



# Alteration of splicing factors' expression during liver disease progression: impact on hepatocellular carcinoma outcome

Hualin Wang<sup>1,2</sup> · Bouchra Lekbaby<sup>1,2</sup> · Nadim Fares<sup>3</sup> · Jeremy Augustin<sup>1,2</sup> · Tarik Attout<sup>1,2</sup> · Aurelie Schnuriger<sup>1,2,4</sup> · Anne-Marie Cassard<sup>5</sup> · Ganna Panasyuk<sup>6,7</sup> · Gabriel Perlemuter<sup>5,8</sup> · Ivan Bieche<sup>9</sup> · Sophie Vacher<sup>9</sup> · Janick Selves<sup>10</sup> · Jean-Marie Péron<sup>10</sup> · Brigitte Bancel<sup>3</sup> · Philippe Merle<sup>3</sup> · Dina Kremsdorf<sup>1,2</sup> · Janet Hall<sup>3</sup> · Isabelle Chemin<sup>3</sup> · Patrick Soussan<sup>1,2,4</sup>

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## Abstract

**Purpose** Trans-acting splicing factors (SF) shape the eukaryotic transcriptome by regulating alternative splicing (AS). This process is recurrently modulated in liver cancer suggesting its direct contribution to the course of liver disease. The aim of our study was to investigate the relationship between the regulation of SFs expression and liver damage.

**Methods** The expression profile of 10 liver-specific SF and the AS events of 7 genes associated with liver disorders was assessed by western-blotting in 6 murine models representing different stages of liver damage, from inflammation to hepatocellular carcinoma (HCC). Relevant SFs (PSF, SRSF3, and SRSF6) and target genes (*INSR*, *SRSF3*, and *SLK*) modulated in mice were investigated in a cohort of 179 HCC patients.

**Results** Each murine model of liver disease was characterized by a unique SF expression profile. Changes in the SF profile did not affect AS events of the selected genes despite the presence of corresponding splicing sites. In human HCC expression of SFs, including the tumor-suppressor SRSF3, and AS regulation of genes studied were frequently upregulated in tumor versus non-tumor tissues. Risk of tumor recurrence positively correlated with AS isoform of the *INSR* gene. In contrast, increased levels of SFs expression correlated with an extended overall survival of patients.

**Conclusions** Dysregulation of SF expression is an early event occurring during liver injury and not just at the stage of HCC. Besides impacting on AS regulation, overexpression of SF may contribute to preserving hepatocyte homeostasis during liver pathogenesis.

**Keywords** Alternative splicing · Splicing factors · Liver disease · Hepatocellular carcinoma

## Abbreviations

AS	Alternative splicing
SF	Splicing factors
SRSF	Serine/arginine-rich splicing factor

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✉ Patrick Soussan  
patrick.soussan@inserm.fr

<sup>1</sup> INSERM U1135, Centre d'immunologie et de maladie infectieuse, 91 boulevard de l'Hôpital, 75013 Paris, France

<sup>2</sup> Sorbonne Université, Paris, France

<sup>3</sup> Centre de Recherche en Cancérologie de Lyon, UMR INSERM 1052, CNRS 5286, Lyon Cedex 03, France

<sup>4</sup> Département de Virologie, Hôpitaux Est Parisien, Paris, France

<sup>5</sup> Faculté de médecine Paris-Sud, Université Paris-Sud, 94270 Kremlin-Bicêtre, France

<sup>6</sup> Institut Necker-Enfants Malades, Université Paris Descartes, Paris, France

<sup>7</sup> INSERM U1151/CNRS Unité Mixte de Recherche (UMR) 8253, Paris, France

<sup>8</sup> AP-HP, Hôpital Antoine Bécélère, Service d'hépatogastroentérologie, 92140 Clamart, France

<sup>9</sup> Institut Curie-Hôpital, Paris, France

<sup>10</sup> Institut Universitaire de Cancérologie de Toulouse Oncopole, Université Paul Sabatier, Toulouse, France

hnRNP	Heterogeneous nuclear ribonucleoprotein
PSF	Proline-rich splicing factor
SF1	Splicing factor 1
La	Lupus autoantigen
HCC	Hepatocellular carcinoma
NAFLD	Non-alcoholic fatty liver disease
ALD	Alcohol liver disease
T	Tumor
NT	Non-tumor
LPS	Lipopolysaccharide
CCl <sub>4</sub>	Carbon tetrachloride
HFD	High-fat diet
ND	Normal diet
DEN	Diethylnitrosamine
Dgkd	Diacylglycerol kinase, delta
Slk	STE20-like kinase
Fas	TNF receptor superfamily member 6
Col18a1	Collagen, type XVIII, alpha 1
InsR	Insulin receptor
SRSF3	Serine/arginine-rich splicing factor 3
Usp4	Ubiquitin specific peptidase 4

## Introduction

In contrast to prokaryotes, splicing regulation is critical for eukaryotic gene expression to accurately excise introns/non coding sequences and combine exons and thus generate mature mRNAs. In addition, alternative splicing (e.g., exon skipping, intron retention, alternative use of 3' or 5' splice sites...) generates multiple mRNA isoforms, which contribute to the diversity of the eukaryotic transcriptome and proteome [1]. Based on next-generation sequencing (NGS), it has been estimated that up to 95% of mammalian genes could be alternatively spliced, thus generating transcripts with different exon compositions [2], and translated into proteins that are not always detectable by western blot or mass spectrometry [3, 4]. Alternative splicing (AS) is affected in human disease through mutations located either directly in splice sites or within regulatory sequences surrounding exonic/intronic AS domains. In addition, AS mechanisms also rely on ubiquitous transregulatory splicing factors (SFs), consisting of activators or repressors of the spliceosome assembly at splice sites. Transregulatory SFs mainly belong to one of three main families, the serine–arginine-rich (SR), the heterogeneous nuclear ribonucleoproteins (hnRNPs) and the *Drosophila* Behavior Human Splicing (DBHS) proteins or are unique. SFs play a critical role during embryonic development, tissue specificity and throughout the mammalian cell lifespan [5–7]. The investigation of the AS mechanisms remains complex, as there is a plethora of cis-elements and trans-acting SFs implicated in both constitutive and AS events. Nevertheless, variations

in transcript isoforms have been well documented in liver disease, and particularly in hepatocellular carcinoma (HCC), suggesting a contribution of AS regulation in carcinogenesis [8, 9]. The landscape of alternatively spliced variants, recently depicted from RNA-seq libraries generated from more than 400 primary human HCC (including the TCGA LIHC data set) illustrates the frequent and aberrant of AS occurrence in HCC [10].

Splicing factors play an essential role in the AS regulation of genes. In the hypothesis of a direct relationship between transcript expression level and AS dysregulation, SFs might be suitable for prognostic biomarkers in HCC. Nevertheless, contrasting pro- and anti-oncogenic properties of the SF members of the SR family were reported in vitro as well as in animal models during liver disease [11, 12]. *Srsf3*, encodes for a SF with auto-catalytic activity (required for its splicing function) that has been involved in liver homeostasis and in the maturation/proliferation of hepatocytes. Deletion of this SF in hepatocytes disturbed lipid and glucose metabolism and predisposes mice to HCC [12, 13]. In contrast, the oncogenic properties of SRSF1 have been related to the activation of the mTOR pathway and activation of insulin receptor (InsR) AS event [11, 14].

Almost all studies exploring AS regulation have compared the expression profile of tumor (T) and non-tumor (NT) regions. However, human HCC is frequently associated with chronic liver-injuring processes such as inflammation, steatosis and fibrosis related to various etiological factors that could impair the AS machinery. Thus, the alteration of SF expression during the different liver disease stages that precede HCC remains to be investigated. We have recently identified hepatic SFs that are disrupting in vivo the AS regulation of HBV transcripts during liver injury [15]. In the present report, we have expanded this study and investigated the impact of hepatic injury on the expression of a panel of SFs in six different prototypical mouse models of liver disease, ranging from non-alcoholic fatty liver disease to HCC. Finally, we compared both profiles of SFs characterized in mice and in a cohort of HCC patients.

## Materials and methods

### Patients and liver tissue specimens

179 HCC patients treated by surgical resection, included between 1996 and 2014 from three French clinical centers (Lyon Croix Rousse, Toulouse Hospitals and the French National Biological Resource Centers) were retrospectively studied (Agreement # DC-2008-235). For 152 patients, tumor (T) and matched non-tumor liver parenchyma (NT) (at least 2 cm distant from the tumor) were obtained from surgically resected HCCs. For the other 27 patients, only tumor

samples were available. In addition, 11 normal liver tissues, used as control samples, were collected after surgical liver resection for colorectal adenocarcinoma metastasis (Centre Léon Bérard Resource Biological Centre, ministerial agreements #AC-2013-1871 and DC-2013-1870). Liver histological analysis confirmed the diagnosis and characteristics of HCCs in T. Liver fibrosis was assessed in paired NT samples (METAVIR score). Patient characteristics are summarized in Supplementary Table 1.

### Mice and liver pathogenesis models

C57BL/6 J mice (Janvier Labs, France) were maintained under a 12 h light/dark schedule, with food and water ad libitum and treated in accordance with EU regulations on animal care (Directive 86/609/EEC). All procedures were approved by the local animal care and use committee (Agreement A75-14-08; n° 02890 and 02891). Mice were bred in three different animal facilities across France, but were all experimented in the same animal facility with corresponding littermate controls. Methods applied to generate disease models were:

**Liver inflammation:** 2-month old male mice were injected intraperitoneally with equal volumes of phosphate-buffered saline (PBS) or lipopolysaccharide (LPS) (2.5 mg/kg, Sigma) 3 times a week for 9 months. All mice were sacrificed 48 h after the last injection [16].

**Liver fibrosis:** 2-month old male mice were injected intraperitoneally with an equal volume of olive oil or carbon tetrachloride (CCl<sub>4</sub>) (1 ml/kg in oil, Sigma) twice a week for 7 weeks. All mice were sacrificed 72 h after the last injection.

**Non-alcoholic fatty liver disease (NAFLD):** 2-month-old male mice were fed either a normal diet (ND) or a high-fat diet (HFD) in which the energy content of fat was either 12% or 60%, respectively. The HFD diet was made up of lard (34%) and contained saturated fatty acid (8.3%), mono-unsaturated fatty acid (4.5%) and poly-unsaturated fatty acid (5.8%), (Ssniff, France). Animals had free access to either water or water containing 20% fructose (w:v). Mice were fed the diets for 16 weeks.

**Alcoholic liver disease (ALD):** 8-week old female mice were fed a liquid diet adapted from Lieber-DeCarli for 21 days [17, 18] based on the NIAAA model [19]. Briefly, the ethanol diet was based on adding absolute ethanol to a solution of a Lieber-DeCarli powder (Ssniff, Spezialdiäten GmbH, Soest, Germany) in filtered water. After a 7-day period adaptation to the animal facility and a 7-day adaptation period to the semi-liquid diet, mice were given increasing amounts of ethanol for 7 days (1% increase every 2 days). The final concentration of ethanol in this liquid diet was 5% (vol/vol), such as ethanol accounted for 28% of the daily caloric intake. In the control diet, the ethanol was replaced

with an isocaloric amount of maltodextrin (Maldex 150, Safe, France). Alcohol-fed groups were allowed free access to the 5% (vol/vol) ethanol diet for 7 days. Control mice were fed the isocaloric control diet throughout the entire feeding period. During the alcoholization protocol, water was added to the semi-liquid Lieber-DeCarli diet and the animals did not have access to drinking water.

**PTEN-deficient liver cancer:** *Pten*<sup>fl/fl</sup> mouse line has been previously described [20]. Liver-specific *Pten* deletion mice (C57BL/6J, male) develop liver malignancies by 12 months of age [21].

**DEN-induced liver cancer:** 15-day-old male mice were injected intraperitoneally with a single dose of diethylnitrosamine (DEN, 5 mg/kg, Sigma) or an equal volume of PBS. These DEN-administrated mice were monitored for tumor development and survival at 10 months of age.

Additional materials and methods are provided as supporting information.

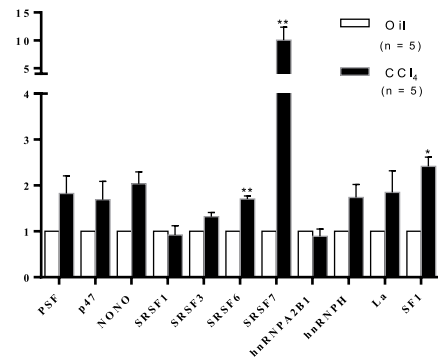
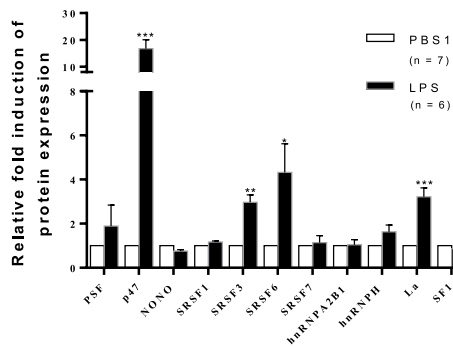
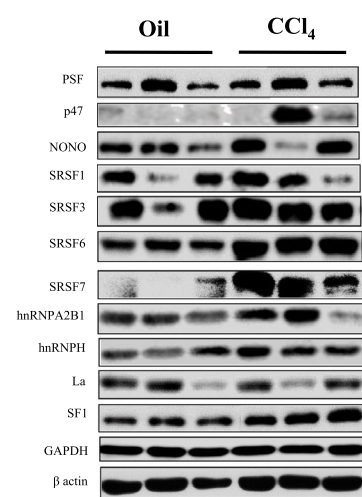
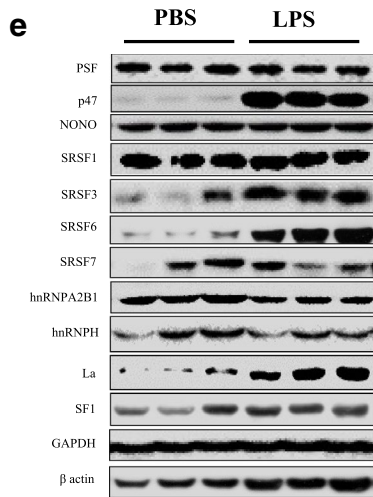
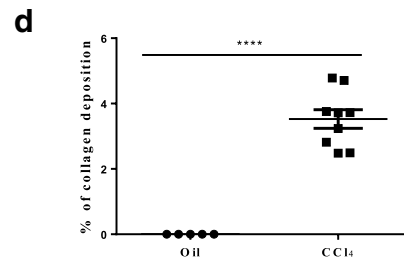
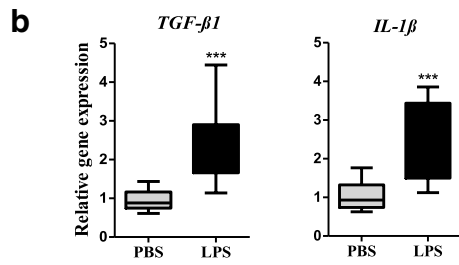
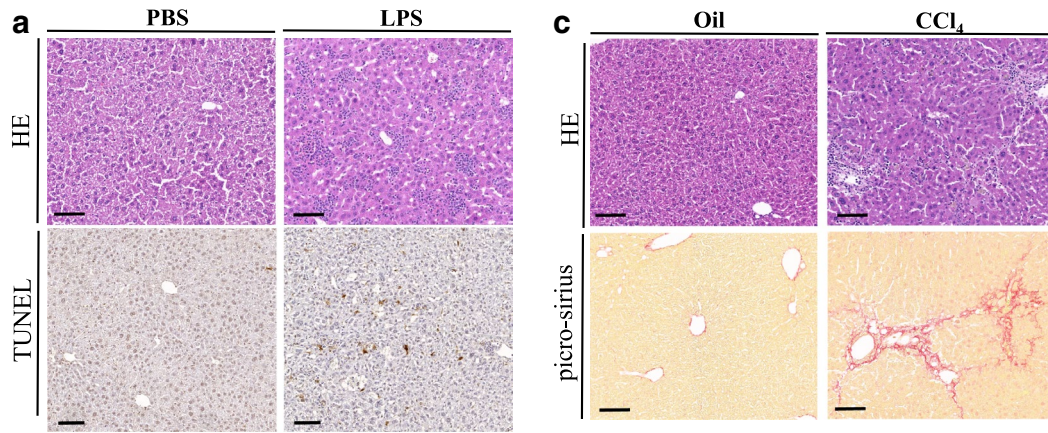
## Results

### Alteration of splicing factors' expression reflects the pathogenesis of mouse liver disease

The expression of 10 liver-specific SFs implicated in vivo in the regulation of AS event of HBV was previously investigated in the liver [15]. This panel was studied in six different C57Bl6 liver genetically or chemically induced injury prototypes including, chronic inflammation, fibrosis, 2 steatosis and 2 cancer models.

Chemically induced liver inflammation was confirmed by the increase level of the cytokines *TGF-β* and *IL-1β* in liver extracts (Fig. 1a, b). Liver fibrosis (CCl<sub>4</sub> treatment) was confirmed by collagen quantification (Fig. 1c, d). The expression level of SFs was measured by western blot from whole-liver extracts of LPS- and CCL<sub>4</sub>-treated and control (treated with PBS or oil, respectively) mice. Among the SFs evaluated by western blot, PSF antibodies revealed a supplementary 47 kDa (p47) band corresponding to the C-terminal cleavage product of PSF (not detected with the antibody directed against the C-terminal domain of PSF, data not shown) [22]. Both complete and cleaved (p47) PSF forms were separately considered in our further analyses. A statistically significant upregulation of p47, SRSF3, SRSF6 and La in LPS-induced inflammation and of SRSF6, 7 and SF1 in CCL<sub>4</sub>-induced fibrosis (Fig. 1e left and right panels, respectively) was detected.

Micro- and macro-steatosis, induced by HFD and alcohol intoxication, respectively, were characterized by elevated levels of serum ALT and intra-hepatic triglycerides (Fig. 2a, b). Significant increase of NONO, SRSF6, hnRNPA2B1 and hnRNPH and decrease of SRSF3 level were found in





**Fig. 1** The expression of splicing factors in murine models of liver inflammation and fibrosis. **a** Upper: representative (upper) H&E staining and lower: immunohistochemical (lower) detection of cell apoptosis by TUNEL assay in histological liver section (bar 100  $\mu$ m) of male mice treated with PBS/LPS for 9 months. **b** Cytokines' mRNA expression in the liver of mice treated with PBS ( $n=12$ ) or LPS ( $n=12$ ) for 9 months. **c** Representative (upper) H&E and (lower) PicroSirius-stained histological liver section (bar 100  $\mu$ m) of male mice treated with oil/CCl<sub>4</sub> for 7 weeks. **d** Quantification of collagen deposition in histological liver sections of mice treated with oil ( $n=5$ ) or CCl<sub>4</sub> ( $n=9$ ) for 7 weeks, each dot represents a mouse. **e** Left upper panel: representative expression of SFs in the livers of mice treated with PBS/LPS for 9 months. Left lower panel: quantification of the intensity of bands using imageJ, normalized to GAPDH and compared to littermate controls. Right upper panel: representative expression of SFs in the livers of mice treated with oil/CCl<sub>4</sub> for 7 weeks. Right lower panel: quantification of the intensity of bands using imageJ, normalized to GAPDH and compared to littermate controls. Data shown are the mean  $\pm$  SD. Unpaired  $t$  test: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

HFD-fed mice. In alcohol-fed mice, an overexpression of PSF, p47, SRSF7 and La was reported (Fig. 2c).

Both liver cancers were confirmed by macroscopic and microscopic data (HE staining) (Fig. 3a, b). Low levels of alpha-fetoprotein (AFP) suggested a predominant cholangiocarcinoma incidence in Pten-HKO mice while a higher level of AFP was associated with HCC in the DEN-treated mice (Fig. 3c). Despite the advanced stage of liver cancer in both models, only 2 SFs were significantly modulated, p47 and SRSF6 appeared upregulated in the Pten-HKO model, while p47 and hnRNPA2B1 were downregulated in DEN-induced HCC (Fig. 3d).

Overall our data showed that a specific signature of SFs' expression characterized each liver disease model studied. Among the SFs assessed, PSF/p47 and SRSF6 were more frequently deregulated while SRSF1, reported with oncogenic abilities, was never modified in liver injury models [11, 12]. The modulation of SFs' expression in murine liver disease models (Fig. 3e), ranging from 50% in the NAFLD model (5/10) to 20% in the two liver cancer models (2/10), was preferentially associated with an upregulation (85%) in the pathological conditions.

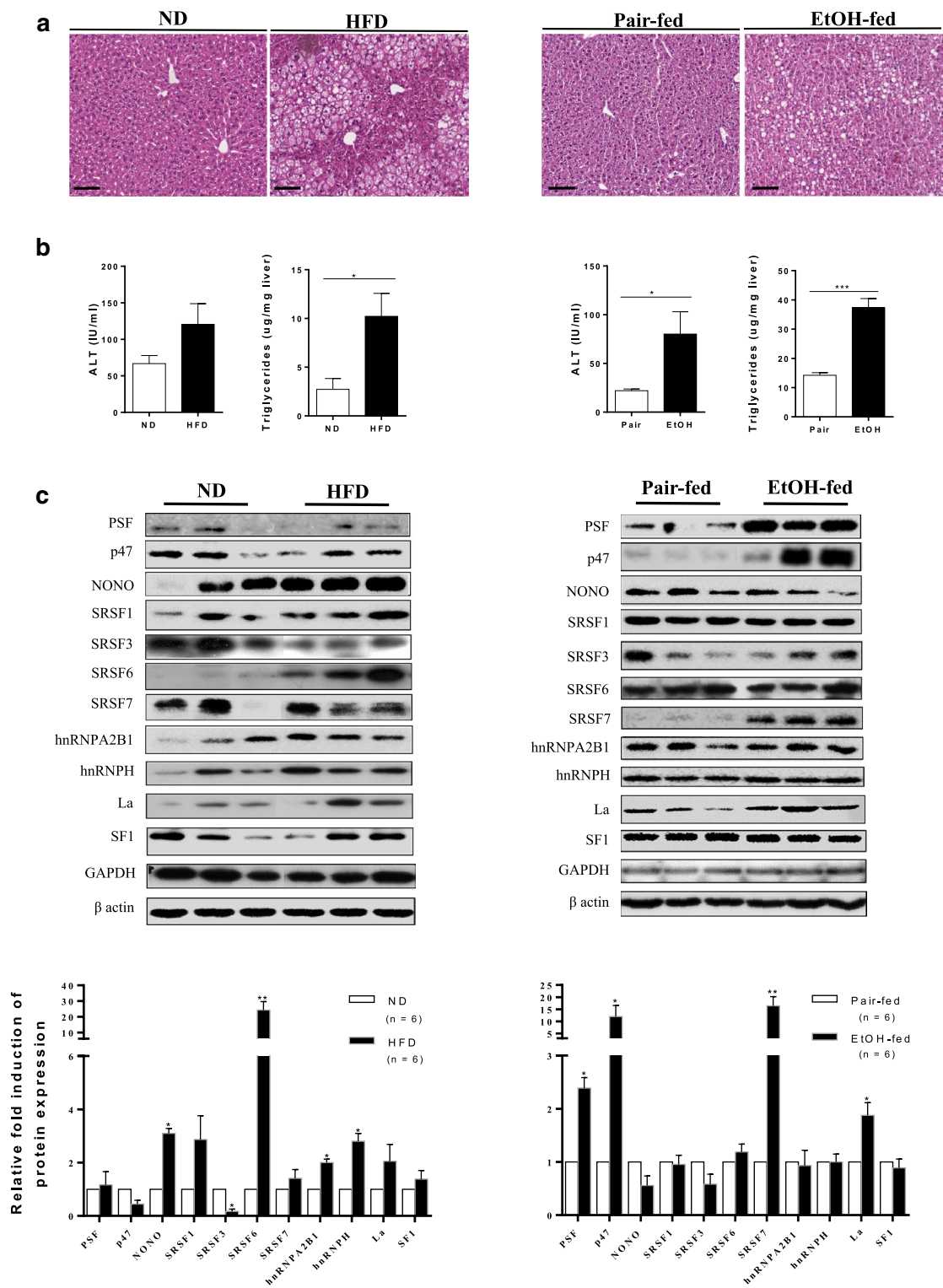
### Impact of the splicing factor signature associated with mouse liver disease on gene expression

Next, we investigated the impact of SFs modulation on the AS regulation. Considering the auto-catalytic splicing activity of SRSF3 (exon 4 skipping) and taking in account the down- or upregulation of SRSF3 protein level in HFD and LPS treated mice, we first studied AS affecting SRSF3 [12, 13]. In this purpose, both spliced (overlapping primers) and unspliced (encompassing primers) RNA isoforms of *Srsf3* were quantified and the ratio of the spliced (S)/

unspliced (L) forms was calculated (Fig. 4, upper left panel). Our data showed no modification of the AS regulation of *Srsf3* despite the modulation of expression of transcript as well as protein level (encoded by the spliced isoform) in both HFD and inflammatory liver disease (Fig. 4 and S1). We next investigated the AS regulation of 6 supplementary genes reported from the EASANA RNA splicing website and implicated in liver disease [7, 23]. We explored the AS regulation of *Slk* and *Dgkd* that are inversely regulated by the ESRP2 factor and associated with hepatocyte differentiation and proliferation [24]; *Fas* exon 3, leading to the excision of one dead domain related to apoptosis; *Collagen 18a1*, for which the frizzled domain excised in the spliced isoform negatively correlates with the  $\beta$ -catenin and adipogenesis activity [25]; *Usp4*, a suppressor of metabolic disorders related to NAFLD [26–28] and; *Insr* encoding for Insr-A (S) preferentially expressed in embryonic tissues [29, 30] or Insr-B (L) detected in adult liver [31]. Both of latter isoforms promote the metabolic effects of insulin in the liver (suppression of gluconeogenesis, promotion of lipogenesis) and, an additional mitogenic effect was reported for InsR-A [31].

Our data showed that inflammation and fibrosis injuries were associated with an increase of *Slk* and a decrease of *Dgkd*-spliced isoforms (Fig. 4). In addition, the AS event of *Fas* was reduced upon inflammation (apoptosis) and activated in the hepatic fibrosis model (necrosis). In the other models, AS regulation of these three genes remained unchanged. Despite a reduction of the *Coll18a1* and *USP4* transcript levels in the 2 models of steatosis (Fig. S1), AS of these two genes remained unaltered across the different prototypes of liver disease. One exception was observed for *Coll18a1* in HFD (Fig. 4). Finally, with the exception of HFD, all types of liver damage resulted in an increase of *Insr* AS event, suggesting an impact on hepatic regeneration and not only, as previously reported, in liver carcinogenesis (Fig. 4) [29]. In addition and in line with the acquisition of insulin resistance upon steatosis, transcription level of both *Insr* isoforms was decreased in HFD and in ALD-treated mice (Fig. S1).

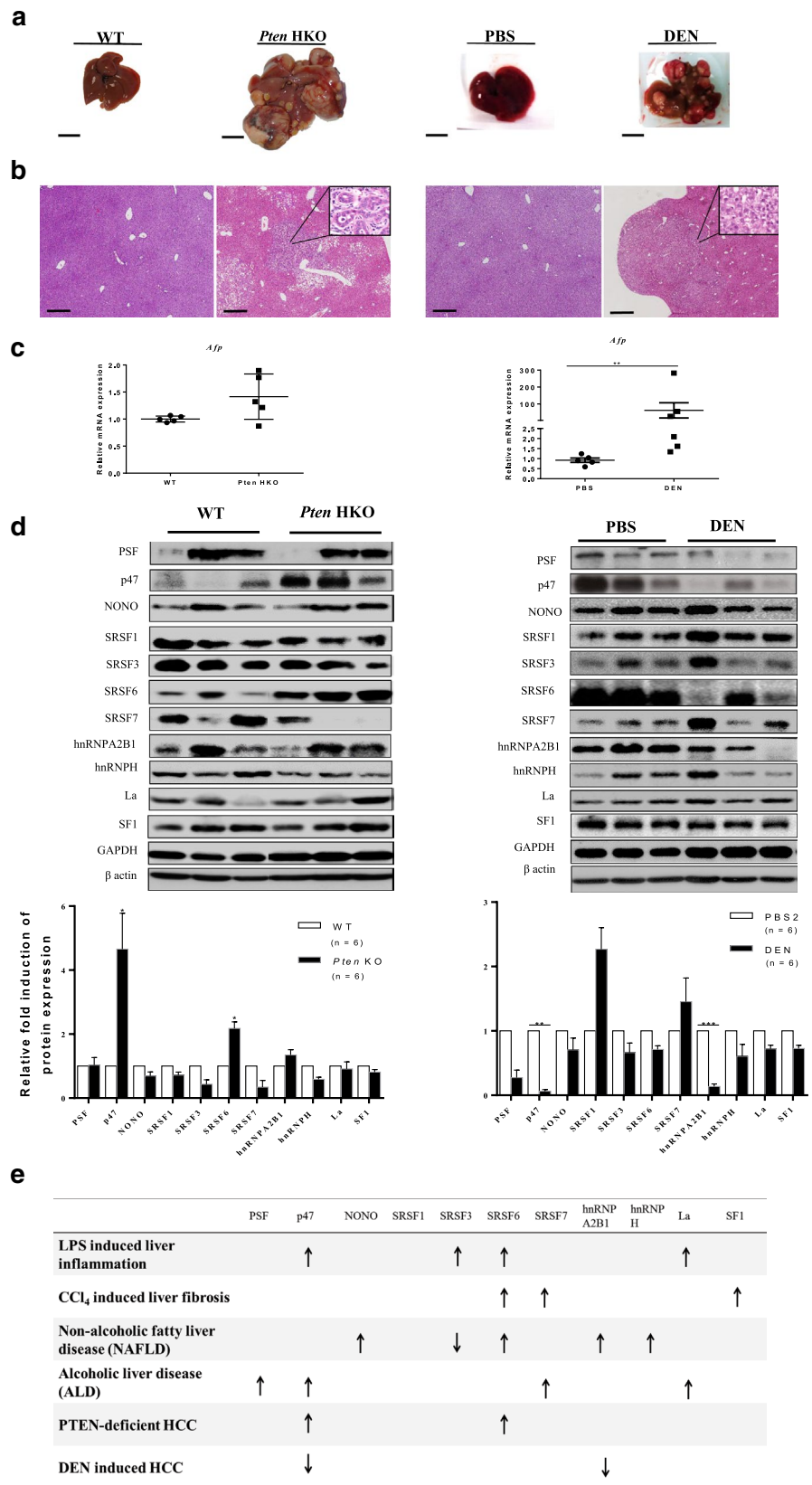
Taken together, our data provided evidence for a differential regulation of AS in pathological liver conditions. Nevertheless, a direct relationship between the expression profile of SFs and the regulation of AS remains challenging to establish. This is well illustrated by *Insr* AS, which appeared activated upon all kinds of liver injury despite contrasting levels of SFs expression. In contrast, AS of *SRSF3* was not modified despite numerous splice-recognition sites surrounding the exon 4 of the transcript and the dysregulation of its protein expression in LPS- and HFD-treated mice (Fig. S2) [32].

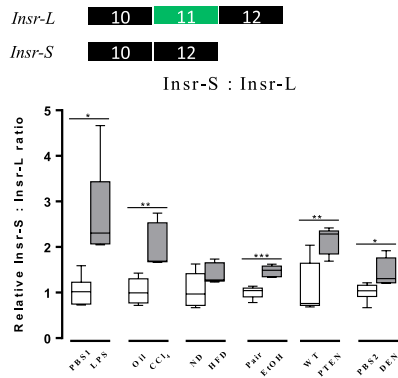
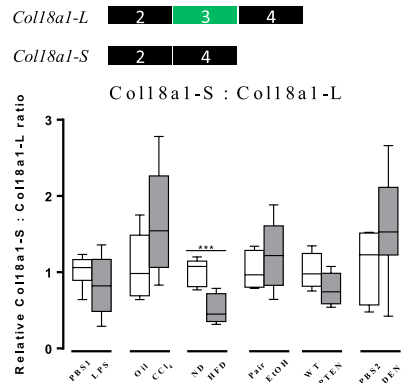
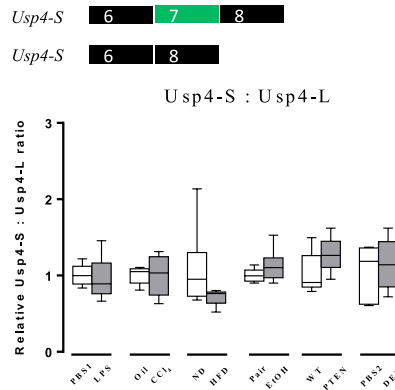
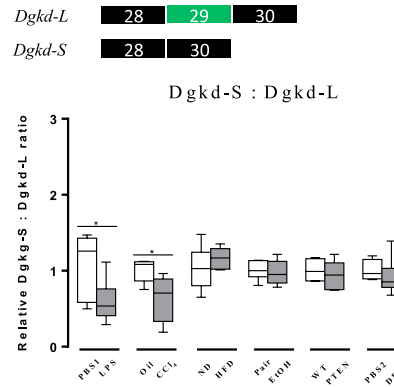
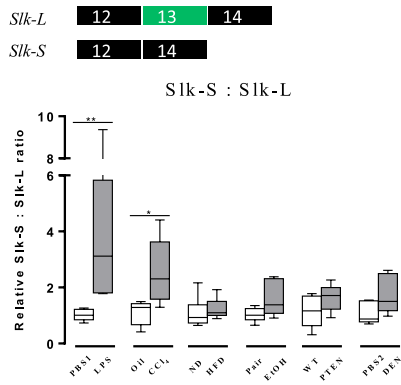
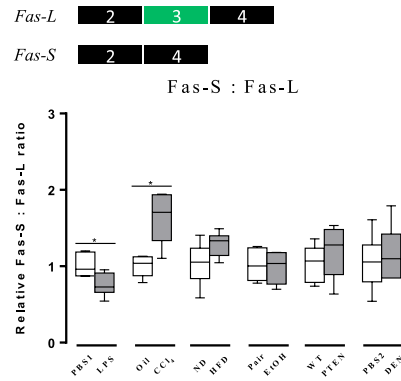
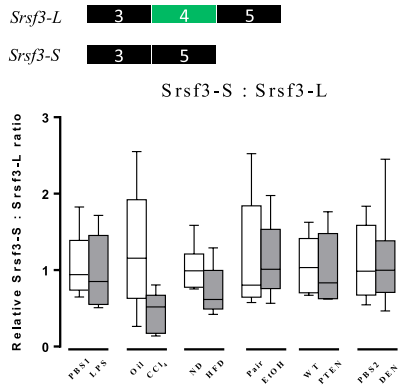


**Fig. 2** Splicing factors' expression in liver steatosis. **a** Representative H&E-stained histological liver section (bar 100  $\mu$ m) of male mice fed with normal (ND)/high-fat diet (HFD) (left) and female mice fed with normal/ethanol (EtOH) diet (right). **b** Quantification of serum ALT and liver triglycerides. Experiments were performed on ND ( $n=6$ ), HFD ( $n=5$ ), pair-fed ( $n=6$ ) and EtOH-fed ( $n=6$ ) mice. **c** Represent-

ative expression of SFs in livers of mice fed with ND/HFD (left) or normal/EtOH diet (right) (upper panel). The intensity of bands was quantified using imageJ, normalized to  $\beta$ -actin and compared to littermate controls (lower panel). Data shown are the mean  $\pm$  SD. Unpaired  $t$  test: \* $p < 0.05$  and \*\* $p < 0.01$

**Fig. 3** Splicing factors' expression in liver cancers. **a** Representative images of livers of 12-month-old WT/*Pten*-HKO null (left) or 10-months old PBS/DEN treated male mice (right). Scale bar: 1 cm. **b** Representative H&E-stained histological liver section (bar 400  $\mu$ m) and  $\times 10$  magnification box from 12-month-old WT/*Pten*-null mice (left) or PBS/DEN-treated mice at 10-months old (right). **c** Quantification of *Afp* mRNA expression level in liver tissues. Each dot represents a mouse; Mann–Whitney *U* tests:  $*p < 0.01$ . **d** Representative SF expression in the liver of 12-month-old WT/*Pten*-null mice (left upper panel) or PBS/DEN-treated mice at 10-months old (right upper panel). Bands were quantified using imageJ, normalized to GAPDH and compared to their own littermate controls (lower panel). Data are represented as mean  $\pm$  SD. Unpaired *t* test:  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$ . **e** Summary of the splicing factors' expression signature (compared with control littermate mice) observed according to liver disease models studied. Arrows indicate statistically significant increase or decrease level of protein expression (unpaired *t* test)







**Fig. 4** Alternative splicing regulation of target genes in liver diseases. The ratio of spliced isoform (S) to unspliced isoform (L) was quantified for selected target genes in murine liver disease models. The schema on the top of each bar graph shows the exon-skipping event studied for each gene. Green: alternatively spliced cassette exon; black: constitutive exons. Data in box plots are normalized to GAPDH. The Y axis represents the ratio between isoforms (S/L). The number of mice in each group: PBS1 ( $n=6$ ), LPS ( $n=6$ ), oil ( $n=5$ ), CCl<sub>4</sub> ( $n=5$ ), ND ( $n=6$ ), HFD ( $n=6$ ), pair ( $n=6$ ), EtOH ( $n=6$ ), WT ( $n=5$ ), Pten-HKO ( $n=5$ ), PBS2 ( $n=6$ ), DEN ( $n=6$ ). Unpaired  $t$  test: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

### Expression of splicing factors and alternative splicing of target genes in human HCC

The frequent alteration of the SFs PSF/p47 and SRSF6 expression in murine liver disease models led us to investigate their profile in a cohort of human HCC. In addition, because of its tumor-suppressor activity and its HCC predisposition seen in SRSF3-depleted mice, SRSF3 expression was also studied [12, 13]. Clinical status and etiological risk factors for the 179 HCC patients are summarized in Supplementary Table 1. For 152 patients, T and matched NT liver parenchyma were compared. In addition, 11 surgically resected normal liver tissues from colorectal adenocarcinoma metastasis patients were used as control tissue for comparison purposes.

The transcription level of the cell proliferation marker MKi67 was first compared in control, NT and T. As expected, proliferation was higher in T versus NT samples (Fig. 5a). In addition, a significant increase in the relative expression for PSF, SRSF6, and SRSF3 was observed in T compared to NT (although SRSF6 was not detected in 50% of both T and NT human samples) (Fig. 5b). These changes contrast with the downregulation observed in mouse DEN-induced HCC model. However, in patients, liver cirrhosis-associated HCC (71% viral hepatitis, 65% alcohol, 43% NASH) could account for this difference. We thus, compared the regulation of SFs expression according to the cirrhotic status. In cirrhotic patients, a significant increase of SRSF6 was detected associated with an unchanged level of PSF and SRSF3 (Fig. 5c). Notably, this profile is comparable in mice with CCl<sub>4</sub>-induced cirrhosis. To examine the influence of the liver microenvironment on SFs expression, we assessed their expression with the other etiological risk factors of HCC. In line with the ALD/HFD mouse models, PSF and SRSF3 were significantly highly expressed in alcohol- compared to NASH-associated HCC (Figs. 3e, 5d, Supplementary Table 4A).

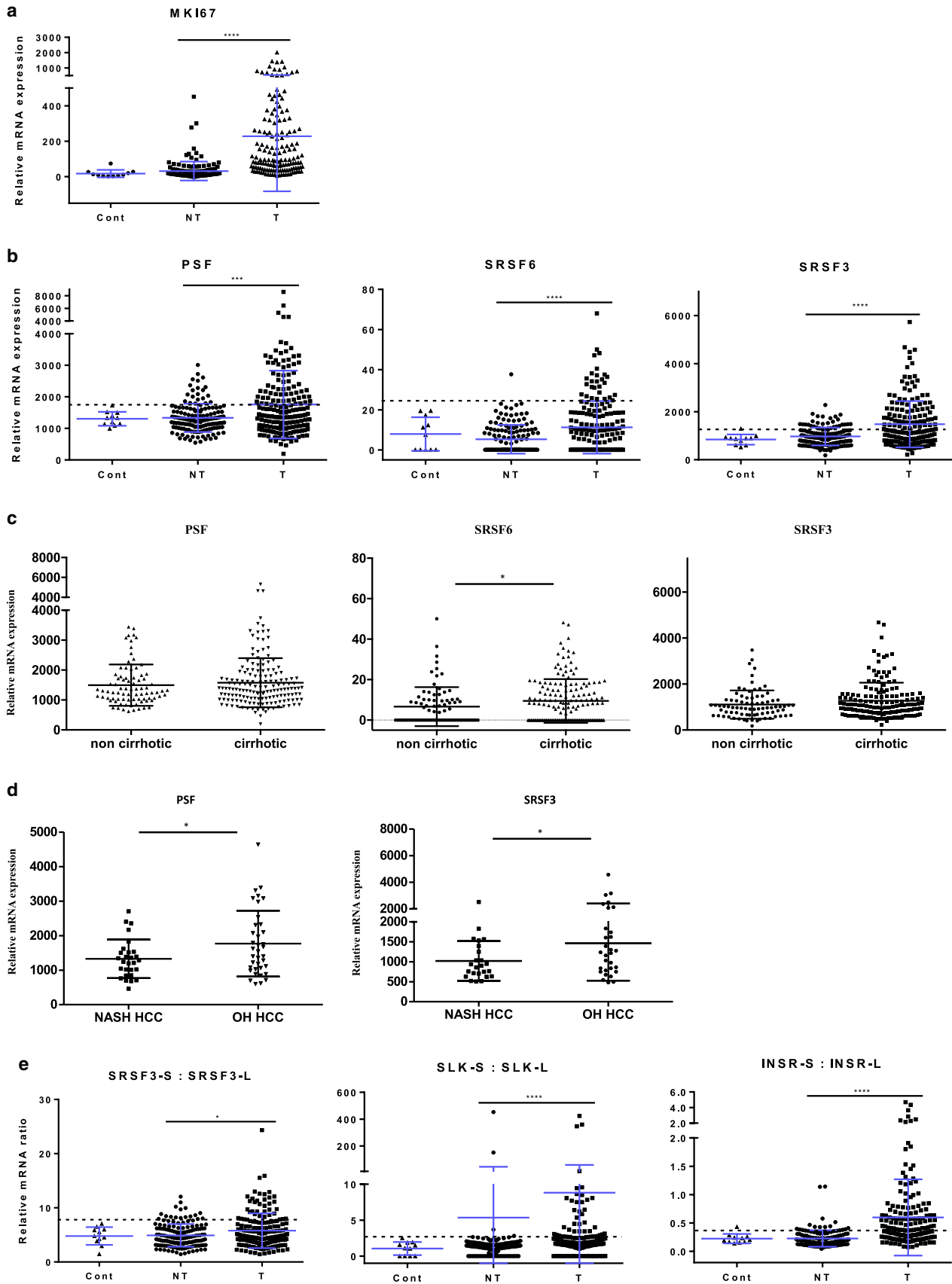
Taken together, these data suggest that the SFs' modulation in human HCC is related to the liver microenvironment. Nevertheless, the role of the SFs' upregulation in HCC remains to investigate.

### Alternative splicing regulation and clinical significance of SFs overexpression in HCC

We first investigated, in human HCC, the impact of the SFs on the AS regulation of the SLK, SRSF3, and INSR genes, previously explored in mice. Splicing regulation of these genes was significantly activated in T compared to NT samples (Figs. 5e and S3). In contrast to the data obtained from mouse models, impact of SRSF3 on its own AS regulation was detected in the human HCC tissues (Fig. S4). An overall significant correlation was also detected between SFs' expression and AS activation of SLK and INSR (Fig. S4). However, these correlations remained very weak ( $r_{\text{pearson}}$  ranging from 0.29 to 0.47), even for SRSF3. In addition, the distribution of HCC according to etiological risk factors showed that activation of SRSF3 AS in T was restricted to the HBV-related HCC (Supplementary Table 4B). Moreover, the overexpression of PSF and SRSF3 in alcohol compared to NASH-related HCC (Fig. 5d) had no impact on the AS regulation of the selected targets (Fig. S5). Taken together, our data in human HCC confirmed the contribution of SFs on the AS mechanism but also highlighted the fragile relationship, already observed in mice. Beside AS regulation, we wondered whether overexpression of SFs in mice as well as in human liver disease might contribute to the severity of HCC.

We thus, compared the expression level of SFs with the clinical parameters of HCC patients. We first, defined a cut-off expression value (mean + 2SD, dotted line in Fig. 5b, e) using 11 control liver samples. The risk of tumor recurrence was assessed from 0 to at least 60 months post-surgical resection (Fig. S6). As previously reported, we confirmed that liver cirrhosis is a strong risk factor of HCC recurrence (Fig. S6A). Whilst the modulation of SFs expression was not a predictive marker for tumor recurrence, an elevated risk was observed in patients with higher level of the INSR and SLK but not SRSF3 spliced isoforms (Fig. S6). In contrary to the results obtained for SLK, multivariate cox analysis validated the independence between the AS activation of INSR and liver cirrhosis ( $p=0.01$ ) for the risk of tumor recurrence.

The relationship between SFs/AS regulation and overall survival (OS) of HCC patients was considered from 6 (to exclude post-surgical related adverse events) to 60 months post-HCC resection. As previously reported and in line with the tumor recurrence, occurrence of liver cirrhosis (and tumor size > 5 cm) at baseline significantly reduced the OS of patients (Figs. 6a and S7). Unexpectedly, upregulation of PSF and to a lesser extent, SRSF3 and SRSF6, was associated with a longer OS ( $p=0.01$  for PSF and a trend for both SRSF3 and SRSF6) (Fig. 6b). As observed for patients without cirrhosis, the improvement of OS associated with PSF overexpression was mainly observed 24 months



**Fig. 5** Relative mRNA expression of splicing factors and target genes in Control, Non-Tumor and Tumor liver samples from HCC patients. Expression level of transcripts in HCC tumors (T), non-tumorous liver parenchyma (NT) and normal liver tissues (Cont) were quantified by RT-qPCR analysis. Expression level of **a** MKI67, **b** splicing factors in liver specimens from HCC patients. (C) Comparison of SFs expression in non cirrhotic ( $n=82$ )/cirrhotic patients ( $n=162$ ) from T+NT samples. **d** PSF and SRSF3 increased in HCC-related to alcohol ( $n=38$ ) compared to NASH ( $n=28$ ) a profile similar to the corresponding mouse models of liver steatosis. Data are normalized to the GUSB house-keeping gene and represented as mean  $\pm$  SD. **e** Ratio of splicing isoforms [Spliced (S)/Unspliced (L)] of SRSF3, SLK and INSR selected genes in liver specimens from HCC tumors (T), non-tumorous liver parenchyma (NT) and normal liver tissues (Cont). This ratio was obtained after quantification by RT-qPCR analysis of spliced (S) and long unspliced (L) isoforms. The dashed lines represent cutoff values for the overexpression of each parameter defined using the expression level in the control liver samples (mean + 2SD). Each dot represents a liver biopsy; T vs NT: MannWhitney  $U$  tests. \* $p < 0.05$ , \*\*\* $p < 0.001$

post-surgery. In contrary, OS was not impacted by the regulation of AS of the INSR, SLK or SRSF3 genes (Fig. 6b). Multivariate cox analysis for extended OS showed an independent relationship between the level of PSF expression and liver cirrhosis status ( $p=0.02$ ).

Altogether, our data highlight the regulation of SFs and AS regulation in liver cancers. However, and as observed in mice, the direct relationship between the regulation of expression of SFs and the impact on AS events remains challenging to establish. Unexpectedly, the overexpression of SFs and more particularly PSF was strongly associated with a better survival after HCC resection.

## Discussion

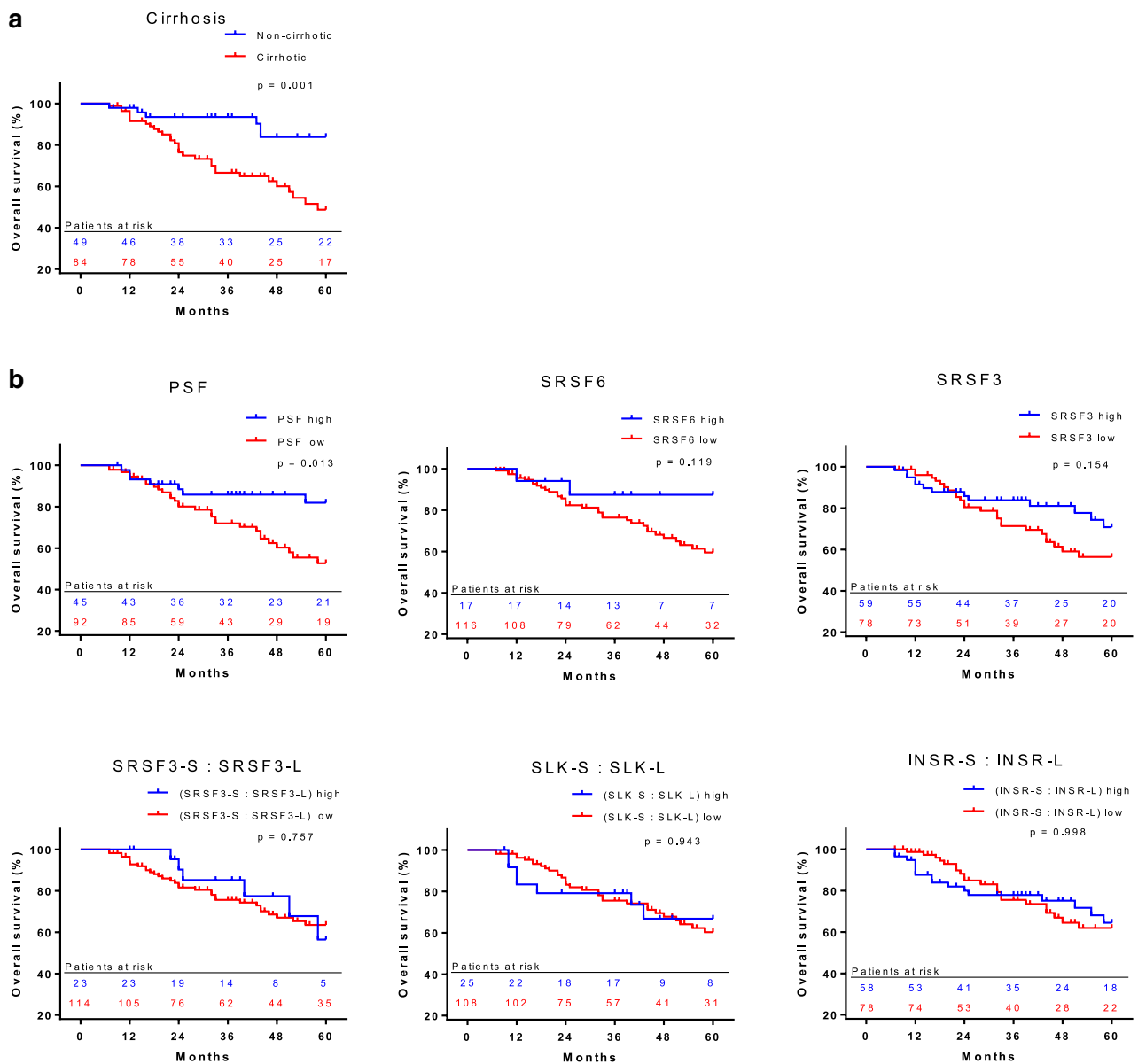
More than 800 000 people die each year following primary liver cancer of which HCC covers around 85% of cases [33]. HCC usually arises after years of inflammation, fibrosis or, as more recently emerged, steatosis associated with chronic hepatitis, alcohol consumption or obesity. Aberrant AS events in liver cancer have been frequently described in numerous genes including *INSR*, *TP53* [8–10]. Almost all of these AS events in HCC have been associated with cell transformation [34]. However, and as illustrated in our data, deciphering the regulation of the spliceosome machinery during liver disease progression remains complex. To address the impact of SF expression on AS events during liver injury, we compared the profiles of a panel of SF- and AS-regulated genes in 6 murine liver disease models. We showed that each liver injury provided a unique signature of SFs modulation compared to their respective control littermates. However, this signature was not associated with a specific pattern of AS regulation of genes, as illustrated for *INSR* or *SRSF3*, suggesting further impact of SF during

liver disease. This hypothesis was reinforced by the weak strength of correlation between SFs and AS regulation in HCC patients.

Nevertheless, alteration of AS regulation of genes may contribute to the progression of liver disease. Indeed, previous data reported that upregulation of *INSR*-A splicing (S) isoform promotes liver cancer by mitogenic activity related to IGF-II binding [29, 35, 36]. Our data in mice confirm that activation of the AS of *INSR*, leading to *INSR*-A expression, was effective in liver cancer. However, in our mouse models, the AS activation of *INSR* was already observed in the inflammatory as well as in the fibrosis liver stages, suggesting that related overexpression of the *INSR*-A may also contribute to the compensatory regeneration effect post-liver injury and not only in the carcinogenic process. In contrary, elevated activity of *INSR*-B in insulin-associated to metabolic pathway accounted for the reduction of the *INSR* splicing ratio in the pancreas of type 2 diabetes patients [37]. In HFD-treated mice, a model for obesity, the *INSR* splicing ratio remained constant despite a significant reduction of both spliced and unspliced RNA isoforms. This result suggests that decreased expression of both receptors in the liver participates in the insulin resistance observed in NAFLD, independent of splicing regulation.

The decrease of SLK AS regulation, linked to the increase of DGKD and both under the dependence of *ESRP2* SF expression, is implicated in post-natal liver maturation [24]. Little is known about the functional activity of the SLK and DGKD isoforms in liver disease. Our data obtained in murine liver inflammation and fibrosis models showed an increase of AS of SLK. Thus, this AS activation of SLK could contribute to the progression to HCC. Indeed, comparable regulation was previously reported in *SRSF3* KO mice leading to HCC [12]. Furthermore, AS activation of SLK is considered as an indicator of the epithelial–mesenchymal transition [38], through the control of *ESPR2* [39]. However, despite this upregulation in HCC, no association with the grade of cancer was observed in our studied patients (data not shown). The contribution of the SLK AS regulation in the early events of the carcinogenic process clearly requires further studies.

Considering the reported oncogenic properties of *SRSF1* [11, 14], its expression level remained unexpectedly unchanged regardless of liver-injury models. Despite tumor-suppressor properties related to *SRSF3* expression in the liver [12, 40], this SF was never downregulated in mouse or in human liver cancers. On the contrary, *SRSF3* was upregulated in HCC patients. This intriguing upregulation, mainly observed in HBV-associated HCC (Supplementary Table 4B) as well as in patients with higher AFP levels (data not shown), suggests that *SRSF3* overexpression may attempt to maintain hepatocyte metabolic function and differentiation despite HCC context [12, 13].



**Fig. 6** Overall survival observed in HCC patients in relation to SF levels. Cumulative overall survival rates (%) of HCC patients according to **a** the presence/absence of liver cirrhosis, **b** the intratumoral expression level of SFs (upper panel) and ratio (S/L) of selected splicing target isoforms (lower panel) sorted as high (blue)>cut-

off value>low (red); the cutoff value defined as the mean+2 SD of control values. Follow-up was studied from 6 to 60 months post-HCC resection. Survival curves were analysed by the Kaplan–Meier method and  $p$  values were calculated using the log-rank test

In mouse models, PSF and SRSF6 were more frequently observed among the modulated SFs (preferentially upregulated). This dual upregulation was confirmed in human HCC despite lack of frequent detection of SRSF6, reflecting species specificity in its expression profile. Overexpression of PSF and SRSF6 in tumor tissues was corroborated in data generated from 371 HCC and 50 non-tumor liver samples extracted from the TCGA Research Network. In addition, higher expression of PSF was already reported in human prostate cancer leading to the aberrant regulation of the

spliceosome machinery [41]. Indeed, PSF can regulate AS through the recruitment of the polypyrimidine tract-binding proteins, or small nuclear ribonucleoproteins using a mechanism not yet clearly defined. Unexpectedly, in our HCC cohort, PSF overexpression was correlated with an extended survival post-tumor resection, independent of liver cirrhosis. In addition, a trend towards improved survival was also depicted for SRSF6 and SRSF3. Finally, modulation of SFs expression in liver inflammation, fibrosis and steatosis, stability of SRSF1, upregulation of SRSF3 in HCC and, the

association between PSF and OS suggests a positive role of these deregulations during liver disease development. This hypothesis regarding the protective role of SFs in liver is reinforced by the additional properties of SFs in DNA repair or in oncogenic gene repression [42, 43].

In conclusion, in murine models, modulation of SFs expression depicts the different stages of liver disease, from inflammation to cancer. In patients and in mouse models, upregulation of SFs seems not preferentially related to AS regulation but provides a new insight into their accurate contribution during liver injury. Intricate relationship between the expression of SFs and the regulation of AS highlights the requirement of new tools to further decipher the impact of SFs regulation in the spliceosome machinery and the significance in liver disease progression leading to HCC.

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**Author contributions** Experimental conception and design of these experiments HW, JH, IC, and PS. Conduction the experiments: HW, BL, NF, ACS, GP, JA, IB, SV, and PS. Analysis of the data: HW, NF, TA, AS, GP, DK, JH, IC and PS. Provide biological samples: JS, JMP, BB, PM. Wrote the manuscript: HW, JH, DK and PS.

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## Compliance with ethical standards

**Conflicts of interest** Hualin Wang, Bouchra Lekbaby, Nadim Fares, Jeremy Augustin, Tarik Attout, Aurelie Schnuriger, Anne-Marie Cas-sard, Ganna Panasyuk, Gabriel Perlemute, Ivan Bieche, Sophie Vacher, Janick Selves, Jean-Marie Péron, Brigitte Bancel, Philippe, Dina Kremsdorf, Janet Hall, Isabelle Chemin, Patrick Soussan have no conflict of interest to declare.

**Informed consent** Signed informed patient consent was obtained before surgery.

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