers obtained for the controls, which are arbitrarily set to 0.

4. Loss of STRAP regulates cell cycle progression

Both Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) enrichment analysis revealed that many metabolic processes were reduced in activity in the STRAP knock-out clones. In addition, this analysis showed that cell cycle progression was significantly affected, as a result of STRAP deletion in both Huh6 as well as Huh7 (Figure 4A and B). This was in line with the general slower growth of all knock-out clones observed during routine culture (data not shown). To further explore the role of STRAP in cell cycle regulation, we tested the cell cycle distribution of Huh6, Huh7 and PLC/PRF/5 by flow cytometry. STRAP knock-out Huh6 cells were significantly enriched in G0/G1 (clone KO#4) or G2/M phases (clone KO#1.33 and #36) compared to control clones. Huh7 showed a significant and uniform accumulation in the G0/G1 phase and a reduction in G2/M phase in all three independent STRAP knock-out clones. Also in PLC/PRF/5, the number of cells in the G2/M phase was significantly lower in the STRAP knock-out clone compared to control cells (Figure 4C). These results suggest that STRAP is required for a proper regulation of the cell cycle in HCC cells.

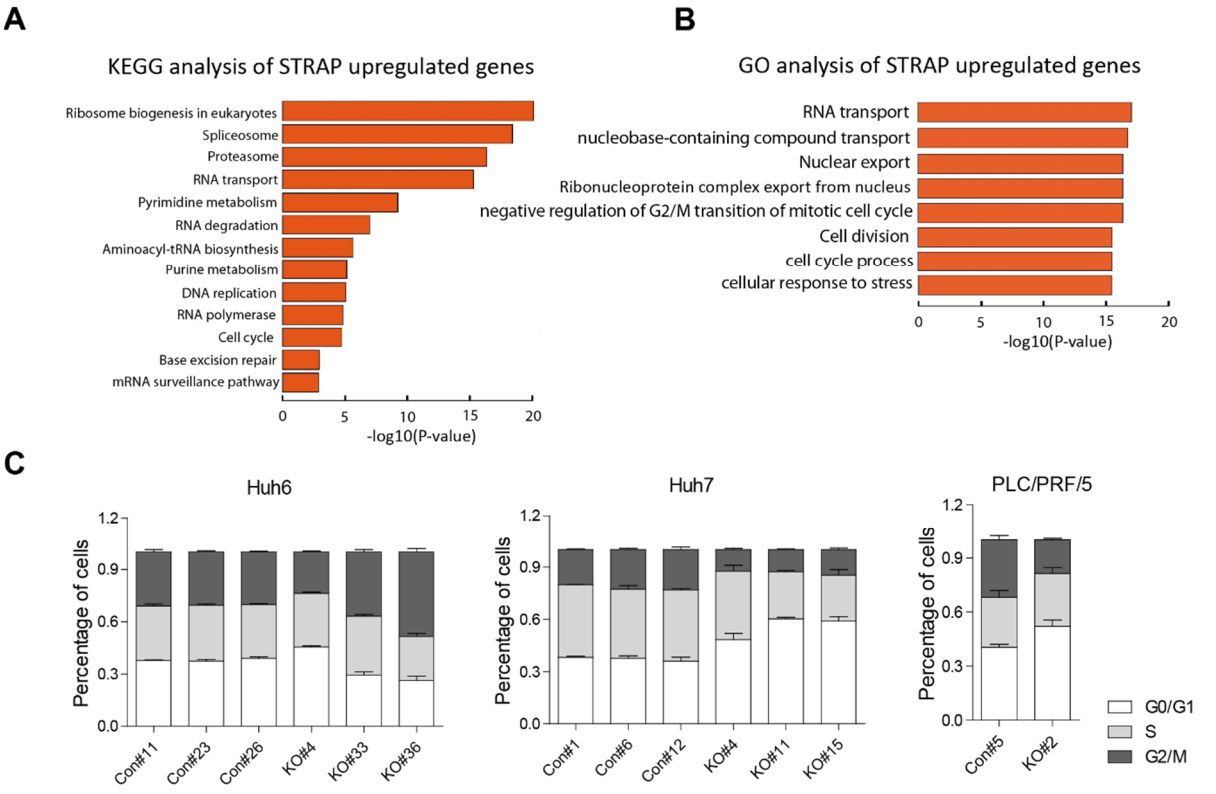

Figure 4. Loss of STRAP affects cell cycle progression. (A) Gene Ontology and (B) KEGG Pathway Gene Set Enrichment analysis of genes significantly higher expressed in the control clones. X-axis indicates *P*-value in the $-\log_{10}$ scale. (C) Flow cytometry shows that deletion of STRAP alters the cell cycle progression in Huh6, Huh7 and PLC/PRF/5 clones. (mean ± SD, n=2, two times).

5. Loss of STRAP perturbs the formation of colonies

To assess the role of STRAP on the reproductive viability of HCC cells, a colony formation assay was employed with the STRAP knockout-clones and controls thereof. We observed that loss of STRAP dramatically decreased not only the number but also the size of Huh6 colonies. Similarly, Huh7 and PLC/PRF/5 showed fewer and hardly noticeable colonies in the STRAP knock-out clones (Figure 5). These results indicate that STRAP is important for an efficient outgrowth of single HCC cells.

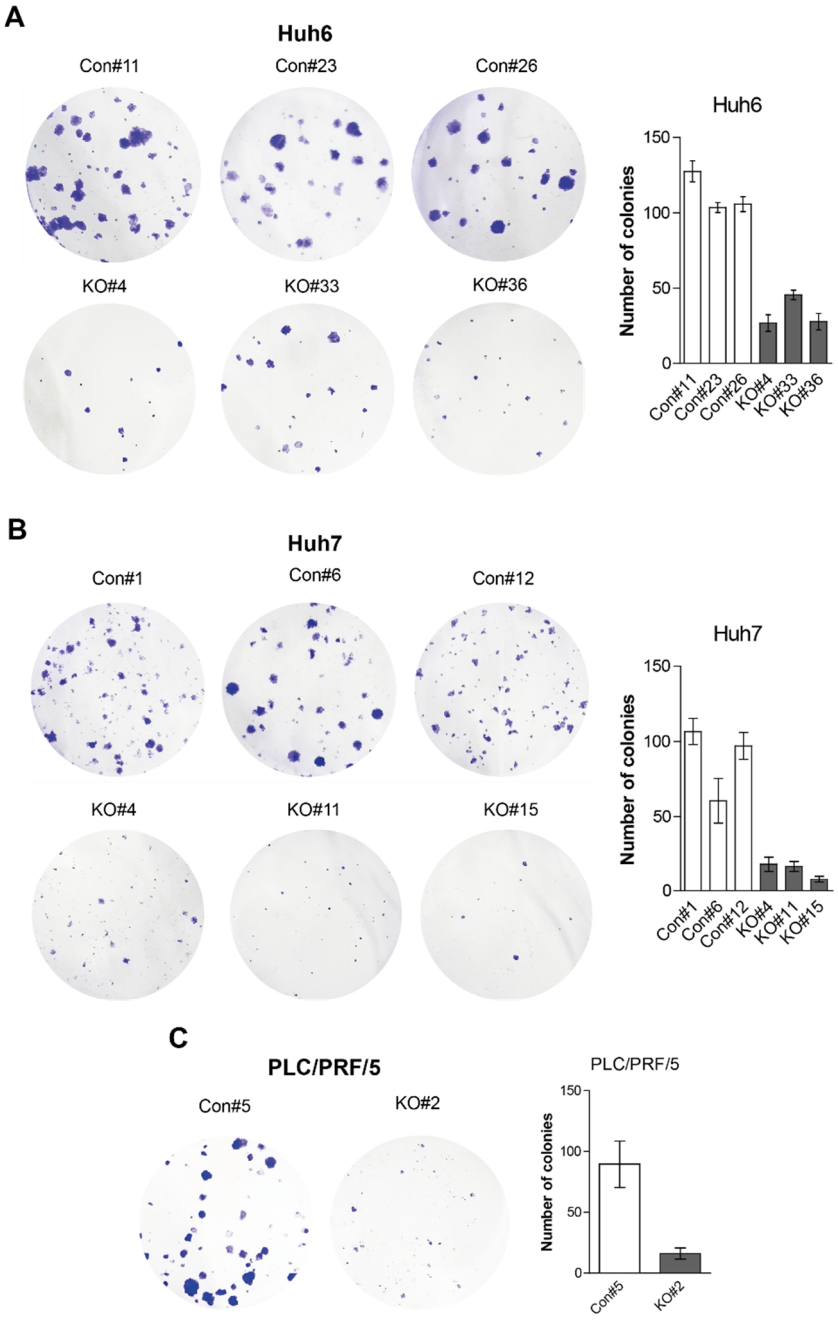

Figure 5. Loss of STRAP perturbs the capacity of colony formation in HCC cell lines. (A,B,C) Deletion of STRAP dramatically inhibits the colony formation of Huh6, Huh7 and PLC/PRF/5 cell lines. The number of colonies in each well was counted (mean ± SD, n=4 for each clone).

6. Loss of STRAP attenuates Wnt/β-catenin signaling activity

It has been reported that upregulation of STRAP correlates with increased Wnt/β-catenin signaling activity in colorectal cancer (16). We wondered whether this also holds true for HCC cells, most of which are known to also depend on β-catenin signaling for sustaining optimal growth (23). To this aim, we evaluated the expression change within the RNA-seq data of several liver specific β-catenin signaling target genes reported previously, i.e. *AXIN2*, *LGR5*, *MYC*, *CCND1*, *GLUL*, *RGN* and *BIRC5* (also known as Survivin) (24). In Huh7 all these genes were downregulated in the STRAP knock-out clones with the exception of *RGN* and *GLUL*. In the *CTNNB1* mutant Huh6 cells the differences were less obvious but most genes showed a trend towards lower expression (Figure 6A).

To confirm the effect of STRAP on Wnt/β-catenin signaling activity using a more sensitive method, we employed a β-catenin reporter assay. Loss of STRAP led to a robust reduction of β-catenin signaling activity in the Huh6 and Huh7 cells as well as a notable decrease in the PLC/PRF/5 knock-out clone (Figure 6B). Using siRNA mediated knockdown of *STRAP*, similar reductions in β-catenin signaling were observed in the original Huh6, Huh7 and PLC/PRF/5 lines and three additional HCC lines, indicating that STRAP is required to maintain optimal β-catenin signaling in most, if not all, HCC lines (Figure 6C).

STRAP has been shown to bind to GSK3β around the catalytic domain, thereby reducing the N-terminal phosphorylation of β-catenin (16, 25) and subsequently increasing the active signaling pool of β-catenin, i.e. unphosphorylated at S33/ S37/T41. Hence, we investigated the amount of the active β-catenin signaling pool present in the HCC clones. As shown in Figure 6D, Huh6 and PLC/PRF/5 showed reduced levels of active β-catenin in the STRAP knock-out clones, whereas no change was observed in Huh7.

Besides N-terminal phosphorylation, the signaling strength of β-catenin is also regulated by phosphorylation at its C-terminus. For example, S675 phosphorylation by protein kinase A (PKA) has been shown to increase Wnt signaling by recruiting transcriptional co-activators (20, 26-28). To investigate whether STRAP promotes Wnt/β-catenin signaling through indirectly affecting the phosphorylation of S675, we tested its levels. As indicated in Figure 6E, reduced levels of phosphorylated β-catenin at S675 were observed in the STRAP knock-out clones of Huh6 and PLC/PRF/5 while no clear change was seen in the Huh7 cell line. Thus, in Huh6 and

PLC/PRF/5 loss of STRAP is accompanied by lower levels of unphosphorylated N-terminal β-catenin and reduced phosphorylation at its C-terminus, both features that are associated with reduced signaling, whereas no alteration is observed in Huh7.

Together, these findings suggest that increased STRAP enhances Wnt/β-catenin signaling in HCC cells by inhibiting N-terminal phosphorylation and favoring C-terminal phosphorylation of β-catenin.

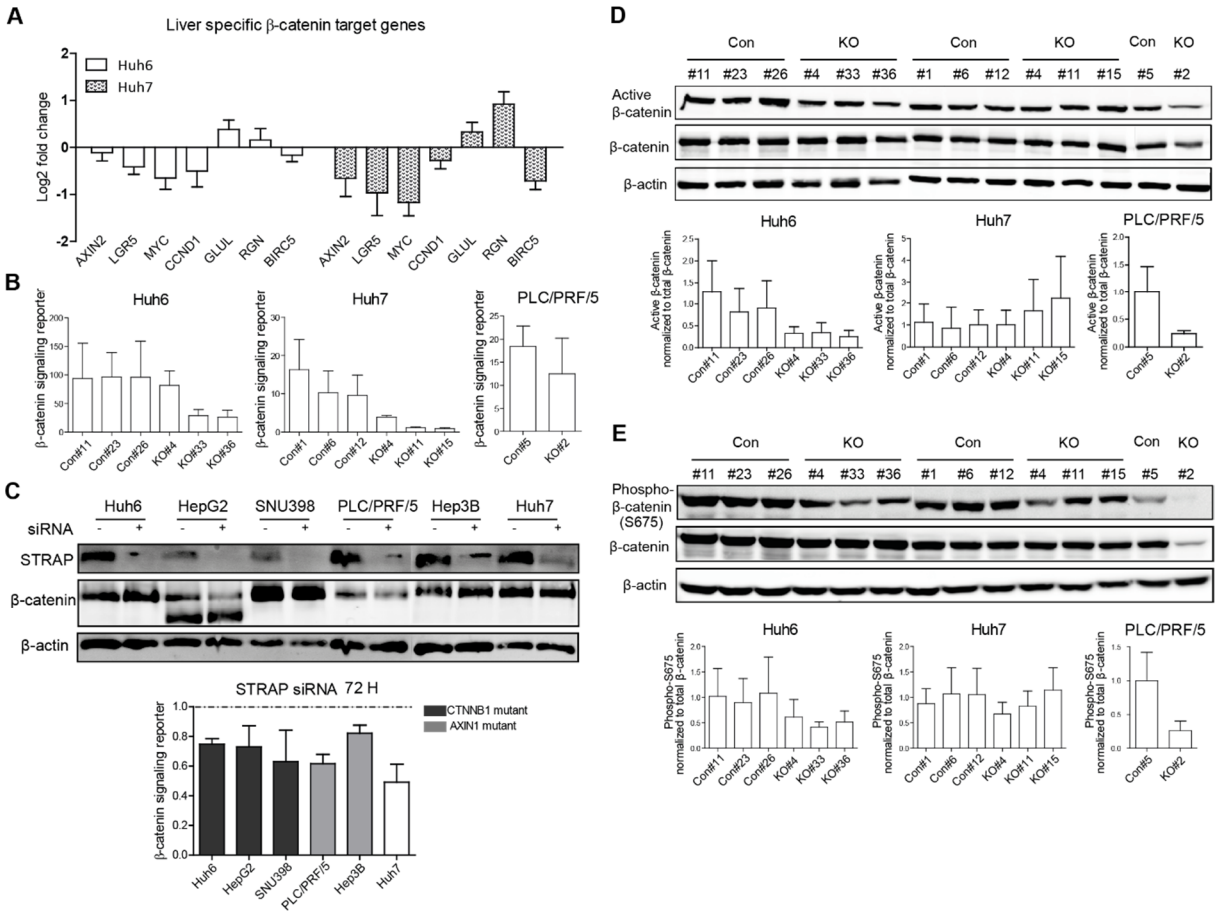

Figure 6. Loss of STRAP reduces Wnt/β-catenin signaling activity in HCC cell lines. (A) Log_2 fold change of liver specific target genes of Wnt/β-catenin signaling in STRAP knock-out clones derived from the RNA-seq data. (B) β-catenin luciferase reporter assay showing reduced β-catenin signaling activity in STRAP knock-out clones of Huh6, Huh7 and PLC/PRF/5 cell lines compared to controls (mean ± SD, n = 2, two times). The β-catenin reporter activities are depicted as WRE/MRE ratios. (C) SiRNA mediated knockdown of STRAP suppresses β-catenin signaling activity in HCC cell lines (mean ± SD, n = 2, two times). Values are depicted relative to the WRE/MRE ratios obtained for the controls, which are arbitrarily set to 1. (D) Deletion of STRAP decreases the abundance of active β-catenin (unphospho-Ser33/Ser37/Thr41) in Huh6 and PLC/PRF/5 cells. Values are depicted relative to the signal intensities obtained for total β-catenin (mean ± SD, n = 3 for each clone). (E) The level of phosphorylated β-catenin at S675 is diminished in STRAP knock-out Huh6 and PLC/PRF/5 cell lines. Values are depicted relative to signal intensities obtained for total β-catenin (mean ± SD, n = 3 for each clone).

7. Loss of STRAP associates with reduced stemness and increased differentiation markers

Wnt/β-catenin signaling is essential for the homeostatic self-renewal and proliferation of the hepatic stem/progenitor cells (29). In particular, Wnt/β-catenin driven AXIN2+ (30) and LGR5+ (31) cells have been identified as stem cells that self-renew and give rise to mature hepatocytes. Therefore, loss of STRAP and the resulting reduction in β-catenin signaling may lead to reduced expression of stem/progenitor cell markers. We observed a clear reduction at the transcription level of *AXIN2* and *LGR5* and other liver progenitor markers (*SOX9*, *CD44* and *PROM1/CD133*) (32) in STRAP knock-out Huh6 cells. In Huh7, expression of *AXIN2*, *LGR5* and *PROM1* but not *SOX9* and *CD44* were decreased (supplemental Figure S6A). Conversely, STRAP deletion increased most liver differentiation related genes, such as *ALB*, *AFP* and *HNF4A* (33, 34) in Huh6 and more obviously in Huh7 cells (Supplemental Figure S6B). Hence, the enhanced STRAP expression observed in HCC may contribute to stem cell maintenance and dedifferentiation of liver tumor cells.

Discussion

STRAP has been identified as a scaffolding protein that is upregulated in breast, lung and colorectal cancers, and was shown to promote their growth (10-12). Here we show that also in most hepatocellular cancers increased levels of STRAP can be observed. Furthermore, knock-out experiments in three different HCC cell lines showed that its expression is required to support optimal growth. Although these experiments also demonstrate that STRAP is not absolutely essential for HCC cell viability, its loss strongly impairs cell-cycle progression and the formation of colonies from single cells. Mechanistically we provide evidence that many signaling pathways and metabolic processes are affected following STRAP loss, including the Wnt/β-catenin signaling pathway.

Given the well-known importance of β-catenin signaling to sustain HCC growth, we have investigated this pathway in more detail (23, 24). Knock-out/down of *STRAP* resulted in reduced β-catenin signaling in all six HCC lines investigated, regardless of the underlying *CTNNB1* or *AXIN1* mutation. This is largely in line with the observation of Yuan et al for colorectal cancer cells (16). Mechanistically, STRAP has been shown to bind to the catalytic site of GSK3β, effectively resulting in reduced N-terminal phosphorylation and reduced breakdown of β-catenin (16, 25). Hence, loss of STRAP is expected to result in more GSK3-

mediated phosphorylation of β-catenin, which is in accordance with the notable decrease in non-phosphorylated (active) β-catenin observed in Huh6 and PLC/PRF/5 cell lines. In these lines a second mechanism appears active by which STRAP increases overall β-catenin signaling. PKA-mediated phosphorylation of β-catenin at S675, previously shown to result in increased signaling (20, 26-28), is higher in the clones that have retained STRAP expression. This result suggests that STRAP may be involved in modulating PKA activity through yet unknown mechanisms. Nevertheless, Huh7 cells apparently share neither mechanism for STRAP to support Wnt/β-catenin signaling, suggesting alternative routes involved. As Wnt/β-catenin signaling can be fine-tuned at multiple levels (35), uncovering the exact mechanism specifically for Huh7 is outside the scope of this current manuscript. Whichever the exact mechanism, our results show that the increased STRAP expression observed in most HCCs will support their growth by increasing overall β-catenin signaling.

The Wnt/β-catenin signaling pathway is well-known for its role in (cancer) stem cell maintenance (29-31, 36, 37). Accordingly, the reduced β-catenin signaling following knock-out of STRAP, is associated with lower expression of the β-catenin regulated stem cell markers *AXIN2* and *LGR5*. In addition, several other liver progenitor markers (*SOX9*, *CD44* and *PROM1/CD133)* are reduced in expression, whereas differentiation markers are elevated. These results suggest that one mechanism by which elevated STRAP expression supports HCC growth is to shift the balance towards the induction of stem cell features.

Besides its role in fine-tuning β-catenin signaling, STRAP has also been linked to various other cellular processes likely contributing to HCC cell viability (9, 14). One of the first functions attributed to STRAP was its role in inhibiting TGF-β signaling (13). In the normal liver this signaling pathway has a crucial role in limiting hepatocyte proliferation and inducing differentiation (38). Likewise, TGF-β signaling is considered to act as a tumor suppressor during the early stages of liver tumor formation by inducing cell-cycle arrest and apoptosis. As such, the elevated STRAP levels that we observe in HCC may contribute to tumor growth by restraining the tumor-suppressive effects of TGF-β, which is supported by the re-activation of several TGF-β anti-proliferative (CDKN1A, CDKN2B, CDKN1C and EIF4EBP1) and pro-apoptotic (BIK, BCL2L11, DAPK1, FAS and GADD45B) target genes (39, 40) derived from RNA-seq data following STRAP loss in Huh6 and Huh7 cells (Supplemental Figure S6C). However, the role of TGF-β signaling in liver cancer is complicated by the observation that a subset of tumors become resistant to the cytostatic and apoptotic effects of TGF-β, and in fact exploit TGF-β to

support their growth, migration and invasion during later stages (38, 41). This indicates that the specific contribution of STRAP to TGF-β-mediated tumor suppression has to be evaluated on a case-by-case basis.

In summary, we show that most HCCs show upregulation of STRAP expression. Our *in vitro* analyses suggest that its elevated expression is important to support optimal growth by affecting a variety of metabolic processes and signaling pathways of known importance for liver cancer. Especially, its contribution to increase Wnt/β-catenin signaling is likely to be a major effector of its tumor-promoting role.

Supplementary Figures and Tables

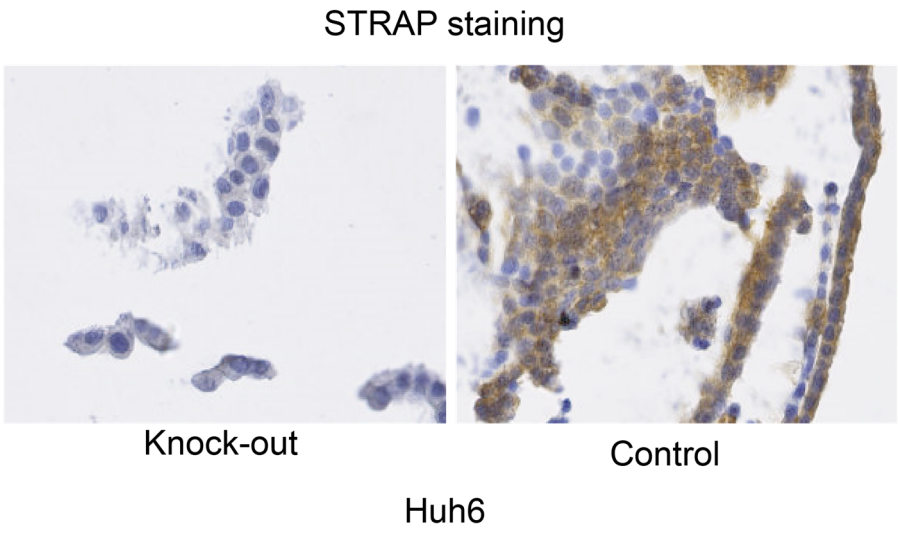

Figure S1. Specificity of STRAP antibody tested on a STRAP knock-out Huh6 clone and control thereof. For this purpose, both cell lines were formalin-fixed and embedded in paraffin.

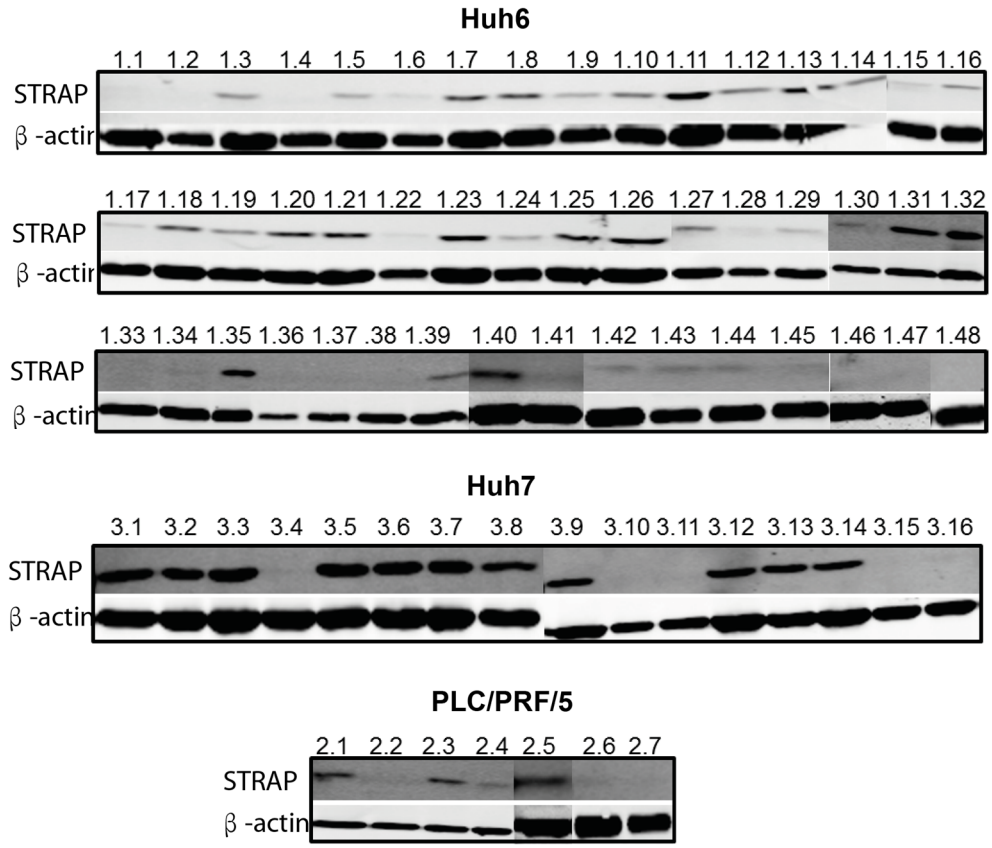

Figure.S2. Levels of STRAP protein in all clones grown successfully from single cells following CRISPR/Cas9 mediated gene editing.

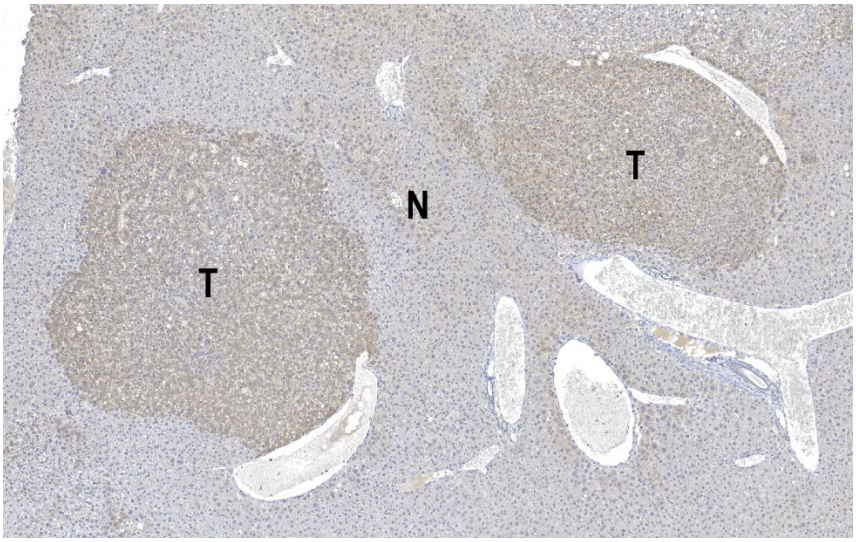

Figure S3. Elevated expression of STRAP in DEN-induced mouse liver tumors (T) compared with flanking normal liver tissue (N).

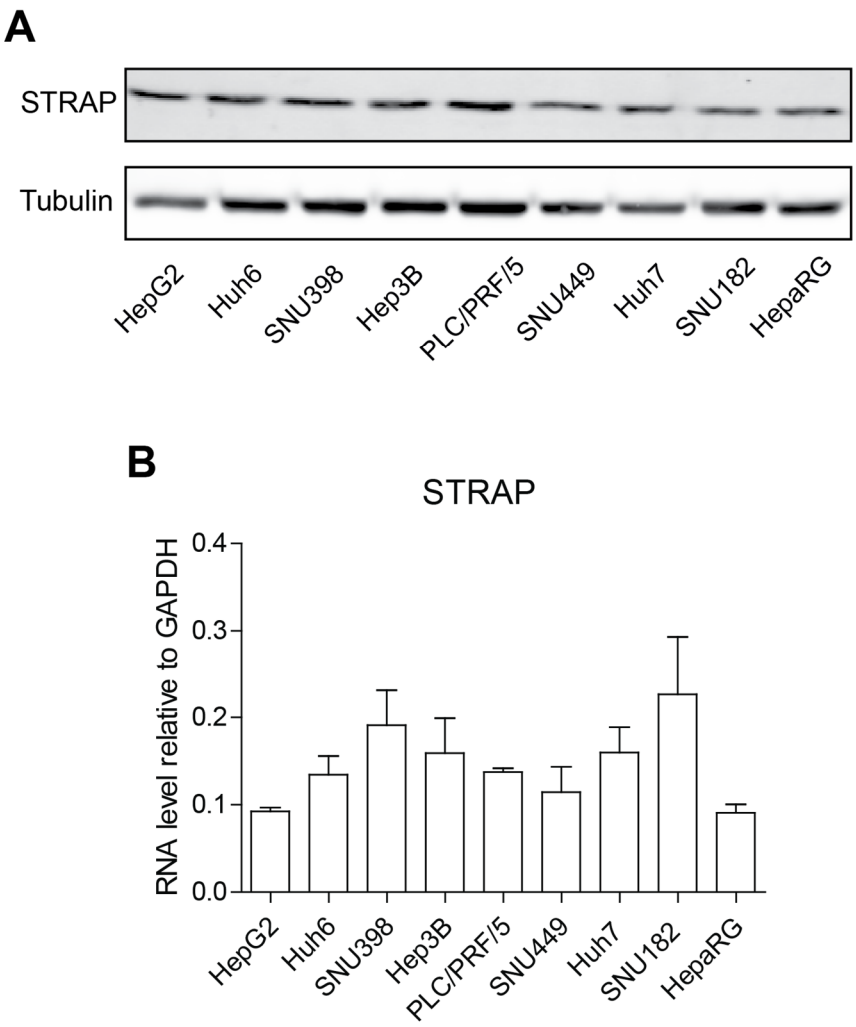

Figure S4. Baseline levels of STRAP protein and RNA in a panel of 9 HCC cell lines.

Exons	Clones	AA alteration	Size	Sequences
1	Wild type	-	-	CACGCGACCCGTGGTTGATTTGGCCTTCAGTGGCATCACGCCTTATGGGTATTTCT TAATCAGCTGGCCACACGCGACCCGTGGTTGAATT
1	Huh6 KO#33	Insertion	+81	CACGCGACCCGTGGTTGA**CCCCGTCTCCCTGGCTTTAGCCACCTCTCCATCCTCTT** **GCTTTCTTTGCCTGGACACCCCGTTCTCCTGTGGATTCGGGTA**TT
1	PLC KO#2	Deletion	-5	CACGCGACCCGTGGTTG:::::GCCTTCAGTGGCATCACGCCTTATGGGTATTTCT TAATCAGCTGGCCACACGCGACCCGTGGTTGAATT
2	Wild type	-	-	TGCAACACTGAATAAGGATGCCACCAAAGCAGCTACAG
2	Huh6 KO#4	Deletion	-1	TGCAACACTGA:TAAGGATGCCACCAAAGCAGCTACAG
		Insertion	+1	TGCAACACTGAA**A**TAAGGATGCCACCAAAGCAGCTACAG
		Deletion	-22	TGCAACAC::::::::::::::::::::::AGCTACAG
2	Huh6 KO#36	Insertion	+2	TGCAACACTGAA**AA**TAAGGATGCCACCAAAGCAGCTACAG
		Insertion	+1	TGCAACACTGAA**A**TAAGGATGCCACCAAAGCAGCTACAG
2	Huh7 KO#4	Insertion	+1	TGCAACACTGAA**A**TAAGGATGCCACCAAAGCAGCTACAG
2	Huh7 KO#11	Insertion	+1	TGCAACACTGAA**A**TAAGGATGCCACCAAAGCAGCTACAG
		Deletion	-1	TGCAACACTGA:TAAGGATGCCACCAAAGCAGCTACAG
		Insertion	+~300	from Ecoli and other unknown sources
2	Huh7 KO#15	Insertion	+1	TGCAACACTGAA**A**TAAGGATGCCACCAAAGCAGCTACAG
		Deletion	-1	TGCAACACTGA:TAAGGATGCCACCAAAGCAGCTACAG

Figure S5. Sequence alterations of selected STRAP knock-out clones. All control clones showed only the wild-type sequence. The insertion of 81 nucleotides in Huh6 KO#33 results in additional 27 amino acids within the WD40 region, which apparently makes the protein highly unstable as demonstrated by no detectable protein using two antibodies.

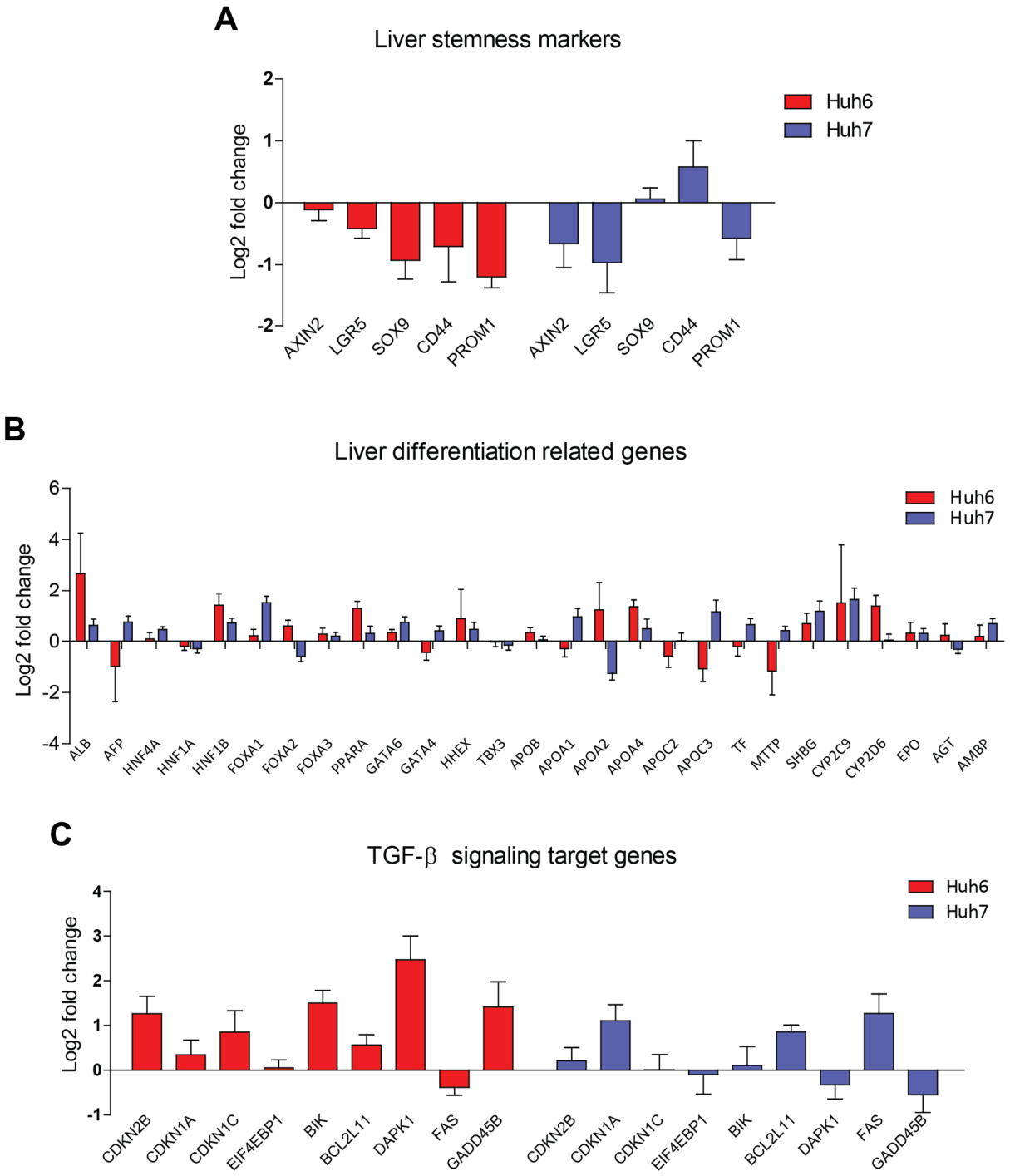

Figure S6. Log2 fold change of liver stemness markers (A), liver differentiation related genes (B) and TGF-β signaling target genes (C) in STRAP knock-out clones compared to controls. The results are presented as log2 fold change ± Standard Error, n=3.

Table S1. Selected *STRAP* sgRNAs

Species	Gene target	gRNA	Sequence
Human	STRAP exon2	sgRNA1	TTGGGGTGCAACACTGAATA
	STRAP exon1	sgRNA2	AATCAACCACGGGTCGCGTG
	STRAP exon1	sgRNA3	CACGCGACCCGTGGTTGATT

Table S2. Primer sequences of *STRAP* used for Sanger sequencing

Exons	Forward Sequence (5′~3′)	Reverse Sequence (5’~3’)
Exon1	CCCTTCTTTTCCTGTTGCC	GTGTTGGCTCTCATCTCAG
Exon2	GGTGGTAGTTAAATAGCTG	TGGGATCAAACATGCGTTC

Table S3. Primer sequence used for Next gene sequencing

Gene	Forward Sequence (5′~3′)	Reverse Sequence (5’~3’)
STRAP	Adapter A-Barcode-CCCTTCTTTTCCTGTTGCC	Adapter PI-Barcode-GTGTTGGCTCTCATCTCAG

Table S4. Primer sequences used for qRT-PCR

Gene	Forward Sequence (5′~3′)	Reverse Sequence (5’~3’)
DCDC2	ACTTGGACATAGGAGAAATCAAGA	CGAGCTGACACGTTGATCCT
NTHL1	TATGAGGGCTCGGACAGTGA	TTTGGTTTGGCTGGAGAGCA
RPS26	AAACATAGTGGAGGCCGCAG	CACATACAGCTTGGGAAGCAC
OLR1	CCTTGCTCGGAAGCTGAATG	TCTCCATGCCAGATCCAGTC
ABHD13	CCGGCGACACCCGAG	ACAAAGTTCCACAGCATCCAG
KLHL42	GGCCTCCATGAACCAGAAGA	GTTCCGGTCTCTGGTAGTGTAT
ZNF853	AGCAGGAAATGCTCCACCAG	GTGGACTGCTGTTCCTCTCC
LGR5	ACACGTACCCACAGAAGCTC	CTAAAAGCCTGGACGGGGAT

Table S5. Symmetric Measures

	Value	Asymp.Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	,609	,064	8,453	,000
N of Valid Cases	115			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Table S6. Genes information

Gene	Protein	
STRAP	STRAP	A scaffolding protein without enzymatic function exerting regulatory functions on a variety of cellular processes
CTNNB1	β-catenin	A dual function protein involved in regulation and coordination of cell–cell adhesion and gene transcription
AXIN1	AXIN1	To form a destruction complex with APC, GSK3 and CK1α leading to the degradation of β-catenin
AXIN2	AXIN2	To form a destruction complex with APC, GSK3 and CK1α leading to the degradation of β-catenin
APC	APC	A tumor suppressor, negatively regulating β-catenin by forming a destruction complex with AXIN1/2, GSK3 and CK1α
GSK3	GSK3	A Ser/Thr kinase
CSNK1A1	CK1a	Kinase with preferential acidic protein targets
LGR5	LGR5	The receptor of R-spondin family of stem cell factors to potentiate Wnt/β-catenin signaling
ASK1	MAP3K5	Mitogen-activated protein kinase
PI3K	PIK3CA	Phosphatidylinositol 3-kinase
PDK1	PDK1	Pyruvate dehydrogenase kinase
DCDC2	DCDC2	A protein with two doublecortin peptide domains binding to tubulin and enhancing microtubule polymerization
NTHL1	NTHL1	A bifunctional DNA glycosylase that has an associated beta-elimination activity
RPS26	40S ribosomal protein S26	A ribosomal protein as a component of the 40S subunit
OLR1	OLR1	The protein binds, internalizes and degrades oxidized low-density lipoprotein
ABHD13	ABHD13	Unknown
KLHL42	KLHL42	Unknown
ZNF853	Zinc finger protein 853	A protein contains the zinc finger, a structural motif, for the coordination of one or more zinc ions in order to stabilize the fold.
MYC	MYC	A multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation
CCND1	Cyclin D1	A member of highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle

GLUL	glutamate-ammonia ligase	It catalyzes the synthesis of glutamine from glutamate and ammonia in an ATP-dependent reaction
RGN	Regucalcin	It may have an important role in calcium homeostasis
BIRC5	BIRC5/Survivin	This protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death
SOX9	SOX-9	A transcription factor
CD44	CD44	*CD44 participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis.*
PROM1	Prominin-1/CD133	The precise function of CD133 remains unknown, it has been proposed to act as an organizer of cell membrane topology
ALB	Albumin	Its main function is to regulate the Oncotic pressure of blood
AFP	alpha fetoprotein	Alpha-fetoprotein expression in adults is often associated with hepatoma or teratoma
HNF4A	HNF4A/ NR2A1	HNF4A is a nuclear transcription factor
HNF1A	HNF1A	A transcription factor expressed in organs of endoderm origin
HNF1B	HNF1B	HNF1B *is a nuclear transcription factor*
FOXA1	FOXA1/ HNF-3A	A transcriptional activator for liver-specific transcripts such as albumin and transthyretin
FOXA2	FOXA2/ HNF-3B/ TCF-3B	A transcriptional activator for liver-specific transcripts such as albumin and transthyretin
FOXA3	FOXA3/ HNF-3G/ TCF-3G	A transcriptional activator for liver-specific transcripts such as albumin and transthyretin
PPARA	PPARα/NR1C1	A transcription factor and a major regulator of lipid metabolism in the liver
GATA6	GATA6	This protein preferentially binds (A/T/C)GAT(A/T)(A) of the consensus binding sequence.
GATA4	GATA4	A member of the GATA family of zinc finger transcription factors
HHEX	HHEX	A member of the homeobox family of transcription factors, many of which are involved in the development of liver, thyroid, forebrain etc.
TBX3	TBX3	A member of T-box family which are the transcription factors involved in the regulation of developmental processes
APOB	Apolipoprotein B	Apolipoprotein B is the primary apolipoprotein of chylomicrons, VLDL, IDL, and LDL particles
APOA1	Apolipoprotein A1	Apolipoprotein A1 is the major protein component of high density lipoprotein particles in plasma.
APOA2	Apolipoprotein A2	The second most abundant protein of the high density lipoprotein particles
APOA4	Apolipoprotein A4	Apolipoprotein A4 is secreted into circulation on the surface of newly synthesized chylomicron particles
APOC2	Apolipoprotein C2	A component of very low density lipoproteins and chylomicrons
APOC3	Apolipoprotein C3	A component of very low density lipoprotein
TF	Transferrin	Transferrins are iron-binding blood plasma glycoproteins that control the level of free iron (Fe) in biological fluids
MTTP	MTTP	This protein plays a central role in lipoprotein assembly
SHBG	SHBG/SSBG	A glycoprotein that binds to the two sex hormones: androgen and estrogen
CYP2C9	CYP2C9	An important cytochrome P450 enzyme with a major role in the oxidation of both xenobiotic and endogenous compounds

CYP2D6	CYP2D6	A member of the cytochrome P450 mixed-function oxidase system, is one of the most important enzymes involved in the metabolism of xenobiotics in the body
EPO	Erythropoietin/ hematopoietin	A hormone that induces red blood cell production
AGT	Angiotensinogen	Angiotensin is a peptide hormone that causes vasoconstriction and a subsequent increase in blood pressure
AMBP	AMBP	AMBP interacts with CD79A
CDKN1A	$p21^{Cip1}$	A cyclin-dependent kinase inhibitor (CKI) that is capable of inhibiting all cyclin/CDK complexes
CDKN2B	CDKN2B	A cyclin-dependent kinase inhibitor
CDKN1C	CDKN1C	A tight-binding inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation
EIF4EBP1	4E-BP1	Interaction of this protein with eIF4E inhibits complex assembly and represses translation
BIK	Bcl-2-interacting killer	Interaction of this protein with cellular and viral survival-promoting proteins, such as BCL2 and the Epstein-Barr virus enhances programmed cell death
BCL2L11	BCL2L11	Interaction of this protein with other members of the BCL-2 protein family, including BCL2, BCL2L1/BCL-X(L), and MCL1, activates apoptosis
DAPK1	DAPK1	A positive mediator of gamma-interferon induced programmed cell death
FAS	Fas cell surface death receptor	The Fas receptor is a death receptor on the surface of cells that leads to programmed cell death (apoptosis)
GADD45B	GADD45B	GADD45B responds to environmental stresses by mediating activation of the p38/JNK pathway and is involved in the regulation of growth and apoptosis

Abbreviations

STRAP, serine-threonine kinase receptor-associated protein; HCC, hepatocellular carcinoma; CRC, colorectal cancer; TMA, tissue microarray; siRNA, gene knockdown by small interfering RNA; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; qRT-PCR, quantitative real-time polymerase chain reaction; FDR, false discover rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; WRE, Wnt Responsive Element; MRE, Mutant Responsive Element; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel; PVDF, Polyvinylidene difluoride; sgRNA, Single guide RNA; NGS, Next-generation sequencing; PI, Propidium Iodide; FACS, Fluorescence activated Cell Sorting; GEO, Gene Expression Omnibus. Supplemental Table S6 depicts all used gene names and their functions.

Author contributions

WW performed the majority of experimental work as well as data analysis and authored the manuscript. SL, PL, WC and ML assisted with part of experiments. KS provided TMA slides and helped with scoring STRAP staining. HW analyzed the raw data generated by RNA sequencing. MH assisted with the single cell sorting. MP, MB and QP reviewed and improved the manuscript. RS coordinated the project and participated in authoring of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Chapter 6

A novel tissue-based ß-catenin gene and immunohistochemical analysis to exclude Familial Adenomatous Polyposis among children with hepatoblastoma tumors

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List of Abbreviations:

BWS	Beckwith-Wiedemann syndrome
DAB	3,3'-diaminobenzidine
FAP	Familial Adenomatous Polyposis
FFPE	formalin-fixed paraffin-embedded
HB	Hepatoblastoma
HCA	hepatocellular adenoma
HCC	hepatocellular carcinoma
IHC	Immunohistochemistry
LFS	Li-Fraumeni Syndrome
NF1	Neurofibromatosis type I
PBS	Phosphate-Buffered Saline
S/T	Serine/Threonine
STR	Short Tandem Repeats

Abstract

Background: The Wnt/β-catenin pathway plays a central role in the pathogenesis of most hepatoblastomas, i.e. up to 60-80% carry activating *CTNNB1* mutations. Hepatoblastomas can however also be the first manifestation of familial adenomatous polyposis (FAP). As this is a severe disease, it is important for the patient and related family members to firmly exclude FAP at an early stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, which is associated with 10-20% false-negative results. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis, which complements germline mutation screening to exclude the diagnosis FAP among HB patients.

Methods: Tumor tissues of 18 hepatoblastoma patients including 3 FAP cases were subjected to *CTNNB1* exon3 mutational analysis and immunohistochemistry comparing staining patterns for total and exon3 specific β-catenin antibodies.

Results: Our novel tissue-based method reliably identified all three FAP patients. Their tumors were characterized by a wild-type exon3 sequence and a comparable nuclear staining for both antibodies. In contrast, the non-FAP tumors carried missense *CTNNB1* mutations combined with a clearly reduced staining for the exon3 antibody, or complete loss of staining in case of lesions with exon3 deletions.

Conclusion: We have successfully established and validated a novel ß-catenin gene and immunohistochemical diagnostic method, which, when combined with routine germline DNA testing, allows the exclusion of the diagnosis FAP among HB patients.

Key words: Hepatoblastoma, genetic counseling, familial adenomatous polyposis (FAP), Wnt/β-catenin signaling, *CTNNB1*, *APC*

Introduction

Hepatoblastoma (HB) is the most common pediatric liver malignancy with an estimated incidence of 1 per 100,000 children, mainly affecting children during their first 3 years of life.[1,2] HBs are believed to originate from hepatic progenitor cells that acquire malignant transformation during embryogenesis. Histologically they show similarity to immature hepatocytes of the developing liver.[3,4] Most HBs are believed to be of sporadic nature, but about 10-20% have been associated with genetic defects such as Beckwith-Wiedemann syndrome (BWS) or familial adenomatous polyposis (FAP).[2,5,6]

The Wnt/β-catenin signaling pathway is recognized as having a central role in the pathogenesis of hepatoblastomas.[4] The β-catenin protein is a key effector of the pathway, affecting cellular decisions such as stem cell maintenance and cell proliferation through modulating the expression of specific target genes. In normal cells, β-catenin is involved both in cell adhesion, when located at the cell membrane, and in transcriptional regulation, when present in the nucleus. In the absence of upstream Wnt signaling, β-catenin is phosphorylated at N-terminal Serine/Threonine (S/T) residues by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumor suppressor, scaffold proteins AXIN1, AXIN2 and AMER1, and the kinases GSK3 and CK1α.[7-11] Phosphorylated β-catenin is then ubiquitinated, leading to its proteasomal degradation. When cells are exposed to Wnt ligands, this β-catenin breakdown complex is temporarily inhibited, leading to the stabilization of β-catenin. As a result, it translocates into the nucleus and associates with members of the TCF/LEF family of transcription factors, thus regulating the expression of specific downstream Wnt/β-catenin target genes.

In several tumor types, this pathway is constitutively activated due to 'loss of function' mutations of the *APC*, *AXIN1* or *AXIN2* genes leading to inefficient β-catenin degradation and its intracellular stabilization. Other tumors carry oncogenic β-catenin (*CTNNB1*) mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation. Important for our study, disease-causing mutations in the *APC* and *CTNNB1* genes have been shown to occur in a mutually exclusive manner in all tumor types studied so far.[12] These mutations lead to aberrant stabilization of β-catenin, which

constitutively activates downstream Wnt/β-catenin target genes and triggers a genetic program resulting in tumor formation.

HBs are characterized by a high proportion (60-80%) of activating mutations within the *CTNNB1* gene, either by point mutation or deletions encompassing exon 3.[13-22] The deletions represent more than 60% of the somatic *CTNNB1* mutations and vary in size from small intra-exonic deletions to larger ones extending up to part of exon 2 or 4. All of them have in common that they result in in-frame deletions leading to mutant β-catenin that is resistant to degradation.

Somatic APC mutations have been rarely reported in HBs.14,16,17,23 However, familial adenomatous polyposis (FAP) patients carrying a germline APC mutation, present with a 750-7500-fold increased risk of hepatoblastoma development in comparison to the general population.2,24-27 FAP is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. Untreated, the risk of colorectal cancer in FAP patients approaches 100%. The average age when colorectal polyps are detected is about 15 years.28 Colonoscopy and surgery in adolescence prevent colorectal cancer formation in FAP. When FAP is diagnosed in time, health benefits and increased life expectancy can be achieved. Importantly, in children hepatoblastoma can be the first manifestation of FAP within a family, especially given the high proportion (10-25%) of de novo germline mutations occurring in the APC gene.29-31 This is of importance for the management of the child itself and its possible future offspring, but might also have implications for yet asymptomatic parents and siblings. For this reason, it has been advocated that all HB patients with unknown family history, should be referred for APC germline mutation detection,6,32-34 using either combination of currently available methods such as next-generation or Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA). These methods will uncover up to 80-90% of disease-causing APC mutations,35,36 but inherent to any diagnostic method will lead to false-negative results. Examples include complex insertions and deletions, chromosomal translocations, or when the patient is a low-mosaic carrier of the APC mutation.37,38 Moreover, sequence analysis will identify genetic variants with uncertain disease-causing relevance. Given the severity of the FAP syndrome a thorough exclusion of carrier status is highly desirable. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis, which complements the

germline mutation screening to exclude the diagnosis FAP among HB patients. We also discuss options how it can be implemented in current clinical practice.

Materials and Methods

Patients

From the period 1995-2013, in total 23 pediatric liver tumors were available for analysis from the pathology archive of the Erasmus MC. Tissues were obtained from the initial operative procedure and embedded in paraffin after formalin fixation. The medical records and family history of patients were analyzed. Patient and tumor characteristics are provided in Table 1. All samples were revised by a GI-pathologist (MD) according to a recently proposed consensus classification for pediatric liver tumors.[39]

Immunohistochemistry (IHC)

Immunohistochemical analyses for total β-catenin (1:200, clone-14, BD-Transduction Laboratories) and non-phosphorylated Ser33/37/Thr41 β-catenin (1:400, #8814, Cell Signaling Technology) were performed in an automated stainer (Benchmark-Ultra, Ventana Medical Systems, Tucson, AZ). Sections were deparaffinized and pre-treated with standard cell conditioning 1 solution (CC1) at 100°C for 64 min, followed by incubation with the specified antibodies at 37°C for 60 min. The antibodies were visualized with the OptiView IHC DAB detection kit.

Tumor DNA and RNA isolation and *CTNNB1* mutation analysis

Tumor DNA was extracted by microdissection from formalin-fixed paraffin-embedded (FFPE) tissue fragments using proteinase-K and 5% Chelex®-100 Chelating Resin (BioRAD, #1432832), as previously described.[40] Sequence analysis of CTNNB1 exon 3 was performed by bi-directional sequencing of PCR-amplified fragments using M13-tailed forward and reverse primers (Supplemental Material S1). To test for genomic deletions of CTNNB1 exon3, two independent PCRs were performed using primers located within exon2 and exon4. Details of the PCR and sequencing reactions and RA isolation are provided in Supplemental Material S1.

Cell lines

Short Tandem Repeat-verified cell lines used in this study were cultured as previously described.[41] Preparation of paraffin-embedded cell line blocks is described in Supplemental Material S1.

Generation of β-catenin variant expression vectors and transfection

Expression vectors for N-terminal FLAG-tagged β-catenin variants were generated using the pcDNA-5'UT-FLAG vector as basis (kindly provided by Dr. Veronique Lefebvre, Lerner Research Institute, Cleveland OH, USA). Wild-type, S33Y and exon3 deleted variants of human *CTNNB1* were cloned using the Gibson assembly method (NEB). The G34V, S37F, T41A and S45P variants were generated by Q5® site-directed mutagenesis (NEB), using the wild-type clone as basis. Primers are available upon request. All plasmids were fully sequence verified. Next, HEK-293 cells seeded in 6-well plates were transiently transfected with Fugene-HD (Promega) using 1µg of plasmid DNA. After two days, cells were lysed in Laemmli sample buffer with 0.1M DTT and heated for 5-10 minutes at 95˚C.

Western blot assay

The western blot assay and quantification for the FLAG-tag, total and non-phosphorylated Ser33/37/Thr41 β-catenin were performed basically as previously described, using all antibodies at a 1:1000 dilution.[41]

Results

Patient and tumor characteristics

In total 23 pediatric liver tumors were included in the study (Table 1), of which 18 were hepatoblastomas (HB), 4 hepatocellular carcinomas (HCC) and one hepatocellular adenoma (HCA). In accordance with literature, most HB patients were diagnosed during their first three years of life. The youngest age at diagnosis was 0.5 years, whereas the oldest patient was 9.7 years. Hepatoblastoma patients HB-4, HB-14 and HB-29 had been diagnosed with FAP previously. The HCC detected in HB-15 was diagnosed in a child belonging to a Li-Fraumeni family (LFS), i.e. *TP53* germline mutation, whereas HB-32 was observed in a patient with Neurofibromatosis type I (NF1). Hypomethylation of LIT1 was reported for patient HB-19, although no other signs of BWS were observed.

Immunohistochemistry for total β-catenin

Immunohistochemistry for β-catenin showed nuclear accumulation of β-catenin in one HCC (HB-8), while in the remaining three HCCs and the HCA an exclusive membranous pattern was observed (Table 2). All HBs showed evidence of nuclear staining, including the ones derived from the FAP patients. No nuclear β-catenin was detected in normal pre-existing hepatocytes.

Table 1. Patient and tumor characteristics

Sample	Gender	Age (years)	HB/HCC	Pathology	Known genetic predisposition
HB-4	F	5.5	HB	Epithelial (too few tumor cells for proper evaluation)	Familial Adenomatous Polyposis
HB-6	M	0,6	HB	Epithelial mixed	
HB-7	M	2,1	HB	Epithelial fetal	
HB-8	M	5,9	HCC	Classic	
HB-11	F	1.2	HB	Epithelial mixed	
HB-13	F	0.5	HB	Epithelial mixed	
HB-14	M	3.5	HB	Epithelial fetal	Familial Adenomatous Polyposis
HB-15	F	9.7	HCC	Classic	Belonging to Li-Fraumeni family (TP53)
HB-16	F	15.8	HCC	Fibrolamellar	
HB-17	M	9.7	HB	Epithelial fetal with low mitotic activity	
HB-18	F	11.4	HCC	Fibrolamellar	
HB-19	F	1.7	HB	Epithelial fetal with low mitotic activity	Hypomethylation of LIT1 (no signs of BWS)
HB-20	M	1,1	HB	Mixed epithelial-mesenchymal, no teratoid	See note*
HB-21	M	0.8	HB	Epithelial mixed	
HB-22	M	1.0	HB	Epithelial mixed	
HB-23	M	9.4	HB	Epithelial fetal	
HB-24	M	1.8	HB	Epithelial fetal, mitotically active	
HB-25	F	16.3	HCA		See note*
HB-27	F	7.3	HB	Epithelial pleomorphic	
HB-28	F	1.1	HB	Mixed epithelial-mesenchymal, teratoid	
HB-29	M	1.6	HB	Mixed epithelial-mesenchymal, teratoid	Familial Adenomatous Polyposis
HB-30	F	0.8	HB	Mixed epithelial-mesenchymal	
HB-32	F	1.1	HB	Mixed epithelial-mesenchymal, no teratoid	neurofibromatosis type I

HCA; Hepatocellular Adenoma, HB; Hepatoblastoma, HCC; Hepatocellular Carcinoma

CTNNB1 mutation analysis

Sequence analysis of exon 3 of *CTNNB1* succeeded in 22/23 tumors. In 8/18 HBs point mutations were detected within or in close proximity to the region encoding the S/T residues required for proteolytic breakdown of β-catenin (Table 2). As expected, no mutation was detected in all three FAP-derived HBs and in the HCC and HCA samples without nuclear

staining. Also no mutation was detected within the single HCC with nuclear accumulation of β-catenin.

Hepatoblastomas are however characterized by a high proportion of genomic deletions partially or completely encompassing exon 3,[13-21] most of which will be missed by the regular exon 3 sequence analysis. Therefore, we attempted two genomic PCRs on DNA isolated from the FFPE samples, using a common forward primer in exon 2 and two reverse primers, respectively at the 5'- and 3'-side of exon 4. The latter can detect the reported deletions.

Table 2. *CTNNB1* mutational status and β-catenin IHC pattern of pediatric liver cancers

Sample	HB or HCC	genetic predisposition	Nucleotide change detected in genomic DNA	RNA alteration	Amino Acid alteration	C-ter β-cat IHC	S33/37/T41 β-cat IHC
HB-15	HCC	LFS	None			Membrane only	Membrane only
HB-16	HCC		None			Membrane only	Membrane only
HB-18	HCC		None			Membrane only	Membrane only
HB-25	HCA		None			Membrane only	Membrane only
HB-4	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-14	HB	FAP p.R1114*	none			Nuclear	Nuclear
HB-29	HB	FAP p.Q1062*	None			Nuclear	Nuclear
HB-6	HB		c.97T>C		p.S33P	Nuclear	Nuclear
HB-17	HB		c.86C>T + c.94G>T		p.S29F + p.D32Y	Nuclear	Moderate Nuclear
HB-22	HB		c.94G>A		p.D32S	Nuclear	Nuclear
HB-7	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-13	HB		c.101G>T		p.G34V	Patchy nuclear	Faintly nuclear
HB-20	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-21	HB		c.101G>T		p.G34V	Nuclear	Faintly nuclear
HB-28	HB		c.121A>G		p.T41A	Nuclear	Patchy nuclear
HB-8	HCC		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-11	HB		Not analyzable	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-19	HB	LIT1	None	±215 bp del	p.?	Nuclear	Negative
HB-23	HB		None			Nuclear	Membrane only
HB-24	HB		None	r.14_241del	p.A5_A80del	Nuclear	Negative
HB-27	HB		None			Nuclear	Membrane only
HB-30	HB		c.98_238del	r.98_238del	p.G34_A80del	Nuclear	Negative
HB-32	HB	NF1	c.14-202_241+69del	r.14_241del	p.A5_A80del	Nuclear	Negative

At large, four different staining patterns were observed using the C-terminal and S33/37/T43 β-catenin antibodies. Combined with the genomic mutation analysis the most likely β-catenin-related mechanism contributing to tumor formation is color-coded as follows:
No evidence of mutations leading to β-catenin activation, APC mutation or β-catenin D32/S33 amino acid alterations, β-catenin G34 amino acid alteration, β-catenin exon 3 deletion.
"Nuclear" means a combination of nuclear, cytoplasmic and membranous staining. "Negative" means no staining visible in tumor cells.
The following abbreviations are used: HCA-Hepatocellular Adenoma, HB-Hepatoblastoma, HCC-Hepatocellular Carcinoma, FAP-familial adenomatous polyposis, LFS-Li-Fraumeni syndrome, LIT1-LIT1 hypomethylation, NF1-Neurofibromatosis Type-1, IHC-Immunohistochemistry.

Immunohistochemical identification of β-catenin alterations

Given the poor quality of DNA extracted from FFPE material, we sought for an alternative method to identify samples with genomic *CTNNB1* deletions. All these mutations generate mutant β-catenin proteins that are more resistant to proteolytic breakdown. Hence, the great majority of β-catenin protein present within a *CTNNB1* mutant cell is represented by the mutant protein. This will be especially the case for the nuclear compartment as most of the wild-type protein is prevented from entering the nucleus through degradation by the APC/AXIN breakdown complex. We hypothesized that an immunohistochemical analysis using a β-catenin antibody specifically recognizing the N-terminal S/T residues, could distinguish between APC- and β-catenin mutant tumors. In APC-mutant tumors wild-type β-catenin accumulates in the nucleus that can still be detected by such an antibody, whereas (partial) deletion or mutation of the β-catenin S/T residues would prevent detection.

To validate this approach, we first tested a panel of cell lines with known *CTNNB1* mutations using a C-terminal β-catenin antibody in comparison with one raised against a peptide corresponding to residues surrounding S37. Western blot analysis using the C-terminal antibody robustly demonstrated β-catenin in all cell lines, including the shortened mutant product present in HepG2 cells (Figure 1A). The non-phospho S33/37/T41 antibody readily detected the wild-type β-catenin present in the APC-mutant line SW480. Also the S45 mutant β-catenin present in HCT116 and LS174T was demonstrated at high intensity compared with the C-terminal antibody. In contrast, the exon 3 deleted product of HepG2 and S37/T41 alterations completely abrogated detection, while S33 and G34 alterations resulted in reduced detection. The latter may represent binding to wild-type β-catenin present in these cells and/or retained affinity for the mutant protein.

To investigate residual binding affinity for mutant β-catenin by the S33/37/T41 antibody, we transiently expressed N-terminal FLAG-tagged β-catenin variants in HEK-293 cells (Figure 1B). Deletion of exon 3, and S37F/T41A alterations completely abrogated binding. In contrast, the S33Y variant was identified at comparable levels to the wild-type protein, while the G34V variant is detected with ±40% reduced signal intensity. In conclusion, the S33/37/T41 antibody retains affinity for S33/G34 alterations when present in denatured form on western blot, while S37/T41 changes and deletion of exon 3 prevent its binding.

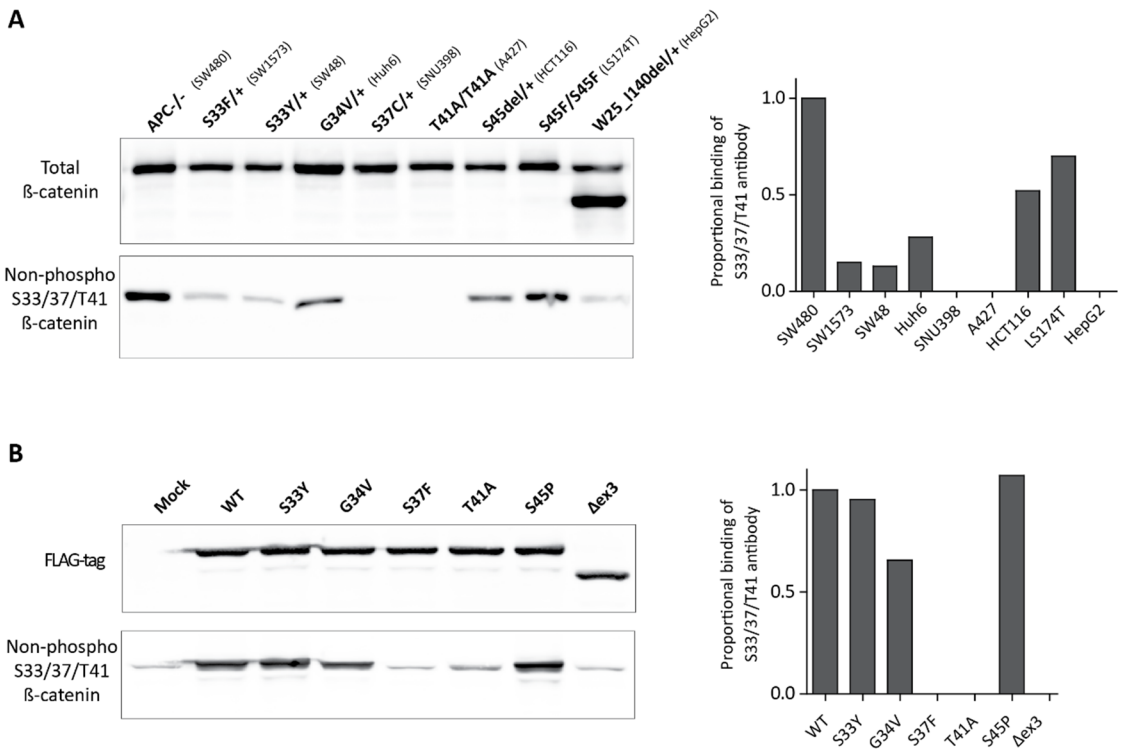


Figure 1. Western blot validation of the non-phospho S33/37/T41 β-catenin antibody. (A) Western blot analysis of one APC mutant and eight β-catenin mutant cell lines using a C-terminal β-catenin antibody (upper lanes) in comparison with one raised against a peptide corresponding to residues surrounding S37 (lower lanes). Amino acid alterations and zygosity status are depicted on top. Name of cell line is in brackets. Deletion of exon 3 and alterations of S37 and T41 abrogate binding. Reduced signal for S33 and G34 variants suggests lowered affinity, although residual binding to remaining wild type protein cannot be formally excluded. S45 changes are largely unaffected. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the *APC*-mutant SW480 cell line set arbitrarily to one. (B) Transient expression of FLAG-tagged wild type and S/T mutant β-catenin variants shows that S33Y β-catenin can still be recognized by the S33/37/T41 antibody. The G34V variant shows a ±40% reduced signal intensity. The other variants confirm the cell line analysis. The weaker band with lower molecular weight visible in all lanes is the endogenous β-catenin protein produced by HEK-293 cells. Proportional binding of the S33/37/T41 antibody is shown on the right with the ratio of the wild type protein set arbitrarily to one.

Next, we evaluated this antibody for immunohistochemical purposes using paraffin blocks containing formalin-fixed β-catenin mutant cell lines. In line with the western blot data, S45 mutant HCT116 and LS174T showed a comparable staining pattern for both antibodies (Figure 2). In strong contrast, all other β-catenin mutant lines showed a clearly reduced overall staining using the S33/37/T41 antibody. The S37C and T41A mutant lines are completely negative. In the S33 mutant lines SW48 and SW1573 nuclear staining can be observed in some cells, although with reduced intensity. The exon 3 deletion mutant HepG2 line showed an exclusive membranous staining, most likely resulting from detection of the remaining wild-type protein. Also in the G34V mutant Huh6 line no obvious nuclear staining was observed. Surprisingly, IHC staining of this variant is much weaker than anticipated from the western blot analysis, most likely resulting from reduced antibody affinity when present in its “native”

form in FFPE sections and/or partial phosphorylation of S37/T41 residues. In conclusion, the S33/37/T41 β-catenin antibody can reliably identify the cell lines with exon 3 *CTNNB1* mutations, with the exception of S33 and S45 alterations.

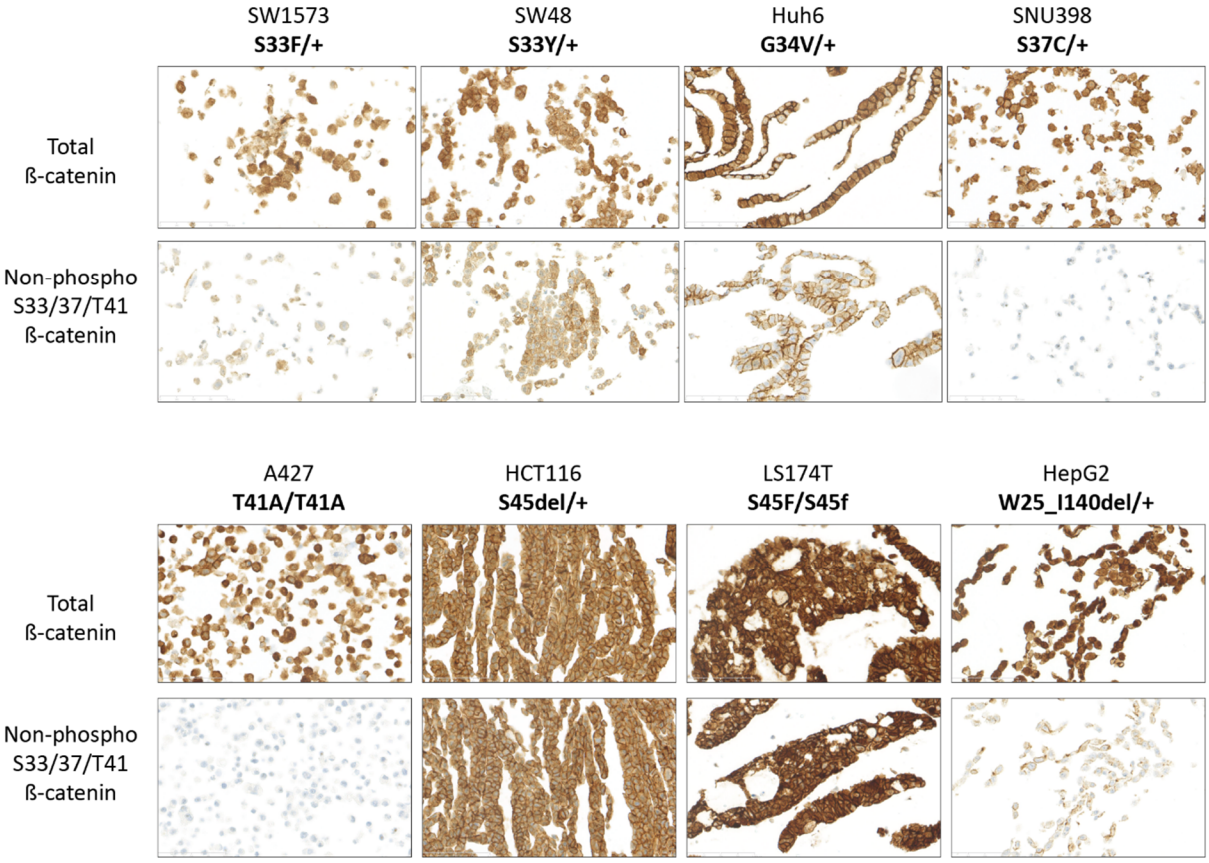


Figure 2. Immunohistochemical validation of the non-phospho S33/37/T41 β-catenin antibody. Formalin-fixed and paraffin-embedded cell pellets of the β-catenin mutant cell lines were stained with the C-terminal (top rows) and S33/37/T41 (bottom rows) β-catenin antibodies. Staining patterns are largely in line with western blot analysis with the exception of the relatively weak staining pattern observed for the G34V protein present in Huh6. HepG2 shows an exclusive membranous staining with the S33/37/T41 antibody, most likely reflecting the remaining wild type protein. Both S33 mutant cell lines retain some nuclear detection, albeit slightly reduced. S45 variant cell lines show identical staining patterns with both antibodies. Original magnification 400x.

Next, the S33/37/T41 antibody was applied to the tumor sections and compared with that of total β-catenin (Figure 3, Supplemental Figure S1). At large, four different staining patterns were observed. The tumors with exclusively membranous β-catenin were stained in an identical fashion with the S33/37/T41 antibody (Figure 3A,B and S1). Importantly, all three FAP-associated lesions showed basically identical staining patterns with both antibodies as well, i.e. a high proportion of tumor cells showing evidence of nuclear accumulation (Figure 3C,D and S1). This pattern was also observed in lesions with D32 and S33 mutations, albeit with slightly lower intensity for the S33/37/T41 antibody (Figure 3E,F and S1). The third pattern is observed in HBs with G34V mutation. These showed a strongly reduced overall

staining using the S33/37/T41 antibody. However, occasionally tumor cells with nuclear staining can be clearly observed, especially in regions with heavy staining for total β-catenin (Figure 3G,H and S1). This pattern was also observed in HB-28, the only lesion with a T41 mutation, in which a heavy overall β-catenin staining is accompanied by occasional nuclear-positive cells using the S33/37/T41 antibody (Figure S1). As the T41A alteration cannot be detected by this latter antibody, the nuclear staining most likely results from wild-type protein induced to enter the nucleus. Lastly, in both HBs with proven genomic exon 3 deletions virtually no tumor cell staining was detectable (Figure 3I,J and S1). A similar pattern was also identified in six additional samples with no identifiable point mutation, suggesting that in total 7 out of 18 HB samples and one HCC sample carry genomic exon 3 deletions. In two of these samples some residual membranous staining was visible (HB-23 and HB-27, Figure S1).

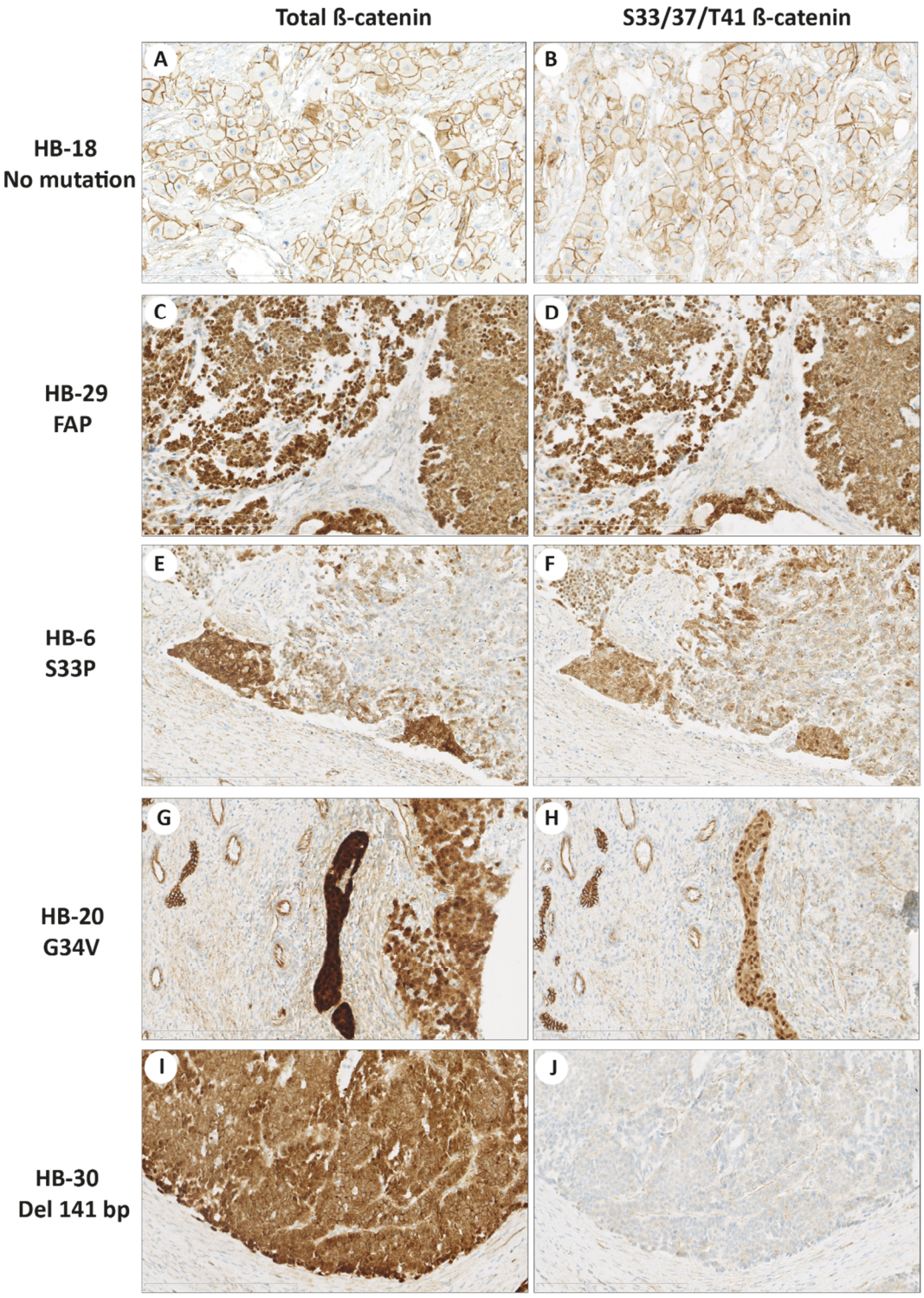


Figure 3. Immunohistochemical identification of β-catenin alterations in tumor samples.
FFPE sections of pediatric liver tumors were stained with the C-terminal (left column) and S33/37/T41 (right column) β-catenin antibodies. (A,B) Comparable membranous staining in the fibrolamellar hepatocellular carcinoma (HB-18) with no evidence of β-catenin mutation or activation. (C,D) HB-29 derived from a FAP patient shows identical nuclear and cytoplasmic accumulation of wild type β-catenin using both antibodies. (E,F) In sample HB-6 a p.S33P mutation was detected, which shows nuclear staining with both antibodies, albeit slightly weaker using the S33/37/T41 antibody. (G,H) Sample HB-20 carrying a p.G34V mutation shows clear nuclear β-catenin accumulation in tumor sections. Staining with the S33/37/T41 antibody is strongly reduced or entirely lost in tumor cells, while normal structures on the left are stained in an identical fashion. (I-L) The strong nuclear and cytoplasmic staining of sample HB-30 with genomic deletion of exon 3, is completely lost using the S33/37/T41 antibody. Original magnification 200x.

Confirmation of exon 3 deletion by RT-PCR

To confirm that these latter samples indeed express mutant *CTNNB1* mRNA (partially) lacking exon 3, we performed RT-PCR on RNA isolated from paraffin sections. Successful RNA isolation was possible in 6 samples. In 4 out of 6 samples a 127 bp product was observed indicative of a complete absence of exon 3 (Figure 4). In sample HB-30 a shortened 214 bp product was observed, which after sequencing was confirmed to carry the same intra-exonic 141 bp deletion already observed during the genomic DNA analysis. Sample HB-19 showed a shortened reproducible product of about 140 bp, indicating that an approximate 215 bp deletion is present in the cDNA. In conclusion, all 6 samples with negative S33/37/T41 β-catenin nuclear staining and absence of exon 3 point mutation, showed evidence of (partial) exon 3 deletion on RNA level.

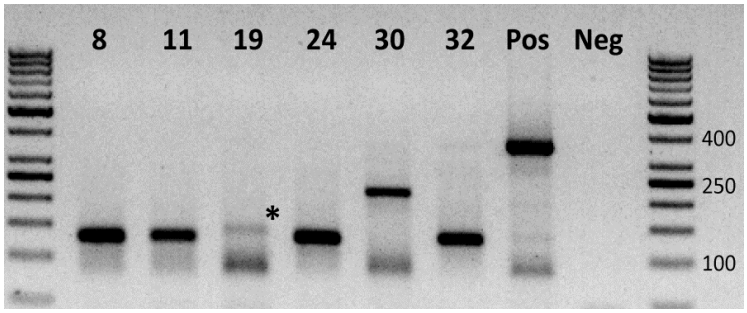


Figure 4. Confirmation of exon 3 deletion by RT-PCR.
RNA was isolated from 6 HBs with immunohistochemical evidence of (partial) exon 3 loss, and subjected to RT-PCR using primers in exon 2 and 4. In 4 out of 6 samples a 127 bp product was observed indicative of a complete absence of exon 3 (HB-8, HB-11, HB-24 and HB-32). Sample HB-19 showed a reproducible shortened product of approximately 140 bp (*). In sample HB-30 the intra-exonic 141 bp genomic deletion was confirmed on RNA level. Positive control is RNA isolated from normal tissue.

Discussion

Most HBs are believed to be of sporadic nature, but about 10-20% have been associated with genetic defects such as Beckwith-Wiedemann syndrome or familial adenomatous polyposis.[2,5,6] For this reason, it has been advocated that all HB patients with unknown family history, should be referred for genetic counseling.[6] Among the genetic syndromes associated with hepatoblastoma development, FAP is one of the most common with an estimated frequency between 5-10%.[2,16] As this is a severe disease, it is important for the patient itself but also for yet asymptomatic family members to determine their carrier status at an early stage. Especially a firm exclusion of an underlying *APC* germline mutation will prevent the unexpected emergence of polyposis or colorectal cancer at a later stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, but inherent to any

diagnostic method this will occasionally lead to false-negative results. In case of classical polyposis phenotypes the *APC* mutation detection rate is around 80-90%,[35,36] meaning that a possible 10-20% of FAP patients may be missed by routine genetic testing. This leads to the highly undesired scenario in which apparent *APC* mutation negative patients develop polyposis later. For this reason, Aretz et al. proposed for all *APC*-negative HB patients to undergo colonoscopy around age 15 and one at age 25, and to also offer colonoscopy to the parents.[2] The downside of this approach is that many genuine *APC*-negative individuals will be exposed to unneeded medical investigations, associated with anxiety and considerable costs. Here, we establish and validate a tissue-based β-catenin gene and immunohistochemical analysis that complements the germline mutation screening to exclude the diagnosis FAP among HB patients. Following successful confirmation in an independent series, its application will lead to a near-complete exclusion of FAP among HB patients.

Our method is based on the observation that tumor-driving *CTNNB1* and *APC* mutations occur in a mutually exclusive nature in all tumor types studied so far.[12] As such, the identification of a somatic activating *CTNNB1* mutation in a HB patient directly reduces the risk of carrying a germline *APC* mutation to that of the general population. By combining mutational analysis of *CTNNB1* exon3 and IHC comparing staining patterns for total and S33/37/T41 β-catenin, we could reliably identify all three FAP patients in our cohort of 18 HB cases. Their tumors are characterized by a comparable nuclear staining for both antibodies and wild-type exon 3 sequence. In fact, the IHC analysis by itself can already select a large proportion of HBs that carry an activating *CTNNB1* mutation, and thus have no increased risk of an underlying *APC* germline mutation. Our epitope and immunohistochemical analysis suggests that all amino acid alterations and genomic deletions encompassing the G34-T41 region would prevent or strongly reduce binding of the S33/37/T41 antibody. Reviewing the literature for the type of *CTNNB1* mutations reported in HBs, about 85% of mutations (198/234) fulfil this criterion.[13-21] The main exceptions are D32/S33 amino acid alterations, while S45 mutations are rarely observed in HB (3/234). Although the IHC analysis can already identify most HB patients with *CTNNB1* mutations, we nevertheless propose to also include the exon 3 mutation analysis on tumor DNA. This will identify point mutations outside the epitope of the S33/37/T41 antibody (D32, S33 and S45), and provides a confirmation of the *CTNNB1* mutational status.

As *CTNNB1* mutations are the predominant mutation in pediatric liver cancers (up to 80%), most patients are unlikely carriers of a germline *APC* mutation. A small subset of cancers arise without apparent β-catenin activation, i.e. the ones with exclusively membranous β-catenin staining and wild-type sequence. As it is highly unlikely that such a β-catenin nuclear-negative tumor arises in a FAP patient, these are also unlikely to carry a germline *APC* mutation. Anecdotally, somatic *APC* and *AXIN1*/*AXIN2* mutations have been reported in hepatoblastomas that also lead to tumors in which wild-type β-catenin accumulates in the nucleus.[15,16,22] These rare tumors would lead to *APC* germline negative patients, whereas the tumor analysis indicates an underlying FAP syndrome. Below we discuss how to deal with this small subset of HB tumors.

How to implement our method in current clinical practice? As HBs can be a manifestation of several genetic syndromes, all patients should be offered genetic counseling and evaluated for syndromic features such as the macrosomia and organomegaly associated with BWS. In the absence of such features and a negative family history, a genetic mutation analysis is warranted. This should include at least the *APC* gene, but may also include other genetic cancer predisposition syndromes such as LFS and NF1.[42,43] Simultaneous with or preceding the counseling process, we propose to combine a routine histopathological evaluation of the tumor tissue with both β-catenin stainings described here. In addition, tumor DNA should be isolated from the FFPE-material and evaluated for *CTNNB1* exon3 mutations, which can also be done as part of a larger next-gen sequencing panel.

In most cases the genetic and tumor tissue analyses will corroborate each other. Identification of a germline *APC* mutation will be accompanied by a comparable staining with both antibodies and wild-type *CTNNB1* sequence in the tumor tissue, while *APC* germline negative cases will show somatic activating *CTNNB1* mutations and reduced staining with the S33/37/T41 antibody. However, given the approximate 10-20% false-negative *APC* mutation analyses, occasionally tumors will be identified showing nuclear accumulation of wild-type β-catenin without an identifiable *APC* germline mutation. As these cases are at increased risk to develop FAP at a later stage, colonoscopies of the index patient and its parents can be performed, as suggested by Aretz et al.[2] Using our diagnostic method, this can however be restricted to the subset of *APC* germline negative patients that express wild-type β-catenin in the tumor. Alternatively, the somatic mutation analysis of these tumors can be extended to

identify inactivating somatic *AXIN1/2* or *APC* mutations. When *AXIN1* or *AXIN2* mutations are identified this also excludes the diagnosis FAP. In the case of a "somatic" *APC* mutation more caution is needed as this may indicate a missed germline mutation that is present for example in a low-mosaic fashion in the patient.

Our approach can also be extended to other extracolonic FAP manifestations, such as pilomatricomas and desmoids. These lesions are also characterized by high frequencies of somatic β-catenin mutations, meaning that most are of sporadic nature.[44-50] About one third of *CTNNB1* mutations reported for desmoids are at codon S45, which are missed by the IHC method described here, but if desired this analysis can be extended by including an antibody specifically recognizing this epitope (e.g. #19807, Cell Signaling technology).

In conclusion, we have successfully established and validated a novel ß-catenin gene and immunohistochemical diagnostic method, which following confirmation in an independent series, allows the near-complete exclusion of the diagnosis FAP among HB patients, when combined with routine germline DNA testing.

Supplemental Methods

Detailed PCR and sequencing protocol for *CTNNB1* mutation analysis

PCR products from tumor DNA were generated in a 15µl reaction mixture including 1µl of each primer (10µM) and 7.5µl KAPA2G Robust Hot Start ReadyMix (KAPA-Biosystems, #KK5702). The PCR reaction was performed with a thermocycler (Biometra, Göttingen, Germany) with an initial denaturation step (95˚C) for 3 min., followed by 35 cycles of denaturation (95˚C) for 15 sec., annealing (60˚C) for 15 sec. and extension (72˚C) for 15 sec. PCR products were sequenced with M13-forward primer and M13-reverse primers using the BigDye® Terminator-v3.1 Cycle Sequencing kit (Applied Biosystems™, ThermoFisher Scientific, Waltham, MA, USA). Sequences were analyzed on an ABI-3730 DNA Analyzer (Applied Biosystems™). Data were analyzed with Mutation Surveyor® software (SoftGenetics) and compared with the public sequences of GenBank (NG_013302.1 or NM_001904.3)

Tumor RNA isolation and analysis

RNA was extracted by microdissection from FFPE tissue fragments using the RNeasy®-FFPE Kit (Qiagen, Cat No./ID: 73504) according to the manufacturer's instructions. cDNA was generated basically according to the manufacturer's instructions (Thermo Scientific, #K1612) using 20pmol of M13-tailed gene-specific reverse primers CTNNB1-Ex3del-R4.1 and CTNNB1-Ex3-4del-R4.2 (Supplemental Table 2). Next, to detect *CTNNB1* deletions at cDNA level, two independent PCRs were performed with the FAM-labeled CTNNB1-Ex2-F forward primer and M13R-CTNNB1-Ex3del-R4.1 and M13R-CTNNB1-Ex3-4del-R4.2 reverse primers. PCR conditions were as described above.

Cell lines

Cell lines SW1573, SW48, SW480, Huh6, SNU398, HCT116 and HEK-293 were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco) complemented with 10% fetal calf serum (FCS) (Hyclone, Lonan, Utah). A427 and LS174T were cultured in RPMI-1640 medium (Invitrogen-Gibco), respectively with 10% and 5% FCS. HepG2 was cultured on fibronectin/collagen/albumin-coated plates (AthenaES) in Williams-E medium (Invitrogen-Gibco) complemented with 10% FCS. All media were supplemented with 100IU/ml penicillin and 100µg/ml streptomycin. Identity of all cell lines was confirmed by Short Tandem Repeat

genotyping. *CTNNB1* mutation status was confirmed in all cell lines by Sanger sequencing and was consistent with those reported at COSMIC (http://cancer.sanger.ac.uk).[38]

Preparation of paraffin blocks from cell lines

For each cell line 2-5 15cm tissues culture dishes were grown to near-confluency. Next, the cells were scraped with a rubber policeman, collected and centrifuged. The resulting pellet was washed with PBS once, followed by the addition of 15ml of PBS-buffered 10% formalin. After at least 2 hrs fixation, cells were transferred to 2ml tubes and spun down for 3 minutes at 300G. The cell pellet was mixed with a 1% agarose solution in PBS, maintained at 50˚C before use, by gently stirring with a pipette tip or flame-closed Pasteur pipette. Following a cool-down on ice, a small hole was burned in the bottom using a heated needle, after which the agarose plug was released by pressurized air. The agarose plug was placed in buffered formalin again and embedded in paraffin according to routine protocols. Fixation time for all cell lines was at least 6hrs and a maximum of 24hrs.

Supplementary Tables and Figures

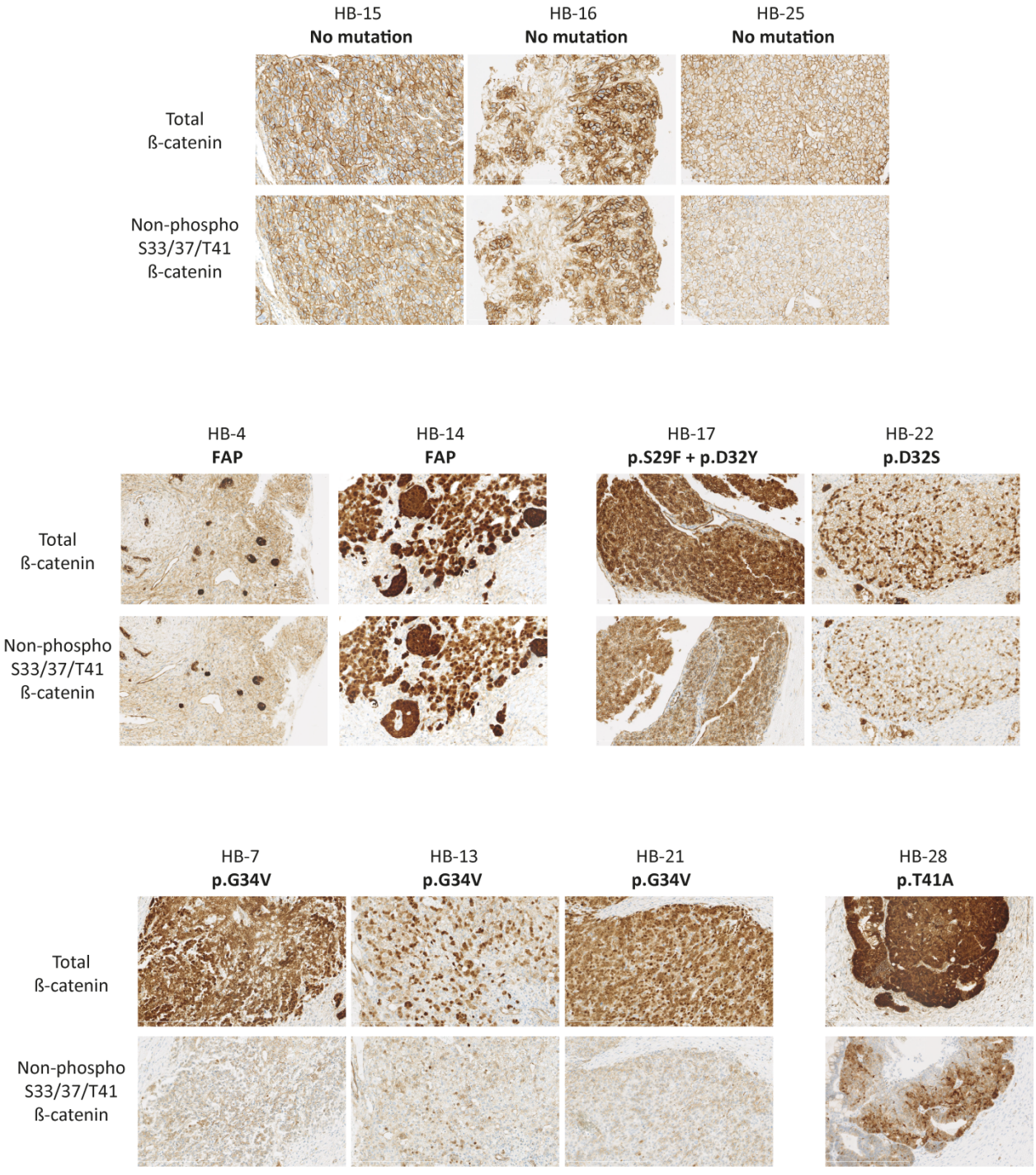
Supplemental Table 1 **Primer sequences for amplifying genomic *CTNNB1***

Primer	Nucleotide sequence	Expected size (without M13)
CTNNB1-AMPL7153305149-Ex3-F	M13F-CTGATTTGATGGAGTTGGACATGG	160
CTNNB1-AMPL7153305149-Ex3-R	M13R-TCCACATCCTCTTCCTCAGGATT	
CTNNB1-Ex2F1	GAAAATCCAGCGTGGACAATG	980
CTNNB1-Ex4R2	TGCCCTCATCTAATGTCTCAG	
CTNNB1-Ex2F2	TGGACAATGGCTACTCAAG	945
CTNNB1-Ex4R1	AACATAGCAGCTCGTACCCTC	
M13F	TGTAAAACGACGGCCAGT	
M13R	CAGGAAACAGCTATGACC	

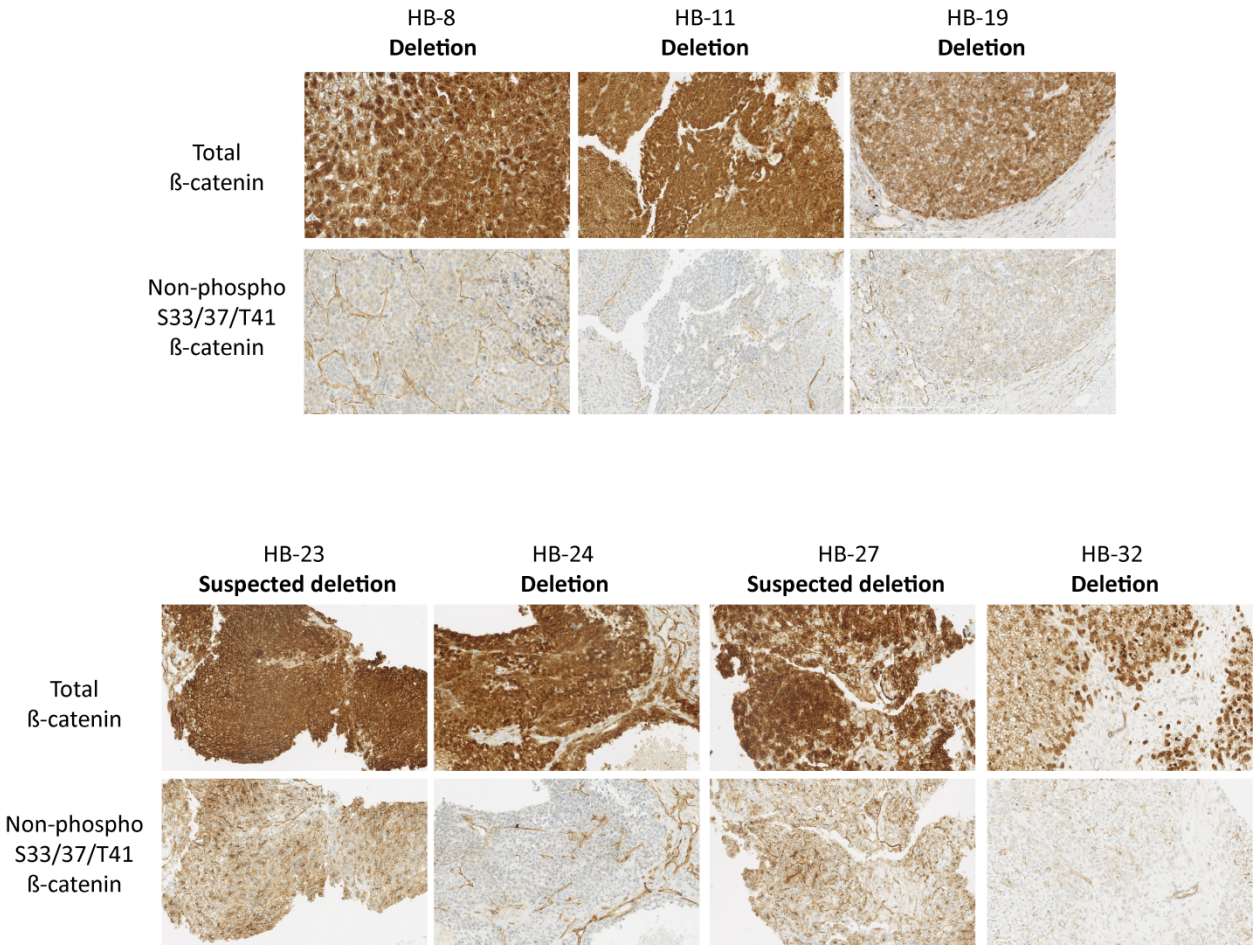
Supplemental Table 2 **Primer sequences for analyzing *CTNNB1* cDNA**

Primer	Nucleotide sequence	Expected wild type product size (bp)
CTNNB1-Ex2-F	GTTTTGAAAATCCAGCGTGGACAAT	
CTNNB1-Ex3del-R4.1	M13R-TCATCTAATGTCTCAGGGAACATAGCAGCTCGTACCCTC	355
CTNNB1-Ex3del-R4.2	M13R-AGTTTTGTCAGTTCAGGGATTGCA	517

Supplemental Figure 1, part 1



Supplemental Figure 1 continued



Supplemental Figure 1.

Immunohistochemical identification of β-catenin alterations in remaining tumor samples.

FFPE sections of remaining pediatric liver tumors were stained with the C-terminal (top rows) and S33/37/T41 (bottom rows) β-catenin antibodies. Identified mutation status is reported for each sample.

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Chapter 7

SUMMARY

Wnt/β-catenin signaling is important for the proper development of all multicellular organisms and is a critical regulator for most types of epithelial stem cells. Therefore, it is not surprising that dysregulation of Wnt/β-catenin signaling is observed in many cancers (1). Also, in hepatocellular carcinoma (HCC), frequently aberrant activation of this signaling pathway has been identified, for example by acquired activating β-catenin or inactivating AXIN1 mutations. Besides direct mutational activation, β-catenin signaling can also be modulated through various other mechanisms. One example is provided by the viral proteins expressed during chronic HBV or HCV infection, both prominent risk factors for the development of HCC. In **Chapter 2**, we comprehensively reviewed the current biomedical knowledge on the role of the Wnt/β-catenin signaling pathway in the progression from chronic hepatitis C infection to cirrhosis and HCC, and explored potential hypotheses as to the mechanisms involved. In this review, we first summarize the relative mutation frequencies of Wnt/β-catenin signaling elements in HCC cohorts reported previously. Activating mutations in *CTNNB1* are the most prevalent in general and especially in HCV-related HCC (>25%). Next, we discuss the mutual interaction between HCV infection and Wnt/β-catenin signaling. Both the HCV viral core and NS5A proteins provoke activation of Wnt/β-catenin signaling. HCV infection is also associated with increased EGFR and FGF signaling, both contributing to the cirrhotic and tumorigenic process by enhancing the PI3K/AKT and Ras/Raf/MEK/ERK pathways, but also by further increasing β-catenin signaling. Thus, HCV infection exploits multiple mechanisms leading to enhanced β-catenin signaling. In turn, stimulated Wnt/β-catenin signaling paves the way for progression of hepatitis C during inflammation and fibrosis to eventually cirrhosis and HCC.

Despite of the development of a large number of molecular inhibitors targeting Wnt/β-catenin signaling during recent decades, little is known about the response to these inhibitors of HCC cells carrying different genetic mutations relevant to aberrantly activated Wnt/β-catenin signaling. In **Chapter 3,** we tested the Wnt secretion inhibitor IWP12 in our HCC cell line panel. First, using siRNA mediated knock-down of β-catenin we showed that the majority of HCC cell lines were dependent on β-catenin signaling for optimal cell growth. Blocking Wnt secretion by treatment with the IWP12 porcupine inhibitor reduced growth of most HCC cell lines, which was confirmed by knock-down of WLS essential for Wnt secretion. However, none of the *CTNNB1* and *AXIN1* mutant HCC cell lines were affected at the β-catenin signaling level. This was shown both by a β-catenin reporter assay and RNA level of *AXIN2* (a well-known

target gene of β-catenin signaling) upon alterations of Wnt ligand exposure, neither by blocking Wnt secretion using IWP12 nor by the addition of WNT3A ligand. This suggests that the expressed oncogenic β-catenin or mutant AXIN1 proteins determine overall signaling levels in a dominant fashion. Furthermore, IWP12 treatment did not induce autophagy or endoplasmic reticulum (ER) stress, which may have resulted from the accumulation of Wnt ligands within the ER. In accordance with our results, Covey et al. also have shown that knocking down porcupine in various tumor cell lines reduced their growth through a Wnt-independent pathway with no clear explanation revealed (2). Similarly, most CRC cell lines tested as a comparison in various assays, showed dependency on β-catenin signaling and inhibition of cell growth after Wnt secretion blockage, either by IWP12 or knockdown of WLS. In accordance with liver cancer cell lines, the growth inhibition was not associated with clear alterations in Wnt/β-catenin signaling regardless of the underlying *APC* or *CTNNB1* mutations. Our results suggest that most colorectal and liver cancers with mutations in components of the β-catenin degradation complex do not strongly rely on extracellular Wnt ligand exposure to support optimal growth. Blocking Wnt secretion may however aid in tumor suppression through alternative routes currently unappreciated.

In **chapter 4**, we investigated a second class of molecular inhibitors of β-catenin signaling, that is the Tankyrase inhibitors, and we determined in more detail to which extent AXIN1 and AXIN2 are involved in the regulation of β-catenin signaling in HCC. Together with APC and the kinases GSK3 and CK1α, these proteins form the core of the β-catenin break-down complex. Loss-of-function mutations in AXIN1 and AXIN2 are observed in 10.4% and 3.3% of HCC patients respectively (3). Mutated AXIN1 or AXIN2 are expected to lead to enhanced Wnt/β-catenin signaling, although this has been debated (4-6). The AXIN proteins themselves are also under proteolytic control and are rapidly degraded following poly-ADP-ribosylation by Tankyrase enzymes. Hence, Tankyrases have emerged as therapeutic targets as their inhibition theoretically leads to AXIN accumulation, ultimately expected to result in reduced β-catenin signaling and tumor cell growth. In this chapter, we first investigated in more detail to what extent AXIN1 and AXIN2 are involved in regulating β-catenin signaling in our panel of HCC cell lines. Western blot analysis showed that both AXINs were expressed at low levels in the non-mutant and *AXIN1* mutant cell lines, while they were readily detectable in the *CTNNB1* mutant ones. Tankyrase inhibition for one day resulted in a clear accumulation of AXIN2

exclusively in the *CTNNB1* mutant lines. Surprisingly, no clear accumulation of AXIN1 was observed in any of the lines. Mechanistically, this may be explained by the RNA expression levels that we observed for both genes, as *AXIN1* was expressed at considerably lower levels than *AXIN2* in all HCC lines. Recently, Thorvaldsen et al. showed that sustained protein translation is required for AXIN accumulation (6). Indirectly this also implies that higher *AXIN2* RNA expression levels will lead to a more robust protein accumulation, which is in line with our results. Next, we investigated to what extent both AXIN proteins are involved in regulating β-catenin signaling in HCC. We knocked-down their expression using siRNA, with APC knock-down as a positive control. Simultaneous knock-down of both AXINs in the non-mutant lines indicates that they work in a complementary fashion to regulate β-catenin signaling. As expected, in the *AXIN1* mutant lines *AXIN1* knock-down had no clear effect, while *AXIN2* knock-down resulted in a similar activation of β-catenin signaling as *APC* knock-down. These results show that both AXIN proteins are involved in β-catenin regulation in HCC, arguing against some reports that suggest that *AXIN1* mutations do not contribute to HCC tumor growth through enhancing β-catenin signaling (4, 5). Lastly, we tested whether Tankyrases represent an attractive target to inhibit the growth of HCCs. B-catenin signaling was barely affected following Tankyrase inhibition in the *CTNNB1* mutant cell lines, while it was clearly diminished in all *AXIN1* and most non-mutant lines. Despite this inhibition in β-catenin signaling the growth and colony formation capabilities of these cell lines were at most slightly inhibited by Tankyrase inhibition. These results suggest that Tankyrase inhibitors are not likely going to efficiently affect the growth of HCCs.

In **chapter 5**, we uncovered the importance of oncogenic serine-threonine kinase receptor associated protein (STRAP) to sustain HCC cell growth through enhancing Wnt/β-catenin signaling. STRAP harbors seven WD40-repeat domains and is considered to be a scaffolding protein without enzymatic function that exerts regulatory functions on a variety of cellular processes. Using immunohistochemistry we showed that STRAP was up-regulated in HCC, as previously reported in breast (7), lung (8) and colorectal cancers (8, 9). To study the function of STRAP in more detail, we applied siRNA mediated knock-down on our panel of HCC cell lines and generated knock-out clones using CRISPR/Cas9 gene editing. Loss of STRAP resulted in a strongly impaired growth, altered cell cycle progression and reduced capacity to form colonies from single cells. Furthermore, our findings indirectly (β-catenin target gene

expression) and directly (β-catenin signaling activity using reporter assays) provide evidence that the abundance of STRAP stimulates Wnt/β-catenin signaling activity, thereby supporting optimal HCC cell growth. Mechanistically, others have shown that STRAP binds to the catalytic site of GSK3β via its WD40 domain region, eliciting the compromised ability of GSK3β to phosphorylate the (N-terminal) serine/threonine residues encoded by exon3 of β-catenin (10, 11). Accordingly, we observed reduced levels of active β-catenin in STRAP knock-out clones of Huh6 and PLC/PRF/5. In these lines we also observed reduced levels of C-terminal phosphorylated β-catenin at S675, which is expected to result in more ubiquitination of β-catenin and less recruitment of transcriptional co-activators (12-14). Nevertheless, different from *CTNNB1* mutant Huh6 and *AXIN1* mutant PLC/PRF/5 cell lines, Huh7 apparently does not share any of the above rationales, despite showing reduced β-catenin signaling following STRAP loss. This indicates that STRAP may modulate β-catenin signaling through alternative routes that remain to be uncovered. RNA sequencing analysis of STRAP knock-out clones and controls thereof, also suggests that STRAP could facilitate stem cell self-renewal while simultaneously attenuating cell differentiation. The same analysis also revealed that besides Wnt/β-catenin signaling, many other signaling pathways and metabolic processes were affected following STRAP loss, which is in line with reports demonstrating STRAP's role in regulating other signaling pathways (15-17). Taken together, our results show that STRAP expression is increased in hepatocellular cancers, and provides growth advantage to the tumor cells, among others by enhancing Wnt/β-catenin signaling.

In **chapter 6**, we extended our study to hepatoblastoma (HB), a potentially malignant pediatric liver cancer. The Wnt/β-catenin pathway plays a central role in the pathogenesis of these tumors, i.e. up to 60-80% carry activating *CTNNB1* mutations. Hepatoblastomas can however also be the first manifestation of familial adenomatous polyposis (FAP), which is a hereditary predisposition to develop hundreds to thousands of colorectal polyps ultimately leading to colorectal cancer. FAP patients carry germline *APC* gene mutations leading to enhanced β-catenin signaling following somatic mutation of the remaining wild-type *APC* allele. As this is a severe disease, it is important for the patient and related family members to firmly exclude FAP at an early stage. Current diagnosis largely depends on *APC* germline mutation detection on genomic DNA, which is associated with 10-20% false-negative results. In this chapter, we establish and validate a tissue-based β-catenin gene and

immunohistochemical analysis, which complements germline mutation screening to exclude the diagnosis FAP among HB patients. Important for our study, tumor-driving *CTNNB1* and *APC* mutations occur in a mutually exclusive fashion in all tumor types. Hence, the identification of a somatic activating *CTNNB1* exon3 mutation in a HB patient directly reduces the risk of carrying a germline *APC* mutation to that of the general population. *CTNNB1* mutation analysis in HBs is however complicated by a high proportion (>60%) of genomic deletions, varying in size from small intra-exonic deletions to larger ones extending up to part of exon 2 or 4, which can easily be missed using routine mutation analysis. Here we show that by combining *CTNNB1* exon3 mutational analysis and immunohistochemistry comparing staining patterns for total and exon3 specific β-catenin antibodies, we can faithfully diagnose the great majority of hepatoblastomas. Among 18 lesions, all three FAP tumors were characterized by a wild-type exon3 sequence and a comparable nuclear staining for both antibodies. In contrast, the non-FAP tumors carried missense *CTNNB1* mutations combined with a clearly reduced staining for the exon3 antibody, or complete loss of staining in case of lesions with exon3 deletions. When this method is combined with routine germline DNA testing, it allows the near-complete exclusion of the diagnosis FAP among HB patients.

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Chapter 8

DISCUSSION

HCC is a heterogeneous cancer type in which many etiological factors, geographic differences and long-time pathological processes contribute to the development of a malignant tumor (1, 2). As one of the most important cascades facilitating HCC initiation and progression, aberrantly activated Wnt/β-catenin signaling has become a focus for pharmacological intervention in recent decades. Although various drugs targeting this signaling pathway have been developed and studied in cancers, evidence about the efficiency of these drugs in different HCC subgroups is still lacking. In my thesis, using liver cancer cell lines, I aimed to identify subgroups of HCCs that may benefit from inhibitors that target β-catenin signaling at different cellular levels.

1. Contribution of mutant β-catenin and AXIN1 to aberrant activation of Wnt/β-catenin-signaling pathway in HCC progression

As reported in Chapter 2 and by others, aberrant stimulation of Wnt/β-catenin signaling in HCC patients is mainly due to mutations at the *CTNNB1* (20%-25%) or *AXIN1* (10%) genes (3-5). The oncogenic *CTNNB1* mutations consist of single mutations within exon 3 at the N-terminal phosphorylation residues, making the protein more resistant to proteolytic degradation. For these activating mutations it is well-accepted that they support tumor growth by enhancing β-catenin signaling in a dominant fashion. However, whether *AXIN1* mutations also lead to HCC by enhancing β-catenin signaling is still under debate. In AXIN1 mutant HCCs neither a clear nuclear β-catenin accumulation is observed nor a clear induction of β-catenin target genes (4). Similar observations have been made in mice with a liver-specific deletion of AXIN1 in which also no clear nuclear β-catenin is detected (6). A potential explanation for this apparent lack of β-catenin signaling activation could be functional compensation by its homolog AXIN2. In addition, it becomes more complex by multi-function roles of AXIN1 regulating the activity of c-Myc (7), p53 (8, 9), Smad3 (10) and interacting with other components of centrosome and mitotic spindles (11-14). However, based on our results we believe that AXIN1 loss leads to enhanced β-catenin signaling relevant for tumor growth, albeit in a less dominant way. Firstly, we observe that all three *AXIN1* mutant lines we investigated show higher baselines of β-catenin signaling activity by a β-catenin reporter assay, indicating β-catenin is actively signaling to the nucleus. The signaling activity is however not as high as that in *CTNNB1* mutant lines. Secondly, all these *AXIN1* mutant lines are impaired in their growth following siRNA mediated knockdown of β-catenin, again suggesting that they

require some β-catenin signaling to sustain growth. Additionally, another three lines without mutations relevant to β-catenin signaling, show induced β-catenin reporter activity resulting from siRNA mediated knockdown of AXIN1.

Hence, although β-catenin is a more powerful driver determining the β-catenin signaling activity, the mutated AXIN1 also contributes to the aberrant activation of β-catenin signaling, which is important for tumor initiation and progression in particular HCC subgroups.

2. Alternative treatment for HCC by targeting Wnt/β-catenin-signaling pathway

Due to difficulties for early detection, most HCC patients are diagnosed at advanced stage. For these advanced HCC patients, the current treatment strategy is chemotherapy limited to the multi-kinase inhibitor sorafenib. However, treatment with sorafenib promises a minimal survival benefit of at most a few months in the majority of HCC patients. Hence, alternative treatment options are urgently needed and as explained above targeting the β-catenin signaling pathway may represent an attractive option.

In support of this we observe in our cell line panel a growth reduction of all 9 lines when β-catenin levels are reduced using siRNA mediated knock-down. Other studies have provided similar results in a limited number of cell lines (4, 15), and by reducing β-catenin levels in mouse hepatocarcinogenesis models (16, 17). Which method to choose for reducing β-catenin signaling in the clinical setting is however more challenging. Here we first investigated the Wnt-secretion inhibitor IWP12. This inhibitor was demonstrated to interfere with Wnt secretion and impair the growth of APC and β-catenin mutant CRC cell lines (18). Similarly, our study shows that IWP12 reduced growth of most HCC cell lines, which was confirmed by knock-down of WLS, a second protein also essential for Wnt secretion. Nevertheless, the growth reduction did not result from blocking β-catenin signaling and is also not due to alterations in autophagy or ER stress responses. A potential alternative explanation not investigated by us, could lie in other functions of Wnt ligands not linked to β-catenin signaling. This leads us to the “non-canonical” Wnt signaling pathway, which supports a growth suppressive effect (19, 20). Therefore, interfering with the secretion of this subset of Wnt ligands is expected to support cellular growth, in contrast to the growth suppression that we observed. Another explanation is the mTOR pathway important for cell growth by activating ribosome biogenesis and protein synthesis, which is validated to be stimulated by Wnt signaling depending on GSK3 instead of β-catenin (21). This suggests that mTOR signaling

could be the mechanism underlying the growth reduction following IWP12 treatment, which needs further investigation.

As a second method to reduce β-catenin signaling, we used tankyrase inhibition. B-catenin signaling was barely affected following tankyrase inhibition in the *CTNNB1* mutant cell lines, while it was clearly diminished in all *AXIN1* and most non-mutant lines. This indicates that in the latter cell lines, tankyrase inhibitor works through regulation of AXIN stability while the *CTNNB1* mutant lines do not respond, most likely because of the mutant β-catenin determining β-catenin signaling activity in a dominant fashion. However, no clear effect on cell growth was observed, even in the lines where some reduction in signaling was obtained.

Based on our data, reduction in β-catenin signaling obtained with either method is apparently insufficient to significantly affect the growth of HCC cell lines. This highlights the demand of other inhibitors or combinations thereof to achieve optimal effects in the clinic.

3. Other potential avenues to target Wnt/β-catenin signaling

Conventional inhibitors targeting Wnt/β-catenin signaling are conservatively designed to block the function or interaction of components specifically involved in this pathway. In Chapter 5, we identify STRAP as a new player in enhancing Wnt/β-catenin signaling in HCC. STRAP has been defined as a scaffold protein participating in a variety of signaling pathways in addition to β-catenin. Thus, theoretically blocking the function of STRAP could impair tumor growth through multiple avenues. Whether the STRAP protein is druggable remains however to be seen, although it will most likely be challenging as there is no enzymatic function that can be easily blocked.

As an active pathway during the embryonic process and carcinogenesis, Wnt/β-catenin signaling crosstalks with a number of other signaling pathways. In Chapter 2, we summarized that both EGF and FGF pathways are triggered by Wnt/β-catenin signaling, which in turn stimulate β-catenin signaling through inhibiting GSK3β activity in HCC development (3). For various other pathways including NOTCH (22), NF-κB (23), TGFβ (24) and so on, it has also been demonstrated that they interact with Wnt/β-catenin signaling.

The function of STRAP in regulating Wnt/β-catenin signaling as well as the mutual crosstalk between Wnt/β-catenin signaling and other pathways imply a supplementary therapeutic approach that synergizes with conventional anti-cancer drugs, to hopefully benefit the therapeutic outcomes of HCC patients.

4. Future directions

Although Wnt/β-catenin signaling has been widely investigated and targeted to cure tumors, a systematic study about the efficiency of the prevailing inhibitors for HCC treatment is still lacking. In this thesis, we started *in vitro* experiments (cell lines) with two inhibitors. In the future, this can also be extended to other reported inhibitors of the pathway, possibly in combination with drugs targeting other cellular targets. Based on the data obtained from these *in vitro* studies, *in vivo* HCC models will also be necessary to evaluate the effect of these inhibitors in more physiological conditions. Since the ultimate goal of this study is to ameliorate clinical practice for HCC patients, organoids directly derived from tumors will be an ideal model for large-scale drug screening to identify the sensitive subgroups to specific inhibitors (25).

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Chapter 9

Nederlandse samenvatting

Dutch summary

Samenvatting

Wnt/β-catenine signalering is belangrijk voor de ontwikkeling van alle meercellige organismen en is een essentiële regulator van de meeste epitheliale stamcellen. Daarom is het niet verrassend dat in vele kankers een foutieve regulatie van Wnt/β-catenine signalering gezien wordt (1). Ook in leverkanker wordt veelvuldig een abnormale activatie van deze signaalroute gezien, bijvoorbeeld door het verkrijgen van een activerende β-catenine of inactiverende AXIN1 mutatie. Behalve door middel van mutaties, kan β-catenine signalering ook door diverse andere mechanismen beïnvloed worden. Een voorbeeld zijn de virale eiwitten die tot expressie komen tijdens een chronische infectie met HBV of HCV, beide belangrijke risicofactoren voor het ontstaan van leverkanker. In **hoofdstuk 2** beschrijven we uitgebreid de huidige biomedische kennis over de rol van Wnt/β-catenine signalering in de progressie van chronische hepatitis C infectie naar levercirrose en leverkanker, en bespreken we in meer detail enkele van de mogelijke onderliggende mechanismen. Allereerst benoemen we de relatieve mutatie-frequenties van aan β-catenine signalering gerelateerde genen zoals die in leverkankercohorten gezien worden. Activerende mutaties in *CTNNB1* (coderend voor β-catenine) zijn de meest voorkomende mutaties resulterend in verhoogd β-catenine signalering en dit is vooral het geval in HCV-gerelateerde levertumoren (>25%). Vervolgens bespreken we de interactie tussen HCV infectie en Wnt/β-catenine signalering. Zowel het virale HCV core als het NS5A eiwit dragen bij aan de activatie van β-catenine signalering. HCV infectie is ook geassocieerd met een verhoogde EGFR en FGF signalering. Beide dragen bij aan de vorming van cirrose en tumorvorming door middel van het activeren van de PI3K/AKT en Ras/Raf/MEK/ERK signaalroutes, maar leiden ook tot een toename in β-catenine signalering. Samenvattend, leidt HCV infectie dus via meerdere mechanismen tot een verhoogde β-catenine signalering. Op zijn beurt draagt de geactiveerde β-catenine signalering bij aan de progressie van de initiële hepatitis C infectie naar uiteindelijk cirrose en leverkanker.

Ondanks de ontwikkeling van een groot aantal moleculaire remmers van β-catenine signalering in de afgelopen decennia, is er nog weinig bekend over de effectiviteit van deze remmers op leverkankercellen met specifieke genetische mutaties leidend tot een verhoogde β-catenine signalering. In **hoofdstuk 3** testen we de Wnt secretie remmer IWP12 in ons panel van leverkankercellijnen. Allereerst laten we zien dat door middel van siRNA gemedieerde knockdown van β-catenine, het overgrote merendeel van de leverkankercellijnen afhankelijk

is van β-catenine signalering voor een optimale groei. Het blokkeren van Wnt secretie door middel van een behandeling met de porcupine remmer IWP12, vermindert de groei van de meeste leverkankercellijnen. Dit effect wordt bevestigd met een knockdown van WLS, wat essentieel is voor uitscheiding van Wnt liganden. Geen enkele van de *CTNNB1* en *AXIN1* mutante cellijnen wordt echter beïnvloedt op het niveau van β-catenine signalering. Dit laten we zien met een β-catenine reporter analyse als ook onveranderde *AXIN2* RNA expressie niveaus (een betrouwbaar door β-catenine gereguleerd gen) volgend op veranderingen in Wnt ligand blootstelling, noch door de Wnt secretie te blokkeren met IWP12 noch met de toevoeging van WNT3A ligand. Dit suggereert dat de geexpresseerde oncogene β-catenine en mutante AXIN1 eiwitten het signaalniveau op een dominante manier beïnvloeden. Daarnaast leidt IWP12 behandeling ook niet tot de inductie van autofagie of endoplasmatisch reticulum (ER) stress die mogelijk zou kunnen ontstaan als gevolg van de opstapeling van Wnt ligand in het ER. In overeenstemming met ons resultaat hebben anderen laten zien dat knockdown van porcupine de groei van verschillende cellijnen verminderd op een Wnt-onafhankelijke manier, zonder dat een duidelijke verklaring werd gevonden (2). De meeste darmkankercellijnen die we voor diverse analyses ter vergelijking hebben gebruikt, laten een vergelijkbaar resultaat zien. Ze zijn afhankelijk van β-catenine signalering en laten een remming in groei zien naar het blokkeren van Wnt secretie, zowel m.b.v. IWP12 als WLS knockdown. In overeenstemming met de leverkankercellijnen is die groeiremming niet geassocieerd met een duidelijke verandering in β-catenine signalering en onafhankelijk van de onderliggende *APC* of *CTNNB1* mutatie. Onze resultaten suggereren dus dat de meeste darm- en leverkankercellijnen met mutaties in de β-catenine signaleringsroute, voor een optimale groei niet sterk afhankelijk zijn van extracellulaire blootstelling van Wnt liganden. Het blokkeren van Wnt secretie lijkt echter wel bij te dragen aan de remming van tumorgroei via nu nog onbekende mechanismen.

In **hoofdstuk 4** bestuderen we een tweede categorie van moleculaire remmers van β-catenine signalering, namelijk de Tankyrase-remmers, en bestuderen we in meer detail de bijdrage van AXIN1 en AXIN2 aan β-catenine signalering in leverkanker. Beide eiwitten vormen samen met APC en de kinases GSK3 en CK1α, de belangrijkste onderdelen van het β-catenine afbraakcomplex. Mutaties in AXIN1 en AXIN2 die tot verlies van functie leiden, worden gezien in respectievelijk 10.4% en 3.3% van de levertumoren (3)]. Van deze mutaties wordt verwacht dat die leiden tot een verhoogde β-catenine signalering, hoewel dit door enkele publicaties

wordt betwist (4-6). De AXIN eiwitten staan zelf ook onder proteolytische controle en worden snel afgebroken na poly-ADP-ribosylatie door Tankyrase enzymen. Om die reden zijn Tankyrase-remmers in het vizier gekomen als mogelijke therapie omdat hun remming in theorie leidt tot stapeling van AXIN, uiteindelijk resulterend in verlaagde β-catenine signalering en tumorgroei. In dit hoofdstuk bestuderen we eerst de expressie-niveaus van AXIN1 en AXIN2 in ons panel van leverkankerlijnen. Western blot analyse laat zien dat beide AXIN eiwitten laag tot expressie komen in de niet-gemuteerde en *AXIN1*-gemuteerde cellijnen, terwijl ze gemakkelijk aan te tonen zijn in de *CTNNB1* mutante lijnen. Remming van Tankyrase leidde alleen in de *CTNNB1* mutante lijnen tot een duidelijk opstapeling van AXIN2. Tot onze verrassing was er in geen enkele cellijn een duidelijke stapeling van AXIN1 eiwit aantoonbaar. Een mogelijke verklaring is te vinden in de RNA expressie niveaus van beide genen, omdat *AXIN1* duidelijk lager tot expressie kwam dan *AXIN2* in alle cellijnen. Onlangs hebben Thorvaldsen *et al* laten zien dat er een continue aanmaak van nieuw eiwit nodig is voor AXIN stapeling (6). Indirect betekent dit dat een hogere *AXIN2* RNA expressie zal leiden tot een meer robuuste eiwitstapeling, wat in overeenstemming is met onze resultaten. Vervolgens hebben we onderzocht in welke mate beide AXIN eiwitten betrokken zijn bij de regulatie van β-catenine signalering in leverkanker. Hun RNA expressie werd sterk verlaagd m.b.v. siRNA, waarbij we *APC* knockdown als positieve controle gebruikten. Gelijktijdige knockdown van beide AXIN genen in de niet-gemuteerde cellijnen liet zien dat beide complementair werken in de regulatie van β-catenine signalering. In de *AXIN1* mutante lijnen had *AXIN1* knockdown zoals verwacht geen effect, terwijl *AXIN2* knockdown leidde tot een vergelijkbare activatie van β-catenine signalering als *APC* knockdown. Deze resultaten tonen aan dat beide AXIN eiwitten betrokken zijn bij β-catenine regulatie in leverkanker, wat in tegenspraak lijkt met publicaties die suggereren dat AXIN1 mutaties niet bijdragen aan de groei van leverkanker door het activeren van β-catenine signalering (5). Als laatste testen we in dit hoofdstuk of de remming van Tankyrases een goede benadering is voor de remming van leverkankergroei. Het niveau van β-catenine signalering wordt nauwelijks beïnvloed door Tankyrase remming in de β-catenine mutante lijnen, terwijl dit duidelijk het geval is in de overige lijnen. Ondanks deze remming resulteerde Tankyrase in geen of slechts een minimaal effect op de groei en kolonievormings-capaciteit van de behandelde leverkankercellijnen. Deze resultaten suggereren dat Tankyrase-remmers waarschijnlijk niet een effectieve behandeling van leverkanker zullen vormen.

In **hoofdstuk 5** ontdekken we het belang van het oncogene serine-threonine kinase receptor associated protein (STRAP) voor het ondersteunen van de groei van leverkanker d.m.v. het versterken van β-catenine signalering. STRAP bevat zeven WD40 domeinen en wordt beschouwd als een structureel eiwit zonder enzymatische functie die diverse regulatoire rollen vervult op een groot aantal cellulaire processen. Gebruikmakend van immunohistochemie tonen we een verhoogde STRAP expressie aan in leverkanker, zoals eerder ook al aangetoond is voor borst-, long- en darmkanker (7-9). Om de functie van STRAP in meer detail te onderzoeken, hebben we siRNA gemedieerde knockdown toegepast op ons panel van leverkankercellijnen en knock-out clones gegenereerd m.b.v. CRISPR/Cas9 genmodificatie. Verlies van het STRAP eiwit resulteerde in een sterk verminderde groei, een veranderd celcyclusprofiel en een verminderde capaciteit tot het vormen van kolonies vanuit een enkele cel. Daarnaast tonen we aan dat STRAP de β-catenine signalering en daarmee de groei van leverkankercellen stimuleert, zowel direct gemeten (verhoogde expressie van door β-catenine gereguleerde genen) als indirect (β-catenine signalering gemeten met een reporter-analyse). Anderen hebben laten zien dat STRAP aan het katalytische domein van GSK3β bindt via zijn WD40 domein en daarmee de capaciteit van GSK3β verminderd om β-catenine te fosforyleren op de door exon3 gecodeerde N-terminale serine/threonine residuen(10, 11). In overeenstemming daarmee zien we verlaagde niveaus van actief β-catenine in de STRAP knock-out clones van de Huh6 en PLC/PRF/5 cellijnen. Bovendien zien we in deze lijnen een vermindering van C-terminale β-catenine fosforylatie op S675, wat naar verwachting leidt tot een verminderde rekrutering van transcriptionele co-activatoren (12-14). Afwijkend van de β-catenine mutante Huh6 en AXIN1 mutante PLC/PRF/5 cellijn lijkt in Huh7 geen van deze mechanismen operationeel ondanks een verminderde β-catenine signalering na verlies van STRAP expressie. Dit suggereert dat STRAP β-catenine signalering ook via nu nog onbekende mechanismen kan beïnvloeden. Een RNA sequentie analyse van STRAP knock-out clones en controles suggereert dat STRAP de vernieuwing van stamcellen kan vergemakkelijken en tegelijkertijd de differentiatie van cellen kan afremmen. Dezelfde analyse laat zien dat behalve β-catenine signalering, diverse andere signaalroutes en metabole processen verstoord zijn na het verlies van STRAP expressie, wat in overeenstemming is met andere publicaties (15-17). Samenvattend laten onze resultaten zien dat STRAP verhoogd tot expressie komt in leverkanker en een groeivoordeel geeft aan de tumorcellen, onder andere door het versterken van het β-catenine signaal.

In **hoofdstuk 6** bestuderen we hepatoblastomas, een potentieel kwaadaardige vorm van leverkanker die vooral bij kinderen voorkomt. De Wnt/β-catenine signaalroute speelt een belangrijke rol in het ontstaan van deze tumoren, d.w.z. tussen de 60-80% heeft een activerende *CTNNB1* mutatie. Hepatoblastomas kunnen echter ook de eerste manifestatie zijn van familiaire adenomateuze polyposis (FAP), een erfelijke aanleg leidend tot duizenden dikke darmpoliepen en uiteindelijk darmkanker. FAP patiënten dragen een kiembaan *APC* genmutatie die resulteert in verhoogde β-catenine signalering zodra er een somatische mutatie ontstaat in het overblijvende wild-type *APC* allel. Dit is een ernstige ziekte waarbij het van groot belang is voor de hepatoblastoma patiënt en gerelateerde familieleden, om FAP in een vroeg stadium uit te sluiten. De huidige diagnose is voor een belangrijk deel gebaseerd op kiembaan *APC* mutatie analyse op genomisch DNA, die echter in 10-20% van de gevallen tot een fout-negatieve uitslag leidt. In dit hoofdstuk ontwikkelen we en valideren een weefsel-gebaseerde β-catenine gen en immunohistochemische analyse, die de kiembaanmutatie analyse aanvult om de diagnose FAP bij hepatoblastoma patiënten grondig uit te sluiten. Van belang voor onze studie is de bevinding dat *CTNNB1* en *APC* mutaties die leiden tot tumorvorming, nooit in dezelfde tumor gezien worden. De vondst van een somatische activerende exon3 β-catenine mutatie in een hepatoblastoma patiënt betekent dan ook meteen dat het risico op FAP sterk verlaagd wordt en gelijk is aan die van de gehele bevolking. *CTNNB1* mutatie analyse in hepatoblastomas wordt echter bemoeilijkt door het grote aandeel (>60%) van genomische deleties, die in grootte kunnen variëren van kleine intra-exonische deleties tot grotere, die een deel van exon 2 tot 4 kunnen beslaan. Deze deleties kunnen gemakkelijk gemist worden met een routinematige mutatie analyse. In dit hoofdstuk laten we zien dat door het combineren van exon3 *CTNNB1* mutatie analyse en een immunohistochemische vergelijking van totaal en exon3-specifieke β-catenine antilichamen, we een betrouwbare diagnose kunnen maken voor het overgrote merendeel van de hepatoblastomas. Van de 18 onderzochte monsters, lieten alle drie de FAP-tumoren een wild-type exon3 sequentie zien en een vergelijkbaar kleurpatroon met beide antilichamen. De overige niet-FAP tumoren werden echter gekenmerkt door de identificatie van een missense mutatie en een sterk verminderde kleuring met het exon3 specifieke antilichaam, of een volledig verlies van kleuring in het geval van exon3 deleties. Als deze methode gecombineerd wordt met de routinematige kiembaan *APC* mutatie analyse, dan kan deze leiden tot een nagenoeg volledige uitsluiting van een FAP-diagnose onder hepatoblastoma patiënten.

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Appendix

Acknowledgements

Publications

PhD Portfolio

Curriculum Vitae

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Dear **Zhiyong**, my beloved husband, thanks for your love, support and understanding during the last years. Thank you for taking good care of mine and your parents. Thank you for taking everything without breaking my peaceful life in Rotterdam, allowing me to concentrate on my study. I am a lucky dog to meet you, know you and marry you. And my lovely son, **Luhan**, you are my angel god set to brighten my life. Your every sweet smile and word since you were born is deeply engraved in my brain. Each time I feel tired or blue, your cute face blows away all the upset things and cure my mind. I feel badly sorry for not being able to be around you during your first two years and missing so many of your first times. I cannot wait for the happy life we will live together. I love you both so much!

Publications

1. Wenhui Wang, Lei Xu, Pengyu Liu, Kiran Jairam, Yuebang Yin, Kan Chen, Dave Sprengers, Maikel P. Peppelenbosch, Qiuwei Pan, Ron Smits. Blocking Wnt Secretion Reduces Growth of Hepatocellular Carcinoma Cell Lines Mostly Independent of β-Catenin Signaling. Neoplasia. 2016;18(12):711-723.

2. Wenhui Wang, Qiuwei Pan, Gwenny M. Fuhler, Ron Smits, Maikel P. Peppelenbosch. Action and function of Wnt/β-catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma. J Gastroenterol 2017;52(4):419-431.

3. Wenhui Wang, Shan Li*, Pengyu Liu*, Kostandinos Sideras, Harmen J. G. van de Werken, Marieke van der Heide, Wanlu Cao, Marla Lavrijsen, Maikel P. Peppelenbosch, Marco Bruno, Qiuwei Pan and Ron Smits. Oncogenic Serine-Threonine Kinase Receptor Associated Protein sustains hepatocellular carcinoma cell growth through enhancing Wnt/β-catenin signaling. (Molecular Cancer Research, under revision)

4. Wenhui Wang, Pengyu Liu, Shan Li, Marla Lavrijsen, Maikel P. Peppelenbosch, Ron Smits. Evaluation of AXIN1 and AXIN2 as targets of tankyrase inhibition in hepatocellular carcinoma cells. (In preparation)

5. Hendrikus J. Dubbink, Iris H.I.M. Hollink*, Carolina Avenca Valente*, Wenhui Wang*, Pengyu Liu, Michail Doukas, Max M. van Noesel, Winand N.M. Dinjens, Anja Wagner, Ron Smits. A novel tissue-based β-catenin gene and immunohistochemical analysis to exclude Familial Adenomatous Polyposis among children with hepatoblastoma tumors. Pediatric Blood&Cancer, DOI:10.1002/pbc.26991. (* equal contribution)

6. Wenshi Wang, Yuebang Yin, Lei Xu, Junhong Su, Fen Huang, Yijin Wang, Patrick P. C. Boor, Kan Chen, Wenhui Wang, Wanlu Cao, Xinying Zhou, Pengyu Liu, Luc J. W. van der Laan, Jaap Kwekkeboom, Maikel P. Peppelenbosch and Qiuwei Pan. Unphosphorylated ISGF3 drives constitutive expression of interferon-stimulated genes to protect against viral infections. Sci Signal. 2017;25;10(476).

7. **王文辉**，汪芳裕，魏娟，沈芸竹等. CpG 甲基转移酶诱导紧密连接蛋白 7 和 8 基因表达下调对人结肠癌 HT-29 细胞增殖和凋亡的影响.中华肿瘤杂志.2013, 35(6).

8. **王文辉**,汪芳裕,沈芸竹等.CpG 甲基转移酶诱导人结肠癌细胞凋亡的实验研究.医学研究生学报. 2012, 25(9): 905-908.

PhD Portfolio

Name PhD Student	Wenhui Wang
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2013 - June 2018
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Copromotor	Dr. Ron Smits

General Courses

- 2013 Biomedical Research Techniques XIII
- 2013 International course on laboratory animal science (Art. 9)
- 2014 Photoshop and Illustrator CS6 workshop
- 2015 Workshop Writing Successful Grant Proposals
- 2016 Course on biomedical English writing course for MSc and PhD-students
- 2016 Basic and translational oncology
- 2016 Next generation sequencing training: CLC Workbench / Ingenuity Variant Analysis Workshop

National and International Conferences

- 2015, Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands. (Poster presentation)
- 2016, 9th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, The Netherlands (oral presentation)
- 2016 The 24th Biennial Congress of the European Association for Cancer Research, Manchester, United Kingdom (poster presentation)
- 2017, EASL HCC SUMMIT, Geneva, Switzerland (ePoster presentation)

Academic Awards

- 2013, China Scholarship Council (CSC) Scholarship (File No. 201306300027)
- 2016, Erasmus Trustfonds travel grant (Reference: III A 97095.78/16.0366/evt)
- 2017, Young Investigator Travel Awards (EASL HCC SUMMIT 2017)

Curriculum Vitae

Wenhui Wang was born on August 24, 1986, in Datong, Shanxi Province, China. She attended primary, middle and high school in Datong.

In 2005, she moved to Taiyuan in Shanxi Province and started her undergraduate study. She majored in Clinical Medicine (5 years) in the Shanxi Medical University. She obtained her Bachelor degree of Medicine in 2010 and started master research in the same year in Nanjing University, Jiangsu Province, China. She studied in the College of Medicine under the supervision of Prof. Fangyu Wang. Her main focus was to study the function of claudin proteins in the development of colorectal cancer. She obtained the Master degree of Medicine in 2013.

In 2013, with the support of China Scholarship Council, she got an opportunity to start her PhD research at the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, the Netherlands. Under the supervision of Prof. Maikel P. Peppelenbosch and Dr. Ron Smits, she mainly focused on the therapeutic improvement of hepatocellular carcinoma (HCC) by targeting Wnt/β-catenin signaling. She was dedicated to elucidating the important role of Wnt/β-catenin signaling in HCC therapy.