Splicing factor SF3B1 is overexpressed and implicated in the aggressiveness and survival of hepatocellular carcinoma

Juan L. López-Cánovas[a,b,c,d], Mercedes del Rio-Moreno[a,b,c,d], Helena García-Fernandez[a,b,c,d], Juan M. Jiménez-Vacas[a,b,c,d], M Trinidad Moreno-Montilla[a,b,c,d], Marina E. Sánchez-Frias[a,c], Víctor Amado[a,e,f], Fernando L-López[a,b,c,d], Marcos F. Fondevila[g,d], Rubén Ciria[a,h], Irene Gómez-Luque[a,h], Javier Briceño[a,h], Rubén Nogueiras[g,d], Manuel de la Mata[a,e,f], Justo P. Castaño[a,b,c,d], Manuel Rodriguez-Perálvarez[a,e,f], Raúl M. Luque[a,b,c,d], Manuel D. Gahete*[a,b,c,d].

[a] Maimónides Institute of Biomedical Research of Córdoba (IMIBIC), Córdoba, 14004, Spain.

[b] Department of Cell Biology, Physiology and Immunology, University of Córdoba, Córdoba, 14004, Spain.

[c] Reina Sofía University Hospital, Córdoba, 14004, Spain.

[d] CIBER Pathophysiology of Obesity and Nutrition (CIBERobn), Córdoba, 14004, Spain.

[e] Department of Hepatology and Liver Transplantation, Reina Sofía University Hospital, Córdoba, 14004, Spain.

[f] CIBER Hepatic and Digestive Diseases (CIBERehd), Córdoba, 14004, Spain.

[g] Department of Physiology, CIMUS, University of Santiago de Compostela-Instituto de Investigación Sanitaria, Santiago de Compostela, 15782, Spain.

[h] Unit of Hepatobiliary Surgery and Liver Transplantation, University Hospital Reina Sofia, Cordoba, 14004, Spain

*Corresponding author: Manuel D. Gahete. Department of Cell Biology, Physiology and Immunology. Maimónides Institute of Biomedical Research of Córdoba (IMIBIC). Av. Menéndez Pidal s/n. 14004-Córdoba, Spain. 957 213 737. bc2gaorm@uco.es

Keywords: Liver cancer, spliceosome, KLF6-SV1, Pladienolide-B, xenograft.

Abbreviations:

CLL: Chronic lymphocytic leukemiaç

FFPE: Formalin-Fixed Paraffin-Embedded

HCC: Hepatocellular carcinoma

IHC: Immunohitochemistry

MDS: myelodysplastic syndrome

SEM: Standard error of the mean

SFs: Splicing factors

SF3B1: Splicing factor 3B subunit 1

siRNA: small interfering RNA

SVs: Splicing variants

Funding Sources: Instituto de Salud Carlos III, co-funded by European Union (ERDF/ESF, "Investing in your future") [PI17/02287, CP15/00156, PI16/00264], MINECO/MECD (BFU2016-80360-R), Junta de Andalucía (BIO-0139), and CIBERobn.

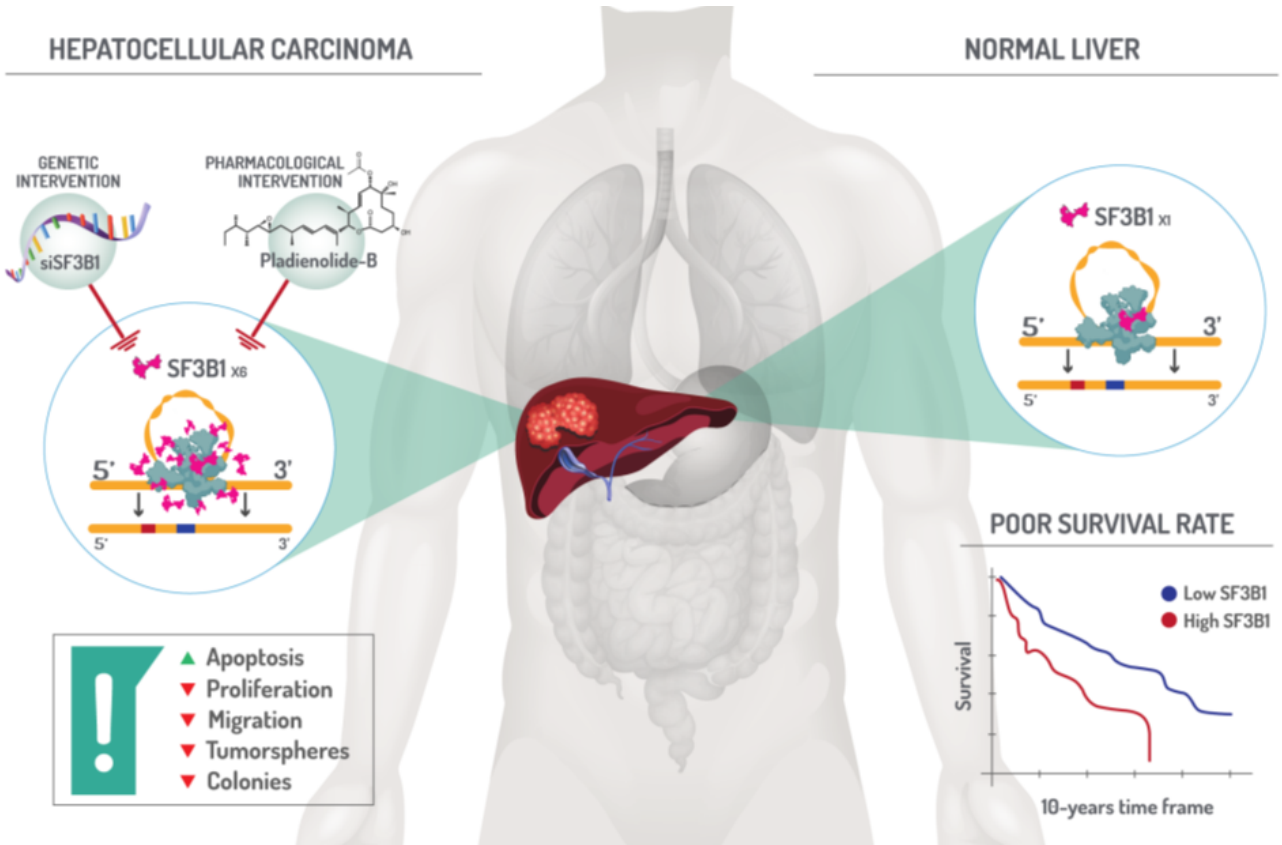
Statement of Ethics: The study protocol was approved by the Reina Sofia University Hospital Ethics Committee, according to institutional and Good Clinical Practice guidelines (Protocol number PI17/02287) and in compliment with the declaration of Helsinki. Written informed consent was obtained from all patients or their relatives.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Acknowledgements: Special thanks to GraphicalAbstractDesign.com for assistance with graph and image design.

Abstract

Splicing alterations represent an actionable cancer hallmark. Splicing factor 3B subunit 1 (SF3B1) is a crucial splicing factor that can be targeted pharmacologically (e.g. pladienolide-B). Here, we show that SF3B1 is overexpressed (RNA/protein) in hepatocellular carcinoma (HCC) in two retrospective (n=154 and n=172 samples) and in five in silico cohorts (n>900 samples, including TCGA) and that its expression is associated with tumor aggressiveness, oncogenic splicing variants expression (KLF6-SV1, BCL-XL) and decreased overall survival. In vitro, SF3B1 silencing reduced cell viability, proliferation and migration and its pharmacological blockade with pladienolide-B inhibited proliferation, migration, and formation of tumorspheres and colonies in liver cancer cell lines (HepG2, Hep3B, SNU-387), whereas its effects on normal-like hepatocyte-derived THLE-2 proliferation were negligible. Pladienolide-B also reduced the in vivo growth and the expression of tumor-markers in Hep3B-induced xenograft tumors. Moreover, SF3B1 silencing and/or blockade markedly modulated the activation of key signalling pathways (PDK1, GSK3b, ERK, JNK, AMPK) and the expression of cancer-associated genes (CDK4, CD24) and oncogenic SVs (KLF6-SV1). Therefore, the genetic and/or pharmacological inhibition of SF3B1 may represent a promising novel therapeutic strategy worth to be explored through randomized controlled trials.



1. Introduction

Primary liver cancer represents a heterogeneous pathology that encompasses diverse cancer types from different origins, representing the fourth most common cancer type worldwide[1]. Hepatocellular carcinoma (HCC) is the most prevalent primary liver tumor (75%) and it is the most frequent cause of death in patients with chronic liver disease. Indeed, HCC occurs in the context of liver cirrhosis in the vast majority of patients, and any aetiology of liver disease including alcohol consumption, chronic viral hepatitis and non-alcoholic steatohepatitis may increase the risk of HCC[1]. Despite routine screening strategies with liver ultrasound every 6 months[2, 3], most HCC patients are currently diagnosed at advanced stages, wherein currently available or emerging systemic therapies (mainly multikinase inhibitors, antiangiogenics and checkpoint inhibitors) still have a limited impact on overall survival[3]. Therefore, a better understanding of the mechanisms underlying HCC development and progression deems mandatory to identify new diagnostic, prognostic and therapeutic targets.

It is now widely accepted that all cancers share a discrete set of common hallmarks, including the alteration or loss of key elements regulating normal cell physiology. In this context, altered or aberrant expression of splicing variants (SVs) is emerging as a novel cancer hallmark, tightly linked to growth, progression and drug resistance in many cancer types[4]. Actually, previous studies in HCC have demonstrated that certain SVs, such as those from the CCDC50, AURKB, KLF6, or FN1 genes, are involved in liver carcinogenesis, thus suggesting that an altered splicing process could contribute substantially to HCC development and progression[5].

Alternative splicing is an intricate process controlled by the spliceosome, a macromolecular ribonucleoprotein complex that cooperates with hundreds of splicing factors (SFs) to catalyse this core cellular function. Indeed, the splicing machinery is indispensable for the appropriate modulation of gene expression, and its dysregulation can generate an aberrant landscape of alternative SVs[6]. In this context, the splicing factor 3B subunit 1 (SF3B1) is a central spliceosome component, which constitutes the U2 snRNP complex together with other factors, such as SF3a or the 12S RNA unit[7]. SF3B1 is crucial for the appropriate splicing process and its mutations are frequent in malignant cells from patients with myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL), breast cancer and pancreatic cancer, which can perturb gene expression programs that contribute to cancer[8]. Importantly, SF3B1 has been shown to be an actionable target that can be blocked by different drugs, among which pladienolide-B is particularly attractive as it acts as the precursor of the

102 majority of SF3B1 inhibitors[8], and has been proven as an useful therapy in gastric and prostate cancers as
103 well as in CLL[9-11]. Accordingly, in the present study we aimed to explore the putative dysregulation and the
104 functional role of SF3B1 in HCC, as well as the potential utility of the genetic and/or pharmacological blockade
105 of SF3B1 in reducing HCC progression using several *in vitro* approaches, animal models and human tissue
106 samples.

107 ## 2. Materials and methods

108 ### 2.1 Patients and samples

109 The study protocol was approved by the Reina Sofia University Hospital Ethics Committee, according to
110 institutional and Good Clinical Practice guidelines (Protocol number PI17/02287) and in compliment with the
111 declaration of Helsinki. Informed consent was obtained from all patients or their relatives. Two independent
112 retrospective cohorts of samples from patients with HCC who underwent surgical resection or liver
113 transplantation were included: 1) Cohort-1: Formalin-Fixed Paraffin-Embedded (FFPE) samples encompassing
114 paired HCC and non-tumor adjacent tissue (n=86); and, 2) Cohort-2: snap-frozen samples comprising HCC
115 tissue (n=57), non-tumor adjacent tissue (n=47), cirrhotic liver samples (n=46) and normal liver samples from
116 autopsies (n=5). All these samples were obtained from the Andalusian Biobank (Cordoba Node), evaluated by
117 liver histology and the diagnosis was confirmed by two independent, experienced pathologists. Clinical data
118 from patients was collected from electronic medical reports.

119 ### 2.2 Reagents

120 For *in vitro* and *in vivo* administration, pladienolide-B (Santa Cruz, Heidelberg, Germany) was resuspended in
121 DMSO (Sigma-Aldrich, Madrid, Spain). For *in vitro* administration, sorafenib (LC Laboratories, Woburn,
122 USA) was dissolved in DMSO. In any case, DMSO in the final solution did not exceed 0.2% (v/v). For *in vivo*
123 testing, sorafenib was dissolved in Cremophor EL/ethanol (50:50)[12].

124 ### 2.3 Cell lines and treatments

125 HepG2, Hep3b and SNU-387 (HB-8065) cell lines were purchased from ATCC (Manassas, USA) and cultured
126 as recommended. Cells were maintained at 37°C and 5% CO2, periodically validated by short tandem repeat
127 analysis (GenePrint, Promega, Barcelona, Spain) and tested for mycoplasma contamination[13-16]. THLE-2
128 cells were cultured according to manufacturer instructions[17]. In each experiment, cells were treated with

pladienolide-B (10^{-7}, 10^{-8} and 10^{-9}M), sorafenib (5 μM)[18] or their combination. Positive [IGF-1 (10^{-6}M)] and negative [Paclitaxel (10^{-7}M)] controls were used.

2.4 SF3B1 silencing by specific siRNAs

A specific small interfering RNA (siRNA) for SF3B1 (s23851, Thermo Fisher, Madrid, Spain) and a commercial negative control (scramble; Thermo Fisher) were used. For transfection, 120,000 SNU-387 cells and 150,000 Hep3b or HepG2 cells were seeded and transfected with 100nM of SF3B1 siRNA (siSF3B1) using Lipofectamine RNAiMAX reagent (Thermo Fisher).

2.5 RNA isolation and retrotranscription

Total RNA from FFPE tissues was isolated using the Maxwell FFPE Purification Kit (Promega), total RNA from frozen tissues was isolated using the AllPrep DNA/RNA/Protein Kit (Qiagen, Madrid, Spain), and total RNA from cell lines was isolated using TRI Reagent (Sigma-Aldrich). RNA extraction was followed by DNase treatment[13-15, 19]. The amount and purity of RNA recovered were determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher). RNA (1μg) was reverse transcribed using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher).

2.6 RNA expression analysis by microfluidic-based qPCR dynamic array and conventional qPCR

RNA expression levels of SF3B1, molecular markers, SVs and housekeeping genes were determined by a microfluidic-based qPCR dynamic array[15, 20] in tissue samples and by conventional qPCR in cell lines and xenografted tumors. Specific primers for human transcripts (Supplemental table 1) were designed with Primer3 software (Applied Biosystems, Foster City, CA). Preamplification, exonuclease treatment, and qPCR dynamic array were implemented using the Biomark System following manufacturer's instructions (Fluidigm, San Francisco, CA). Conventional qPCR was carried out using the Stratagene Mx3000p system with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA)[13, 14, 19]. In the case of tissue samples, the expression level of each transcript was adjusted by a normalization factor obtained from the expression levels of two housekeeping genes (ACTB and GAPDH) using Genorm 3.3[21]. In the case of *in vitro* assays and the *in vivo* preclinical model, the expression level of each transcript was adjusted by ACTB expression. In all cases, these housekeeping genes exhibited a stable expression among experimental groups.

2.7 In vitro assays

The determination of cell viability, proliferation, migration, apoptosis, formation of clones and tumorospheres was performed as previously reported (Supplemental material and[13, 14, 19, 22]).

2.8 Xenograft model

Experiments with xenografted mice were carried out according to the European Regulations for Animal Care under the approval of the university/regional government research ethics committees. Eight-week-old male nude Fox1nu/Foxn1nu mice (Janvier Labs, Le Genest-Saint-Isle, France) were subcutaneous grafted in both flanks with $5x10^6$ Hep3b cells (n=16 mice; n=32 tumors) in 50µl of basement membrane extract (Trevigen, Gaithersburg, MD). Tumor growth was monitored twice per week for 2 months by using a digital caliper. At the third week post grafting, when the tumors were visible, mice were treated with vehicle, pladienolide-B, sorafenib or their combination (n=4 mice/treatment; n=8 tumors/treatment). Sorafenib (30 mg/kg) was administered dissolved in drinking water for 3 days[12]; while the water of the rest of mice was treated with cremophor/ethanol vehicle. Pladienolide-B (10^{-8}M, based on the results obtained *in vitro* assays) was intratumorally injected the second day of sorafenib treatment[23]. General wellbeing and body weight evolution of the mice was not affected by the treatments. After euthanasia, each tumor was dissected and snap-frozen or fixed and sectioned for histopathologic examination. Tumor necrosis was evaluated after hematoxylin and eosin staining and nuclear Ki67 staining by immunohistochemistry by expert pathologists.

2.9 Western Blotting

HCC cells were processed to analyze protein levels by western blot after 24 hours of pladienolide-B exposure, as previously described[11]. Briefly, 200,000 cells were seeded in 6-well plates, and proteins were extracted using prewarmed (65°C) SDS-DTT buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT, and 0.005% bromophenol blue). Then, proteins were sonicated for 10 seconds and boiled for 5 minutes at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated overnight with the specific antibodies for SF3B1 (ab172634, Abcam, Cambdridge, UK), phospho-ERK (#4370S, Cell Signaling), phospho-JNK (AF1205, R&D-Systems, Abingdon, UK), phospho-AMPKα (Thr172; 40H9; #2535, Cell Signaling), phospho-GSK-3-beta (Ser9; D3A4; #9322, Cell Signaling), phospho-PDK1 (Ser241; #3061, Cell Signaling), and TUBB (#2128S, Cell Signaling) as well as with the appropriate secondary antibody, HRP-conjugated goat antirabbit IgG (#7074S, Cell Signaling). Proteins were detected using an

enhanced chemiluminescence detection system (GEHealthcare, Madrid, Spain) with dyed molecular weight markers (Bio-Rad, Madrid, Spain). A densitometry analysis of the bands obtained was carried out with ImageJ software, using TUBB protein levels or total protein loading (Ponceau staining) as normalizing factor.

2.9 SF3B1 IHC analysis

Immunohistochemistry (IHC) analysis was performed in a representative set of FFPE samples that presented paired, adjacent non-tumor and tumor regions from patients diagnosed with HCC (n=16). SF3B1 monoclonal antibody (ab172634, Abcam, Cambridge, UK) at 1:250 dilution was used[11]. Two independent pathologists performed histopathologic analysis of the tumors following a blinded protocol. In the analysis, +, ++, +++ indicate low, moderate, and high staining intensity.

2.10 In silico analysis of SF3B1 expression in HCC cohorts

To analyse the expression level of SF3B1 and survival curves in TCGA, GEPIA2 was used. To analyze the expression levels of SF3B1 in other *in silico* cohorts: Wurmbach Liver (10 normal liver vs. 35 HCC)[24], Mas liver (19 normal liver vs. 38 HCC)[25], Roessler Liver (21 normal liver vs. 22 HCC) and Roessler Liver 2 (220 normal liver vs. 225 HCC)[26], the Oncomine database was used.

2.11 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM), as fold-change (log 2) or relative levels compared with the corresponding controls (set at 100%). Data were evaluated for heterogeneity of variance using the Kolmogorov–Smirnov test and, consequently, parametric (Student t) or nonparametric (Mann-Whitney U) tests were implemented. Spearman's or Pearson´s bivariate correlations were performed for quantitative variables according to normality. Significant relation between categorized mRNA expression and patients survival was studied using the long-rank-p-value method. P-values smaller than 0.05 were considered statistically significant. All statistics analyses were performed using the GraphPad Prism 6.0 software (La Jolla, CA, USA)

3. Results

3.1 SF3B1 is overexpressed in HCC, correlated with oncogenic splicing variants and associated with overall survival.

SF3B1 was significantly overexpressed in two independent cohorts of HCC samples (Table 1) compared with non-tumor adjacent tissues and cirrhotic samples (shown in Fig. 1A). These results were additionally

corroborated *in silico* in 4 cohorts of HCC samples (shown in Supplemental Fig. 1). SF3B1 was also overexpressed in HCC samples compared with normal liver in the TGCA dataset (shown in Fig. 1B). In addition, SF3B1 expression was higher in the three liver cancer cell lines used (HepG2, Hep3b and SNU-387) compared with the normal-like hepatocyte-derived THLE-2 (shown in Supplemental Fig. 2). SF3B1 expression in HCC samples from Cohort-1 and Cohort-2 was directly correlated with that of three oncogenic SVs, KLF6-SV1, CCDC50S and BCL-XL (shown in Fig. 1C; R>0.370; p<0.01), whose expression was elevated in HCC samples (shown in Supplemental Fig. 3). Consistently, SF3B1 protein levels assessed by IHC were markedly higher in HCC compared to non-tumor adjacent regions (shown in Fig. 1D).

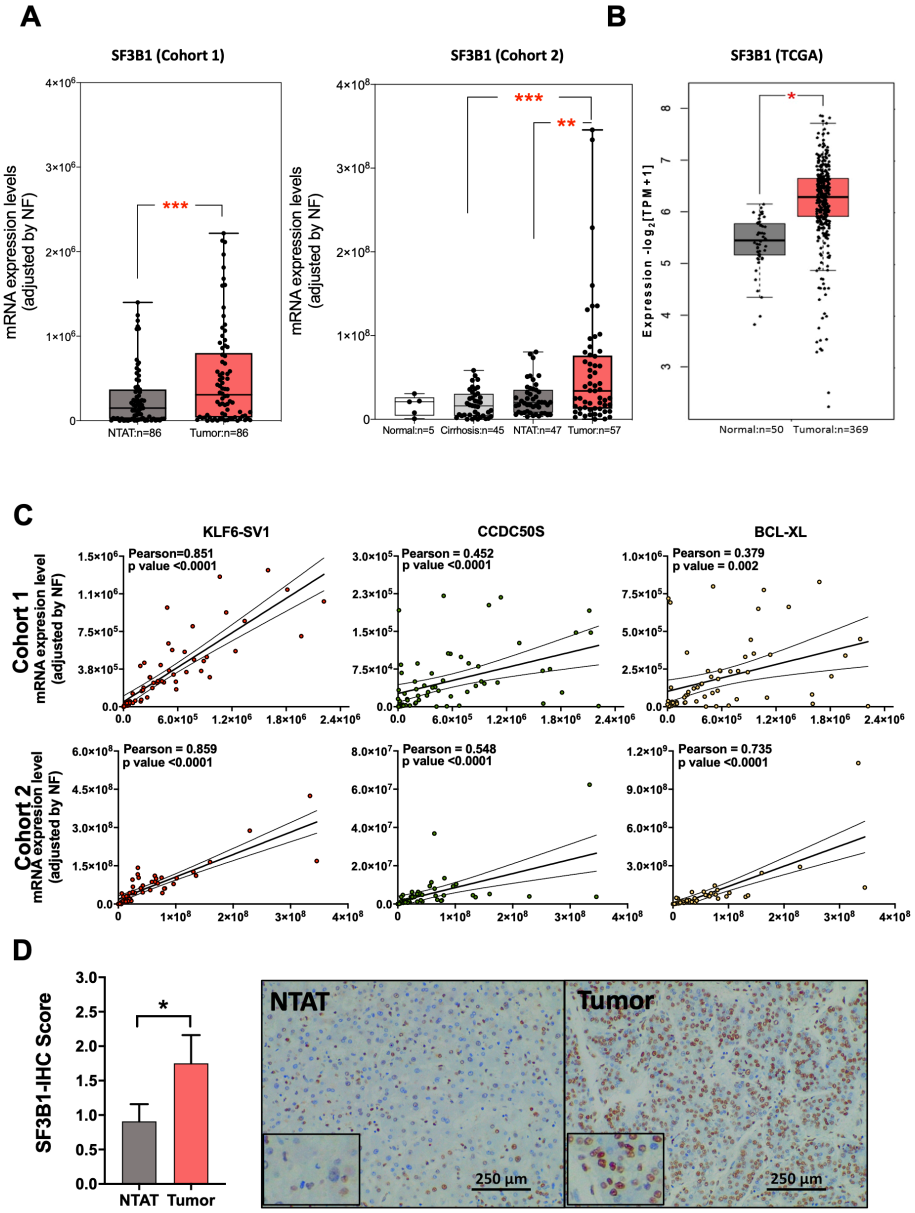


Figure 1. SF3B1 is overexpressed in HCC and correlates with the expression of oncogenic splicing variants. (A) Expression level of SF3B1 in two retrospective cohorts of HCC patients [Cohort-1: FFPE samples (n=86 patients), and Cohort-2: frozen tissues (n=126 samples)]. Data are presented as mean ± SEM. (B) Expression level

of SF3B1 in HCC samples and normal livers from TCGA cohort. Data represent log_2 of fold change. (C) Correlations between the expression of SF3B1 and oncogenic splicing variants in HCC samples from both retrospective cohorts. (D) Immunohistochemical SF3B1 score in NTAT and tumor samples (n=16 patients from Cohort-1). Representative images (X20 objective) are depicted. Asterisks (* p<0,05; ** p<0,01; *** p<0,001) indicate statistically significant differences. NTAT means non-tumor adjacent tissue.

Of note, SF3B1 expression levels were correlated with tumor diameter, number of nodules, tumor differentiation grade and microvascular invasion, but not with aetiology of the underlying liver disease in Cohort-2 (shown in Fig. 2A). This information was not fully available for Cohort-1 and only correlation between SF3B1 and number of nodules was found (shown in Supplemental Fig. 4). Remarkably, SF3B1 was associated with overall survival (shown in Fig. 2B).

Specifically, higher SF3B1 expression levels correlated with lower overall survival rates in TCGA (HR=1.6, p=0.006) and also tended to correlate in Cohort-1 (HR=1.99, p=0.063), whereas this effect was less pronounced in Cohort 2 (HR=3.4, p=0.134), which is comprised by less aggressive HCC with better prognosis (Table 1).

Table 1.

	Cohort 1	**Cohort 2**
Patients [n]	**86**	**57**
Age, y [median (IQR)]	**60,6 (64-67)**	**61,2 (55-67,25)**
Etiology [n (%)]		
- **HCV**	**30 (36,1)**	**17 (25,8)**
- **Alcohol**	**21 (25,3)**	**17 (25,8)**
- **HBV**	**11 (13,3)**	**5 (7,6)**
- **Other**	**5 (6)**	**8 (12,1)**
- **HCV + Alcohol**	**9 (10,8)**	**10 (15,2)**
- **HBV + Alcohol**	**1 (1,2)**	**1 (1,5)**
- **HCV + other**	**3 (3,6)**	**1 (1,5)**
- **Alcohol + other**	**0(-)**	**2(3)**
Histological differentiation [n (%)]		
- **Well differentiated**	**30 (35,3)**	**33 (51,6)**
- **Moderately differentiated**	**50 (58,8)**	**26 (40,6)**
- **Poorly differentiated**	**5 (5,9)**	**5 (7,8)**
Portal Hypertension [n (%)]	**44 (51,2)**	**39 (59,1)**
Microvascular invasion [n (%)]	**33 (39,8)**	**23 (35,4)**
Treated before surgery [n (%)]	**23 (26,4)**	**38 (57,6)**
Recurrence [n (%)]	**39 (47)**	**20 (30,3)**
Death [n (%)]	**50 (61)**	**16 (24,2)**

Table 1. Demographic and clinical parameters of HCC patients.

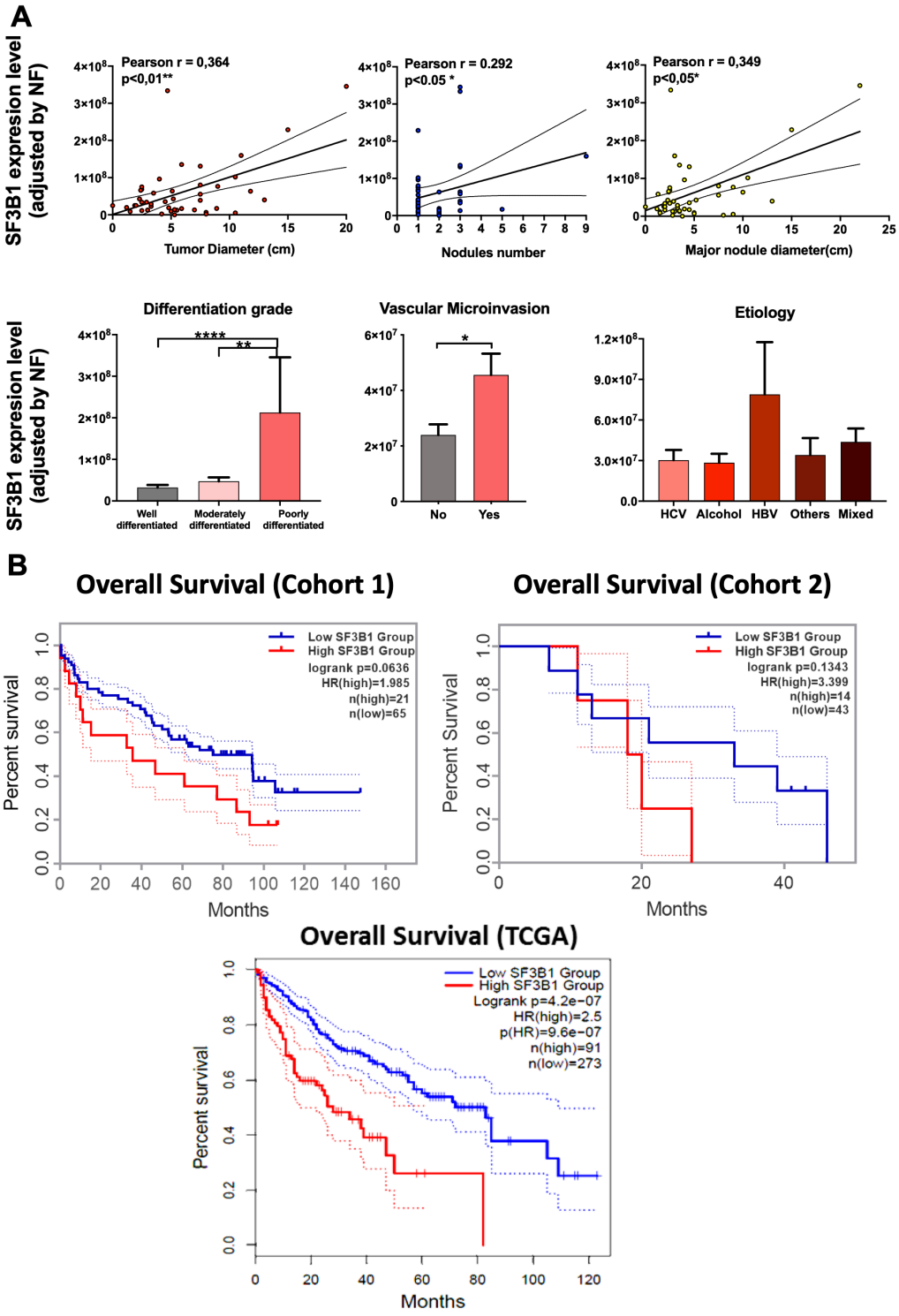


Figure 2. SF3B1 expression is associated to clinical aggressiveness and poor survival of HCC patients. (A) Correlations and associations of SF3B1 expression with aggressiveness parameters in HCC patients from Cohort-2. (B) Overall survival of patients from Cohort-1, Cohort-2 and TCGA categorized by the mRNA expression levels of SF3B1 (high group = 25% patients with higher expression vs. low group = rest of patients) determined by long-rank-p-value method. The asterisks (* p<0,05; ** p<0,01; **** p<0,0001) indicate statistically significant differences. HR means hazard ratio.

3.2 SF3B1 silencing reduced aggressive features of HCC cell lines.

To analyse the implication of SF3B1 in HCC physiopathology, a specific siRNA (siSF3B1) was used to significantly reduce the expression levels of SF3B1 [mRNA (shown in Fig. 3A, confirmed in HepG2, Hep3b and SNU-387) and protein (shown in Fig. 3B, confirmed in Hep3b)] compared to scramble-transfected cells. The silencing of SF3B1 significantly reduced cell viability (shown in Fig. 3C), cell proliferation in a time-dependent manner (shown in Fig. 3D), and cell migration (shown in Fig. 3E) in the three cell lines.

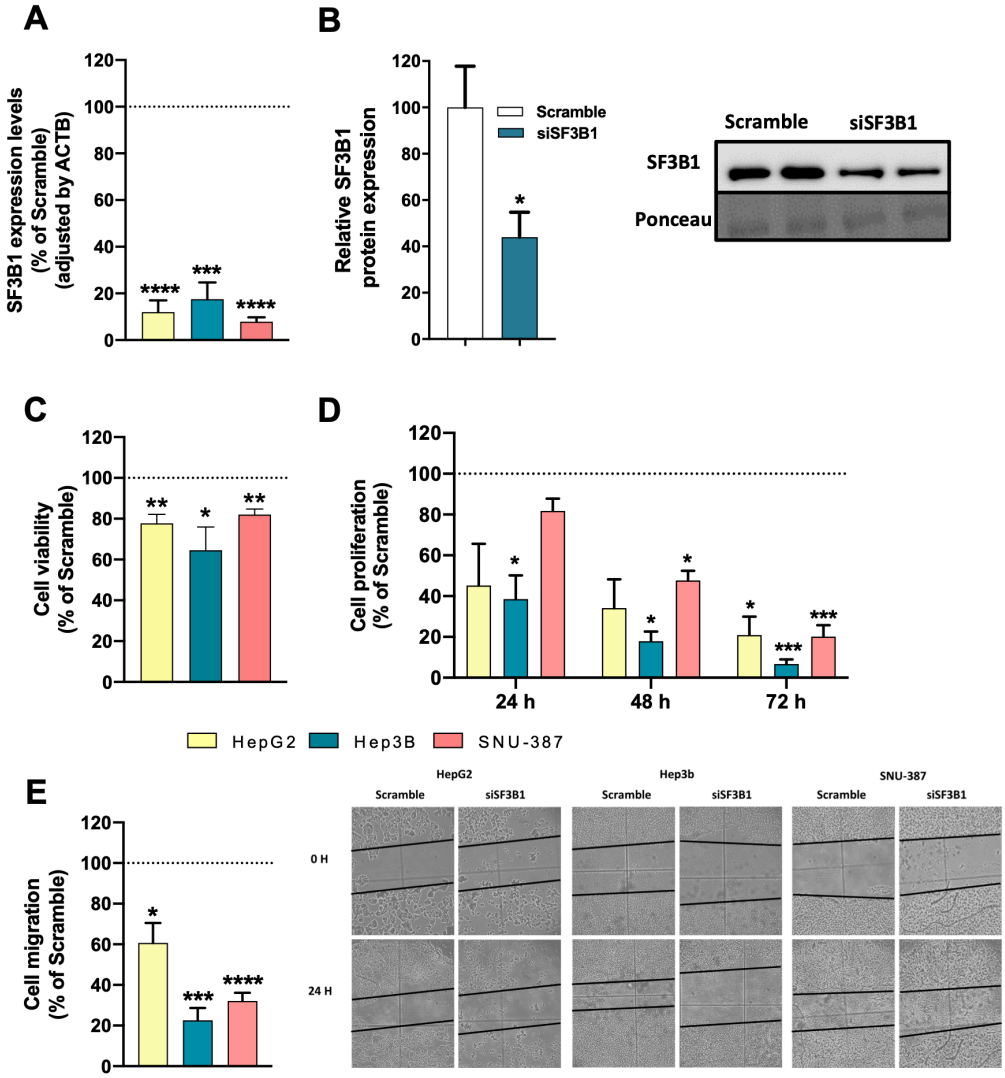


Figure 3. SF3B1 silencing decreases aggressiveness features of HCC cell lines. Validation of siRNA-mediated SF3B1 silencing at mRNA (A) and protein (B) levels in HCC cell lines. (C) Cell viability of SF3B1-silenced cells compared to scramble-treated cells. (D) Proliferation of SF3B1-silenced cells compared to scramble-treated cells. (E) Migration of SF3B1-silenced cells compared to scramble-treated cells. Representative images of cell migration after 24h. Data are presented as mean ± SEM from n=3-5 independent experiments. Asterisks (* $p<0,05$; ** $p<0,01$; *** $p<0,001$; **** $p<0,0001$) indicate statistically significant differences.

3.3 Pharmacological blockade of SF3B1 with pladienolide-B reduced aggressive features of HCC cell lines

Pladienolide-B, a pharmacological inhibitor of SF3B1, had a strong inhibitory, dose-dependent effect on cell proliferation in the three HCC cell lines, while it only exerted a modest effect on the normal-like hepatocyte-derived cell line (THLE-2) (shown in Fig. 4A and in Supplemental Fig. 5). In particular, the three HCC cell lines exhibited a similar dose- and time-dependent response to pladienolide-B, in that 10^{-9}M dose did not have a significant effect on cell proliferation, whereas at 10^{-8}M and 10^{7}M pladienolide-B clearly inhibited cell proliferation, especially at 48-72h (shown in Fig. 4A). In the case of THLE-2 cells, only a 10^{-7}M dose reduced cell viability, while 10^{-9}M and 10^{-8}M doses did not exert any significant inhibition (shown in Fig. 4A). For these reasons, the 10^{-8}M dose was selected for subsequent experiments.

Wound-healing assays demonstrated that pladienolide-B treatment significantly reduced migration capacity of the three HCC cell lines (shown in Fig. 4A and B). In addition, tumorosphere formation was markedly reduced in response to pladienolide-B. Indeed, mean size of tumorospheres was reduced in the three HCC cell lines after 10 days post pladienolide-B treatment (shown in Fig. 4A and C). Moreover, clonogenic assays demonstrated that the number of colonies formed was significantly lower in pladienolide-B treated cells in comparison with vehicle-treated cells (shown in Fig. 4A and D). Finally, DAPI staining revealed that pladienolide-B significantly increased apoptosis in HCC cells (shown in Fig. 4A and E).

In experiments combining pladienolide-B (10^{-8}M) and sorafenib (5μM), pladienolide-B did not reduce viability of THLE-2 cells after 72h, whereas sorafenib significantly reduced it (shown in Fig. 4F). In contrast, pladienolide-B had a comparable effect to that exerted by sorafenib in terms of proliferation in the three HCC cell lines (shown in Fig. 4F). Of note, combination of sorafenib and pladienolide-B exerted significantly more pronounced effects than sorafenib alone in the most aggressive cell lines Hep3b and SNU-387 cells (shown in Fig. 4F).

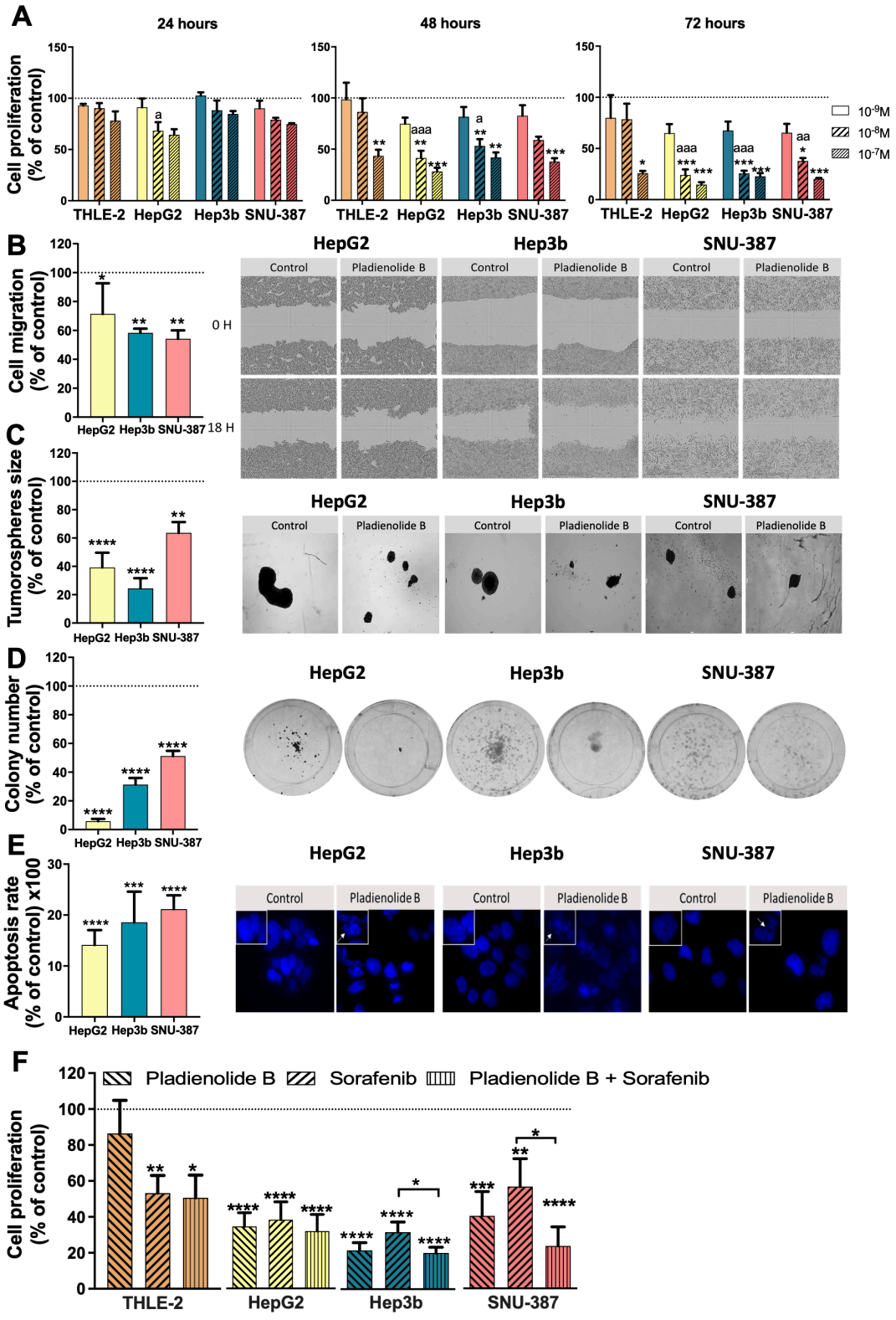


Fig. 4. Pharmacological blockade of SF3B1 by pladienolide-B decreases aggressiveness features of HCC cell lines. (A) Dose-response proliferation of pladienolide-B-treated THLE-2, HepG2, Hep3b and SNU-387 cells compared to vehicle-treated cells. (B) Cell migration of pladienolide-B (10nM)-treated cells compared to vehicle-treated cells. (C) Representative images of cell migration after 18h. (D) Mean tumorosphere size of pladienolide-B (10nM)-treated cells compared to vehicle-treated cells. (E) Representative images of tumorospheres formed

after 10 days. (F) Number of colonies formed in pladienolide-B (10nM)-treated cells compared to vehicle-treated cells. (G) Representative images of colonies formed after 10 days. (H) Apoptosis rate of pladienolide-B (10nM)-treated cells compared to vehicle-treated cells. (I) Representative images of stained nuclei after 24h. (J) Proliferation rate of THLE-2, HepG2, Hep3b and SNU-387 cells after 72h of treatment with pladienolide-B, sorafenib or their combination compared to vehicle-treated cells. Data are presented as mean ± SEM from n=3-5 independent experiments. The asterisks (* p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001) indicate statistically significant differences.

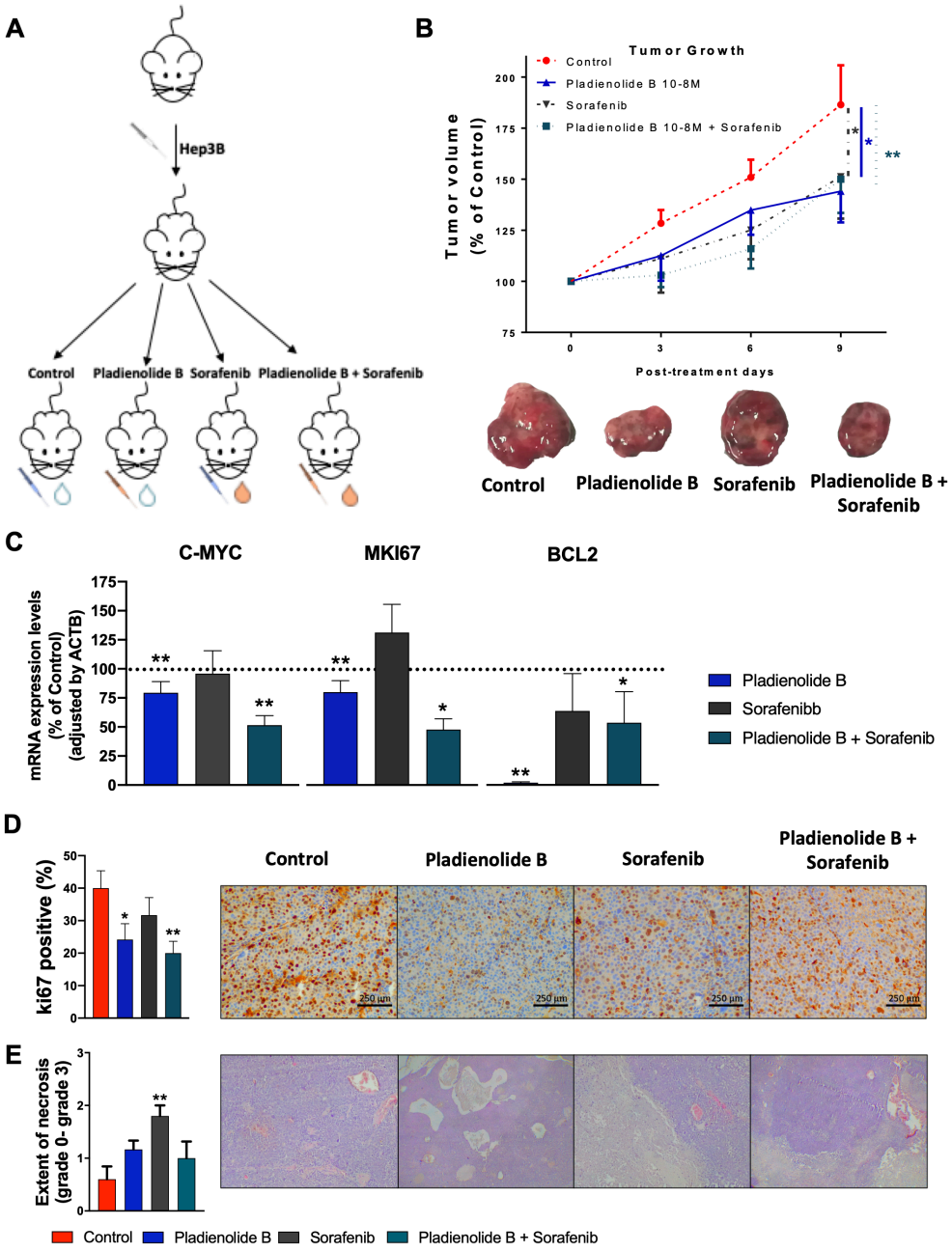


Fig. 5. Pharmacological blockade of SF3B1 by pladienolide-B decreases HCC cells growth *in vivo*. (A) Diagram showing the *in vivo* experimental design. At the third week post grafting, mice were treated with vehicle, pladienolide-B, sorafenib or their combination (n=4 mice/treatment; n=8 tumors/treatment). (B) Growth rate of

tumors was estimated during 9 days after treatment. Representative images of control and treated tumors are depicted. (C) Expression of key tumor-related genes in tumors treated with pladienolide-B, sorafenib or their combination treatment. (D) Percentage of Ki67 positive nuclei in tumors treated with pladienolide-B, sorafenib or their combination treatment. Representative images of control and treated tumors are depicted. (E) Necrosis level in the xenografted tumors. Representative images of control and treated tumors are depicted. The asterisks (* $p<0,05$; ** $p<0,01$) indicate statistically significant differences.

3.4 Pladienolide-B reduced the *in vivo* growth of xenografted HCC cells in nude mice.

The effect of pladienolide-B on *in vivo* tumor growth was evaluated in Hep3b-induced xenografts (shown in Fig. 5A). Remarkably, a single intratumor dose of pladienolide-B significantly reduced the growth of established s.c. tumors compared to vehicle-treated tumors (shown in Fig. 5B). The effect of pladienolide-B was comparable to that exerted by orally administered sorafenib and their co-administration did not seem to enhance the individual effects of each drug. In line with this, pladienolide-B treatment (and co-administration with sorafenib) reduced the expression of key tumor-related genes (MKI67 and C-MYC), as well as that of the apoptosis regulator (BCL2) (shown in Fig. 5C). Consistently, tumors treated with pladienolide-B treatment (and co-administration with sorafenib) exhibited reduced Ki67 staining levels (reduced number of positive nuclei) in the nucleus compared with vehicle-treated tumors (shown in Fig. 5D). Histopathologically, sorafenib treated s.c tumors had more necrosis than vehicle-treated tumors (shown in Fig. 5E).

3.5 SF3B1 silencing or blockade modulate phosphorylation of key signalling pathways, expression of aggressiveness markers and oncogenic splicing variants.

Molecular mechanisms associated to SF3B1 silencing and blockade were explored in Hep3b cell line. First, pladienolide-B (10^{-8} M) treatment modulated the phosphorylation of important tumor-related pathways in cancer after 24 hours in culture. In particular, pladienolide-B reduced the phosphorylation levels of PDK1, GSK3β, JNK and AMPKα, while increased the phosphorylation levels of ERK (shown in Fig. 6A). In addition, pladienolide-B (10^{-8} M) treatment in cell cultures and xenografted tumors and/or SF3B1 silencing reduced the expression of different tumor markers such as CDK4, CDK6 or CD24 (shown in Fig. 6B). Interstingly, SF3B1 pharmacologic blockade and/or silencing induced a selective reduction of key oncogenic SVs found to be correlated with SF3B1 expression in human HCC samples. Indeed, *in vitro* blockade or silencing of SF3B1

reduced the expression of KLF6-SV1 and BCL-XL (shown in Fig. 6C), while *in vivo* pladienolide-B treatment reduced the expression of KLF6-SV1 in Hep3b-derived tumors (shown in Fig. 6C).

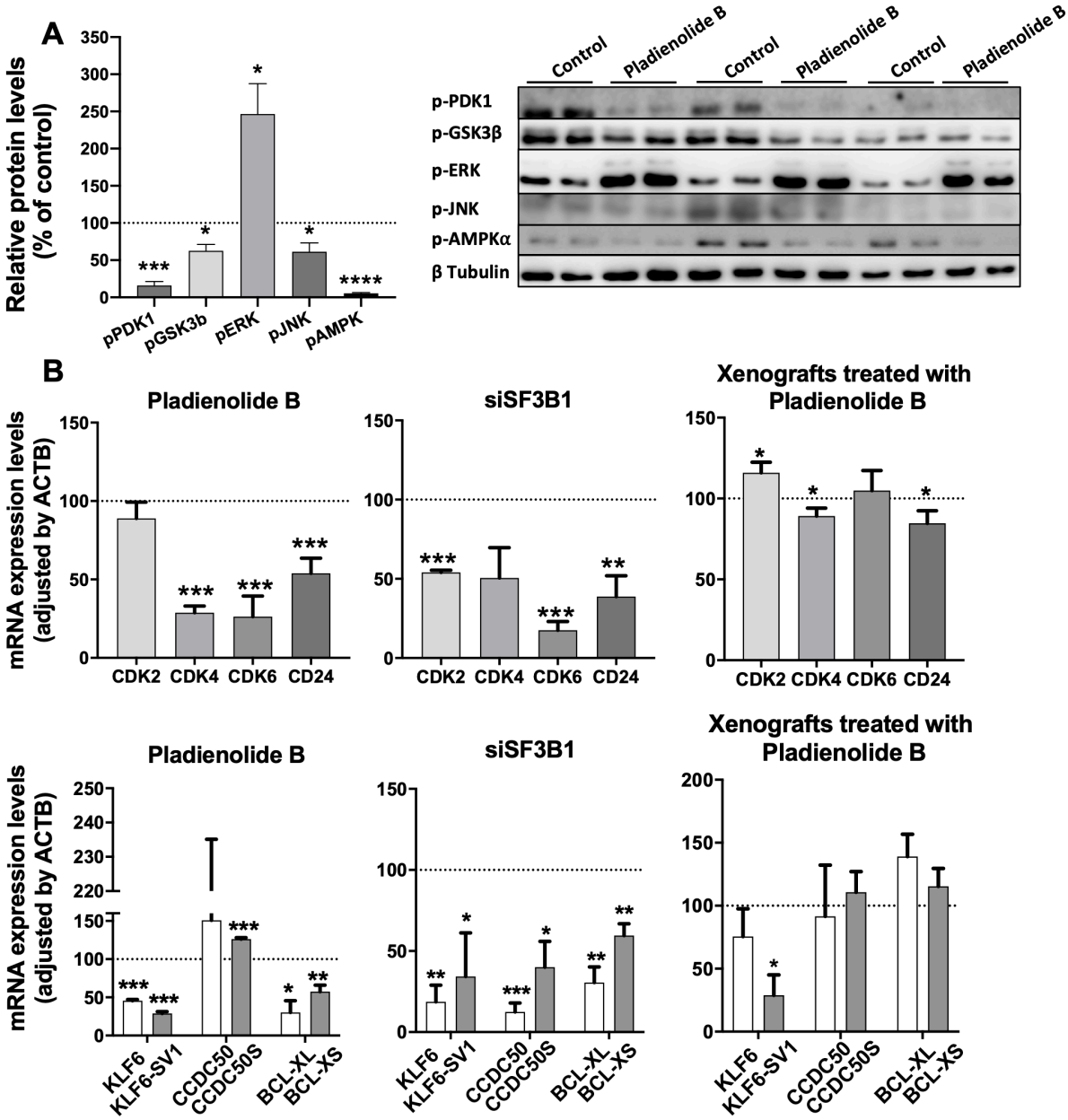


Fig. 6. SF3B1 silencing/blockade modulates the phosphorylation of key signalling pathways and the expression levels of key tumor-associated genes and oncogenic splicing variants. Phosphorylation levels of key tumor-related signaling pathways (PDK1, GSK3b, ERK, JNK and AMPK) in Hep3b cells treated with pladienolide-B during 24h (A). Expression levels of key tumor-associated genes (B) or oncogenic splicing variant and native transcripts (C) in pladienolide B-treated Hep3b cells (left panel), siSF3B1-treated Hep3b cells (middle panel) and pladienolide B-treated Hep3b xenografted tumors (right panel) compared to control (scramble or vehicle)-treated conditions. Data are presented as mean ± SEM from n=3-5 independent experiments. The asterisks (* p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001) indicate statistically significant differences.

4. Discussion

The present study provides novel data demonstrating that the spliceosome component SF3B1 is overexpressed in HCC, wherein its expression is associated to key clinical parameters of aggressiveness and, most importantly, with patient overall survival. We also demonstrate that SF3B1 silencing reduces tumorigenic capacity of different liver cancer cell lines (HepG2, Hep3b and SNU-387) and that its pharmacological blockade with pladienolide-B exerts a strong tumor cell-specific abrogation of aggressiveness features *in vitro* (reduction of proliferation, migration, formation of colonies and tumorospheres and increment in apoptosis) and *in vivo* (in a preclinical HCC model of Hep3b-induced xenografts). Interestingly, our results also suggest that the oncogenic role of SF3B1 and the anti-tumorigenic potential of its silencing/ blockade may be related to the alteration in the expression pattern of key tumor-associated SVs, such as KLF6-SV1 (shown in Fig.7).

SF3B1, an essential splicing element[7, 27], is the most frequently mutated component of the spliceosome in cancer. The recurrent somatic mutations of this factor in multiple cancers suggests that this gene may play a significant role in the development and/or progression of tumor cells[8]. However, most of these studies have been focused on the presence and consequences of SF3B1 mutations in cancer, while very few studies have explored the putative alteration of SF3B1 expression and its implications[11]. This is also the case of liver cancer, wherein different studies have demonstrated that SF3B1 displays mutation rates similar to those identified as driver mutations in other cancers[28], and that advanced stages of HCC show increased frequency of SF3B1 mutations[29]. In this study, we unveiled a novel, relevant aspect of SF3B1 in liver cancer. Indeed, we comprehensively show that SF3B1 is consistently overexpressed (mRNA/protein levels) in HCC samples from different cohorts of patients (two retrospective cohorts of HCC samples and five *in silico* HCC datasets, including the TCGA), which demonstrates that, despite the high heterogeneity observed in HCC[30], alterations in SF3B1 (mutations and/or overexpression) may represent a common hallmark in this pathology. These novel results in HCC compare favourably with a recent study derived from our laboratory showing the overexpression of SF3B1 in prostate cancer[11], and also fits with a report demonstrating the presence of anti-SF3B1 autoantibodies in serum samples from HCC patients[31]. As suggested also by previous studies[30, 31], our results reinforce the contention that alterations in SF3B1 may be causally linked to the genesis and early development of a proportion of HCC cases, as well as to their aggressive progression. Indeed, we demonstrate herein that SF3B1 silencing in different liver cancer cell line models reduces key functional parameters, such

as cell viability, proliferation or migration. This is in accordance with previous studies in myeloid cell lines and prostate cancer cells, wherein SF3B1 knockdown or silencing resulted in the inhibition of aggressiveness features[11, 32]. In addition, this study also shows novel evidence demonstrating that SF3B1 is associated to more aggressive and poor prognosis HCC, inasmuch as patients with higher expression levels of SF3B1 presented tumors with larger size, elevated number of nodules and increased microvascular invasion rates. In addition, SF3B1 expression was associated with shorter survival, particularly in Cohort-1 which was characterized by increased microvascular invasion rates and tumor recurrence rates after surgery, thus suggesting that SF3B1 inhibition could be particularly useful in advanced stages of HCC in which therapeutic options are scarce and prognosis remains unsatisfactory[1].

Interestingly, SF3B1 is an actionable component of the spliceosome, and several groups have developed highly specific inhibitors of SF3B1, which can effectively inhibit spliceosome function and consequently disrupt normal RNA splicing. In general, these products (pladienolides, spliceostatins, sudemycins, etc.) have been demonstrated to be potent antitumor agents, and minimally toxic to normal cells[8, 33]. Importantly, it has been shown that these SF3B1 blockers may be also useful in cancer cells with SF3b1 mutation, wherein the effect can be even higher than in cells with wild-type SF3B1[34]. In this context, our study demonstrates that the pharmacological inhibition of SF3B1 using pladienolide-B can exert strong antitumor actions in HCC cells. Specifically, we found that pladienolide-B exerted antitumor *in vitro* effects in three different liver cancer cell lines, and that these effects were more pronounced in the most aggressive cell lines (Hep3b and SNU-387). In striking contrast, pladienolide-B effect was virtually negligible in normal hepatocyte-derived cells, especially when compared with the *in vitro* effect of sorafenib. In particular, similar doses of pladienolide-B blocked proliferation of the cancer cell lines, while not affecting relevantly viability and proliferation of normal-like THLE-2 cells. In addition, pladienolide-B treatment markedly influenced various functional features of cancer cell biology by: 1) inhibiting migration capacity, likely through modulation of key cyclin-dependent kinases[35], such as CDK4 or CDK6, which regulate transition from G1 to S phase, and are important in tumoral processes; 2) increasing the apoptotic rate; and 3) inhibiting the capacity to form colonies and tumorospheres, an important feature related to the control of cancer stem cells viability and the progression of cancer. Overall, our results agree with and extend those from other studies showing that pladienolide-B can exert antitumor effects on cervical carcinoma[36], prostate cancer[11], gastric cancer[9], or CLL[10], and demonstrate that the

blockade of SF3B1 could be a suitable pharmacological target to control liver cancer cell progression with potentially low side-effects on adjacent normal cells. Indeed, the pharmacological blockade of SF3B1 with pladienolide-B modulates classical cancer-related signalling pathways such as PDK1[37], GSK3β[38], ERK[39], JNK[40-42], AMPKα[43], which could help to explain some of the changes observed herein (CMYC expression, proliferation or migration modulation, etc), and provides further mechanistic foundation to the association between SF3B1 inhibition and anti-tumor effects.

In HCC patients, trans-arterial chemoembolization has been the standard of care in intermediate-stage HCC patients, while sorafenib has been the first systematic therapy available for advanced HCC[2, 3]. Remarkably, this study demonstrates that an intratumor injection of pladienolide-B can drastically reduce tumor progression in a preclinical model of Hep3b-induced xenograft tumors, an effect that was comparable to that exerted by oral administration of sorafenib at a standard dose[12]. Although the combined treatment between pladienolide-B and sorafenib *in vivo* did not exert an additive effect, we observed that their combined *in vitro* treatment improved the effects observed by sorafenib, especially in the most aggressive cell lines. This could be of great relevance in that a vast proportion of sorafenib-treated patients are unresponsive to the treatment or develop resistance[44], and second-line treatments such as Regorafenib, Nivolumab or Lenvatinib are needed in these patients[45]. In this sense, a recent study indicated that pladienolide-B significantly increases the sensitivity of cancer cells to cisplatin[46], which together with the results presented herein suggest that pharmacological inhibitors of SF3B1, including pladienolide-B, may be considered as novel therapeutic options worth to be explored for HCC patients. In this sense, there is a vast number of small molecules that act as splicing modulators (natural and derivates), including Pladienolide B, the one studied herein, FR901464 family (including Spliceostatin A, Meayamycin and thailanstatins), derivates of Pladienolide B (including E7107, HB-8800 and FD-895) and GEX1 family (including herboxidiene), whose common target is SF3B1. All of them modulate splicing and have antiproliferative and/or pro-apoptotic activities. Some of them (E7107 and HB-8800) have been explored in clinical trials[47]. Similarly, others small molecule acting as splicing modulators can target aadditional splicing factors, such as RBM39. In particular, arylsulfonamides E7820, indisulam, tasisulam and chloroquinoxaline promote the binding of RBM39 to E3 ligase substrate receptor DCAF15 and degrade the splicing factor by cutting the 3′ splice site-recognizing protein U2AF2[48]. Alternatively, the splicing process can be modulated by antisense oligonucleotides. These molecules bind to pre-mRNA

418 sequences, preventing their recognition by spliceosome components or splicing factors. This approach is
419 selective by the recognition of specific target sequences. Moreover, these treatment will represent an advantage
420 in liver diseases since a problem of this approach in others pathologies is delivering oligonucleotides to
421 particular tissues, as they often accumulate in the liver and kidney[47]. For example, SF3B4 has been reported
422 as early biomarkers of HCC[49] and could be used as target to antisense oligonucleotide to reduce the harmful
423 effect of its overexpression.
424 Finally, many studies have tried to elucidate the exact consequences of SF3B1 inhibition; however, the results
425 generated are not completely consistent and seem to be cell type- or drug-dependent. In general terms, initial
426 reports indicated that intron retention events may be the major consequences of SF3B1 inhibition[50], although
427 more recent studies suggest that exon skipping may be the predominant aberrant splicing event[51]. In any case,
428 pharmacological blockade of SF3B1 leads to the alteration of the pattern of SVs and recent studies suggest that
429 the antitumor action of SF3B1 inhibition may be related to its capacity to reduce the expression of certain
430 oncogenic SVs in a cancer and/or cell type dependent manner[11, 52]. In the case of liver cancer, we have
431 shown that among all the oncogenic SVs previously observed to be altered in HCC samples (SVs from CCDC50,
432 BRMS1, DNMT3b, AURKB, MDM2, TENSIN2, MAD1, KLF6, SVH or FN1 genes[5]), the expression of
433 SF3B1 was correlated with the alteration in the expression pattern of KLF6-SV1[53], CCDC50S[54] and BCL-
434 XL[55] in HCC samples from two independent cohorts, suggesting a putative link between SF3B1 and these
435 oncogenic SVs in HCC. Indeed, further analysis demonstrated that the expression of KLF6-SV1, a SV found to
436 be overexpressed in HCC and shown to exert an important oncogenic role in different cancer types[53], was
437 reduced in response to SF3B1 silencing and blockade *in vitro* and *in vivo*. Therefore, these findings strongly
438 suggest that the antitumorigenic potential of SF3B1 silencing/blockade in HCC may be related to the alteration
439 of the splicing pattern of KLF6 gene.
440 When viewed together, our results demonstrate that SF3B1 is consistently overexpressed in HCC, where it is
441 associated with expression of key oncogenic SVs related with aggressiveness in this pathology, especially
442 KLF6-SV1. Moreover, our data demonstrate that dysregulation expression of SF3B1 may also be involved in
443 the development, progression, and aggressiveness of HCC, wherein this factor could represent a novel,
444 alternative prognostic biomarker and/or potential therapeutic target. In this sense, we also demonstrate that
445 pladienolide-B, a SF3B1 inhibitor, alters the expression pattern of oncogenic SVs *in vitro* and *in vivo*, and

446 reduces malignant features in liver cancer cells, suggesting its potential role as a novel actionable target to
447 increase the therapeutic arsenal against HCC.

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