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Antagonism between wild-type and mutant β-catenin controls hepatoblastoma differentiation via fascin-1

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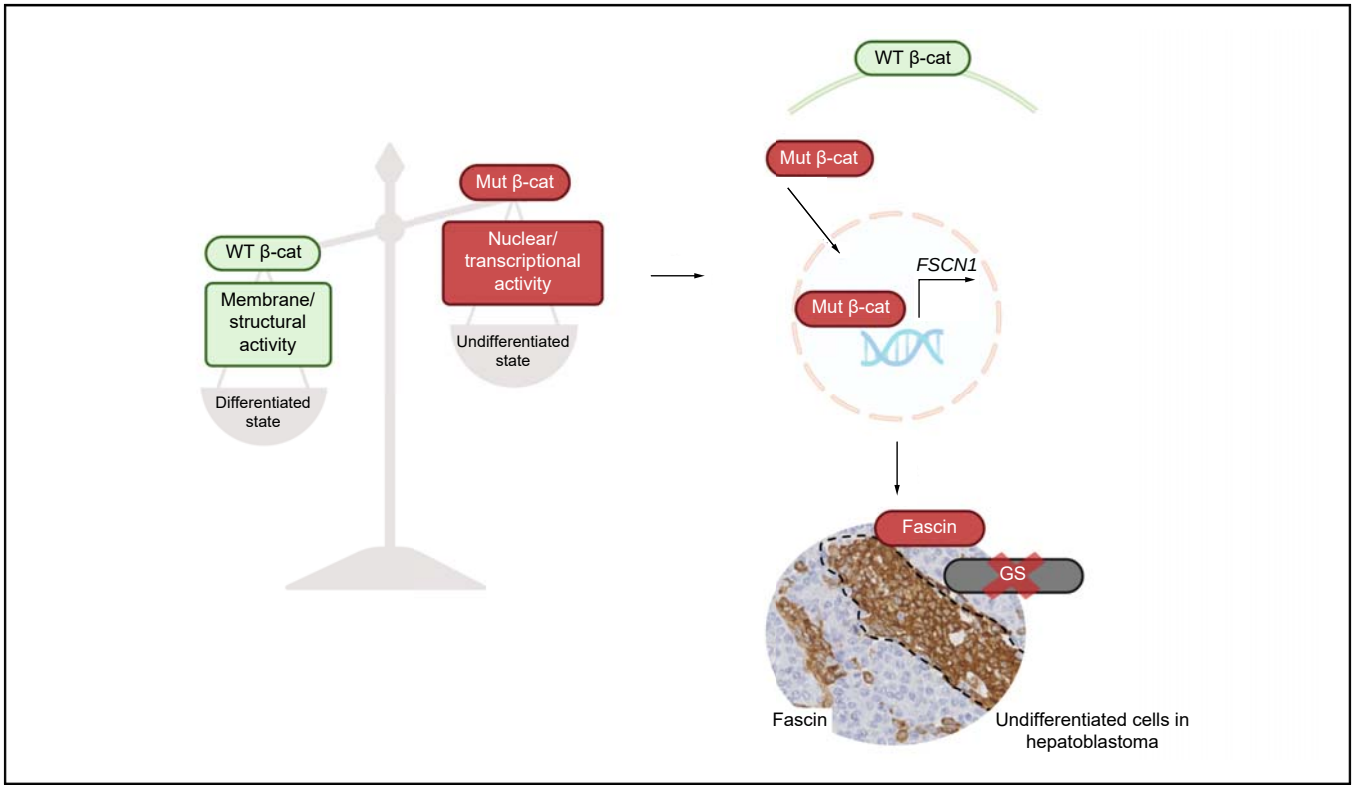
Graphical abstract



Highlights

- A dual β-catenin knockdown HepG2 model allows study of independent structural and transcriptional activities of β-catenin.
- WT and mutated β-catenins play antagonistic functions in hepatoblastoma cells.
- Fascin-1 is a target of mutated β-catenin in tumour hepatocytes.
- Fascin-1 expression is high in β-catenin-activated undifferentiated tumours in mice.
- Fascin-1 expression is a marker of immature cells in human hepatoblastomas.

Impact and implications

The *FSCN1* gene, encoding fascin-1, was reported to be a metastasis-related gene in various cancers. Herein, we uncover its expression in poor-prognosis hepatoblastomas, a paediatric liver cancer. We show that fascin-1 expression is driven by the mutated beta-catenin in liver tumour cells. We provide new insights on the impact of fascin-1 expression on tumour cell differentiation. We highlight fascin-1 as a marker of immature cells in mouse and human hepatoblastomas.

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Research article

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Antagonism between wild-type and mutant β-catenin controls hepatoblastoma differentiation via fascin-1

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Background & Aims: β-catenin is a well-known effector of the Wnt pathway, and a key player in cadherin-mediated cell adhesion. Oncogenic mutations of β-catenin are very frequent in paediatric liver primary tumours. Those mutations are mostly heterozygous, which allows the co-expression of wild-type (WT) and mutated β-catenins in tumour cells. We investigated the interplay between WT and mutated β-catenins in liver tumour cells, and searched for new actors of the β-catenin pathway.

Methods: Using an RNAi strategy in β-catenin-mutated hepatoblastoma (HB) cells, we dissociated the structural and transcriptional activities of β-catenin, which are carried mainly by WT and mutated proteins, respectively. Their impact was characterised using transcriptomic and functional analyses. We studied mice that develop liver tumours upon activation of β-catenin in hepatocytes (APC^{KO} and $\beta\text{-catenin}^{\Delta exon3}$ mice). We used transcriptomic data from mouse and human HB specimens, and used immunohistochemistry to analyse samples.

Results: We highlighted an antagonistic role of WT and mutated β-catenins with regard to hepatocyte differentiation, as attested by alterations in the expression of hepatocyte markers and the formation of bile canaliculi. We characterised fascin-1 as a transcriptional target of mutated β-catenin involved in tumour cell differentiation. Using mouse models, we found that fascin-1 is highly expressed in undifferentiated tumours. Finally, we found that fascin-1 is a specific marker of primitive cells including embryonal and blastemal cells in human HBs.

Conclusions: Fascin-1 expression is linked to a loss of differentiation and polarity of hepatocytes. We present fascin-1 as a previously unrecognised factor in the modulation of hepatocyte differentiation associated with β-catenin pathway alteration in the liver, and as a new potential target in HB.

Impact and implications: The *FSCN1* gene, encoding fascin-1, was reported to be a metastasis-related gene in various cancers. Herein, we uncover its expression in poor-prognosis hepatoblastomas, a paediatric liver cancer. We show that fascin-1 expression is driven by the mutated beta-catenin in liver tumour cells. We provide new insights on the impact of fascin-1 expression on tumour cell differentiation. We highlight fascin-1 as a marker of immature cells in mouse and human hepatoblastomas.

Introduction

β-catenin is an evolutionary conserved protein that plays a dual role in cells. It is the key effector of the canonical Wnt pathway, acting as a transcriptional cofactor with the lymphoid enhancer factor/T-cell factor (LEF/TCF).[1] In addition, β-catenin plays a central role in cadherin-mediated cell adhesion. In epithelial cells, in the absence of Wnt signalling, β-catenin is associated to E-cadherin at cell–cell junctions, and is maintained at low cytoplasmic levels through its destruction complex of Axin, adenomatosis polyposis coli (APC), and glycogen synthase 3β kinase (GSK-3β). GSK-3β phosphorylates β-catenin, causing its degradation by the proteasome. The destruction complex is disrupted by the Wnt signal, whereupon the cytoplasmic stabilised β-catenin translocates in the nucleus, where it drives the transcription of target genes. Thus, β-catenin is endowed with two main functions: a structural function at cell–cell adhesion, and a transcriptional function in the nucleus. An imbalance in

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the signalling properties of β-catenin may lead to deregulated cell growth, adhesion, and migration, resulting in tumour development and metastasis. However, given the dual function of β-catenin, it is difficult to distinguish whether it is specifically the structural function or the transcriptional function of β-catenin that is involved in cellular processes.

In the liver, the Wnt/β-catenin pathway plays a number of important roles in the regulation of embryonic and postnatal development, zonation, metabolism, and regeneration.[2] This pathway is also strongly involved in hepatocarcinogenesis. Its aberrant activation arises from mutations in the *CTNNB1* gene encoding β-catenin or in components of the degradation complex, such as *AXIN* and *APC*. *CTNNB1* alterations are identified in up to 80% of human hepatoblastomas (HBs), the primary hepatic malignancy in children, and in 30–40% of hepatocellular carcinomas (HCCs).[3,4] Although HBs are complex tumours with various histologic components (epithelial, mesenchymal, foetal, and embryonal) within the same tumour,[5] molecular analyses nonetheless described two subtypes: the foetal C1 subtype with favourable outcome, and the proliferative poorly differentiated C2 subtype characterised by an intense nuclear staining of β-catenin.[3] The C2 subtype has recently been split into subgroups, C2A and C2B, with the C2A subgroup containing more proliferative tumours.[6] The majority of β-catenin mutations affect exon3 at GSK3β phosphorylation sites, constitutively activating the Wnt pathway. Interstitial deletions in this exon are highly prevalent in HBs, whereas point mutations are more common in HCCs.[7] Interestingly, most of mutations in exon3 are monoallelic, leaving a wild-type (WT) allele in tumour cells. We therefore attempted to address the interplay between WT and mutated β-catenins in liver tumour cells. To do so, we used the human HB HepG2 cell line, which exhibits a heterozygous deletion of 348 nucleotides in exon3, resulting in an abundant truncated form of β-catenin and a smaller amount of WT β-catenin.[4] The large deletion removes both the GSK-3β phosphorylation sites and the binding site for α-catenin, which is the β-catenin partner in E-cadherin-mediated cell adhesion. Thus, both WT and mutated (Δaa25-140) forms of β-catenin co-exist in these cells. The interplay between the two has never been explored. We therefore designed an RNA interference approach to specifically knock down (KD) WT and/or mutated β-catenin, and to address their reciprocal role in hepatocyte differentiation. Using this approach, we dissociated the structural and transcriptional activities of β-catenin, which are carried mainly by the WT and the mutated proteins, respectively. Transcriptomic and cellular analyses revealed that both functions play an antagonistic role in tumour hepatic cell differentiation. For the first time, molecular characterisation revealed *FSCN1* (fascin-1) as a target of β-catenin, controlling tumour hepatocyte differentiation state and proliferation. We also found that fascin-1 expression is associated with undifferentiated β-catenin-mutated tumours in mice, which are close to human HBs. Using human samples, we showed that fascin-1 is specifically expressed in the embryonal component of HBs. Thus, we described *FSCN1* as a β-catenin target gene associated with hepatic tumours of poor outcome, such as poorly differentiated HBs.

Materials and methods

Cell culture

The human HB cell line, HepG2, and human HCC cell lines, Hep3B, Huh7, and SNU398, were purchased from the American Type Culture Collection (see Supplementary CTAT table). HB cell line Huh6 was generously provided by C. Perret (Paris, France). All cell lines were cultured as previously described.[8,9] Cell line authentication was performed using short tandem repeat analysis, and absence of mycoplasma contamination in cell culture media was tested every week.

Mouse samples

We collected tumoural and non-tumoural livers from mouse transgenic models with hepatic β-catenin activation. All animal procedures were approved by the ethical committee of Université de Paris according to the French government regulation. The $Apc^{fs\text{-}ex15}$ and β-$catenin^{\Delta ex3}$ mouse tumours were obtained from compound $Apc^{flox/flox}$/TTR-Cre^{Tam} and β-$catenin^{ex3\text{-}flox/flox}$, respectively, injected with 0.75 mg tamoxifen or with 5×10^8 i.p. Cre-expressing adenovirus, as previously described.[10–12] All the mice were maintained at the animal facility with standard diet and housing. They were followed by ultrasonography every month until tumour detection, thereafter ultrasound imaging was continued every 2 weeks. Table S1 describes the cohort of mice used for this study.

Patient samples

Liver tissues were immediately frozen in isopentane with Snapfrost and stored at -80 °C until used for molecular studies. Samples were obtained from the Centre de Ressources Biologiques (CRB)-Paris-Sud (BRIF N°BB-0033-00089) with written informed consent, and the study protocol was approved by the French Government and the ethics committees of HEPATOBIO (HEPATOBIO project: CPP N°CO-15-003; CNIL N°915640). Liver samples were clinically, histologically, and genetically characterised (Table S2). Among the 20 cases, 12 were classified as C1, three as C2A and five as C2B in Grosset's previous study.[6]

Statistical tests

Data were reported as the mean ± SEM of at least three experiments. Statistical significance (p <0.05 or less) was determined using Student's t test or analysis of variance (ANOVA) as appropriate and performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Values of p are indicated as follows: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001; $^{****}p$ <0.0001; n.s., non-significant.

Results

The dual β-catenin knocked-down HepG2 model

An exon3 region is deleted on one allele of the *CTNNB1* gene in HepG2 cells.[4] A high amount of a truncated form of 76 kDa is therefore co-expressed with a 92-kDa full-length β-catenin (Fig. 1). We designed small interfering RNAs (siRNAs), named 'siβcat-WT', 'siβcat-mut', and 'siβcat-both', to respectively, specifically target the WT, the mutated form of β-catenin, or both (Fig. 1A). Each siRNA efficiently knocked down their targeted protein (Fig. 1B and Fig. S1A). At the mRNA level, siβcat-WT and siβcat-mut reduced the amount of β-catenin transcripts by half. As expected, siβcat-both led to a near full elimination of β-catenin in HepG2 cells (Fig. 1C).

Using the TOP-flash reporter system, we characterised the transcriptional activity of β-catenin relative to the silencing of both alleles. Whereas silencing the WT allele did not impact TCF/LEF reporter activity, silencing the expression of the mutated allele or of both alleles strongly inhibited it (Fig. 1D). Moreover,

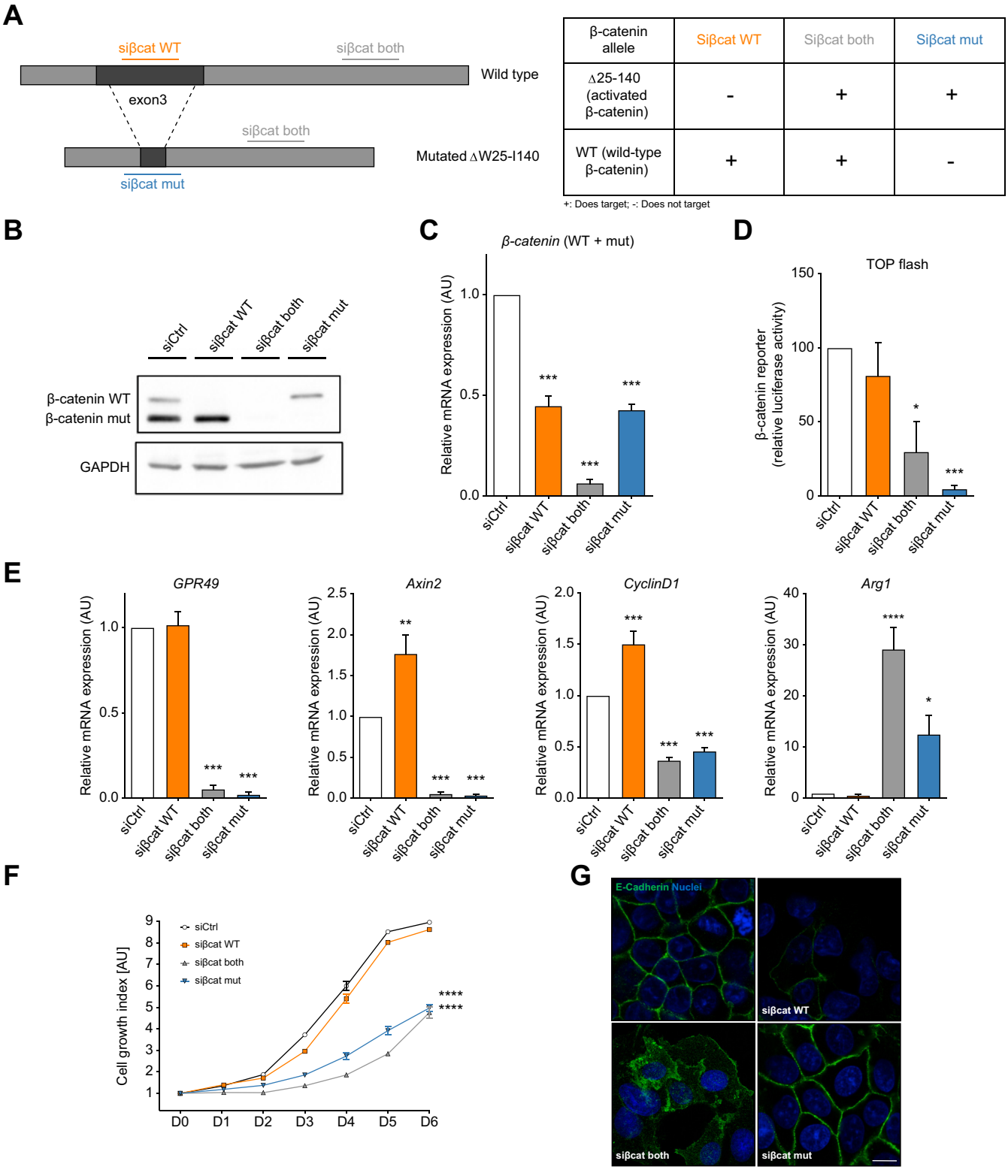

Fig. 1. The HepG2 dual β-catenin KD model. (A) Schema showing the different siRNAs used (B–E) HepG2 cells were transfected with indicated siRNAs (B) Protein extracts were analysed using anti-β-catenin and GAPDH antibodies. Note that because of the W25–I140 deletion, mutated β-catenin migrates faster and is more abundant than the WT β-catenin (C) β-Catenin mRNA expression was analysed by qRT-PCR (D) Promoter activity was evaluated by luciferase reporter assays with TCF responsive reporter. Shown is the mean relative luciferase activity, normalised to *Renilla* luciferase and compared with siRNA control transfected cells (E) mRNA levels of indicated genes were analysed by qRT-PCR. Shown is the relative mRNA level compared with control transfected cells (C–E) Each graph shows the quantification of three independent experiments. Error bars indicate S.E.M. (n = 3). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 by one-way ANOVA (F) HepG2 cells transfected with indicated siRNAs were seeded in 96-well plates and total biomass, reflecting the number of cells, and was assayed every day. Each time point was performed in triplicates. Error bars indicate S.E.M. (n = 3). ****p <0.0001 by one-way ANOVA (G) HepG2 cells transfected with indicated siRNAs were fixed and stained with anti-E-cadherin antibodies (green) and Hoechst (blue). Scale bar: 10 μm. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; TCF, T-cell factor; WT, wild-type.

expressions of positive targets of β-catenin, such as GPR49, Axin2, and cyclinD1, were strongly inhibited by the application of siβcat-mut and siβcat-both (Fig. 1E). The opposite result was consistently obtained for negative targets such as Arg1 (Fig. 1E). The data therefore suggest that, in HepG2 cells, the TCF-dependent transcriptional activity of β-catenin is mainly carried by the mutated form of β-catenin.

Because of this impact on β-catenin targets, and specifically on CyclinD1, HepG2 cell growth was found to be highly dependent on a mutated β-catenin expression, while being insensitive to the knockdown (KD) of the WT form of β-catenin (Fig. 1F). These data were confirmed on spheroids (Fig. S1B), showing that the mutated β-catenin is required for HepG2 cell growth in 2D and 3D environments. The mutated form of β-catenin would thus appear to be a strict requirement for HepG2 cell growth, which is consistent with the idea of oncogene addiction.

The large deletion present in HepG2 cells also removes the α-catenin binding site of β-catenin. We thus found that the silencing of the mutated allele of β-catenin did not impact E-cadherin localisation at cell–cell junctions. Interestingly, we found that in an obverse manner WT β-catenin KD strongly affected E-cadherin engagement at adherens junctions in HepG2 cells (Fig. 1G). Consequently, we observed that HepG2 cells acquire a more migrating and invasive phenotype upon silencing of WT β-catenin (Fig. S1C and D). In cells lacking the expression of both β-catenins, E-cadherin staining appears to be strongly affected with a more intracellular localisation (Fig. 1G). These results suggest that the structural role of β-catenin is altered only upon WT β-catenin KD. This conclusion is further supported by our observations of β-catenin localisation (Fig. S1E). In control HepG2 cells, β-catenin localised at cell–cell junctions, in the cytoplasm and in nuclei. Those stainings which were lost when cells were transfected with siβcat-both. In cells transiently transfected with siβcat-mut, remaining β-catenin, that is WT β-catenin, was enriched at cell–cell junctions, but failed to localise in the cytoplasm and in nuclei. In contrast, in cells transfected with siβcat-WT, remaining β-catenin, that is mutated β-catenin, continued to be cytosolic and less at the plasma membrane (Fig. S1E). Thus, in HepG2 cells, WT β-catenin appears to be mainly involved in adherent junctions, whereas mutated β-catenin appears to be preferentially involved in the regulation of gene expression. Based on these observations, this dual β-catenin KD HepG2 model allows the two functions of β-catenin in the same cellular background to be distinguished: the membrane/structural activity mediated by the degradable WT β-catenin and the nuclear/transcriptional activity mediated by the mutated β-catenin. Thus, this model provides a suitable method of addressing the structural and the transcriptional functions of β-catenin independently.

WT and mutated β-catenin have distinct gene expression patterns

To study the involvement of each β-catenin allele in gene expression, we performed a transcriptional analysis of HepG2 KD cells (Tables S3–5). The expression of β-catenin and known-β-catenin targets (Fig. S2A) was altered in a way similar to that previously observed by qRT-PCR (Fig. 1C and E), thus validating our data. Global analysis demonstrated that WT β-catenin KD cells and mutated β-catenin KD cells have distinct gene expression patterns. The silencing of both alleles led to a gene expression pattern closer to the mutated than the WT β-catenin KD, suggesting a dominant impact of the oncogenic β-catenin on gene expression in HepG2 cells (Fig. S2B). A Venn diagram of our observations shows that only a small proportion of genes were altered by both the silencing of WT and of mutated β-Catenin (Fig. S2C). Hoping to understand how WT β-catenin may regulate gene expression, we performed gene set enrichment analysis (GSEA). GSEA revealed that the genes that upregulated upon WT β-catenin depletion showed significant overlap with genes associated with the transforming growth factor-β and Hippo pathways (Fig. S2D). Indeed, the transcription factors TEAD1 and TEAD2, and the target genes of the Hippo pathway, were found to be upregulated by the silencing of WT β-catenin (Fig. S2E and F). These data are consistent with Piccolo's work which shows that depletion of the sole WT pool of β-catenin is sufficient to promote TAZ activity in HepG2 cells.[13] We also used FuncAssociate 3.0 to analyse alterations in biological functions (Table S6). Removal of the WT β-catenin led to a decrease in the expression of genes involved in metabolic processes. Strikingly, we observed the opposite effect upon removal of the oncogenic β-catenin. Given that metabolic functions are key features of differentiated hepatocytes, these alterations led us to explore the impact of both β-catenin functions on cell differentiation.

Alteration of hepatocyte differentiation and polarity upon β-catenin knock-downs

Differentiated hepatocytes are characterised by the expression of specific markers, including xenobiotic-metabolising enzymes, transporters, transcription factors, and bile canaliculi molecules. Interestingly, we observed a mirror image alteration upon the silencing of either the WT or the mutated allele of β-catenin. Hepatocyte markers were found to be upregulated by mutated β-catenin silencing and downregulated by WT β-catenin silencing (Fig. 2A). Given that hepatocyte nuclear factor-4 alpha (HNF4α) is the major transcription factor involved in hepatocyte differentiation, we pursued our exploration of the impact of β-catenin KD on HNF4α signalling. Using qRT-PCR analysis, we observed only a slight decrease of HNF4α expression upon WT β-catenin KD, whereas it is not significantly altered by the silencing of the oncogenic β-catenin (Fig. 2B). However, we observed a significant increase of HNF4α transcriptional activity when the oncogenic β-catenin was silenced. This observation was made using an ApoC3 promoter reporter assay (Fig. 2C) and expression analysis of HNF4α transcriptional positive targets (Fig. S3A–C and Fig. 2D). The removal of WT β-catenin slightly decreased the expression of HNF4α target genes. As described earlier,[14] this result confirms our belief that transcriptional activity of β-catenin may repress the hepatocyte differentiation programme of HNF4α. Taken as a whole, these results demonstrate that the structural function of β-catenin, supported mainly by the WT β-catenin, is necessary to maintain a differentiated state of hepatocytes, and that the inhibition of the transcriptional activity of oncogenic β-catenin reverses the dedifferentiation programme of HepG2 cells.

Given that differentiated hepatocytes are polarised epithelial cells endowed with the capacity to produce and excrete bile into a specialised structure called bile canaliculus (BC), we further analysed the effect of β-catenin KD on BC markers. Similar to the alteration described above for hepatocyte markers, we observed a mirror image alteration of the expression of junction- and polarity-associated genes (Fig. S3D and E), such as JAM-A (*F11R* gene), connexin-32 (*GJB1* gene), and claudin-1 (*CLDN1* gene)

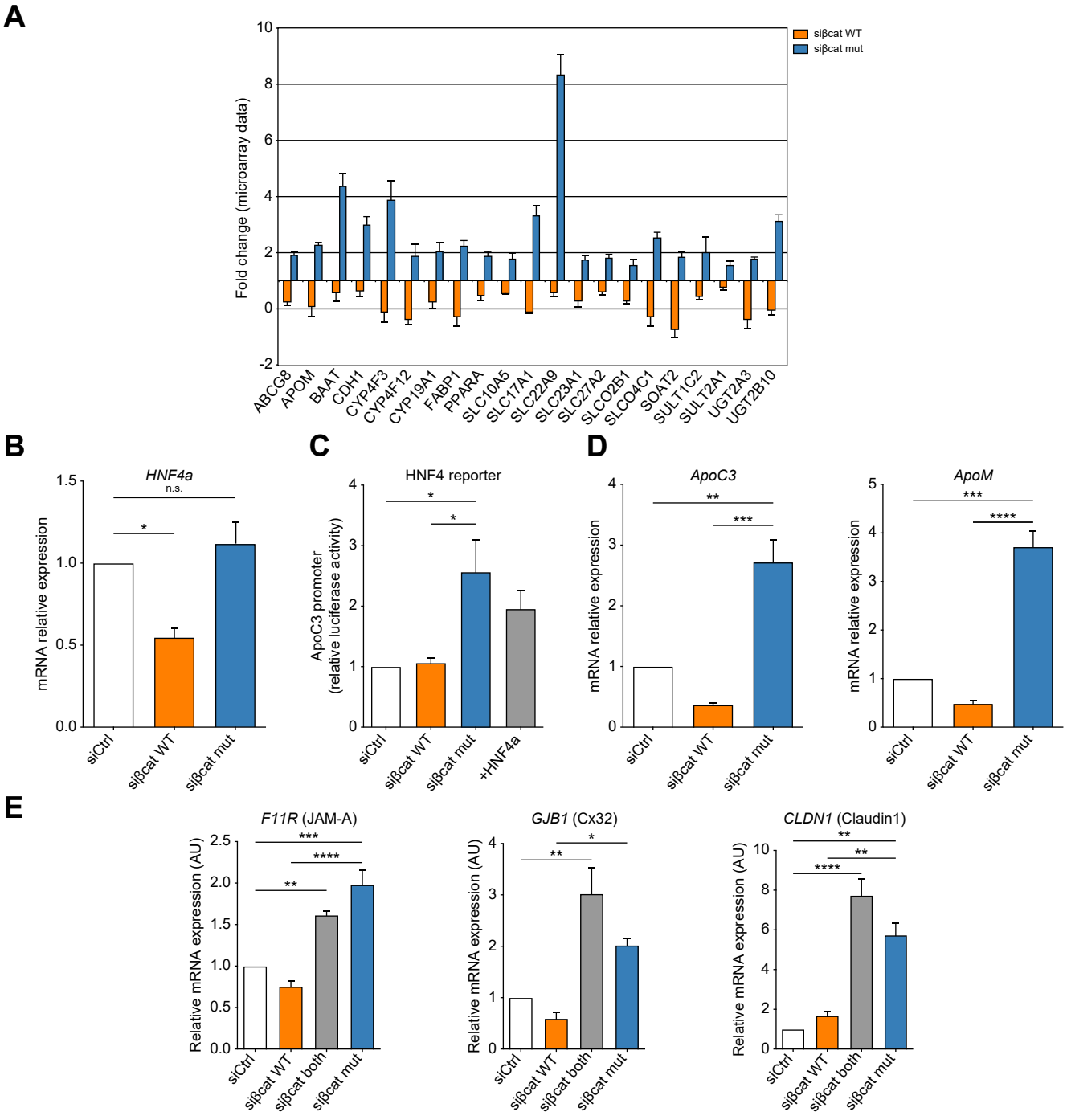

Fig. 2. Antagonistic impact of WT and mutated β-catenins. (A) The graph shows the relative expression of differentiated hepatocyte markers, upon silencing of both alleles of β-catenin in HepG2 cells, extracted from the transcriptomic analysis (B) Indicated siRNA were transfected in HepG2 cells, and *HNF4A* relative mRNA level was analysed. Shown is the relative mRNA level compared with control transfected cells (C) HepG2 cells transfected with indicated siRNAs were transfected with HNF4a responsive luciferase reporter. Shown is the mean relative luciferase activity, normalised to *Renilla* luciferase and compared with siRNA control transfected cells (D) Relative mRNA levels of *APOC3* and *APOM*, both positive transcriptional targets of HNF4a were analysed by qRT-PCR (E) Alteration of the expression of polarity markers upon silencing of both alleles of β-catenin in HepG2 cells. The graph shows the relative expression of the indicated genes analysed by qRT-PCR (B–E) Each graph shows the quantification of three independent experiments. Error bars indicate S.E.M. (n = 3). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001; n.s., non-significant by one-way ANOVA. HNF4α, hepatocyte nuclear factor-4 alpha; siRNA, small interfering RNA; WT, wild-type.

(Fig. 2E, Fig. S3D and E). Given that HepG2 cells retain the ability to form BC in culture,[15] we addressed the influence of β-catenin on their maintenance, using confocal microscopy by staining F-actin and radixin (Fig. 3A). As for cells exhibiting BC, the depletion of mutated β-catenin induced an increase in their number, whereas the depletion of WT β-catenin induced a decrease in their number (Fig. 3A and B). We performed super-resolution stimulated emission depletion (STED) microscopy on HepG2 cells stained with phalloidin, to visualise BC with a resolution better than confocal (Fig. 3C and Fig. S4A). Quantification of BC

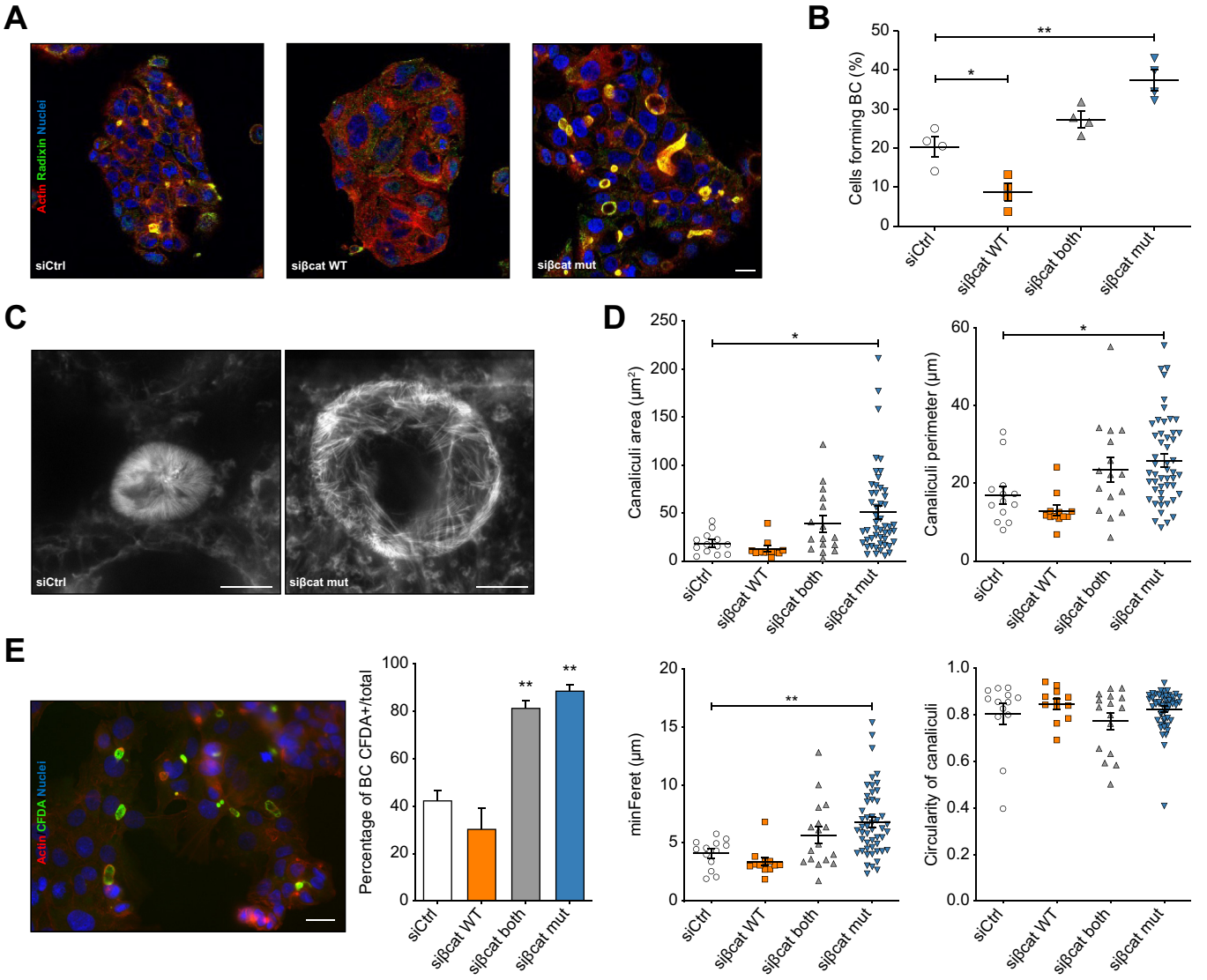

Fig. 3. Alteration of bile canaliculi formation upon silencing of β-catenin in HepG2 cells. (A) HepG2 cells transfected with indicated siRNAs were fixed and stained with phalloidin (red), anti-radixin antibodies (green) and Hoechst (blue). Scale bar: 15 µm (B) Quantification of the percentage of cells forming BC in the conditions described in (A). Graph shows the quantification of four independent experiments, where at least 100 cells were observed per experiment (C) siRNA transfected HepG2 cells were fixed, stained with phalloidin-ATTO and observed by STED microscopy. Scale bar: 5 µm (D) BC features (area, perimeter, circularity, min Feret [diameter]) were quantified by imageJ on STED images performed as described in (C). Each dot corresponds to one BC (E) Quantification of BC functionality by using CFDA incorporation. Life-Act (red) expressing HepG2 cells were treated with CFDA (green). Scale bar: 25 µm. Graph shows the quantification of three independent experiments (B, D and E) Error bars indicate S.E.M. (n = 4 for B, n = 3 for D and E). *p <0.05; **p <0.01 by one-way ANOVA. BC, bile canaliculus; CFDA, 5-carboxyfluorescein diacetate; siRNA, small interfering RNA; STED, stimulated emission depletion; WT, wild-type.

features demonstrated that mutated β-catenin KD increases their size in terms of area, perimeter and diameter, whereas their circularity remains unchanged (Fig. 3D), with the number of cells engaged in the formation of each canaliculus also being found to increase (Fig. S4B). Finally, we examined their ability to translocate 5-carboxyfluorescein diacetate (CFDA), a fluorescent substrate of ABC transporters, into the apical lumen. The removal of the mutated form of β-catenin increased the percentage of CFDA-positive BC, demonstrating that they were fully functional (Fig. 3E). Thus, WT and mutated β-catenins can be said to act antagonistically on hepatocyte BC formation. These results highlight the fact that WT β-catenin continues to act as a gatekeeper of differentiation and polarity in tumour hepatocytes.

Fascin-1, as a target of β-catenin, involved in hepatocyte dedifferentiation

Searching for genes that may impact hepatocyte differentiation and polarity, we focused on *FSCN-1*, which was found to upregulate upon WT β-catenin KD, and to downregulate upon mutated β-catenin KD (Fig. S5A). *FSCN-1* encodes fascin-1, an actin-bundling protein, which is normally not expressed in epithelial cells. Unlike villin, an actin-bundling protein associated with BC microvilli, fascin-1 is absent in mature hepatocytes. Moreover, fascin-1 is expressed in tumours including HCC,[16] and is described as a transcriptional target of β-catenin in breast, gastric, and colon cancers.[17–19] First, we analysed fascin-1 protein expression in HB and HCC cell lines with different β-catenin

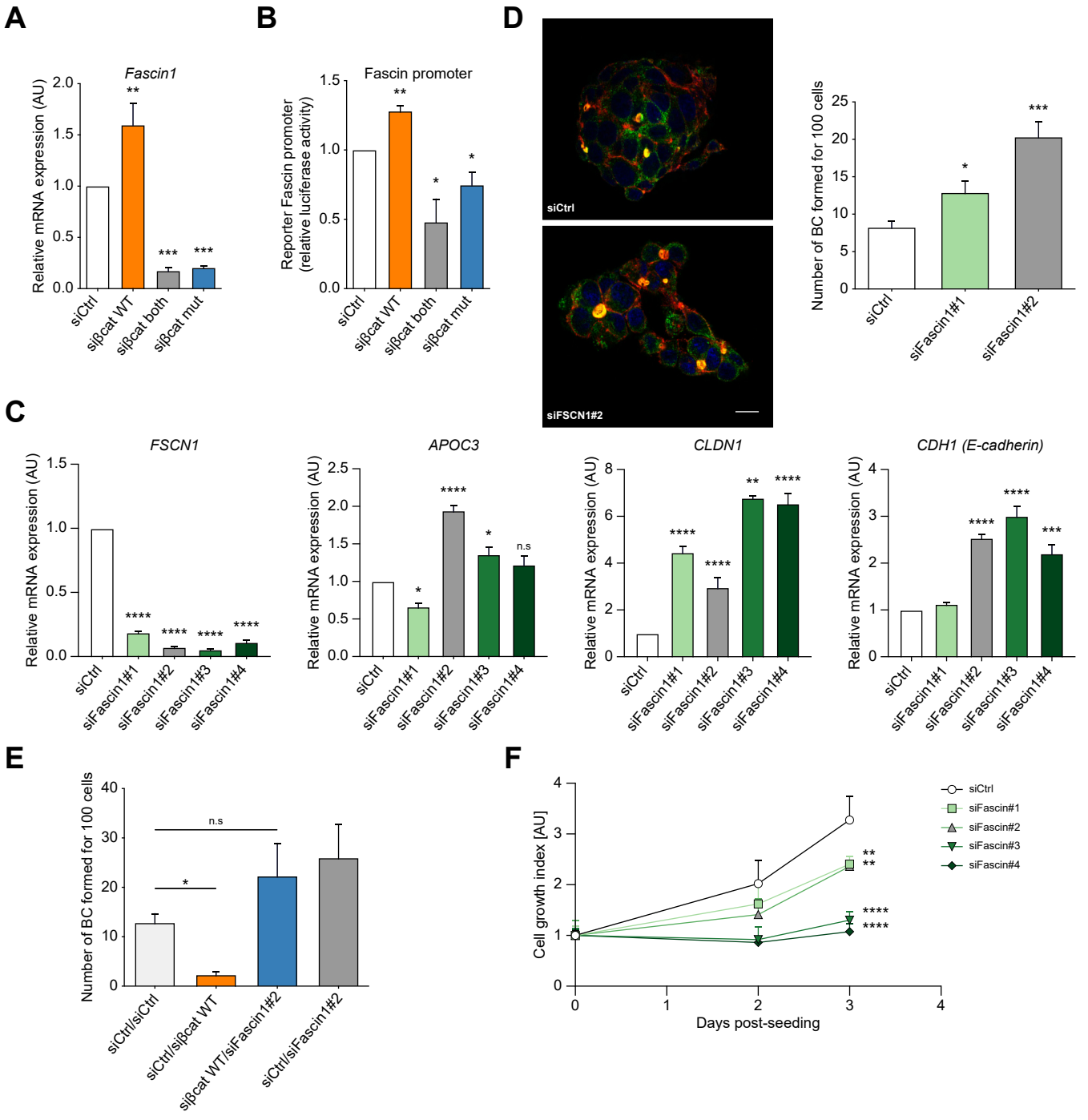

Fig. 4. Fascin-1, a target of β-catenin, alters hepatocyte differentiation status. (A) Fascin-1 mRNA expression upon depletion of β-catenin analysed by RT-qPCR in HepG2 cells (B) Activity of fascin-1 promoter studied by reporter luciferase assay. Shown is the mean relative luciferase activity, normalised to *Renilla* luciferase and compared with control siRNA transfected cells (C) mRNA levels of *FSCN1*, *APOC3*, *CLDN1*, and *CDH1* upon treatment of HepG2 cells with siRNA targeting fascin-1 (siFascin1#1, #2, #3, and #4) (D) siRNA transfected HepG2 cells were fixed and stained with phalloidin (red), anti-radixin antibodies (green) and Hoechst (blue). Scale bar: 15 μm. The graph shows the quantification of three independent experiments where the number of BC formed for 100 cells are indicated (E) Experiments were performed as described in (D) with co-transfection of indicated siRNAs (F) HepG2 cell growth was monitored upon KD of fascin-1 using the indicated siRNAs (A–F) Graphs show the quantification of at least three independent experiments. Error bars indicate S.E.M. (n = 4 for A, n = 5 for B, and n = 3 for C–F). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001; n.s., non-significant by one-way ANOVA. BC, bile canaliculus; KD, knockdown; siRNA, small interfering RNA; WT, wild-type.

status (Fig. S5B). We found a greater expression of fascin-1 in cell lines bearing-*CTNNB1* deletion (HepG2) or mutations (SNU398 and Huh6), as compared with non-mutated (Huh7, Hep3B) cell lines. The level of fascin-1 protein was positively correlated to the β-catenin protein level, which accumulates upon mutations (Fig. S5C). As a β-catenin transcriptional target, fascin-1 mRNA

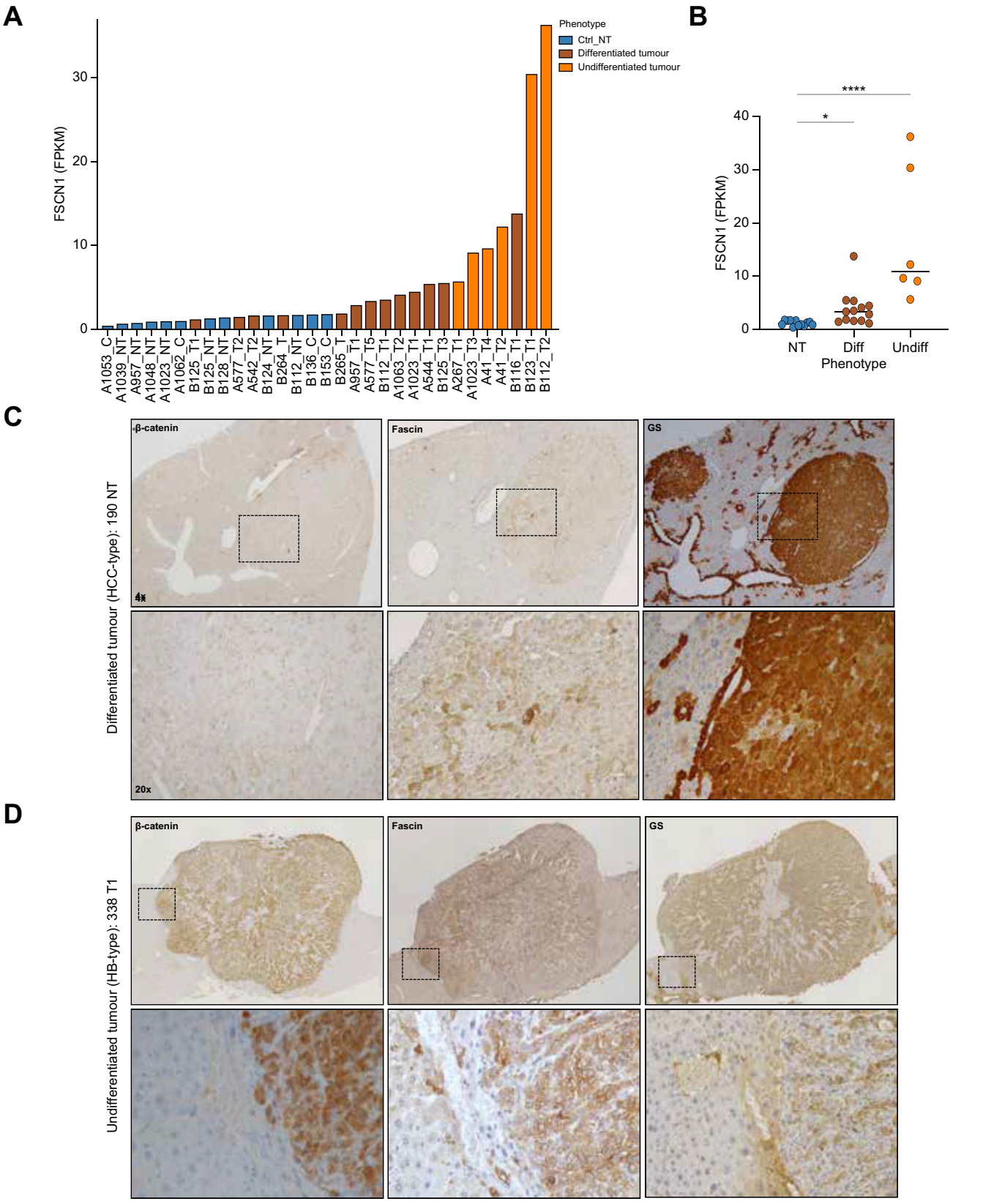

Fig. 5. Fascin-1 expression in murine β-catenin-mediated tumours. (A, B) Data were extracted from RNAseq performed on mouse hepatic tumours induced either from APC KO or β-catenin Δexon3 expression in livers. *FSCN-1* expression is shown in non-tumoural samples (n = 12) and in differentiated (n = 13) and undifferentiated (n = 6) tumours. Levels of significance: $^{*}p$ <0.05; $^{****}p$ <0.0001; by one-way ANOVA test and Kruskal-Wallis post-test (C) Representative immunohistochemistry images of β-catenin, fascin-1, and glutamine synthetase (GS) in differentiated (HCC-type) (C), and undifferentiated (HB-type) (D) murine tumours. Boxed regions are enlarged in the zoom images. APC, adenomatosis polyposis coli; HB, hepatoblastoma; HCC, hepatocellular carcinoma; KO, knockout; NT, non-tumoural.

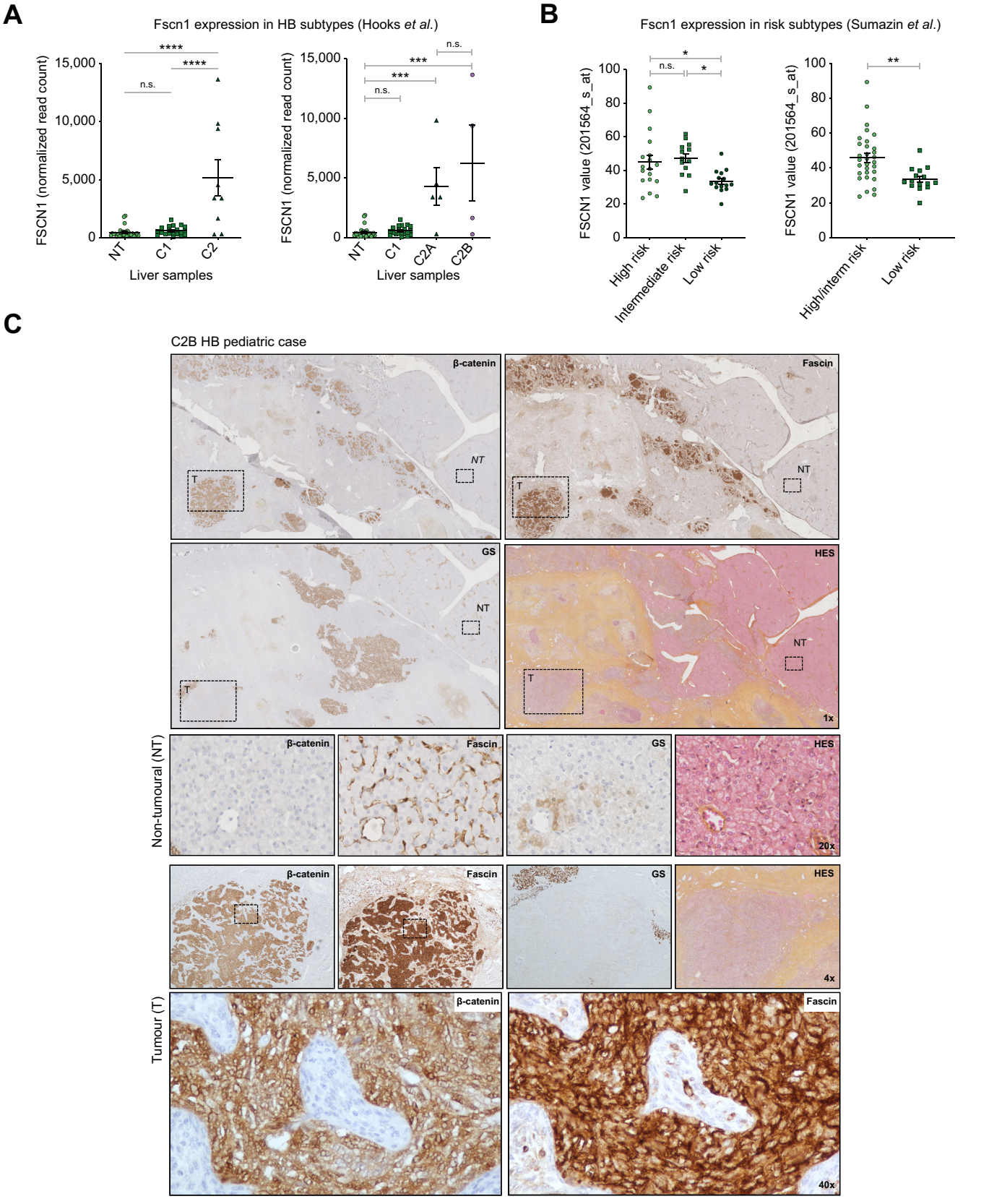

Fig. 6. Fascin-1 expression in human HBs. (A) Data were extracted from RNAseq performed on human HBs by Hooks *et al.*[6] FSCN-1 expression is shown in non-tumoural samples (n = 30) and in C1 (n = 20) and C2A or C2B (n = 9) tumours. Levels of significance: ***p <0.001; ****p <0.0001; n.s., non-significant by one-way ANOVA test (B) Data were extracted from RNAseq performed on human HBs by Sumazin *et al.*[21] *FSCN-1* expression is shown in high-risk and/or intermediate-risk or low-risk HB tumours. Levels of significance: *p <0.05; **p <0.01; n.s., non-significant by one-way ANOVA test (left-hand graph) or unpaired t test (right-hand graph) (C) Representative immunohistochemistry images of β-catenin, fascin-1 and GS and HES staining in a C2B HB paediatric case. Boxed regions are enlarged in the zoom images. GS, glutamine synthetase; HES, haematoxylin-eosin saffron; NT, non-tumoural; T, Tumoural.

expression was strongly inhibited upon treatment of HepG2 cells with siRNAs siβcat-mut and siβcat-both (Fig. 4A). As previously observed for Axin2 and CyclinD1 (Fig. 1E), fascin-1 expression was slightly but significantly upregulated upon removal of WT β-catenin. These alterations were also detected at the protein level (Fig. S5D). Using a luciferase assay with fascin-1 promoter, we demonstrated that this regulation occurs at the transcriptional level, with an antagonistic regulation of promoter activity which occurs upon the silencing of either WT or mutated β-catenin (Fig. 4B). Thus, *FSCN1* shows itself to be a target gene of β-catenin in tumour hepatocytes. We then considered the possibility that fascin-1 expression might play a role in the alteration of hepatocyte differentiation. The silencing of fascin-1 in HepG2 cells led to increased ApoC3, claudin1, and E-cadherin mRNA expressions (Fig. 4C). The same tendency was observed in HCC cell lines, Hep3B and Huh7 (Fig. S6A and B). We also found that fascin-1 KD (Fig. S6C) led to a two-to threefold increase in the number of BC in HepG2 cells (Fig. 4D). Moreover, the depletion of fascin-1 upon inhibition of WT β-catenin expression has a restorative effect on the formation of BC (Fig. 4E), demonstrating that fascin-1 is one of the effectors responsible for the impact of β-catenin on BC formation. In the same way, the overexpression of fascin-1 induced a decrease in BC formation, as compared with the control (Fig. S6D). Finally, consistent with the gain of differentiation observed with fascin-1 KD, we observed a strong inhibition of cell growth upon fascin-1 silencing (Fig. 4F). In parallel, in these conditions, we also found a slight decrease of the invasive properties of fascin-1 KD HepG2 cells (Fig. S6E). Taken together, these results demonstrate that the level of fascin-1 regulated by β-catenin alters hepatocyte differentiation status.

High fascin expression in undifferentiated tumours in mice

Next, we sought to confirm fascin-1 as a β-catenin target in liver tumours in mice. For this, we used mouse models that mimic β-catenin dependent tumorigenesis, such as the APC loss-of-function and the Δexon3 β-catenin models. These models have been shown to lead to the development of phenotypically undistinguishable liver tumours within about 10 months.[12,20] Interestingly, two phenotypically distinct tumours, defined as differentiated and undifferentiated tumours, were observed in these mice.[12,20] The well-differentiated tumours were characterised by hepatocyte-like tumour cells that maintain a β-catenin-induced expression of glutamine synthetase (GS) and in which nuclear β-catenin is present. The undifferentiated tumours were composed of small cells with basophilic nuclei, and showed a strong expression of nuclear β-catenin.[12] RNA-Seq analysis showed that these tumours lose the expression of GS, which is reminiscent of a loss of differentiation (Fig. S7A). We therefore analysed fascin-1 expression in this mouse cohort (Table S1). We found that fascin-1 expression increased in both well-differentiated and undifferentiated types of tumours, as compared with normal liver (Fig. 5A and Fig. S7B). Doing so, we again highlighted that fascin-1 expression was higher in undifferentiated samples than in differentiated ones (Fig. 5B). Moreover, fascin mRNA expression was found to correlate positively with various markers of undifferentiated tumours such as *MMP2*, *VIM*, *HIF1A*, and *YAP1*, and was found to correlate negatively with markers of differentiated tumours such as *HNF4a*, *APOC3*, and *GJB1* (Fig. S7C). Finally, as a *bona fide* β-catenin target, fascin-1 expression correlated positively with the levels of *CTNNB1*, *LEF1*, and *TCF4* (Fig. S7C). These new findings were confirmed by immunohistochemistry performed on both types of murine tumours obtained from a new cohort of APC knockout (KO) mice. As described previously,[12] the well-differentiated tumours were characterised by the nuclear localisation of β-catenin, along with a high expression of GS (Fig. 5C), whereas the undifferentiated tumours were characterised by a GS expression ranging from lower to near-absent (Fig. 5D). Fascin-1 staining, which is absent from the non-tumoural hepatocytes, was found to have increased in both types of tumours. However, whereas its expression is low in differentiated tumours, fascin expression was high in undifferentiated tumour cells, with a cytoplasmic and membranous staining. In mice, fascin-1 expression may therefore be said to be a marker of β-catenin-induced undifferentiated tumours. Given that those murine tumours were found to be transcriptionally close to human mesenchymal HBs,[12] we were prompted to explore fascin-1 expression in human HBs.

Fascin-1 is a marker of embryonal contingent in human HB

To analyse fascin-1 expression in human HBs, we first resorted to public datasets.[3,6,21] Doing so, we found that fascin-1 mRNA is specifically expressed in the C2-subtype of HBs that corresponds to poor-prognosis tumours, with no obvious difference between C2A and C2B subtypes (Fig. 6A). As observed in mice, fascin-1 mRNA expression correlated negatively with markers of differentiated hepatocytes such as *HNF4a*, *APOC3*, *GJB1*, and *CLDN1* (Fig. S8A). Moreover, in the cohort used by Sumazin *et al.*[21] to classify HBs in three risk-stratifying molecular subtypes, we found that fascin-1 is significantly more expressed in high-risk and intermediate-risk tumours than in low-risk tumours, indicating that high-fascin expression is associated with poor-prognosis subgroups (Fig. 6B). We then used immunohistochemistry to confirm the expression of the fascin-1 protein in HBs. Whereas fascin-1 was not expressed in normal hepatocytes, and was restricted to sinusoidal cells in normal and peri-tumoural tissues (Fig. 6C and Fig. S8B), it was expressed in a specific contingent of tumour cells in HBs (Fig. 6C). This fascin-1 staining is highly consistent with our mouse data in showing that fascin-1 is expressed in GS-negative cells and in cells, which are highly β-catenin positive (Fig. 6C and Fig. S8C). These cells may correspond to the embryonal contingent of undifferentiated small cells with basophilic nuclei. However, given that chemotherapy is known to induce alteration of hepatoblastoma cell subtypes, we further used a human sample from a patient that was not treated by chemotherapy before surgery (Fig. S8D). We observed that fascin-1-positive tumour cells are also present as small cuboid or round cells forming solid nests or glands. They look as poorly differentiated epithelial cells, with intermediate phenotype between embryonal and small undifferentiated cells, since anti-hepatocyte is always negative, CK19 unfrequently positive (slightly positive on glands) and vimentin faintly positive. The mesenchymal cells with strong vimentin stain were conversely negative for fascin-1 (Fig. S8E). Foetal areas strongly stained with anti-hepatocyte and anti-GS antibodies were also negative for fascin-1 (Fig. S8F). Taken together, fascin-1 expression can therefore be confirmed as a specific marker of β-catenin-induced primitive cells including embryonal and blastemal cells.

Discussion

β-Catenin performs a structural function at cell–cell junctions and a transcriptional function in the nucleus, but these functions are difficult to dissociate and study individually. Here, we developed the dual β-catenin KD HepG2 model, which allowed

us to perform allele-specific KD, and to address the interplay between the WT and the mutated β-catenin in liver tumour cells. We found that the mutated form of β-catenin is mostly dedicated to the transcriptional function, whereas the WT β-catenin, without Wnt stimulation, is more involved with adhesive function in HepG2 cells. Our model therefore enables the structural and transcriptional functions of β-catenin to be studied independently of each other. Our transcriptomic analysis revealed that the disruption of each function alters gene expression. Consistent with the transcriptional activity of β-catenin, genes that were dysregulated upon removal of mutated β-catenin were coherent with a Wnt/β-catenin signature. By contrast, genes altered upon the silencing of the WT allele were endowed with different signalling pathways, including the Hippo pathway. In fact, the structural function of β-catenin, considered transcriptionally irrelevant, had previously been shown to be a potent repressor of the TAZ transcriptional programme.[13] We also described both the transcriptional and the adhesive activities of β-catenin as playing antagonistic roles in tumour hepatocytes. Whereas the structural function of β-catenin is necessary for the maintenance of a differentiated state of hepatocytes, the transcriptional activity of β-catenin induces the dedifferentiation programme of HepG2 cells. The KD of each allele specifically reverts those programmes. As previously published,[14] we found that this antagonism is, in part, a result of the regulation of the transcriptional activity of HNF4α, which is required to maintain a hepatocyte differentiation programme. Thus, the dual β-catenin KD model recapitulates many features previously described for β-catenin-dependent pathways, and is therefore an appropriate means of furthering our understanding of the role of β-catenin in liver carcinogenesis.

We revealed that WT and mutated β-catenins act in an opposite manner on hepatocyte polarity. Normal membrane polarity and the formation of BC are essential for hepatocyte function, and their alteration may lead to numerous diseases, including cholestasis-associated diseases. In the literature, however, the link between β-catenin and BC abnormalities has not been elucidated. On the one hand, cholestasis has been described as a feature of β-catenin-mutated HCCs,[22] but on the other hand liver-specific β-catenin KO mice develop intrahepatic cholestasis associated with BC abnormalities and bile secretory defect.[23] Whether this phenotype is a result of the structural or the transcriptional activity of β-catenin remains largely unknown. Several studies have reported the involvement of cell adhesion molecules, such as E-cadherin or α-catenin, in the maintenance of BC.[24–26] Here, we revealed that mutated β-catenin plays a repressor role by decreasing the number and the size of BC, and that WT β-catenin is important for their maintenance. As demonstrated for the development of bile ducts,[27] β-catenin must be maintained at the right level, as its loss or overactivation is detrimental to BC formation and/or stabilisation.

In addition to this, our work also highlights the involvement of fascin-1 downstream of β-catenin activation. Fascin-1 has been reported as a transcriptional target of β-catenin/TCF signalling in colon cancer cells.[17] However, this regulation of fascin-1 by β-catenin would appear to be cell type-specific, as no regulation was observed in breast cancer cells.[19] In contrast, fascin-1 has been found to induce epithelial-mesenchymal transition of cholangiocarcinoma cells, and to promote breast cancer stem cell function by regulating Wnt/β-catenin signalling.[28,29]

Downstream of β-catenin, we found that fascin-1 expression alters hepatocyte polarity and differentiation status. *In vitro*, the silencing of fascin-1 upregulates epithelial markers and increases BC formation. Fascin-1 is highly expressed in β-catenin-mutated undifferentiated tumours both in mice and in humans, where its expression correlates negatively with differentiated hepatocyte markers and correlates positively with mesenchymal markers. It has yet to be explored how epithelial and mesenchymal gene expression may be regulated by fascin-1, which is an actin-binding protein. One hypothesis is mechanotransduction, given that modulation of the actin cytoskeleton is known to alter gene expression. An alternative hypothesis points to the presence of fascin-1 in a large list of proteins found to bind mRNA, suggesting a potential role of fascin in post-translational regulation.[30]

Our study sheds light on the existence of various differentiation states of tumours upon β-catenin activation. As has been recently described, the expression of mutated β-catenin in mouse hepatocytes can generate either well-differentiated HCC-like tumours or undifferentiated HB-like tumours.[12] GS expression is the gold standard marker of *CTNNB1*-mutated HCC, that is well-differentiated *CTNNB1*-mutated tumour cells. In this study, we propose fascin-1 as a strong marker of undifferentiated *CTNNB1*-mutated tumour cells, negative for GS. The manner or mechanism by which the same β-catenin mutation can generate different gene expression profiles and different tumour types, however, remains a matter of ongoing debate.[31] We have clearly demonstrated that WT β-catenin acts as a gatekeeper of differentiation in tumour hepatocytes. Our data are consistent with the hypothesis that the expression of WT β-catenin may blunt the oncogenic ability of β-catenin mutations in hepatocytes. Specifically, according to this hypothesis WT β-catenin removal favours the Hippo pathway target gene expression, which is also key in the development of HB.[32,33]

Finally, we revealed fascin-1 as a previously unrecognised marker of *CTNNB1*-mutated immature tumour cells, that is the embryonal contingent, in HBs. Even if fascin-1 is enriched in the C2 subclass of HBs, we also found fascin-1 staining in C1 samples, thus indicating the complexity of these tumours. Fascin-1 being mainly associated to HBs with bad prognosis, it may therefore be suitable to consider it as a new actionable target in these liver paediatric tumours.

Abbreviations

APC, adenomatosis polyposis coli; BC, bile canaliculus; CFDA, 5-carboxyfluorescein diacetate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glutamine synthase; GSEA, gene set enrichment analysis; GSK-3β, glycogen synthase 3β kinase; HB, hepatoblastoma; HCC, hepatocellular carcinoma; HNF4α, hepatocyte nuclear factor-4 alpha; KD, knock-down; KO, knockout; LEF, lymphoid enhancer factor; siRNA, small interfering RNA; STED, stimulated emission depletion; TCF, T-cell factor; WT, wild-type.

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Conflicts of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study design: VM. Generation of experimental data: CG, SS, VN, LD, L. Piquet, L. Paysan, TR, NDS, RL, NAC, DD, VL. Analysis and interpretation of data: CG, SS, LD, DD, PBS, BLB, VL, SC, VM. Providing biological samples from HB patients: CFG, AR. Drafting of the manuscript: FS, VL, SB, VM.

Data availability statement

The transcriptomic data are archived in the public GEO data repository under the GEO accession number GSE144107.

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Supplementary data

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Author names in bold designate shared co-first authorship.

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