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Sobolewski, Cyril; Abegg, Daniel; Berthou, Flavien; Dolicka, Dobrochna; Calo, Nicolas Virgile; Sempoux, Christine; Fournier, Margot; Maeder Garavaglia, Christine; Ay Berthomieu, Anne-Sophie; Clavien, Pierre-Alain; Humar, Bostjan; Dufour, Jean-François; Adibekian, Alexander; Foti, Michelangelo

ORIGINAL RESEARCH

S100A11/ANXA2 belongs to a tumour suppressor/oncogene network deregulated early with steatosis and involved in inflammation and hepatocellular carcinoma development

Cyril Sobolewski,[1] Daniel Abegg,[2] Flavien Berthou,[1] Dobrochna Dolicka,[1] Nicolas Calo,[1] Christine Sempoux,[3] Margot Fournier,[1] Christine Maeder,[1] Anne-Sophie Ay,[1] Pierre-Alain Clavien,[4] Bostjan Humar,[5] Jean-François Dufour ,[6] Alexander Adibekian,[2] Michelangelo Foti [1]

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[1]Department of Cell Physiology and Metabolism, University of Geneva Faculty of Medicine, Geneve, GE, Switzerland
[2]Department of Chemistry, The Scripps Research Institute, Jupiter, Florida, USA
[3]Department of Clinical Pathology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland
[4]Visceral and Transplantation Surgery, University Hospital of Zurich, Zurich, Switzerland
[5]Department of Surgery, University Hospital Zurich, Zurich, Switzerland
[6]Department of Hepatology and Clinical Research, University of Bern, Bern, Switzerland

Correspondence to
Professor Michelangelo Foti, Department of Cell Physiology and Metabolism, University of Geneva Faculty of Medicine, Geneve 1206, Switzerland; michelangelo.foti@unige.ch

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ABSTRACT

Objective Hepatocellular carcinoma (HCC) development occurs with non-alcoholic fatty liver disease (NAFLD) in the absence of cirrhosis and with an increasing incidence due to the obesity pandemic. Mutations of tumour suppressor (TS) genes and oncogenes (ONC) have been widely characterised in HCC. However, mounting evidence indicates that non-genomic alterations of TS/ONC occur early with NAFLD, thereby potentially promoting hepatocarcinogenesis in an inflammatory/fibrotic context. The aim of this study was to identify and characterise these alterations.

Design The proteome of steatotic liver tissues from mice spontaneously developing HCC was analysed. Alterations of TSs/ONCs were further investigated in various mouse models of NAFLD/HCC and in human samples. The inflammatory, fibrogenic and oncogenic functions of S100A11 were assessed through in vivo, in vitro and ex-vivo analyses.

Results A whole set of TSs/ONCs, respectively, downregulated or upregulated was uncovered in mice and human with NAFLD. Alterations of these TSs/ONCs were preserved or even exacerbated in HCC. Among them, overexpression of S100A11 was associated with high-grade HCC and poor prognosis. S100A11 downregulation in vivo significantly restrains the development of inflammation and fibrosis in mice fed a choline/methionine-deficient diet. Finally, in vitro and ex-vivo analyses revealed that S100A11 is a marker of hepatocyte de-differentiation, secreted by cancer cells, and promoting cell proliferation and migration.

Conclusion Cellular stress associated with NAFLD triggers non-genomic alterations of a whole network of TSs/ONCs fostering hepatocarcinogenesis. Among those, overexpression of the oncogenic factor S100A11 promotes inflammation/fibrosis in vivo and is significantly associated with high-grade HCC with poor prognosis.

Significance of this study

What is already known on this subject?

- Hepatocellular carcinoma (HCC) and cholangiocarcinoma can arise from non-cirrhotic stages of non-alcoholic fatty liver disease (NAFLD).
- With the pandemic of NAFLD, the incidence of HCC/intrahepatic cholangiocarcinoma (ICC) development in the absence of cirrhosis is expected to dramatically increase in the future.
- The molecular bases of HCC/ICC development in non-cirrhotic livers are poorly known.

What are the new findings?

- Cellular stress associated with hepatic steatosis triggers non-genomic alterations of a whole network of oncogenes and tumour suppressors.
- Among this network, S100A11/ANXA2 overexpression is a hallmark of inflammation/fibrosis, high-grade HCC and correlates with a poor clinical outcome.
- S100A11 downregulation in vivo significantly restrain development of inflammation and fibrosis in mice fed a choline/methionine-deficient diet.
- S100A11 has oncogenic properties in hepatocytes and its expression is associated with hepatocytes-dedifferentiation and epithelial–mesenchymal transition.
- S100A11 is secreted by HCC cells.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) associated with obesity/diabetes[1] encompasses a variety of metabolic/histological liver disorders starting with chronic accumulation of lipids in hepatocytes (steatosis) and cellular stress.[1] With time, the conjunction of various pathological mechanisms (eg, lipotoxicity, endoplasmic reticulum (ER) stress, mitochondrial dysfunctions and epigenetic changes) triggers hepatocytes death and inflammation; a condition referred as non-alcoholic steatohepatitis (NASH).[2] In this inflammatory context, fibrosis can develop and progress towards cirrhosis,[2] a high-risk condition for hepatocellular carcinoma (HCC).[3] Yet, it is well established that not only HCC but also intrahepatic cholangiocarcinoma (ICC) can arise

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Hepatic steatosis/inflammation/fibrosis should be considered as important risk factors for HCC in clinical practice, since it triggers a favourable molecular landscape for HCC/ICC development.
- High expression of S100A11 is a promising diagnostic tool to identify high-grade HCC.
- S100A11 is a novel candidate biomarker for HCC in liquid/solid biopsies.
- S100A11 is a new potential target for immuno-based and other therapies directed against non-alcoholic steatohepatitis/fibrosis and aggressive HCC/ICC.

in the absence of cirrhosis.[4 5] However, whether cellular stress and metabolic disorders feature pre-cancerous lesions and how they prime the liver for HCC/ICC development remains poorly understood.

HCC includes heterogeneous tumours resulting from different aetiologies and genetic alterations. Major gene mutations characterising different HCC types have been identified.[6 7] Unfortunately, current therapeutic strategies are ineffective against most of tumour-promoting pathways mutated in HCC (eg, hTERT, β-catenin, TP53). Of importance, recent evidence indicates that besides gene mutations, metabolic disorders and the tumour microenvironment[8] can trigger epigenetic changes and/or aberrant regulatory mechanisms leading to non-genomic alterations of tumour suppressors (TSs), oncogenes (ONCs) or DNA damage response genes (DDRGs). Aberrant expression/activity of inflammatory/cancer-promoting factors are likely as important as the well-characterised mutations of TSs/ONCs/DDRGs driving hepatocarcinogenesis. Therefore, a better understanding of non-genomic alterations of HCC-related factors may not only uncover new biomarkers but will likely point to novel and druggable therapeutic targets.

The phosphatase and tensin homolog (PTEN) is a relevant example of non-genomic loss of a key TS with NAFLD (eg, steatosis, inflammation/fibrosis). PTEN is frequently deleted, mutated or downregulated in human cancers.[9] Although PTEN mutations frequency in HCC is low (2%–5%),[7] its expression/activity is significantly downregulated with NAFLD/NASH,[10] alcoholism[11 12] and HBV/HCV infections[13] through distinct mechanisms. Evidence further indicates that key drivers of steatosis (eg, FASN, CD36) and inflammation/fibrosis (eg, interleukin 1β, tumour necrosis factor-alpha (TNF-α)) can promote hepatocarcinogenesis.[14–16] Finally, the tumour microenvironment and epigenetic mechanisms can trigger upregulation of ONCs, thereby mimicking activating mutations. ONCs overexpression in the absence of mutations, such as cyclooxygenase-2 (COX2), annexin-A2 (ANXA2), S100 proteins (eg, S100A8, S100A9[17]) or galectin-1[18 19] were indeed reported to have important functions (ie, cell proliferation/survival, EMT, immune escape) in human HCC. While such alterations were mostly identified in tumours, it remains unclear whether they occur early with hepatic cellular stresses associated with steatosis/inflammation/fibrosis preceding malignancy.

Herein, we uncovered a whole network of TSs and ONCs downregulated and upregulated, respectively, with NAFLD seemingly to provide a favourable landscape for HCC development. Among those, we identified the calcium-binding protein S100A11, which has oncogenic properties, is secreted by cancer cells and whose overexpression promotes inflammation/fibrosis and is associated with high-grade HCC with poor clinical outcome.

MATERIALS AND METHODS

Reagents and antibodies

All reagents, commercial kits, antibodies are described in online supplementary materials and methods.

Animals

Animal sources and experimental procedures are described in the online supplementary materials and methods. All experiments were ethically approved by the Geneva Health head office and were conducted in agreement with the Swiss guidelines for animal experimentation.

Proteomic analysis by LC-MS/MS

Liver tissues from 4-month-old Control and LPTENKO mice were processed for liquid chromatography-mass spectrometry (LC-MS/MS). MS data were analysed with MaxQuant (V.1.5.2.8) and searched against the mouse proteome. The methodology and statistics are described in online supplementary materials and methods.

Cell cultures, cell cycle analysis, transfections, proliferation, migration/invasion assays and primary hepatocytes isolations

Human/mouse cell lines, reagents and detailed experimental procedures for cell cultures, cell cycle analysis, primary hepatocytes isolation, siRNAs/plasmid transfection, cell proliferation, migration/invasion assays are described in online supplementary materials and methods.

Real-time PCR analysis

A detailed description of real-time PCR analyses and primers are provided in online supplementary materials and methods.

Western blot, co-immunoprecipitations and ELISA analyses

Description of western blot analyses, co-immunoprecipitation assays, ELISA and primary/secondary antibodies are provided in online supplementary materials and methods.

Histology and human tissue microarrays

Liver tissues were fixed in paraformaldehyde and processed for histological analyses as described in online supplementary materials and methods. S100A11 immunohistochemical staining was performed on human tissue microarrays and evaluated by two independent experimenters including a pathologist as detailed in online supplementary materials and methods.

Bioinformatics analysis

Identification of cancer/HCC-related factor in the proteomic, gene ontology enrichment, interactomes and correlations, Gene Expression Omnibus (GEO), mutations frequencies and survival analyses, as well as exosomes content and transcription factor binding site analyses, were performed using publicly available softwares and databases as described in online supplementary materials and methods.

Statistical analysis

Data are reported as the mean±SD. Student's t-test (two-tailed) were used to determine the statistical significance of means

between two groups. Statistical significances were assessed by one-way analysis of variance for comparisons between more than two groups. The χ^2 independence test was used to evaluate the independence of different variables. For survival analysis, differences between groups (low/high) were assessed with log rank tests. Differences were considered significant when *p<0.05, **p<0.01 or ***p<0.001.

RESULTS

Hepatic steatosis and cellular stress in LPTENKO mice are associated with deregulation of a whole network of TSs/ONCs

Liver-specific PTEN knockout (LPTENKO) mice at adulthood spontaneously develop hepatic steatosis, inflammation/fibrosis and HCC/ICC with ageing.[20 21] To uncover potential alterations of cancer-related factors occurring with steatosis-associated cellular stress or inflammation/fibrosis, we compared the liver proteome of control littermates and steatotic LPTENKO mice (figure 1, online supplementary table S1). We identified >2000 proteins, of which 279 and 340 were, respectively, upregulated and downregulated in LPTENKO livers. Differentially expressed proteins were then compared with public databases of cancer/HCC-related factors. Based on literature screening (online supplementary table S2), 160 cancer-associated factors were modulated in our proteomic analysis, among which *22.22%* displayed oncogenic properties (ONC), *14.81%* were TSs and *3.09%* had both ONC/TS features (figure 1B, online supplementary materials and methods). Among these 160 candidates, we further considered only downregulated TS (n=11), upregulated ONC (n=10) and candidates with either TS or ONC activity (n=5) (figure 1C). Finally, given some heterogeneity observed between mice only the most deregulated TS/ONC candidates (ONC >1.55 and TS <0.66) with a more permissive p value (p<0.2) were retained for further analyses (22 candidates in bold, figure 1C). Gene ontology analysis further indicated that several selected candidates were regulating the lipid metabolism or activating oncogenic pathways, for example, MAPK signalling (online supplementary figure S1). mRNA expression of selected candidates in LPTENKO hepatic tissues indicated that most of the ONCs were upregulated at the mRNA level, while mRNAs expression of TSs was mostly unchanged suggesting a downregulation through post-transcriptional/post-translational mechanisms (online supplementary figure S2A). Publicly available transcriptomic dataset from 3 months old LPTENKO mice further confirmed these data (online supplementary figure S2B). Finally, only candidates, whose protein expression was also deregulated in steatotic mice following a tamoxifen-inducible deletion of PTEN at adulthood (LIPTENKO mice, online supplementary figure S3), were retained for further analyses therefore excluding alterations resulting from adaptive developmental mechanisms (figure 1D).

Taken together, these analyses revealed that steatosis-associated cellular stress in PTEN-deficient mice triggers upregulation of several ONCs (ie, LGALS1, CD36, FASN, ANXA2, ACC and MGLL) and downregulation of TSs (ie, ENDOG, PDCD4 and SBP1). Relevant to our analyses, some of these deregulated TSs/ONCs were previously associated with HCC (eg, LGALS1, ANXA2), while the roles of others (CREG1, ENTPD5 and S100A11) remains unclear (online supplementary table S2).

Deregulated expression of TSs/ONCs accompanies all stages of NAFLD in rodents and humans

Alterations of TSs/ONCs network confirmed in figure 1D was further explored in other mouse models of NAFLD/NASH and using in silico transcriptome data of both rodents and humans with fatty liver disease. As shown in figure 2A–B and online supplementary figure S4, *ob*/*ob* and *db*/*db* mice have steatosis associated with downregulation of SBP1, PDCD4, ENDOG and CREG1 protein level, while mRNA and/or protein expressions of *Lgals1*, *Anxa2*, *Mgll*, *Fasn*, *Acaca*, *Cd36*, *Entpd5* and *S100a11* were upregulated. Mouse hepatic steatosis developing with ageing was also associated with *S100a11*, *Anxa2* and *Lgals1* upregulation (online supplementary figure S5). Interestingly, high-fat-containing diet (HFD)-induced steatosis for 10 weeks was accompanied by only *Anxa2* and *Lgals1* upregulations, which interestingly were prevented if mice were exercising the last 4 weeks of HFD (online supplementary figure S6), Publicly available transcriptomic datasets further confirmed the induction of well-established ONC (*ie, Anxa2, Lgals1*, *Cd36*, *Mgll*) but also *S100a11* and *Entpd5* upregulation in rodent models of genetic/diet-induced hepatic steatosis, inflammation/fibrosis and cirrhosis (figure 2C, online supplementary figure S7, S8 and table S3). Finally, human GEO dataset mining indicated that *ANXA2*, *LGALS1* and *FASN* mRNA expressions were also upregulated with steatosis, whereas *S100A11* expression was significantly increased only with NASH and cirrhosis (figure 2D, online supplementary table S3).

S100A11 promotes hepatic inflammation and fibrosis in mice

Among TSs/ONCs deregulated with NAFLD/NASH, the role of S100A11 is currently poorly known. From our analyses, depending on the mouse model S100A11 expression is heterogeneously upregulated with steatosis, but constantly overexpressed with fibrosis/inflammation in particular in humans (figure 2D). Together, these observations strongly suggest that S100A11 upregulation might represent a key feature and marker of steatosis transition to NASH/fibrosis. To test this hypothesis, we downregulated in vivo in mice S100A11 expression through transduction of hepatotropic adeno-associated virus (AAV8) encoding shRNAs against *S100a11* (figure 3A) and then submitted these mice 2 weeks to a choline/methionine-deficient diet (MCD) (figure 3A). The MCD diet was shown to better recapitulate the various pathological mechanisms triggering NASH pathogenesis and its progression toward severe stages in humans.[22] As shown in online supplementary figure S9A,B, 2 weeks of MCD induced a severe steatosis, inflammation and fibrosis in the liver that was associated with a strong *S100a11*, *Anxa2* and *Lgals1* upregulation. *S100a11* knockdown by shRNA-AAV8 prior to the MCD significantly improves histopathological features (steatosis and fibrosis) induced by the MCD (figure 3B), which was associated with a decreased hepatic triglycerides content and a reduced expression of CD36, a key driver of steatosis (figure 3C–D). Strikingly, genes associated to inflammation (*Il1β*, *Tnfa*, *Itgam*, *Ptgs2*), macrophages (*Itgam*, *Cd44*, *Ccl2*) and cancer-initiating cells recruitment (*Cd44*), fibrosis (*Col1a1*, *Pdgfa*), EMT (ie, *Tgfb1*), apoptosis (ie, *Bax*) and cell proliferation (ie, *Mki67*), were significantly downregulated (figure 3D). Consistent with these analyses, macrophages infiltration was significantly reduced in the absence of S100A11 (figure 3E). Interestingly, *S100A11* expression was reciprocally upregulated by inflammatory mediators in cancer cells consistent with the presence of a NFκB response element within *S100A11*'s promoter (online supplementary figures S9D and S25D).[23]

Together, these data indicate that S100A11 upregulation significantly contributes to inflammation and fibrosis development, which are key drivers of hepatocarcinogenesis. In agreement, GSEA analysis further highlights a positive enrichment for

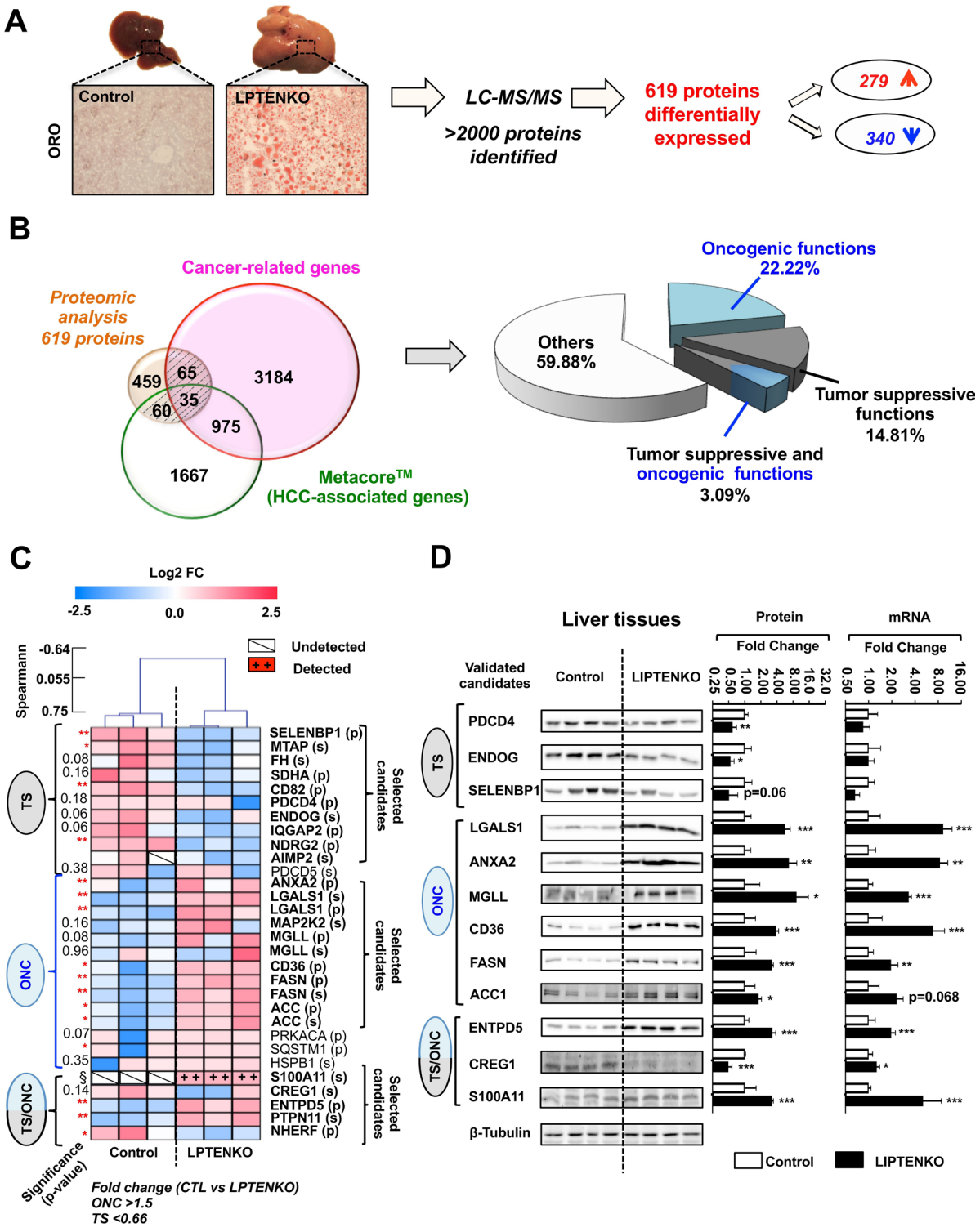

Figure 1 Proteomic analysis of liver tissues from LPTENKO mice. Proteomic analyses (LC-MS/MS) were performed with liver samples from 4-month-old control and LPTENKO mice (n=3 mice/group) (A) Liver anatomy and ORO staining of lipids in liver sections from control and LPTENKO mice. LC-MS/MS process and quantitative results are illustrated. (B) Differentially expressed proteins between control and LPTENKO mice were compared with cancer-related/HCC-associated genes from publicly available databases (online supplementary materials and methods) and Metacore software. Cancer-related factors were classified as ONCs, TSs and *'others'* based on literature (online supplementary table S2). (C) Upregulated ONCs and downregulated TSs in the soluble (s) or pellet (p) fractions are represented in a heatmap (1.5≤fold change ≤0.66; normalised genes/rows). Proteins selected for further investigation are indicated in bold. The similarity between samples was evaluated by a hierarchical clustering based on a Spearman correlation. (D) Representative western blots and qRT-PCR analyses of TS/ONC in a cohort of control and inducible hepatocyte-specific PTEN knockout (LIPTENKO) mice. Quantifications were made on 5–6 mice/group. Cyclophilin-A was used to normalise qRT-PCR analyses. Data represent the mean ± SD. ***p<0.001, **p<0.01 and *p<0.05 (t-test). §Candidates undetected in controls and detected in LPTENKO (NO fold change). HCC, hepatocellular carcinoma; LC-MS/MS, liquid chromatography-mass spectrometry; ONCs, oncogenes; ORO, Oil-red-O; qRT-PCR, quantitative real-time PCR; TSs, tumour suppressors.

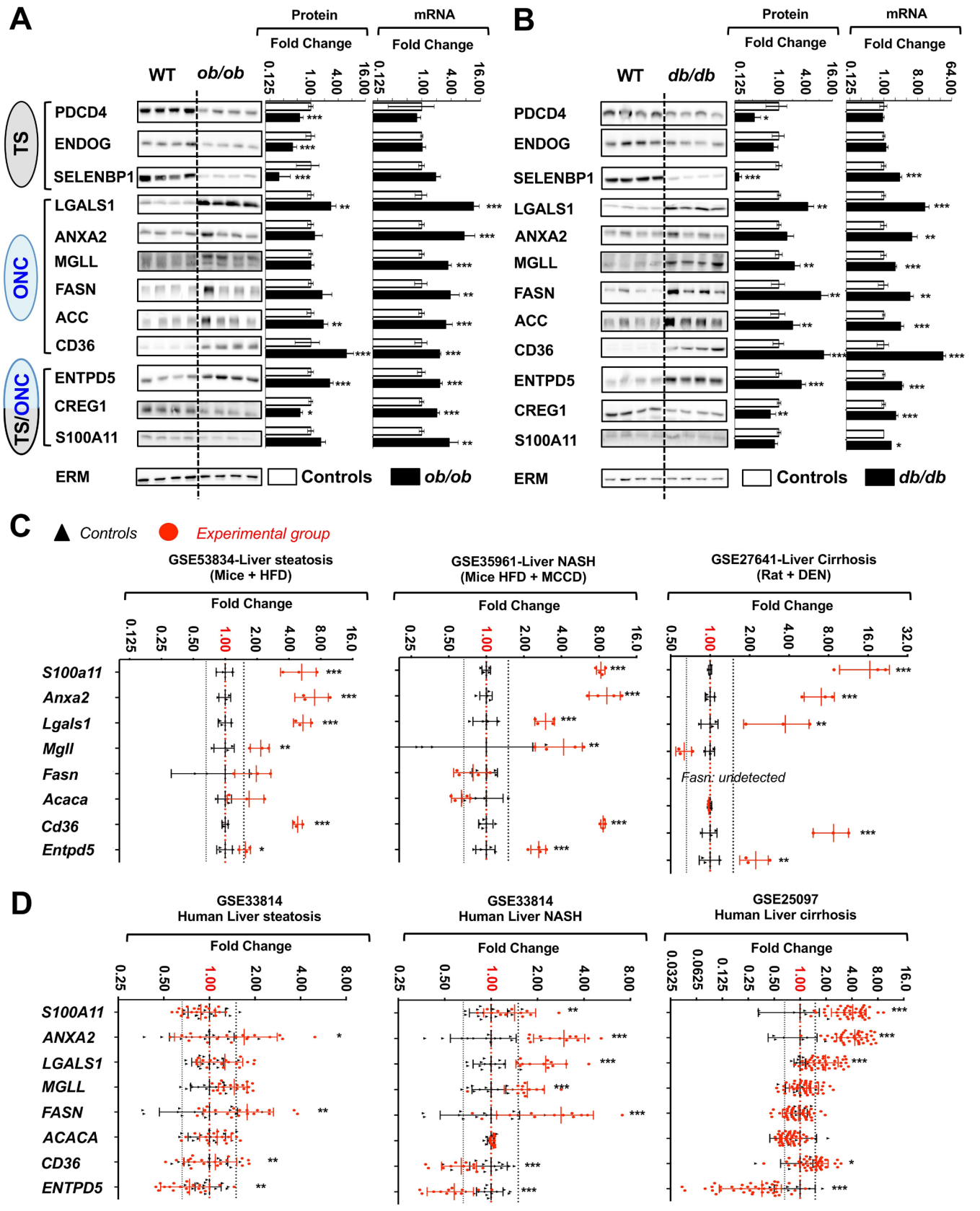

Figure 2 Hepatic TSs/ONCs expression in mice/human models of NAFLD. Representative western blots and qRT-PCR analyses of TS/ONC in 2-month-old *ob/ob* mice (A) and *db/db* mice (B). Quantifications were made on 5–6 mice/group. Cyclophilin-A was used to normalise qRT-PCR analyses. Transcriptomic datasets from Geo database were used to assess hepatic mRNA levels of TSs/ONCs with steatosis, NASH and cirrhosis in mouse/rat models (C) and in human patients (D). Data represent the mean ± SD. ***p<0.001, **p<0.01 and *p<0.05 (t-test). NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ONCs, oncogenes; qRT-PCR, quantitative real-time PCR; TSs, tumour suppressors.

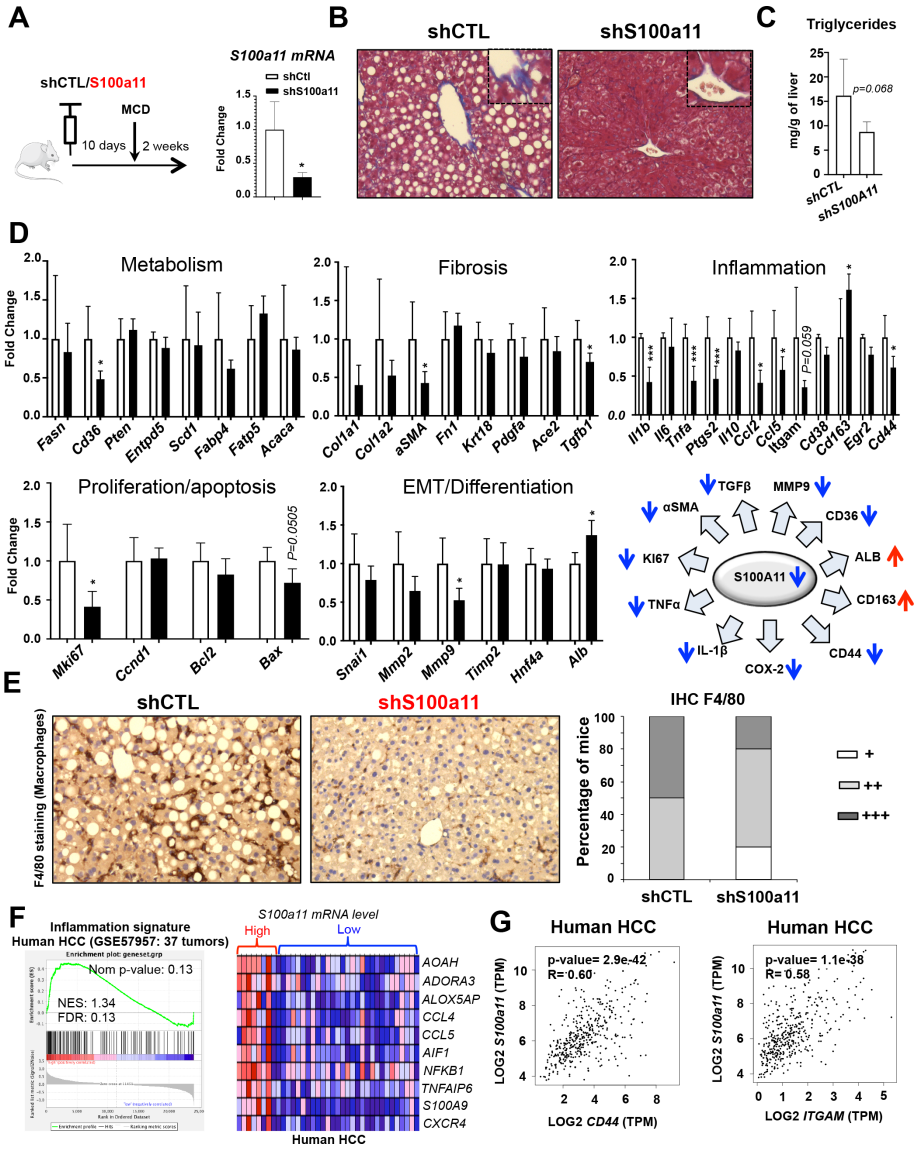

Figure 3 Liver inflammation and fibrosis in mice fed a MCD following in vivo silencing of S100A11 (A) experimental scheme and *S100a11* expression in hepatic tissues. (B) Representative trichrome Masson staining of liver sections from mice transduced with control or shS100a11-expressing hepatotropic AAV8 and fed a MCD for 2 weeks. (C) Hepatic triglycerides content in mice transduced with control or shS100a11-expressing hepatotropic AAV8 and fed a MCD for 2 weeks. (D) qRT-PCR analyses of key regulators of hepatic lipid metabolism, fibrosis, inflammation, proliferation/apoptosis and EMT/differentiation in ex-vivo hepatic tissues of mice transduced with control or *shS100a11*-expressing hepatotropic AAV8 and fed a MCD for 2 weeks. The sun-like graph presents a summary of gene whose expression is significantly modulated by S100A11 silencing. Cyclophilin-A was used to normalise qRT-PCR analyses. (n=4–5 mice for each conditions). Data represent the mean ± SD. ***p<0.001, **p<0.01 and *p<0.05 (t-test). (E) Representative immunostaining of F4/80 (macrophages) and quantifications in liver sections from mice transduced with *control/shS100a11* AAV8 and then subjected 2 weeks to a MCD. Samples were stratified according to the estimated immunoscore (staining: ±*; weak;* ++, *medium;* +++, *strong*). (F) GSEA of a human transcriptomic HCC dataset (GSE57957) for inflammation (Geneset M10617). Only tumour samples were considered for this analysis and segregated for *S100A11* expression (high vs low based on a 80th percentile separation threshold). The top 10 genes up/downregulated in patients with high *S100A11* mRNA level are represented in a heatmap. Nominal p values (nom p value), Normalised enrichment score (NES) and false discovery rate (FDR) q values are indicated. (G) Correlation analyses (Spearman) between mRNA expression of *S100A11* and macrophages markers *CD44* and *ITGAM* (CD11b) in human HCC (TCGA cohort). EMT, epithelial–mesenchymal transition; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; MCD, methyl/choline-deficient diet; NES, normalised enrichment score; qRT-PCR, quantitative real-time PCR. ; TCGA, The Cancer Genome Atlas.

inflammation-associated genes in human HCC overexpressing S100A11 (figure 3F) and expression of inflammatory mediators was strongly upregulated in tumour from LPTENKO mice (online supplementary figure S10). Finally, a positive correlation between macrophages markers (ie, *CD44* and *ITGAM*) and *S100A11* mRNA levels was observed in HCC patients (figure 3G).

S100A11 expression increases with progression of NAFLD towards HCC development

To assess whether alterations of TSs/ONCs occurring with steatosis/inflammation/fibrosis were relevant for hepatocarcinogenesis, we analysed mRNA/protein expressions in hepatic tumours from LPTENKO mice. Aberrant TS/ONC expression

was not only conserved but also even exacerbated in tumourous tissues. *S100a11*, *Anxa2* and *Lgals1* expressions, in particular, were markedly upregulated in fatty hepatocytes and their expressions further increased in HCC from 12-month and 15-month-old PTEN-deficient mice (figure 4A, online supplementary figures S11 and 12). Bioinformatic analyses revealed that these TSs/ONCs are (i) in network through protein–protein interactions or co-expression patterns suggesting a functional link in HCC development (STRING database, figure 4B, online supplementary table S4), (ii) barely mutated in human HCC (cBioPortal database: mutation rate <4%, figure 4C) and (iii) tightly connected to human HCC deregulated pathways and typical hallmarks of cancer (figure 4D and online supplementary figure S13). S100A11/ANXA2 upregulation were further confirmed in hepatic tumourous tissues of various rodent models (figure 4E), as well as in HCC induced by the carcinogen diethylnitrosamine (DEN) (figure 4F and online supplementary figure S14).

ANXA2 oncogenicity in HCC was previously described,[24] but whether S100A11 contributes to hepatic carcinogenesis was poorly investigated. Members of the S100 family were previously reported to contribute to hepatic carcinogenesis and several of them are significantly upregulated with steatosis and HCC in LPTENKO mice (online supplementary figure S15, table S5). Although *S100a11* upregulation is already detectable with steatosis in LPTENKO mice, its expression leaps in tumours at 12 months (mostly adenomas) and further increases when tumours progress from adenoma to HCC in the high inflammatory microenvironment of 12-month to 15-month-old mice (online supplementary figure S10 and S11C). Of note, in vivo downregulation of S100A11 through hepatic transduction of shRNA-AAV8 did not affect early pathological responses (eg, proliferation/apoptosis) induced by DEN 2 days post-exposure (online supplementary figure S15). Together, these observations suggest that S100A11 overexpression is functionally relevant over time mostly on tumour progression and in inflammatory conditions.

S100A11/ANXA2 upregulation is a hallmark of human high-grade HCC with poor prognosis

Protein/mRNA levels of S100A11/ANXA2 are also drastically increased in human/mouse cultured liver cancer cells (figure 5A, online supplementary figure S17). Consistent with the predicted S100A11 network, co-imunoprecipitation assays in human hepatic cancer cells further supported a direct functional interaction between S100A11 and cancer-related partners such as ANXA2, AKT1 or PTEN (online supplementary figure S18). Transcriptome data analyses of paired non-tumoural/HCC tumourous tissues from *490* patients further revealed that *22.65%* and *38.98%* of HCC display *S100A11* and *ANXA2* mRNAs upregulation (*>1.5* fold), respectively, with *63.1%* of S100A11 high-expressing HCC showing concomitant *ANXA2* upregulation (figures 5B, online supplementary figure S19A, table S6). Overexpression of *S100A11/ANXA2* was even more important in human ICC tissues (online supplementary figure S19B).

Consistent with the mRNA upregulation of *S100A11/ANXA2*, analyses of a proteomic dataset of human HCC relative to non-tumoural tissues and of human HCC from The Cancer Genome Atlas (TCGA) confirmed a similar proportion of HCC overexpressing S100A11/ANXA2 at the protein level (online supplementary figure S19C,D). Gene Set Enrichment Analyses (GSEA) of a human HCC transcriptomic dataset further indicated that S100A11 upregulation correlates with significant enrichments of genes associated with poor prognosis and poorly differentiated tumours (figure 5C). We therefore investigated protein S100A11 expression by immunohistochemistry in human HCC tissues microarrays. S100A11 was overexpressed in *15.6%* of tumours as compared with matched non-tumoural tissues (figure 5D). S100A11 was localised both in the cytoplasm and the nucleus of human HCC cells, as well as in cholangiocarcinoma cells and in HepG2/Huh7 cancer cells (figure 5D, online supplementary figure S19D-E and online supplementary table S7-S8). Importantly, S100A11 overexpression was observed mostly in poorly differentiated HCC (figure 5E), as suggested by the GSEA analysis (figure 5C), and further confirmed in (i) a transcriptomic dataset showing that *S100A11/AFP* overexpression can discriminate HCC grade 3 in contrast to other HCC markers such as *GPC3/ANXA2* (online supplementary figure S20A) and (ii) a proteomic dataset of human HCC confirming a tight relationship between S100A11 expression and high-grade HCC (online supplementary figure S20B). Accordingly, *S100A11* expression and the hepatic differentiation marker *HNF4A* were negatively correlated (online supplementary figure S20C). Furthermore, analyses of human HCC from the TCGA indicated that *S100A11/ANXA2* high expressions were associated with stages 2/3 and higher and lower frequencies of *TP53* and *CTNNB1* mutations, respectively; in agreement with specific mutations patterns reported for poorly differentiated HCC[25] (online supplementary figure S20D,E). As previously described for ANXA2,[24] patients displaying *S100A11* upregulation had also reduced overall survival, were resistant to sorafenib-based therapies and a high co-expression of ANXA2 and S100A11 was associated with a more severe prognosis (figure 5F,G).

Finally, significant amounts of circulating S100A11 protein were detected in the *sera* of mice with DEN-induced HCC (figure 5H). Bioinformatic analyses (exoRbase) further revealed that *S100A11/ANXA2* mRNAs are overexpressed in human HCC-derived exosomes (figure 5I). Consistently, both S100A11 and ANXA2 are secreted by human Huh7 cells (online supplementary figure S19F). Altogether, these data suggest that monitoring of S100A11 and ANXA2 expression may predict more accurately clinical outcomes, and that S100A11 is a potential biomarker for poorly differentiated HCC in solid and liquid biopsies.

S100A11 expression is associated with hepatocytes de-differentiation and epithelial–mesenchymal transition

S100A11 upregulation in high-grade HCC suggests a close link between S100A11 expression and hepatocytes de-differentiation or epithelial–mesenchymal transition (EMT). Accordingly, *S100a11* and *Anxa2* expression were strongly induced during de-differentiation of mouse primary hepatocyte (MPH) in culture concomitantly with downregulation of hepatocytes markers (*Serpina1*, *Alb*) (figure 6A, online supplementary figure S21A,B). Conversely, siRNA-mediated S100A11 knockdown in MPH triggered a modest but significant increase of differentiation markers (*Serpina1* and *Hnf4a*), suggesting that S100A11 silencing contributes but is not sufficient to maintain MPH differentiation (figure 6B). Differentiation of HepaRG cells was also accompanied by S100A11 downregulation (figure 6C, online supplementary figure S21C) and in silico analyses of hepatic gene expression during mouse development, indicate that *S100a11* and *Anxa2* expressions in fetal livers are strongly downregulated after birth (online supplementary figure S21D). Consistent with these observations, silencing or overexpressing

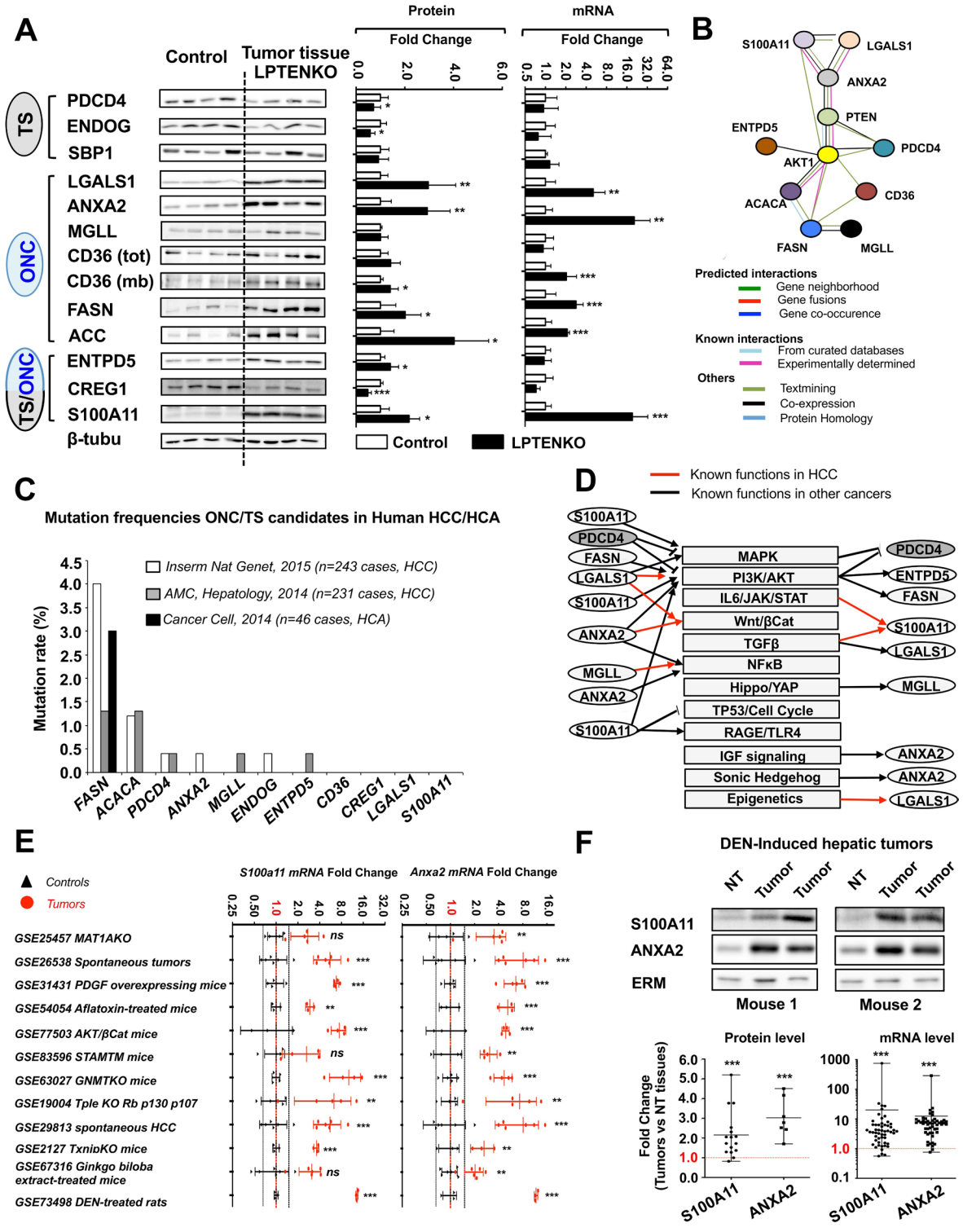

Figure 4 TS/ONC expression in tumours from 1-year-old LPTENKO mice and functional interactome bioinformatic analysis. (A) Representative western blots and qRT-PCR analyses of TSs/ONCs in tumourous hepatic tissues of 1-year-old LPTENKO mice. Quantifications are made on n=4 control mice and 6 LPTENKO mice. Cyclophilin-A was used to normalise qRT-PCR analyses. (B) Bioinformatic analysis of potential association/interactions between TSs/ONCs in humans (string database). (C) The mutation rates of TS/ONC candidates (revealed by our proteomic analysis) in human HCC were obtained with the cBioPortal database using three different studies ('HCC, *HCC, Inserm, Nat Genet 2015*'; 'HCC, *HCC, AMC, Hepatology, 2014*' and 'HCA, *HCA, Cancer Cell, 2014*'). (D) Literature-based classification of TSs/ONCs involvement in HCC-associated signalling pathways. (E) Hepatic mRNA expressions of *S100a11*/*Anxa2* in mouse/rat models of HCC (transcriptomic datasets from Geo database, online supplementary table S6). (F) Representative western blot and protein/mRNA quantifications of S100A11/ANXA2 expression in control hepatic tissues and DEN-induced HCC in 11 months old mice (western blot: n=16 tumours for S100A11 and 7 tumours for ANXA2; for qRT-PCR: n=53 tumours for S100A11 and 51 tumours for ANXA2). Cyclophilin-A was used to normalise qRT-PCR analyses. Data represent the mean ± SD. ***p<0.001, **p<0.01 and *p<0.05 (t-test and one-sample t-test for panel F). DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR; TS/ONC, tumour suppressor/oncogene.

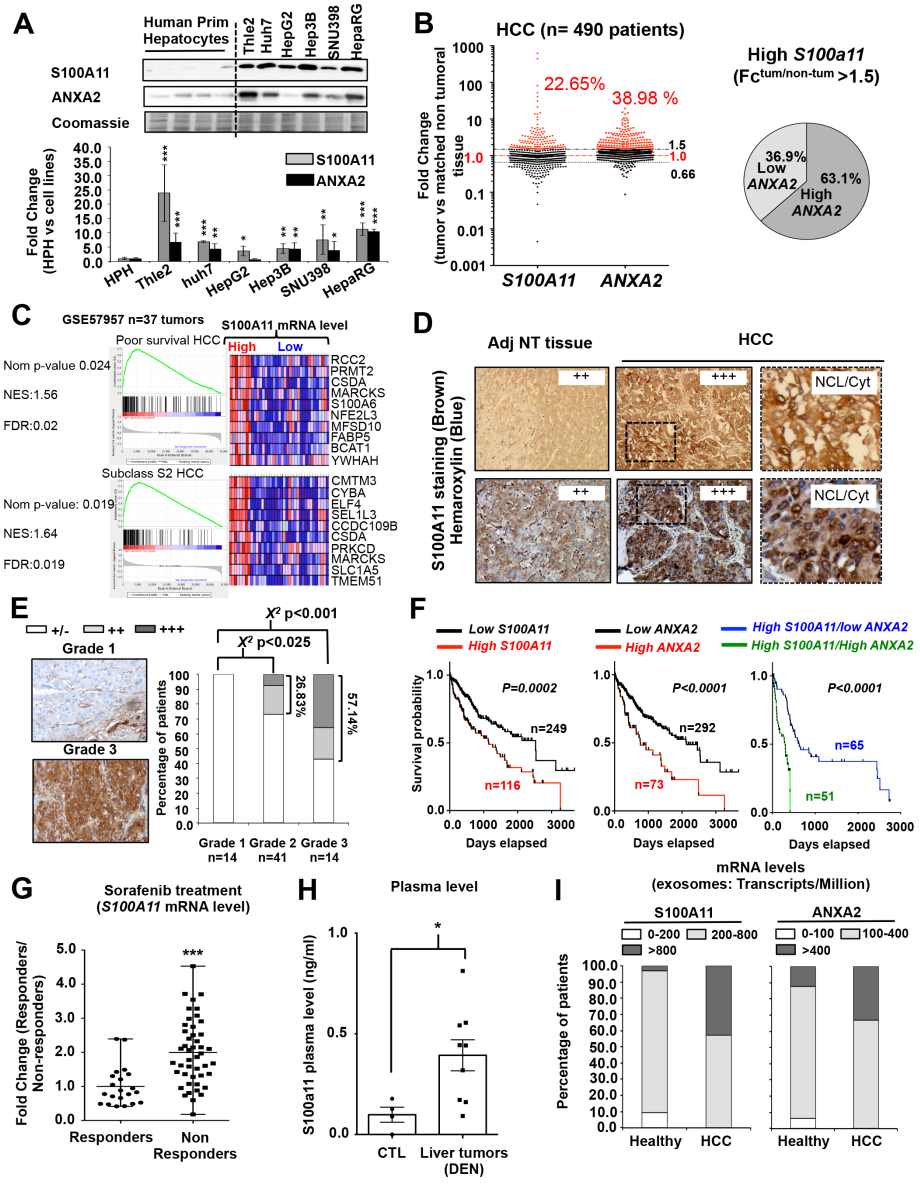

Figure 5 S100A11/ANXA2 expression, secretion and prognostic value in human HCC. (A) Representative western blot and quantifications of S100A11/ANXA2 expression in human primary and cultured hepatic cells. Western blots quantifications were normalised with Coomassie staining (B). Dot-plot and pie chart illustrations of relative fold change of *S100A11*/*ANXA2* mRNA expression between HCC and non-tumoural tissues of 490 patients from transcriptomic datasets of Geo database (online supplementary table S8). (C) GSEA of a human transcriptomic HCC dataset (GSE57957) for genes associated with a poor prognosis and poorly differentiated HCC (Geneset M3268). Only tumour samples were considered for this analysis and segregated for S100A11 expression (high vs low based on a 80th percentile separation threshold). For each gene set, the top 10 genes upregulated in the patients with high *S100A11* mRNA level are represented in the heatmaps. Nominal p values (nom p value), Normalised enrichment score (NES) and false discovery rate (FDR) q values are also indicated. (D) Representative S100A11 immunostaining of HCC from human tissue microarrays. Patients were stratified according to tumoural grade and estimated immunoscore (staining: ±; *weak;* ++, *medium;* +++, *strong*). (E) Representative illustrations of S100A11 expression in high-grade vs low-grade human HCC and Pearson χ^2/Fisher-derived analyses of *S100A11*-high expressing tumours as a function of HCC stage in human tissue microarrays. (F) Patients survival curves as a function of low versus high *S100A11* and/or *ANXA2* expression in tumour (data derived from human protein atlas and TCGA). P values were calculated with log RANK tests. (G) *S100A11* mRNA expression in HCC patients responding or not to sorafenib-based therapy. Data are derived from Geo datasets GSE109211 (online supplementary table S6). (H) ELISA determination of plasma concentrations of S100A11 in 11-months-old DEN-treated mice with HCC. Data represent the mean ± SD. ***p<0.001, **p<0.01 and *p<0.05 (t-test). (I) In silico analysis of *S100A11 and ANXA2* mRNA content in the *sera* of healthy and HCC patients-derived exosomes (exoRbase database). DEN, diethylnitrosamine; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; NES, normalised enrichment score; TCGA, The Cancer Genome Atlas.

HNF4α, a key hepatic transcription factor triggering differentiation, in hepatic cancer cells lead to either induction or repression of S100A11 expression respectively (figure 6D, online supplementary figure S21E). A negative correlation between *S100A11* and *HNF4A* mRNA levels was even observed in human HCC (online supplementary figure S21F) and HNF4α silencing in HepG2 cells decreases S100A11 mRNA stability suggesting a direct, or indirect, transcriptional control of S100A11 by

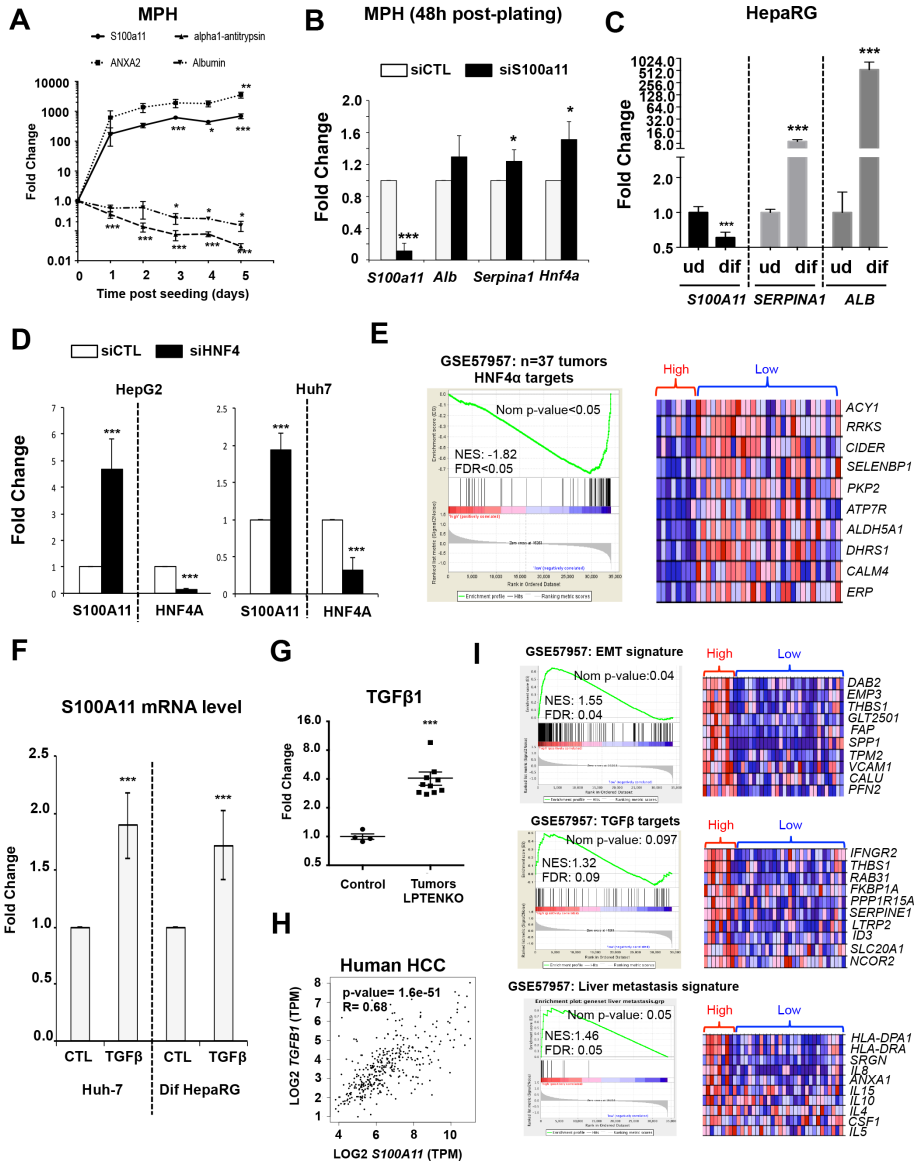

Figure 6 S100A11 is associated with hepatocytes dedifferentiation and EMT. (A) mRNA expression of alpha1-antitrypsin (Serpina1), albumin (Alb), Anxa2 and S100a11 in isolated mouse primary hepatocytes seeded on culture dishes over 5 days. (B) mRNA expression of hepatic differentiation markers and *S100A11* in mouse primary hepatocytes following S100A11 silencing by specific siRNAs 48-hour post-transfection. (C) mRNA expression of albumin, alpha1-antitrypsin (*SERPINA1*) and *S100A11* in undifferentiated (ud) versus differentiated (dif) HepaRG cells. (D) Relative mRNA levels of *HNF4a* and *S100A11* in HepG2 and Huh-7 cells 48-hour post-transfection with a siRNAs against HNF4A. (E) GSEA of a human transcriptomic HCC dataset (GSE57957) for HNF4α targets (Geneset M11410). Only tumour samples were considered for this analysis and segregated for S100A11 expression (high vs low based on a 80th percentile separation threshold). The top 10 genes up/downregulated in the patients with high *S100A11* mRNA level are represented in a heatmap. Nominal p values (nom p value), Normalised enrichment score (NES) and false discovery rate (FDR) q values are indicated. (F) Relative mRNA expression of *S100A11* in Huh-7 and differentiated HepaRG cells exposed to TGFβ1 (10 ng/mL) for 24 hours. (G) mRNA expression of *Tgfb1* (qRT-PCR) in hepatic tissues of 15-month-old LPTENKO mice and their respective controls. (H) Correlation analyses (Spearman) between mRNA expression of *S100A11* and *TGFB1* in human HCC. (I) GSEA of a human transcriptomic HCC dataset (GSE57957) for EMT signature (geneset M5930), TGFβ targets (Geneset M5896) and liver metastasis signature (Geneset M10595). 18S or cyclophilin-A were used to normalise qRT-PCR analyses. Data represent means ± SD of at least three independent experiments. ***p<0.001, **p<0.01 and *p<0.05 (t-test or one-way ANOVA for panel A). ANOVA, analysis of variance; EMT, epithelial–mesenchymal transition; GSEA, gene set enrichment analysis; NES, normalised enrichment score; qRT-PCR, quantitative real-time PCR.

HNF4α (online supplementary figure S21G). Finally, GSEA further support the relevance of this regulation by confirming a negative enrichment of HNF4α target genes in human HCC overexpressing S100A11 (figure 6E).

Likewise, TGFβ, a key inducer of EMT in hepatocytes, triggered S100A11 upregulation in hepatic cells (figure 6F). High levels of *Tgfb1* were also present in tumours of LPTENKO mice overexpressing S100A11 (figure 6G) and *S100A11* and *TGFB1* expression were positively correlated in human HCC (figure 6H). Consistent with these data, GSEA confirmed positive enrichments for gene signatures associated with TGFβ signalling, EMT and metastasis formation in human HCC overexpressing S100A11 (figure 6I). Of note, upregulation of S100A11/ANXA2 in hepatocytes did not directly result from PTEN loss, since

neither in vivo induction of hepatic *PTEN* deletion nor in vitro PTEN knockdown upregulated S100A11/ANXA2 in hepatocytes (online supplementary figure S22).

Together, these data indicate that S100A11 overexpression is tightly linked to hepatocytes de-differentiation and EMT pointing again to S100A11 as a relevant biomarker of poorly differentiated HCC.

S100A11 controls cell proliferation, survival, migration and ER stress

S100A11 functional relevance in classical cancer hallmarks was investigated by silencing S100A11 with specific siRNAs in hepatic cancer cells. S100A11 knockdown significantly reduced proliferation, an effect associated with a modest but significant increase of apoptosis (figure 7A, and online supplementary figure S23 and S24). S100A11 knockdown in HepG2 cells was also accompanied by accumulation of cells in a G0/G1 cell cycle phase, in agreement with the induction of *CDKN1A*(p21)/*CDKN1B*(p27) cell cycle inhibitors and the reduction of the proliferation marker *PCNA* (figure 7B–C). Of importance, S100A11 overexpression in low S100A11-expressing cells (SNU-398) significantly prevented apoptosis induced by clinically relevant anti-cancer drugs such as doxorubicin and sorafenib, whereas S100A11 silencing in high S100A11-expressing cells (HepG2) strongly increased doxorubicin-induced apoptosis (figure 7D–E).

Migration/invasive capacities of HepG2 cells in Boyden chamber assays were also strongly reduced in S100A11-deficient cells (figure 7F) and transcriptomic datasets analyses indicated that S100A11 was overexpressed in high (MHCC97L/HCCLM3) versus poorly (Huh7) metastatic cells (online supplementary figure S26). Consistent with these data, S100A11 silencing in HepG2 cells triggers abnormal formation of membrane ruffles/protrusions and accumulation of ERM actin-binding proteins suggesting actin cytoskeleton dynamic defects (figure 7G).[26 27] Finally, since S100A11 was previously associated with ER calcium homeostasis,[28] we investigated ER stress in S100A11-deficient HepG2 cells. Downregulation of S100A11 activated ER stress responses as supported by the induction of ER-stress associated genes (*CHOP*, *GADD34*), PERK overexpression, phosphorylation of EIF2α/p38MAPK and de-phosphorylation of ERK1/2 (figure 7H–I).

DISCUSSION

Despite intense efforts to understand signalling pathways, genetic mutations and epigenetic alterations driving HCC, the molecular mechanisms underlying HCC development in the absence of cirrhosis remain unclear.[4] Our study identifies a whole network of TSs/ONCs that are severely deregulated already with NAFLD/NASH and remain so during tumourigenesis.

Besides genetic mutations, epigenetic or post-transcriptional deregulations of TS/ONC are important drivers of hepatocarcinogenesis. Identifying relevant non-genomic alterations is of key importance for therapeutic perspectives given that frequent TS/ONC mutations characterising HCC are currently not targetable. Herein, we demonstrate that a whole network of TSs/ONCs is deregulated with early stages of NAFLD, thereby providing a microenvironment amenable to tumour development. Indeed, several members of this network were previously linked to classic cancer hallmarks including inflammation/fibrosis[16] and deregulated signalling pathways in HCC (eg, LGALS1[18] and ANXA2 for the PI3K/AKT and β-cat signalling), as well as to poor clinical outcomes (eg, Galectin-1,[18 19] PDCD4[29 30]), suggesting that these non-genomic alterations represent early important events priming hepatocarcinogenesis.

Perturbations in lipid metabolism are well-recognised hallmarks of HCC. Obesity is an important risk factor for HCC,[31 32] and dietary/genetic obesity in rodents fosters HCC development.[33] At the molecular level, reprogramming of the lipid metabolism occurs to support tumour growth and metastatic dissemination.[34] Aberrant de novo lipogenesis provides cancer cells with continuous lipid supply required for membrane production, energy generation and post-translational protein modification.[35] Consistent with our data, FASN[14] and ACC[36 37] are overexpressed in human HCC, a feature associated with reduced survival. CD36 overexpression further correlates with increased EMT in HCC suggesting a key role for CD36 in HCC migration/invasion[15] akin to human oral carcinomas.[38] Finally, other studies indicated that FASN is required for AKT/Ras-driven hepatocarcinogenesis in vivo,[14] whereas β-catenin-mutated HCC are driven by lipid oxidation.[39] Thus, *FASN*, *ACC1* and *CD36*, whose expression/activity are increased with steatosis likely, act also as potent early drivers of hepatocarcinogenesis.

TSs/ONCs deregulated with steatosis/inflammation-associated cellular stress may also foster carcinogenesis by affecting genomic stability, thereby promoting mutation. For instance, PTEN, functions as a major guardian of the genome integrity,[40] is importantly downregulated with NAFLD/NASH.[10 12 13] Similarly, FASN overexpression in human colorectal cancer cells correlates with microsatellite instability,[41] whereas ANXA2 nuclear accumulation induces chromosomal damages.[42] Together, these observations suggest that hepatic steatosis/inflammation favour genomic instability and the occurrence of mutations relevant for HCC development.

Molecular mechanisms affecting TSs/ONCs hepatic expression with NAFLD/NASH still remain poorly defined except for PTEN.[9 12 43] Epigenetic mechanisms[44] or post-translational modifications might be involved[45] in particular for TSs subject to post-transcriptional downregulation. Future studies are now required to understand how hepatic metabolic stress deregulates these important cancer drivers. Of note, we likely underestimated the impact of NAFLD/NASH on such key tumour drivers because of the limited amount of detectable proteins by proteomic analysis. Moreover, only known cancer-related factors differentially expressed in the LPTENKO proteome were considered, while other proteins with potentially unknown TSs/ONCs activities were discarded.

S100A11 is of particular interest since it is poorly expressed in healthy livers, heterogeneously induced with steatosis, but well upregulated with inflammation/fibrosis and further drastically increased, although almost never mutated, in mouse/human HCC/ICC. In addition, S100A11 overexpression is associated with poor survival, resistance to sorafenib-based therapy and with mutations, histological characteristics and gene signatures of poorly differentiated HCC.[25] Finally, S100A11 is a reliable biomarker of hepatocytes dedifferentiation and EMT, consistent with a previous study suggesting that S100A11 contributes to EMT in cholangiocarcinoma.[46] In this regard, we identified HNF4α, a master transcription factor governing hepatocytes differentiation, as a key, but likely indirect, negative regulator of S100A11. Indeed, no putative HNF4α binding motif are present within S100A11's promoter, but multiple transcription factors under the control of HNF4α and predicted to induce *S100A11* transcription, were upregulated following HNF4α silencing in hepatic cells (online supplementary figure S27).

Interestingly, combined overexpression of S100A11 and ANXA2 worsens the prognosis, indicating that assessing both

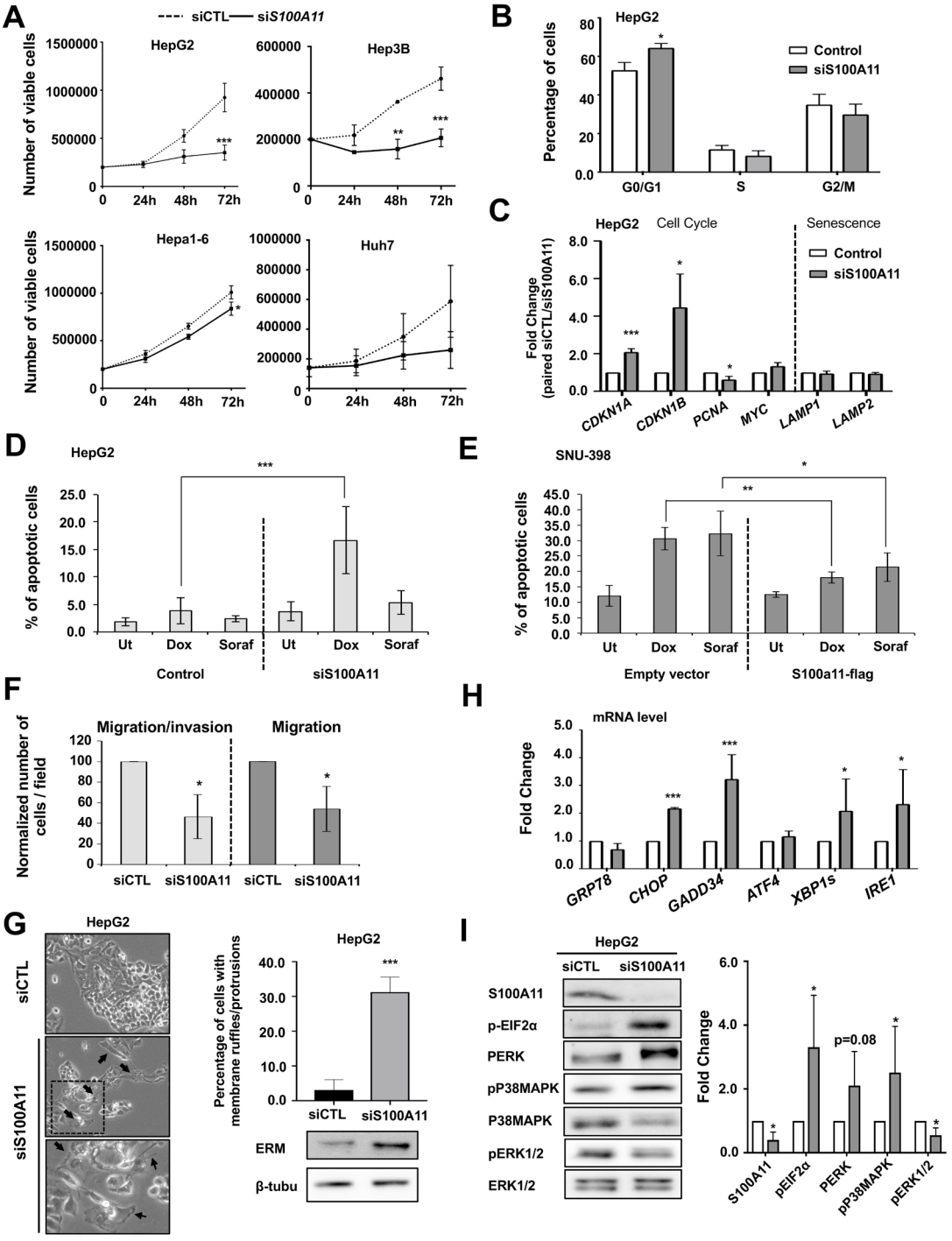

Figure 7 S100A11 affects proliferation, survival, migration/invasion and drug resistance of HCC cells. (A) Proliferation of HepG2, Hep3B, Hepa1-6 and Huh-7 cells over 72 hours following S100A11 silencing by specific siRNAs. Data represent the mean ± SD of five independent experiments. (B) Cell cycle analysis of HepG2 cells following S100A11 silencing by specific siRNAs (72-hour post-transfection). (C) Relative mRNA expression in HepG2 cells of cell cycle regulators (*CDKN1A*, *CDKN1B*, *PCNA* and *MYC*) and senescence markers (*LAMP1* and *LAMP2*) 72-hour post-transfection with specific S100A11 siRNAs. (D) Apoptosis in HepG2 cells having S100A11 silenced by siRNAs for 48 hours and then treated 24 hours with doxorubicin (0.5 µM) or sorafenib (5 µM). Apoptosis was investigated by nuclear morphology analysis. (E) Apoptosis in SNU-398 cells having S100A11 overexpressed following transfection with a plasmid encoding S100A11 for 48 hours and then treated 24 hours with doxorubicin (0.5 µM) or sorafenib (5 µM). Apoptosis was investigated by nuclear morphology analysis. (F) HepG2 cells migration/invasion in Boyden chambers following S100A11 silencing by specific siRNAs. (G) Morphology of HepG2 cells following S100A11 silencing by specific siRNAs (72-hour post-transfection). Percentage of cells displaying membrane ruffles and protrusions are represented in the histogram. Ezrin,Radixin, Moesin (ERM) expression was evaluated by western blot. (H) Relative mRNA expression of ER stress markers in HepG2 cells following S100A11 silencing by specific siRNAs (72-hour post-transfection). (I) Representative western blot analyses of ER stress markers, p38MAPK and ERK1/2 phosphorylations in HepG2 cells following S100A11 silencing by specific siRNAs (72-hour post-transfection). Data represent means ± SD of at least three independent experiments. ***p<0.001, **p<0.01 and *p<0.05 (t-test or one-way ANOVA for panel A). ANOVA, analysis of variance; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma.

biomarkers together might refine patients' outcome predictions. Of note, dedifferentiation of HCC towards a more aggressive cholangiocytic/progenitor phenotype was observed in HCC patients following trans-arterial chemoembolisation.[47] Considering the common alterations between ICC and high-grade HCC (eg, S100A11/ANXA2 overexpression), it is not clear whether these tumours are mixed HCC-CC or poorly differentiated HCC, thereby calling for cautiousness in the diagnosis and medical care of these patients.

Distinct members of the S100A11 family, that is, S100A8/9, were previously reported to promote HCC.[17] In lung,[48] ovaries[49] and colon[50] cancers, an oncogenic role for S100A11 was suggested, but in HCC only sparse and controversial in vitro evidence exist. Indeed, S100A11 silencing in Huh7 cells was described to impair cell migration,[51] whereas others suggested that TGFβ1-mediated nuclear S100A11 localisation inhibits cell cycle progression.[52] Our data provide new in vivo evidence that S100A11 upregulation fosters hepatic inflammation/fibrosis in agreement with a previous report investigating the role of S100A11 in *Toxoplasma gondii* infection.[53] Since inflammation/fibrosis are key drivers of hepatic carcinogenesis,[16] S100A11 upregulation likely fosters tumour development by promoting a favourable inflammatory microenvironment. While molecular mechanisms behind such pro-inflammatory action of S100A11 remain poorly understood, an attractive hypothesis reside in the potential of circulating S100A11 to activate RAGE signalling, a key pathway triggering monocyte/macrophage recruitment/activation and previously reported to occur in other type of cancers.[54] Both our data and available databases confirmed secretion of S100A11 by HCC cells further supporting also the relevance of S100A11 as a circulating biomarker and/or risk factor for the development of high-grade HCC. This is of importance, since biomarkers to detect HCC are still tremendously needed. Indeed, alpha-fetoprotein (AFP) is currently used in the clinic to diagnose HCC and evaluate tumour recurrence following treatment[55]; however, it is uninformative regarding tumour differentiation, malignancy and prognosis.[55] More so, whether AFP is a good serum marker for HCC in non-cirrhotic patients remains to be established.

Among the pleiotropic functions of S100A11, our data also support S100A11 as a key factor driving HCC cells proliferation and migration/invasion, while protecting them from apoptosis and ER stress. Whether S100A11 represents a potential therapeutic target for poorly differentiated HCC remains unknown; however, antitumour molecules such as erlotinib were shown to reduce *S100a11*/*Anxa2* expression in hepatic tissues from CCL4-treated mice (online supplemenary figure S28) and our data advocate an important potentiation of anti-cancer drugs, such as doxorubicin and/or sorafenib, when S100A11 expression remains low in hepatic cancer cells.

Finally, our data confirm a direct interaction of S100A11 with relevant factors for HCC development, that is, AKT1/PTEN and ANXA2. The importance of PTEN/AKT signalling in HCC is well established[56]; however, cancer-related processes governed by S100A11 interaction with ANXA2 are less clear. Our preliminary observations are consistent with previous studies reporting the formation of S100A11/ANXA2 complexes in stressed cells to maintain plasma membrane integrity, for example during cancer cell migration.[57] Other potential functions of S100A11, including (i) paracrine stimulatory effect on RAGE as discussed above,[58] (ii) interaction with nucleolin, which affects genomic stability and DNA repair,[59] (iii) regulation of calcium signalling[28] and (iv) remodelling of actin cytoskeleton in cancer cells migration,[27] might be important for hepatocarcinogenesis. S100A11 may also sustain carcinogenesis through multi-modal mechanisms including binding to intracellular partners (eg, PLP2/TACC1), which have poorly defined roles in HCC but are tightly associated with poor prognosis (online supplementary figure S29, table S9 and S10). Future studies are now required to unravel more precisely the various molecular mechanisms underlying S100A11 functions in HCC.

Altogether, our findings show that in the absence of cirrhosis, hepatic cellular stress associated with steatosis and/or inflammation/fibrosis, trigger non-genomic alterations of key TSs/ONCs, therefore providing a favourable landscape for HCC/ICC development. Identifying transcriptional or post-translational alterations of relevant TSs/ONCs in the absence of any mutations may provide new tracks for therapeutic interventions. This is the case for S100A11, which is strongly deregulated with NASH, highly abundant in liquid/solid HCC biopsies and which plays a key role in carcinogenic processes promoting tumour growth and progression. Therefore, S100A11 may represent not only a novel candidate biomarker for the conjunctional use with AFP but also a new potential target for immuno-based therapies that might even display preventive efficacy.

Twitter Jean-François Dufour @dufour_jf

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Contributors CSo: study concept and design; acquisition, analysis and interpretation of data; drafting of the manuscript; DA, FB, DD, NC, CSe, MaF, CM and A-SA: acquisition, analysis and interpretation of data. P-AC, J-FD, BH and AA: interpretation of data and critical revision of the manuscript for important intellectual content. MiF: study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, funding, study supervision. All authors critically revised the manuscript and approved its final version.

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ORCID iDs
Jean-François Dufour http://orcid.org/0000-0002-8062-1346
Michelangelo Foti http://orcid.org/0000-0001-7199-4135

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