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Abstract:	<p>Background & Aims: The tumour microenvironment shapes tumour growth through cellular communications that include both direct interactions and secreted factors. The aim of this study was to characterize the impact of the secreted glycoprotein ADAMTSL5, whose role in cancer has not been previously investigated, on hepatocellular carcinoma (HCC).</p> <p>Methods: ADAMTSL5 methylation status was evaluated through bisulfite sequencing, and publicly available data analysis. ADAMTSL5 RNA and protein expression were assessed in mouse models and HCC patient samples and compared to data from published datasets. Functional studies, including association of ADAMTSL5 depletion with responsiveness to clinically relevant drugs, were performed in cellular and in vivo models. Molecular alterations associated with ADAMTSL5 targeting were determined using proteomics, biochemistry, and RT-qPCR.</p> <p>Results: Methylome analysis revealed hypermethylated gene body CpG islands at the ADAMTSL5 locus in both mouse and human HCC, correlating with higher ADAMTSL5 expression. ADAMTSL5 targeting interfered with tumorigenic properties of HCC cells in vitro and in vivo, whereas ADAMTSL5 overexpression conferred tumorigenicity to pre-tumoral hepatocytes sensitized to transformation by a modest level of MET receptor expression. Mechanistically, ADAMTSL5 abrogation led to reduction of several oncogenic inputs relevant to HCC, including reduced expression and/or phosphorylation levels of receptor tyrosine kinases MET, EGFR, PDGFRβ, IGF1Rβ, or</p>

	<p>FGFR4. This phenotype was associated with significantly increased sensitivity of HCC cells to clinically relevant drugs, namely Sorafenib, Lenvatinib, Regorafenib. Moreover, ADAMTSL5 depletion drastically increased expression of AXL, accompanied by a sensitization to Bemcentinib.</p> <p>Conclusions: Our results point to a role for ADAMTSL5 in maintaining the function of key oncogenic signalling pathways, suggesting that it may act as a master regulator of tumorigenicity and drug resistance in HCC.</p>
Response to Reviewers:	

ADAMTSL5 is an epigenetically activated gene that confers tumorigenic properties and drug resistance in hepatocellular carcinoma

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Conflict of interest. The authors M.A., T.J.M., R.D., S.S.A., and F.M. are inventors of the patent: *“Method and kit for diagnosing and for treatment of a cancer based on the overexpression of the ADAMTSL5 gene”*.

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Author contributions

M.A.: performed the majority of the experiments with mouse HCC, methylation studies, computational work with human HCC databases, RT-qPCR on human cohort #3 samples, data analysis, and interpretation; provided input on studies and contributed to write the manuscript.

S.K.B.: generated lentivirus, contributed to molecular, biochemical, xenograft, and functional studies with mouse and human HCC cells.

A.A.: prepared samples for secretome studies, contributed to molecular, biochemical, and functional validation of outcomes.

C.S.: performed analysis on human HCC cells, contributed to molecular, functional, and methylation studies, performed bioinformatics analysis of human HCC databases, provided input on studies.

T.J.M.: performed analyses on Alb-R26Met and human HCC samples, data analysis, and interpretation; provided input on studies and on the manuscript.

1 S.R.: generated and characterized immorto-R26Met hepatocytes; performed tumour sphere
2 assays, immunostaining of cells, and Decitabine treatment of *Alb-R26^{Met}* mice; contributed to
3 xenograft and orthotopic studies.
4
5

6
7
8 F.D.: contributed to computational work with human databases, data analysis, and
9 interpretation.
10
11

12
13 S.A.: performed and analysed secretome studies.
14
15

16 R.D.: contributed to establishing the *Alb-R26^{Met}* mouse model, to the generation of lentivirus,
17 to molecular and morphological studies with HCC cells, data analysis and interpretation;
18 provided input on studies.
19
20
21

22
23
24 D.G.: performed and provided input on orthotopic studies, generated *N-Ras^{G12D}:p53null* mouse
25 tumours, prepared human cohort #4 samples, contributed to write the manuscript.
26
27
28

29
30 A.L.: performed orthotopic studies, RT-qPCR on *N-RasG12D:p53null* mouse tumours and on
31 human cohort #4 samples.
32
33
34

35
36 U.H.: provided input on studies and contributed to write the manuscript.
37
38

39 D.S.A.: contributed to studies with human HCC samples (selection of HCC cases, controls,
40 interpretation of staining).
41
42
43

44 S.S.A.: designed and supervised studies on human HCC samples; provided input on studies;
45 contributed to interpret data and to write the manuscript.
46
47
48

49
50 F.M.: designed the study, contributed to experimental work, analysed and interpreted data,
51 ensured financial support, and wrote the manuscript.
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Abstract

Background & Aims: The tumour microenvironment shapes tumour growth through cellular communications that include both direct interactions and secreted factors. The aim of this study was to characterize the impact of the secreted glycoprotein ADAMTSL5, whose role in cancer has not been previously investigated, on hepatocellular carcinoma (HCC).

Methods: *ADAMTSL5* methylation status was evaluated through bisulfite sequencing, and publicly available data analysis. *ADAMTSL5* RNA and protein expression were assessed in mouse models and HCC patient samples and compared to data from published datasets. Functional studies, including association of *ADAMTSL5* depletion with responsiveness to clinically relevant drugs, were performed in cellular and in vivo models. Molecular alterations associated with *ADAMTSL5* targeting were determined using proteomics, biochemistry, and RT-qPCR.

Results: Methylome analysis revealed hypermethylated gene body CpG islands at the *ADAMTSL5* locus in both mouse and human HCC, correlating with higher *ADAMTSL5* expression. *ADAMTSL5* targeting interfered with tumorigenic properties of HCC cells in vitro and in vivo, whereas *ADAMTSL5* overexpression conferred tumorigenicity to pre-tumoral hepatocytes sensitized to transformation by a modest level of MET receptor expression. Mechanistically, *ADAMTSL5* abrogation led to reduction of several oncogenic inputs relevant to HCC, including reduced expression and/or phosphorylation levels of receptor tyrosine kinases MET, EGFR, PDGFR β , IGF1R β , or FGFR4. This phenotype was associated with significantly increased sensitivity of HCC cells to clinically relevant drugs, namely Sorafenib, Lenvatinib, Regorafenib. Moreover, *ADAMTSL5* depletion drastically increased expression of AXL, accompanied by a sensitization to Bemcentinib.

Conclusions: Our results point to a role for ADAMTSL5 in maintaining the function of key oncogenic signalling pathways, suggesting that it may act as a master regulator of tumorigenicity and drug resistance in HCC.

Lay Summary

The environment of cancer cells has profound effects on establishment, progression, and response of a tumour to treatment. Herein, we show that ADAMTSL5, a protein secreted by liver cancer cells and overlooked in cancer so far, is increased in this tumour type, is necessary for tumour formation and supports drug resistance. *Adamtsl5* removal conferred sensitivity of liver cancer cells to drugs used in current treatment. This suggests ADAMTSL5 as a potential marker in liver cancer as well as a possible drug target.

Highlights

- *ADAMTSL5* overexpression in HCC is associated with gene body CGI hypermethylation
- ADAMTSL5 is strongly expressed in a large fraction of human HCC
- Targeting ADAMTSL5 diminishes RTK inputs and interferes with tumorigenicity
- ADAMTSL5 confers tumorigenicity to sensitized, non-transformed liver cells
- Targeting ADAMTSL5 sensitizes HCC cells to drugs currently used in the clinic

Introduction

Hepatocellular carcinoma (HCC) is the fourth-most common cause of cancer-related mortality worldwide and is growing in incidence.[1-3] It is characterized by a remarkable molecular heterogeneity among patients, with distinct (epi)genetic modifications and gene expression profile alterations.[4-6] Globally, 78 percent of liver cancer cases are secondary to chronic hepatitis B or C infections, most frequently associated with cirrhosis, a chronic disease in which fibrosis distorts liver architecture and predisposes to HCC.[6] In addition, HCC can be a sequel of alcoholic liver disease or non-alcoholic fatty liver disease (NAFLD), which are predisposing conditions whose incidence is also growing. While the knowledge of risk factors will improve likelihood of early diagnosis, current diagnostic methods show limited sensitivity and specificity, and most patients diagnosed with HCC are already at an advanced stage.[7] The perils of late diagnosis are compounded by the limited efficacy of current HCC therapies in patients with advanced-stage disease.[4, 8, 9] Sorafenib and Lenvatinib, which are first-line drugs, and second-line drugs Regorafenib, Cabozantinib, and Ramucirumab are the only approved drugs,[10] and they only marginally improve median survival . Moreover, there exist no a priori indicators of expected patient response to these treatments. Thus, there is a clear unmet need for identification of new pathways that could be targeted for therapy and for reliable biomarkers that might enable detection of early stage HCC. As in most cancers, the tumour microenvironment has a key role in HCC establishment, progression, and response to therapy.[6] Several microenvironment components affect composition and assembly of extracellular matrix (ECM), cancer cell interactions with ECM, components of the immune system, and activity of tumour-associated fibroblasts. Among these are secreted and cell-surface proteases, and several secreted molecules that associate with and modify the ECM, called matricellular proteins.[11]

1 A variety of systems (-omics) approaches have been employed for identifying new disease-
2 causing pathways and biomarkers. For example, -omics outcomes were used to stratify patients
3 for their potential response to the available therapeutic options.[8] We recently applied a
4 systems strategy to identify new regulators of tumorigenesis using a clinically relevant HCC
5 model, the *Alb-R26^{Met}* mouse,[12, 13] where we also used in vivo longitudinal imaging to
6 follow tumour evolution.[14] Among highly-ranked genes emerging from methylome analysis,
7 we were intrigued by *Adamtsl5*, a gene not previously linked to cancer. It encodes a secreted
8 glycoprotein having a bipartite structure, with an N-terminal domain similar to members of the
9 ADAMTS (A disintegrin-like and metalloprotease domain containing thrombospondin type 1
10 motif) protease family, and a C-terminal netrin domain. Since it lacks the metalloprotease and
11 disintegrin-like domains of ADAMTS proteases, it lacks catalytic activity.[15] In this regard, it
12 is a unique component of the proteome and could be regarded as a variant netrin, since like the
13 members of that family, its netrin domain is located C-terminally. Little is known about
14 ADAMTSL5, other than that it associates with fibrillin microfibrils formed in cell culture and
15 binds heparin through the netrin domain.[15] Hence, it could participate in matrix assembly,
16 tissue mechanics, and regulation of fibrillin-bound growth factors, as well as bind to
17 proteoglycans located at the cell surface or in the matrix.

18 Here, we identify a major disease and mechanistic context for this unique molecule, showing
19 that it is an epigenetically regulated oncogene in HCC, strongly associated with a subset of
20 human HCC, and more importantly, affecting the efficacy of drugs used to treat HCC. Thus,
21 the outcomes of this study have potential therapeutic value in HCC and highlight ADAMTSL5
22 as both a potential HCC biomarker and target.

Materials and Methods

Data and materials availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD016634.

***Alb-R26^{Met}* mice.** The generation of the *R26^{stopMet}* mice (international nomenclature *Gt(ROSA)26Sor^{tm1(Actb-Met)Fmai}*) carrying a conditional mouse-human chimeric *Met* transgene into the *Rosa26* locus, and the genotyping procedures were previously reported.[16-18]

Human samples. After Institutional Review Board approval, a search in a clinical database was performed for human HCC cases from 2002 to 2007 (Cleveland Clinic, USA; human cohort #5). A total of 43 HCC cases were included. Haematoxylin and eosin stained slides from 10% formalin-fixed paraffin-embedded blocks of these cases were reviewed by a liver pathologist (D.S.A.). A representative block from the tumour was identified and unstained slides on positively charged glass slides were generated for additional stains and RNAscope in situ hybridization. For RT-qPCR analyses, 18 HCC samples from Navarra University (Spain; human cohort #3) and 16 from Montpellier University (France; human cohort #4) were used.

Detailed methods can be found in the **Supplementary Information**

Results

***Adamtsl5* is hypermethylated in its gene body CGI and overexpressed in clinically relevant HCC mouse models.**

Mouse *Adamtsl5* has two CpG islands (CGIs), one in the promoter and the other in the gene body region (Fig. 1A). DNA methylation analyses revealed that *Adamtsl5* was consistently hypermethylated in the gene body CGI in *Alb-R26^{Met}* HCC compared to control livers, without a change in the DNA methylation content of the promoter CGI (Fig. 1B). This alteration was accompanied by an upregulation of *Adamtsl5* mRNA, as shown by RNA-seq analyses (Fig. 1C). Remarkably, treatment of *Alb-R26^{Met}* mice with a DNA-demethylating agent (Decitabine) significantly decreased *Adamtsl5* expression in tumours, accompanied by a consistent reduction in the methylation levels of most CpGs within its gene body (Fig. 1D, S3A, Table S1). *Adamtsl5* expression was upregulated in *Alb-R26^{Met}* tumours both at early stages of liver tumorigenesis and in advanced HCC compared to controls (wild-type or *Alb-R26^{Met}* healthy livers), correlating with increased expression of *Afp* and *Gpc3* (two well-characterized HCC markers), and *MKi67* (a proliferation marker; Fig. 1E). The low levels of *Adamtsl5* we found in the healthy mouse livers was supported by single cell RNA-seq data from the <https://tabula-muris.ds.czbiohub.org/> website,[19] in which *Adamtsl5* mRNA expression was virtually absent in liver cells, similar to *Afp* and *Gpc3*, whereas *Met* was expressed in a high proportion of hepatocytes (Fig. S1). Low *ADAMTSL5* levels in human liver were also observed (Fig. S2).

We analysed *ADAMTSL5* mRNA and protein levels in *Alb-R26^{Met}* tumours compared with control livers. In situ hybridization analysis by RNAScope revealed a consistent *Adamtsl5* mRNA upregulation in *Alb-R26^{Met}* HCC in contrast with adjacent liver tissue, where *Adamtsl5* mRNA was not detected (Fig. 1F). Furthermore, immunohistochemical analysis of proximate sections showed strong *ADAMTSL5* staining in all analysed *Alb-R26^{Met}* HCC, whereas

ADAMTSL5 was not detected in non-tumoral tissue (Fig. 1F). Upregulation of *Adamtsl5* mRNA levels was also found in another mouse model of liver tumorigenesis, in which tumours are generated by hydrodynamic gene transfer of *N-Ras*^{G12D} and Crispr/Cas9 targeting of *p53* (*N-Ras*^{G12D}:*p53*^{null}; Fig. 1G). These results indicate that *Adamtsl5* overexpression is not solely a characteristic of the *Alb-R26*^{Met} HCC model. However, *Adamtsl5* gene body CGI was not hypermethylated in the *Ras*^{G12D}:*p53*^{null} model (Fig. S3B), suggesting the existence of alternative mechanisms of *Adamtsl5* upregulation, which are presently unknown. Thus, upregulation of *Adamtsl5* mRNA and subsequent protein production consistently discriminates HCC from adjacent liver tissue by the early tumorigenic state in at least these two mouse HCC models.

ADAMTSL5 is hypermethylated in the gene body CGI and overexpressed in a large proportion of HCC patients.

Whereas mouse *Adamtsl5* has two CGIs (Fig. 1A), human *ADAMTSL5* has four CGIs: one in the promoter and three in the gene body (Fig. 2A). Analyses using HCC expression data from The Cancer Genome Atlas (TCGA) database (human cohort #1) revealed that *ADAMTSL5* mRNA levels were upregulated in 52% of HCC cases (193/371; Log₂ fold change>1; Fig. 2B). We also examined the methylation status of *ADAMTSL5* in the subgroup of HCC patients for which both methylation and expression data were available (41/371 patients). Strikingly, 86% (18/21) of patients with *ADAMTSL5* overexpression displayed hypermethylation in one of the gene body CGIs (Fig. 2C, Table S2). The incidence of *ADAMTSL5* CGI gene body methylation in HCC patients was further explored in a second cohort of 214 human samples (human cohort #2: GSE56588[20]). 55% (112/204) of these patients shared hypermethylated *ADAMTSL5* gene body CGIs (Fig. S4A, Table S3). Importantly, HCC patients with high *ADAMTSL5* levels were characterised by a shorter overall survival and of disease-free interval (Fig. 2D-E). We found that upregulation of *ADAMTSL5* mRNA levels correlated with alcohol intake rather than with other risk factors such as hepatitis C, hepatitis B, or NAFLD (Fig. 2F, S4B).

Intriguingly, we found *Adamtsl5* upregulation also in mice following alcohol consumption (Fig. S4C), strengthening a possible correlation between *ADAMTSL5* expression and alcohol intake observed in human patients. Interestingly, by searching correlations between *ADAMTSL5* expression levels and genetic alterations in HCC patients, we observed an inverse correlation between *ADAMTSL5* expression and the number of mutated genes or deletions/amplifications (Fig. 2G-H). Additionally, we found a significant difference in altered genes in the group of patients with *ADAMTSL5* overexpression compared to the other two groups (Fig. 2I-J, S5, Table S4-5). Intriguingly, *Dchs1* is a gene predominantly mutated in *ADAMTSL5* overexpressing patients (Fig. 2I, S5). No correlations were found with a series of clinical parameters that were analysed (Fig. S6-8). We also examined whether high *ADAMTSL5* expression correlated with changes of specific markers generally used to classify HCC patients[5], which were previously used to characterise the *Alb-R26^{Met}* HCC model.[12] No significant differences were found among the three HCC subgroups (Fig. S9A, Table S6). Although *Adamtsl5* mRNA was upregulated in the *Alb-R26^{Met}* HCC model, which is characterized by slightly enhanced MET levels, we did not find a correlation between *ADAMTSL5* and *MET* levels in the cohort of HCC patients (Fig. S9B). Collectively, these results indicate that high *ADAMTSL5* levels distinguish patients with specific mutations and with a predominant genetic stability, and may thus independently identify a distinct HCC patient subgroup not already classified by other HCC markers.

Next, we experimentally assessed *ADAMTSL5* mRNA levels in human HCC and control specimens using samples from two independent cohorts (human cohort #3 and #4). RT-qPCR analysis revealed upregulation of *ADAMTSL5* mRNA in 50% (9/18) and 44% (7/16) HCCs compared to adjacent non-tumorous livers (Fig. 3A-B, Table S7), similar to the HCC cohort from TCGA. Collectively, these findings show that *ADAMTSL5* is overexpressed in a large proportion of HCC patients from independent human cohorts. Immunohistochemical analysis

revealed ADAMTSL5 protein staining at moderate to high levels in sections from 30/43 analysed HCC patients, compared to adjacent non-tumour tissue (human cohort #5; Fig. 3C, Table S8). In HCC and adjacent livers, staining for ADAMTSL5 and fibrillin-1 (Fig. S10A-C), a protein previously associated with liver fibrosis, were mutually exclusive.[21] Although non-tumour regions of the liver were consistently ADAMTSL5-negative (Fig. 3C), weak staining was observed in some cirrhotic areas. The diverse staining patterns observed in ADAMTSL5-positive tumours included weak, moderate, or intense staining of tumour cells, intense staining of macrophages bordering necrotic areas and of capillary plexuses in some tumours, and weak staining in vascular smooth muscle cells (Fig. 3D, S10D). RNAscope in situ hybridization revealed *ADAMTSL5* mRNA expression by tumour cells but not by macrophages, contrasting with the immunostaining data (Fig. 3D). These observations suggest that ADAMTSL5 protein is secreted by tumour cells in a significant proportion of analysed HCC tumours, and undergoes subsequent uptake by macrophages.

ADAMTSL5 is required for tumorigenic properties of HCC cells.

We generated *Alb-R26^{Met}* HCC cell lines from distinct tumours[12] (Fig. 4A), which reproducibly showed high mRNA and protein levels of ADAMTSL5 (Fig. 4B, S11A). Consistent with reported secretion by cells,[15] ADAMTSL5 was detected in the conditioned medium of *Alb-R26^{Met}* HCC cells (Fig. 4B). The consistent expression in *Alb-R26^{Met}* HCC cells prompted us to explore whether ADAMTSL5 influences HCC cell tumorigenic properties. *Adamtsl5* expression was reduced by shRNA targeting sequences in *Alb-R26^{Met}* HCC cells (Fig. 4C, S11B-E), which led to a striking switch in cell morphology from epithelial-like to fibroblast-like, and loss of ZO-1 and E-Cadherin expression (Fig. 4D; S11F-H).

We assessed whether the fibroblast-like shape acquired by HCC cells following *Adamtsl5* targeting corresponded to an epithelial-mesenchymal transition (EMT) or to the acquisition of a “fibroblast-like” identity, by following the expression levels of specific markers through RT-

qPCR. HCC^{shAdamtsl5} cells, while losing *Cdh1* (E-cadherin) expression, gained only the expression of one mesenchymal marker, *Snail*, but not of *Cdh2* (N-Cadherin), *Vim* (Vimentin), *Slug*, *Zeb1*, *Zeb2*, and *Twist1* (Fig. 5A, S12A-S13). Concerning the TGF β pathway, beside a slight increase in the expression of *Tgfb1* and *Tgif2* (and *Snail*), we observed similar levels of *Smad2* and *Mtss1* (beside *Twist1*, *Vim*, *CD44*; Fig. 5B, S12B-S13) and a similar response to TGF β 1 stimulation in HCC^{shAdamtsl5} and control cells (Fig. S12G). Interestingly, HCC^{shAdamtsl5} cells acquired expression of fibroblast markers, such as α -*Sma*, *Fsp1* and *Fbn1* (Fibrillin-1; Fig. 5C, S12C-S13). We further explored the fibroblast-like identity and found that HCC^{shAdamtsl5} cells acquired expression of *Col3a1*, *Colla1*, *Pcolce*, which are markers of scar-associated mesenchymal cells (SAMES), while losing *Krt19* expression (Fig. 5E, S12E-S13). Furthermore, HCC^{shAdamtsl5} cells lost expression of *Afp* (HCC marker) as well as of *Prom1*, *Epcam*, and *Cdh1* (considered as cancer stem cell markers; Fig. 5D-F, S12D-F-S13). Collectively, these results indicate that loss of *Adamtsl5* expression in HCC cells confers molecular/morphological fibroblast-like characteristics rather than triggering a bona fide EMT.

Functionally, we found that *Adamtsl5* downregulation interfered with the ability of *Alb-R26^{Met}* HCC cells to form colonies in anchorage-independent assays (Fig. 6A-B, S14A) and tumour spheroids upon growth in self-renewal conditions (Fig. 6C), yet without major changes in cell viability (Fig. S14B). HCC^{shAdamtsl5} cells migrated to a greater degree (Fig. S14C-E), consistent with the acquired fibroblast-like morphology (Fig. 4D), although they were not as invasive as controls (Fig. S14F-G). Interestingly, the ability of HCC^{shAdamtsl5} cells to form colonies and tumour spheroids, but not their morphological change, was significantly restored when they were exposed to conditioned medium from HCC cells overexpressing human *ADAMTSL5* (Fig. 6D, S15).

Next, we assessed *ADAMTSL5* expression and requirement in human HCC cell lines. Analysis of RNA-seq data from a panel of human HCC cells[22] revealed consistently high *ADAMTSL5*

1 levels in all cell lines (Fig. 6E). RT-qPCR and western blot studies performed on a subset of
2 these human HCC cells showed consistent ADAMTSL5 expression, although at variable levels
3
4 (Fig. 6F, S16A). We assessed the ADAMTSL5 requirement of human HCC cells by silencing
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6 it by shRNA in three HCC cell lines, SNU449, MHCC97H, and HLF. *ADAMTSL5*
7
8 downregulation hampered the ability of these cell lines to form colonies in anchorage-
9
10 dependent or -independent growth assays (Fig. 6G-I, S16B-E). Collectively, these findings
11
12 demonstrate that ADAMTSL5 expression is necessary for HCC cells to achieve their full
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14 tumorigenic properties in vitro.
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21 **ADAMTSL5 is required for tumorigenic properties of HCC cells in vivo and its**
22 **overexpression is sufficient to transform sensitized hepatocytes.**
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26 Next, we investigated whether *ADAMTSL5* expression is a requirement for HCC cells
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28 tumorigenicity in vivo using xenografts. *Alb-R26^{Met}* HCC and *Alb-R26^{Met}* HCC^{shAdamtsl5} cells
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30 were injected subcutaneously into the flank of nude mice and tumour formation was followed
31
32 over time. Compared to controls, HCC^{shAdamtsl5} cells showed a striking reduction in tumour size
33
34 and the percentage of animals that developed tumours (Fig. 7A-C). Additionally, we assessed
35
36 the requirement for ADAMTSL5 in an experimental setting that recapitulated the liver tumour
37
38 environment by performing orthotopic injections of *Alb-R26^{Met}* HCC^{shControl} and HCC^{shAdamtsl5}
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40 cells intra-hepatically in mice. Remarkably, in contrast to controls, HCC^{shAdamtsl5} cells did not
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42 form tumours (Fig. 7D, E), consistent with reduced in vitro tumorigenicity, loss of *Afp* and
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44 cancer stem cell marker expression (Fig. 5-6).
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51 Next, we asked whether *ADAMTSL5* overexpression would confer tumorigenicity to cells
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53 otherwise incapable of forming tumours. For this purpose, we chose a cellular context in which
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55 enhanced wild-type MET RTK levels, although not sufficient on their own, provide a sensitized
56
57 genetic background for testing cooperativity with another pro-tumorigenic hit (Fig. 7F).[23]
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59 Whereas *immorto-R26^{Met}* hepatocytes did not form tumours in immuno-compromised mice,
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human *ADAMTSL5* overexpression conferred in vivo tumorigenicity to these cells (Fig. 7G-I). Collectively, these in vivo studies underline the oncogenic properties of ADAMTSL5 in HCC.

ADAMTSL5 expression correlates with high dosage of several oncogenes relevant to HCC.

We performed mass spectrometry for unbiased comparison of the secretome of HCC^{shControl} and HCC^{shAdamtsl5} cells. Excluding ribosome-related proteins frequently detected in such analyses, we found 614 proteins with differential abundance in the two secretomes: 348 upregulated and 266 downregulated in HCC^{shAdamtsl5} cells compared to controls (Fig. 8A-B, Table S9). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, several cancer-related pathways were significantly enriched, such as axon guidance (frequently overrepresented in cancer), ECM-receptor interaction and adhesion components, proteoglycans and glycans (Fig. 8C, Table S9). These pathways appear to be consistent with ADAMTSL5 effects on cell morphology and tumorigenicity, and indicated that loss of tumorigenicity following *Adamtsl5* knock-down was accompanied by depletion of several oncogenic signals. We selected some proteins for biochemical assessment in the secretomes and cell lysates of HCC^{shControl} and HCC^{shAdamtsl5} cells (Fig. 8D, S17A). We found a striking reduction of the RTK MET extracellular domain in the medium and of full-length MET in HCC^{shAdamtsl5} cell lysates (Fig. 7D-E, S17E). Protein levels of EPHA2 and EPHA4, two other RTKs also implicated in HCC,[24, 25] were reduced (although levels of a smaller band detected with an EPHA4 antibody in cell lysates were increased; Fig. 8D-E, S17E). Additionally, UNC5B (a netrin-receptor, which is a prognostic marker of HCC; Fig. S17B) and LPHN3 (Latrophilin3, which interacts with UNC5B and regulates cell adhesion[26]), each a trans-membrane protein, were less abundant in the secretome of HCC^{shAdamtsl5} cells, but remained unchanged in cell lysates (Fig. 8D-E). Furthermore, we found a reduction of cleaved products of FAT1 (which acts as an oncogene in HCC[27]) in the medium, likely resulting from extracellular shedding [28] (Fig.

S17C-D). We also detected an upregulation of biglycan (a small leucine-rich repeat proteoglycan) and Sulf-2 (an extracellular modulator of heparan sulfate that modifies binding sites for signalling molecules) in HCC^{shAdamtsl5} cells (Fig. 8D-E, S17E). Remarkably, in HCC^{shAdamtsl5} cells we found a striking upregulation of AXL (Fig. 8D-E, S17E), a RTK whose upregulation is frequently associated with drug resistance in several tumour types,[29] including HCC response to Sorafenib.[30] Next, we asked whether the changes in protein levels observed occurred at a transcriptional level. RT-qPCR analyses revealed no transcriptional alteration in *Met*, *EphA2*, *Unc5B*, and *Lphn*., whereas *Bgn*, *Sulf2*, and *Axl* were upregulated and *EphA4* was downregulated (Fig. 8F). Intrigued by the remarkable reduction of levels of RTKs relevant to HCC, we analysed the expression and phosphorylation levels of other RTKs with essential roles in HCC tumorigenicity and resistance to treatment. Besides a strong downregulation of MET expression and phosphorylation in HCC^{shAdamtsl5} cells, we found reduced phosphorylation of EGFR, PDGFR β , and IGF1R β (Fig. 8G, Fig. S17E). Moreover, while the protein levels of EGFR and IGF1R β were unchanged, PDGFR β protein levels were increased (Fig. 8G, Fig. S17E), similarly to its mRNA (Fig. 8H). Consistent with the loss of MET and EGFR phosphorylation, two main upstream regulators of GAB1, we observed downregulation of GAB1 phosphorylation (Fig. 8G, Fig. S17E). Additionally, we found depletion of FGFR4 (Fig. 8G-H), another key RTK in HCC.[31] Collectively, these findings suggest that ADAMTSL5 may act as a master regulator of tumorigenicity by affecting oncogenic signalling.

ADAMTSL5 depletion sensitizes HCC cells to drugs used for HCC treatment in the clinic.

The severe depletion of several RTKs relevant to tumorigenicity and resistance of HCC cells to drugs used for HCC treatment in the clinic prompted us to explore whether ADAMTSL5 downregulation influenced HCC cell sensitivity to these drugs. The *Alb-R26^{Met}* HCC cellular system was particularly appropriate for addressing this question because of its resistance to

1 Sorafenib, which was previously reported.[12] We compared the sensitivity of HCC^{shAdamtsl5}
2 and control cells to several multi-kinase inhibitors: Crizotinib, Lenvatinib, Regorafenib, and
3 Sorafenib. Because of the striking upregulation of AXL observed in *Adamtsl5*-downregulated
4 cells, we also assessed the effects of Bemcentinib, an inhibitor of AXL (Table S10). HCC^{shControl}
5 cells were either totally resistant (Lenvatinib) or displayed limited sensitivity when drugs were
6 used at high dosage (Fig. 8I). In contrast, HCC^{shAdamtsl5} cell viability was significantly decreased
7 when cells were exposed to Crizotinib, Lenvatinib, Regorafenib, and to a lesser extent, to
8 Sorafenib (Fig. 8I). Remarkably, Bemcentinib treatment drastically interfered with
9 HCC^{shAdamtsl5} cell viability whereas no effect was observed in control cells (Fig. 8I). Overall,
10 our results illustrate that ADAMTSL5 targeting in HCC cells leads to a profound alteration of
11 several oncogenic inputs such as those linked to RTKs, and confers sensitivity to clinically
12 relevant drugs.
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Discussion

Lack of efficient therapies for HCC provides a strong incentive for seeking new disease pathways and more reliable biomarkers, notably those that can be used for diagnosis of early stages of HCC. Biomarkers for early detection and patient stratification will be even more important in the future, when additional first- and second-line therapies for liver cancer may become available. Here, we identified *ADAMTSL5* upregulation in a large percentage of HCC patients from several independent cohorts: according to RNA-seq data or our RT-qPCR analyses, ranging from 44 to 52% of human HCC patients; according to immunohistochemistry analysis, 70% of HCC patients. *Adamtsl5* upregulation was reproduced in the *Alb-R26^{Met}* HCC model and importantly, its increased expression was already evident during early liver tumorigenesis. *Adamtsl5* upregulation was similarly detected in *N-Ras^{G12D};p53^{null}* mouse liver tumours, indicating that its overexpression is not restricted to a single genetic trigger during mouse tumorigenesis. We have shown, both in mouse and human tumours, that augmented *ADAMTSL5* expression correlates with hypermethylation of its gene body CGI, an epigenetic mechanism that we recently reported to be involved in the regulation of oncogene expression.[13] Thus, our data suggest that hypermethylation of gene body CGI is predictive, although not exclusively, of high *ADAMTSL5* expression in HCC. While these data require further validation in large patient cohorts, they imply a potential clinical significance of *ADAMTSL5* upregulation in a subgroup of patients and might qualify *ADAMTSL5* as a potential HCC biomarker. Interestingly, in situ analysis indicated that *ADAMTSL5* was produced by HCC cells in the *Alb-R26^{Met}* HCC model, as well as in roughly half of human HCC cases. In agreement with the described heterogeneity of HCCs and with mRNA expression observed in about half of the cases annotated by TCGA, we found that a significant proportion of human HCC samples stained negative for *ADAMTSL5*. However, one caveat of analysing biopsies, either for RNA content or by staining, is that of sampling variability, i.e. samples may

1 come from ADAMTSL5-negative areas of heterogeneous, often multifocal tumours that may
2 contain expressing cells elsewhere. Therefore, it is noteworthy that as a secreted protein,
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4 ADAMTSL5 is a potential candidate biomarker that is accessible through liquid biopsy. Indeed,
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6 liquid biopsy can overcome limitations of tissue biopsy, since it is expected to represent the
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8 entire molecular picture of a patient's malignancy. Future studies will determine if measuring
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10 plasma ADAMTSL5 levels and/or hypermethylation of the gene body *ADAMTSL5* CGI in
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12 circulating cell-free DNA could be useful as a HCC biomarker. It will also be important to
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14 ascertain if human HCC can be stratified by ADAMTSL5 status to uncover histological,
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16 precancerous or risk associations, which may contribute to a better-informed choice of
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18 therapeutic options.
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24 We illustrated by a series of in vitro and in vivo assays that increased levels of ADAMTSL5
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26 are associated with tumorigenic properties of HCC cells. Indeed, ADAMTSL5 targeting
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28 interfered with HCC cell anchorage-dependent and -independent growth, tumour spheroid
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30 formation and with orthotopic and subcutaneous xenograft growth. The oncogenic function of
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32 ADAMTSL5 is further supported by our data showing that its overexpression conferred
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34 tumorigenicity to sensitized, non-transformed liver cells. Collectively, these results indicate that
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36 ADAMTSL5, which is produced by HCC cells, may be a key factor determining their
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38 tumorigenicity. Of note, the secreted ADAMTSL5 acts in a non-cell autonomous fashion, as
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40 demonstrated by the partial restoration of the phenotype of HCC^{shAdamtsl5} cells by the
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42 conditioned medium of cells over-expressing ADAMTSL5. Importantly, since the secretome
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44 of ADAMTSL5-expressing cells is profoundly altered, it is thus possible that some of its
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46 oncogenic effects are indirect.
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53 Interestingly, increased *ADAMTSL5* methylation was recently reported in chemotherapy-
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55 resistant acute lymphoblastic leukaemia patients.[32] However, neither the genomic location of
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57 such hypermethylation nor the consequences for *ADAMTSL5* expression in acute lymphoblastic
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leukaemia patients have been investigated. In addition, recent studies implicated ADAMTSL5 in pathologies other than cancer.[33]

In summary, we have shown that *ADAMTSL5*, which is overexpressed in a significant proportion of HCC patients, has an essential role in HCC pathogenesis. Our results suggest that ADAMTSL5 is a potential master regulator of HCC, acting upstream of several key oncogenic pathways, including RTKs, such as MET, EGFR, GAB1, PDGFR β , IGF1R β , and FGFR4. Intriguingly, ADAMTSL5 targeting results in decreased activation of several RTK signals, irrespectively on changes of their protein levels. Strikingly, ADAMTSL5 depletion gives rise to a dramatic increase of AXL. It is tempting to speculate that upregulation of AXL may occur in response to loss of several other RTK inputs. This interpretation is supported by the acquired vulnerability of HCC^{shADAMTSL5} to AXL inhibition, suggesting a novel therapeutic strategy for a defined subset of HCCs. From a general perspective, a number of RTK-targeting drugs are now available and our results suggest that some could be repurposed for treating *ADAMTSL5*-overexpressing HCC, especially when used in combination with agents targeting ADAMTSL5, which remain to be developed.

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Figure Legends

Fig. 1. *Adamtsl5* is hypermethylated in the gene body CGI and overexpressed in *Alb-R26^{Met}* tumours compared with control livers. (A) Schematic representation of the mouse *Adamtsl5* locus (blue: exons; CGIs: green). (B) Methylation levels of the *Adamtsl5* gene body and promoter CGIs in control livers and *Alb-R26^{Met}* tumours. Note significantly higher *Adamtsl5* methylation levels in gene body CGI in *Alb-R26^{Met}* tumours, with no change in the promoter CGI. (C) *Adamtsl5* mRNA levels in *Alb-R26^{Met}* tumours versus controls. (D) Methylation levels of CpGs within the *Adamtsl5* gene body CGI (left) and *Adamtsl5* expression levels (right) in tumours dissected from *Alb-R26^{Met}* mice untreated (red) or treated with decitabine (green). (E) mRNA expression levels of *Adamtsl5*, *Afp*, *Gpc3* (two HCC markers), and *Mki67* (proliferative marker). Note *Adamtsl5* upregulation in *Alb-R26^{Met}* early tumours (smaller than 3mm) and HCC. (F) *Adamtsl5* mRNA (left) and protein (right) in *Alb-R26^{Met}* tumours versus adjacent control livers. (G) Increased *Adamtsl5* mRNA levels in *N-Ras^{G12D}:p53^{null}* mouse tumours versus adjacent livers. n.s.:not significant; *:P<0.05; **:P<0.01; ***:P<0.001 (Mann-Whitney: B,C,E,G,H; Kruskal-Wallis: D).

Fig. 2. *ADAMTSL5* expression correlates with hypermethylated gene body CGIs and with a worse prognosis in human HCC. (A) Scheme of the human *ADAMTSL5* locus (blue: exons; CGIs: green). (B) *ADAMTSL5* mRNA levels in HCC patients (cohort #1). (C) *ADAMTSL5* mRNA (middle) and methylation (bottom) levels in HCC patients. Bottom: Hypermethylated CGI (black line) in the *ADAMTSL5* promoter and gene body in the corresponding patients. Note hypermethylated gene body CGIs in most HCC patients with *ADAMTSL5* overexpression (86%, 18/21). (D-E) Kaplan-Meier curves reporting the probability of the overall survival (D) and disease-free interval (E) according to *ADAMTSL5* levels. (F) Diagram reporting the presence (blue line) of major HCC risk factors. HCC patients with higher *ADAMTSL5* mRNA levels showed a significant, although not exclusive, association with alcohol consumption. (G-

H) Number of mutated genes (G) or deletions/amplifications (H) according to *ADAMTSL5* levels (cohort #1). (I) Heatmap reporting genes mutated only in one or two groups corresponding to *ADAMTSL5* levels, taking into consideration the top 10 ranked positions determined by the percentage of patients carrying mutations in the indicated genes. Red and green: upper and lower gene ranking (corresponding to high and low percentage of patients), respectively. White: absence of mutations in patients. On the right, *TP53* and *CTNNB1* (among the most frequently mutated genes) are also reported. (J) Heatmap reporting genes with copy number alterations in the HCC patient subgroups corresponding to *ADAMTSL5* levels, taking into consideration the top 10 ranked positions determined by the percentage of patients carrying genetic alterations of the indicated genes. Detailed information about altered genes in the three groups are reported in Figure S5 and Table S4-5. Two-way ANOVA followed by Tukey multiple comparison: statistical significance between UP versus DOWN groups, DOWN versus NO CHANGE (I); between UP versus DOWN groups, UP versus NO CHANGE, DOWN versus NO CHANGE (J). $P < 0.0001$.

Fig. 3. *ADAMTSL5* upregulation and *ADAMTSL5* localization in human HCC patient cohorts. (A-B) *ADAMTSL5* mRNA upregulation in HCC samples versus adjacent non-tumorous liver (cohort #3: 50%; cohort #4: 44%). (C) Immunohistochemical analysis revealed strong *ADAMTSL5* levels in HCC regions, but not adjacent non-tumorous liver. *ADAMTSL5* staining was localized to HCC cells and to macrophages in necrotic areas. (D) *ADAMTSL5* protein and mRNA in proximate HCC sections. Note *ADAMTSL5* RNA expression in HCC cells (arrowheads), but not in macrophages (arrows). *ADAMTSL5* protein in macrophages indicates its possible uptake by these cells during clearance of necrotic areas.

Fig. 4. *ADAMTSL5* is expressed in *Alb-R26^{Met}* HCC cells and its downregulation leads to a switch from epithelial- to fibroblast-like characteristics. (A) Scheme of *Alb-R26^{Met}*

HCC^{shAdamtsl5} cell generation. (B) ADAMTSL5 protein levels in cell lysates and in conditioned medium (CM) of HCC3, HCC13, and HCC14. CM from untransfected HEK cells or HEK cells transfected with a human *ADAMTSL5* (hA) was used as the control. (C) Down-regulation of ADAMTSL5 protein levels in *Alb-R26^{Met}* HCC13^{shControl} (cntr) and HCC13^{shAdamtsl5} cells (four clones). Two different *Adamtsl5* shRNAs were used (sh3 and sh1). Equal loading is shown (Ponceau). (D) Immunofluorescence showing loss of ADAMTSL5, ZO1, and E-Cadherin in *Alb-R26^{Met}* HCC13^{shAdamtsl5} cells versus controls. HCC13^{shAdamtsl5} cells acquired a fibroblast-like phenotype.

Fig. 5. ADAMTSL5 downregulation in HCC cells leads to a switch in the expression of several cellular markers. mRNA levels of the indicated genes in *Alb-R26^{Met}* HCC^{shControl} (cntr) and HCC^{shAdamtsl5_clone3.7} (sh3.7) cells. Genes correspond to markers of epithelial/mesenchymal cells (A), TGFβ pathway (B), fibroblasts (C), HCC (D), fibroblast subtypes (E), cancer stem cells (F). *:P<0.05; **:P<0.01; ***:P<0.001 (Student t-test). Heatmaps report downregulated (green), upregulated (red) and unchanged (black) mRNAs, or no expression (grey). Values are reported as Log₂ fold change of expression levels in HCC^{shAdamtsl5_clone3.7} compared to controls (complementary data: Figure S12-S13).

Fig. 6. ADAMTSL5 is required for in vitro tumorigenic properties of mouse and human HCC cells. (A-B) Reduced colony formation by *Alb-R26^{Met}* HCC^{shAdamtsl5} cells in anchorage-independent growth assays. Note significant restoration of tumorigenic properties after exposing HCC^{shAdamtsl5} cells to the HCC secretome with overexpressed hADAMTSL5 (CM+hA). (C) Numbers of tumour spheroids formed by HCC13^{shAdamtsl5} cells and controls. (D) Rescue of tumour spheroid formation upon exposing HCC13^{shAdamtsl5} cells to a HCC secretome with hADAMTSL5 overexpression (CM+hA). (E) *ADAMTSL5* mRNA levels in a panel of human HCC cells, based on data from [22]. (F) ADAMTSL5 protein levels in conditioned

medium and cell lysates (HCC13 cells used as controls). (G-I) *ADAMTSL5* downregulation in SNU449 cells and its effects on cell tumorigenicity. (G) Protein levels in conditioned medium of SNU449 cells carrying a shRNA targeting sequence (sh1 or sh2) versus controls. Equal loading is shown (Ponceau). (H) Numbers of colonies formed by SNU449^{shAdamtsl5} cells in anchorage-dependent growth assays. (I) Photographs and graphs reporting colony numbers and colony size formed by SNU449^{shAdamtsl5} cells in anchorage-independent growth assays. *:P<0.05; **:P<0.01; ***:P<0.001 (Student-t test: A; one-way ANOVA: B,C,D; Mann-Whitney: H,I).

Fig. 7. *ADAMTSL5* expression confers tumorigenic properties to cells in vivo. (A-C) Images of dissected tumours (A) and quantification of mean tumour volume over time (B) and at 8 weeks (C) in mice injected subcutaneously with *Alb-R26^{Met}* HCC13 and HCC^{shAdamtsl5} cells. In (C), grey dots correspond to tumours dissected earlier than 8 weeks for ethical reasons. (D-E) Images of dissected livers (arrows indicate tumours) and quantification of tumour volume at 5 weeks following orthotopic injection with *Alb-R26^{Met}* HCC13^{shControl} and HCC^{shAdamtsl5} cells. (F) Scheme reporting the establishment of *immorto-R26^{Met}* sensitized hepatocytes without and with human *ADAMTSL5* (h-*ADAMTSL5*) expression (*immorto-R26^{Met}* hepa^{overADAMTSL5}). (G-I) Images of mice (G), quantification of mean tumour volume over time (H) and at 11 weeks (I) in mice injected subcutaneously with *immorto-R26^{Met}* control hepatocytes (hepa) and *immorto-R26^{Met}* hepatocytes^{overADAMTSL5} (hepa^{overADAMTSL5}). Arrows indicate tumours. *:P<0.05; **:P<0.01; ***:P<0.001 (Mann-Whitney test).

Fig. 8. *ADAMTSL5* downregulation leads to suppression of several oncogenic signals in HCC cells. (A-B) Volcano plot (A) and heatmap (B), reporting proteins found significantly downregulated or upregulated in the secretome of *Alb-R26^{Met}* HCC^{shAdamtsl5_clone3.7} compared with HCC^{shControl} cells. Orange dots in (A) correspond to proteins biochemically validated by

western blots. (C) Enrichment KEGG pathway for differentially abundant proteins in the secretome of HCC^{shAdamts15_clone3.7} and HCC^{shControl} cells. (D) Graphs reporting the values from mass spectrometry analysis for the biochemically validated proteins (shown in E). 5 independent biological replicates each with 3 experimental replicates were used. Some of the proteins were undetectable because of low levels. (E) Protein levels in the secretome and cell lysates of HCC^{shControl} (cntr) and HCC^{shAdamts15_clone3.7} (sh3.7) cells. For secretome analysis, samples were normalized to protein in cell lysates. (F) mRNA levels of the indicated genes. (G) Protein expression and phosphorylation levels. E and G: quantifications in Table S10D. (H) mRNA levels of the indicated genes. (I) Cell viability in the absence (NT) or in the presence of the indicated drugs. ns.: not significant; *:P<0.05; **:P<0.01; ***:P<0.001 (student t-test: D,F,H; two-way ANOVA: I).

Figure 1 to 8

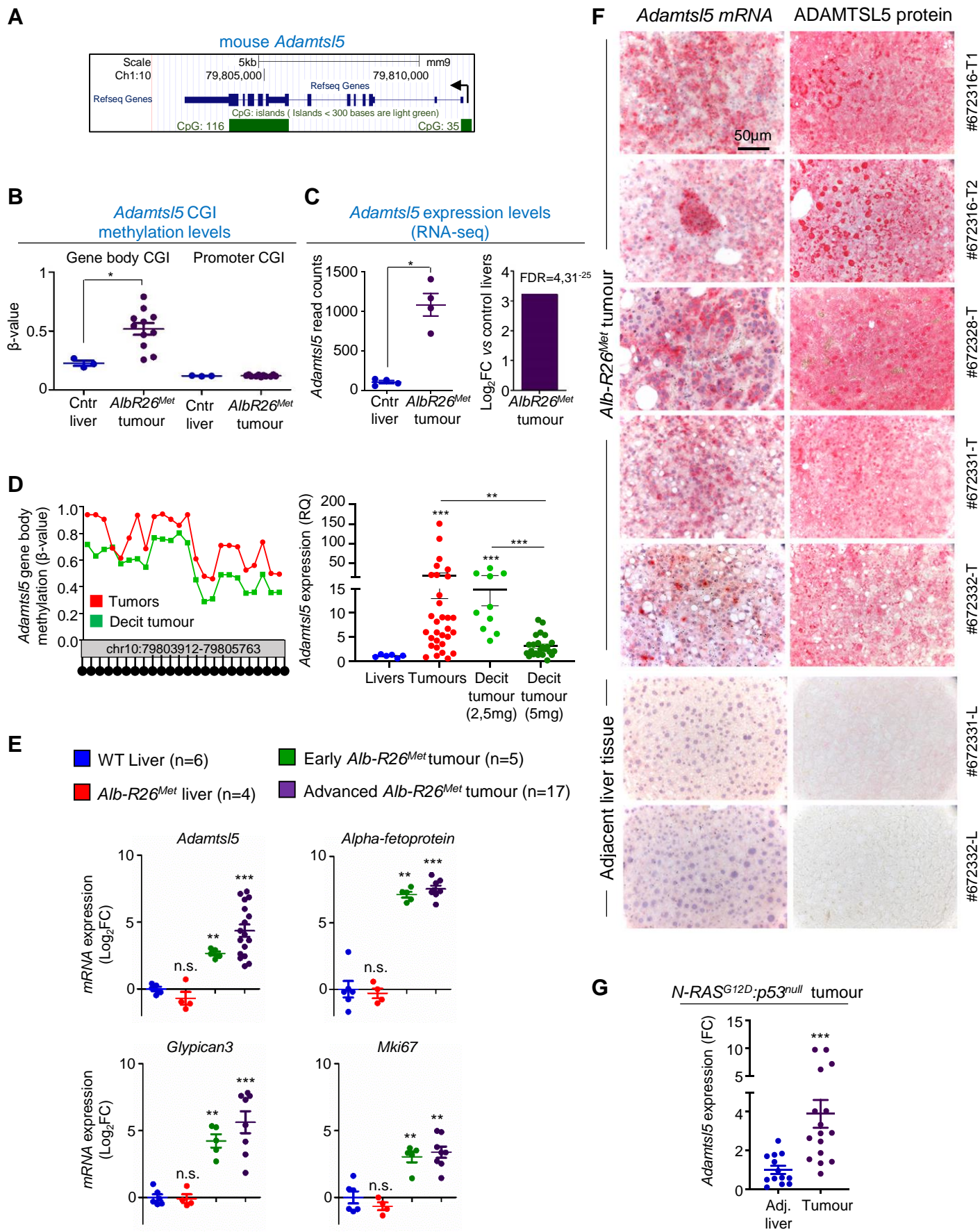


Figure 1

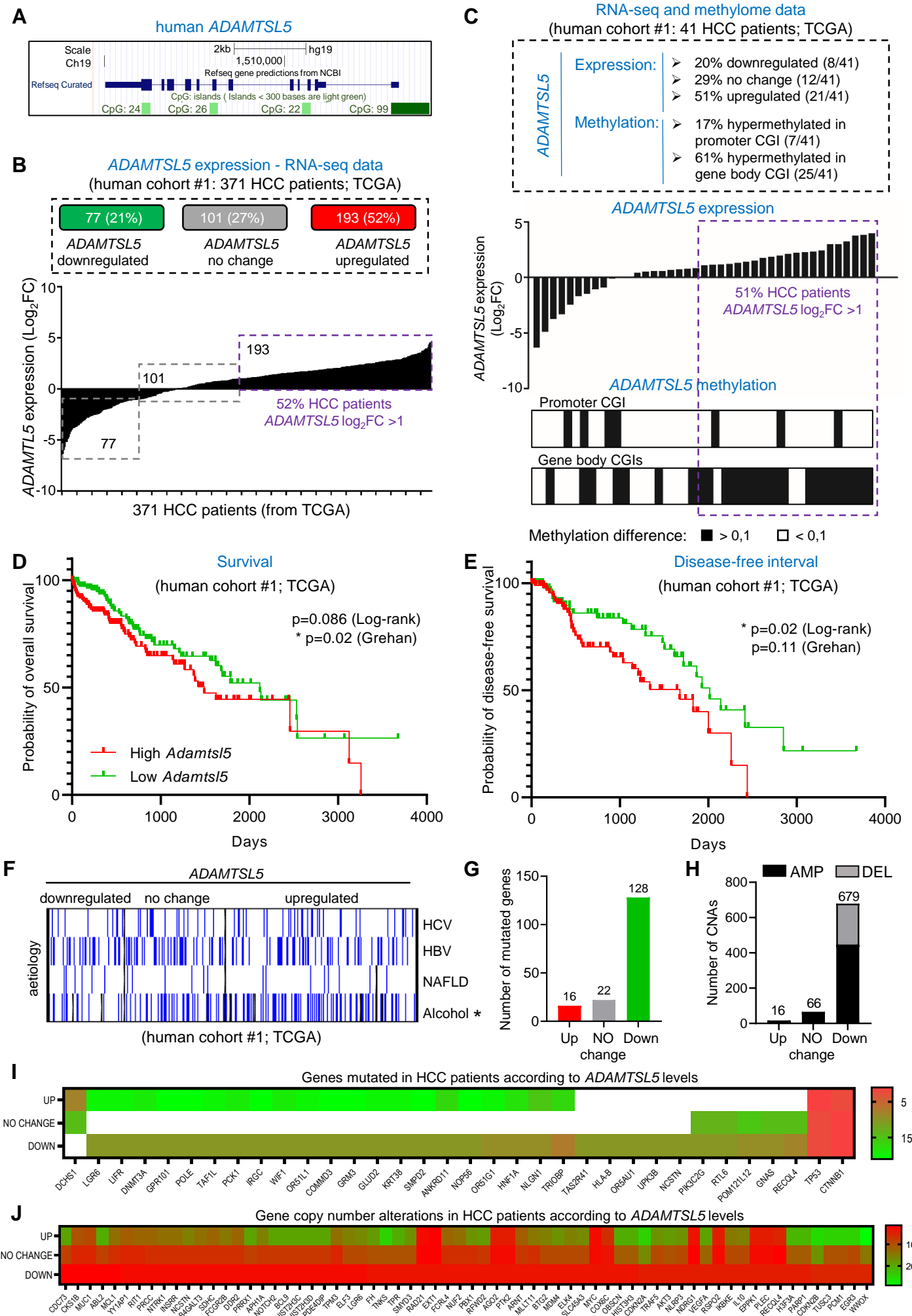


Figure 2

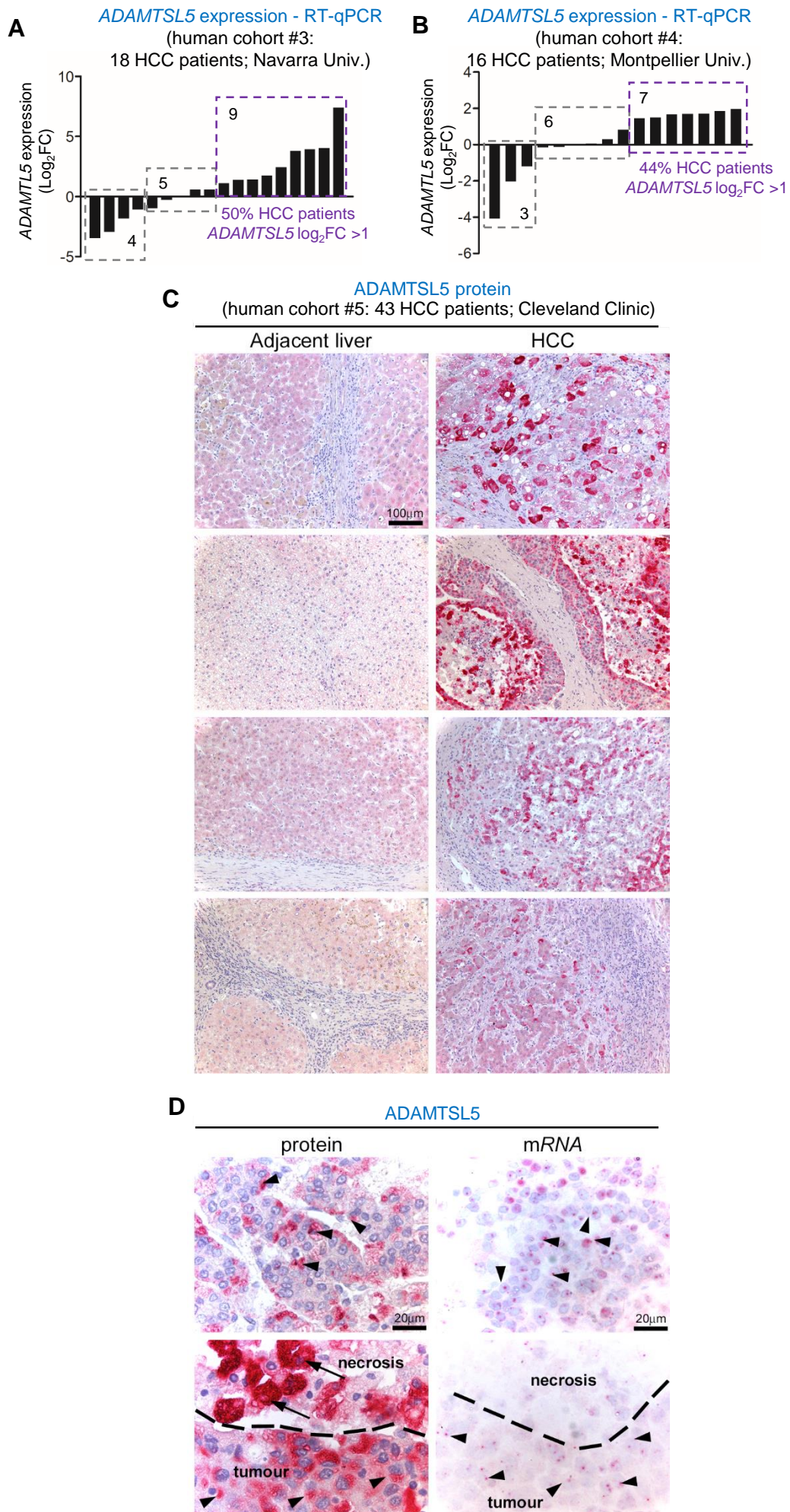


Figure 3

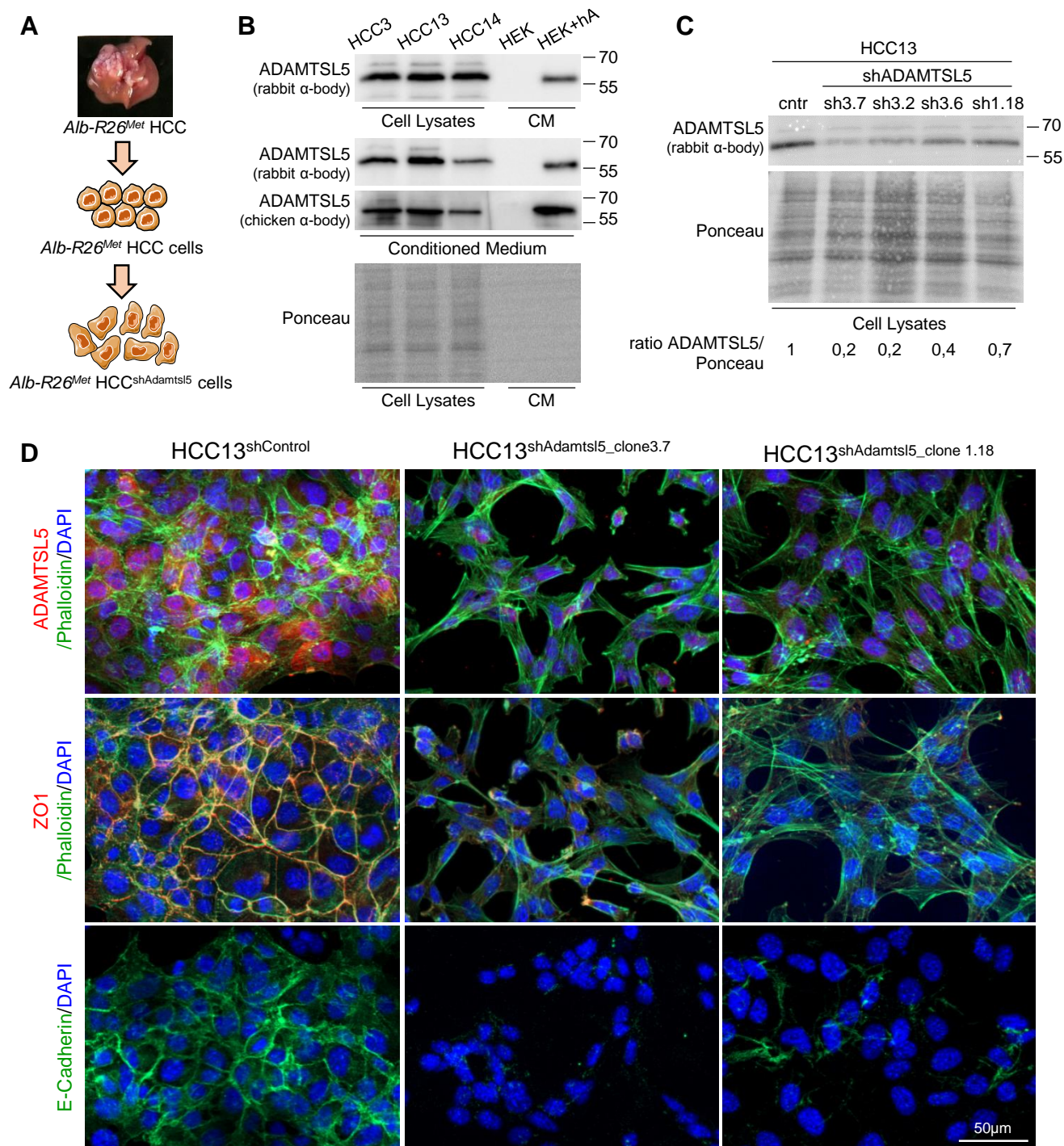


Figure 4

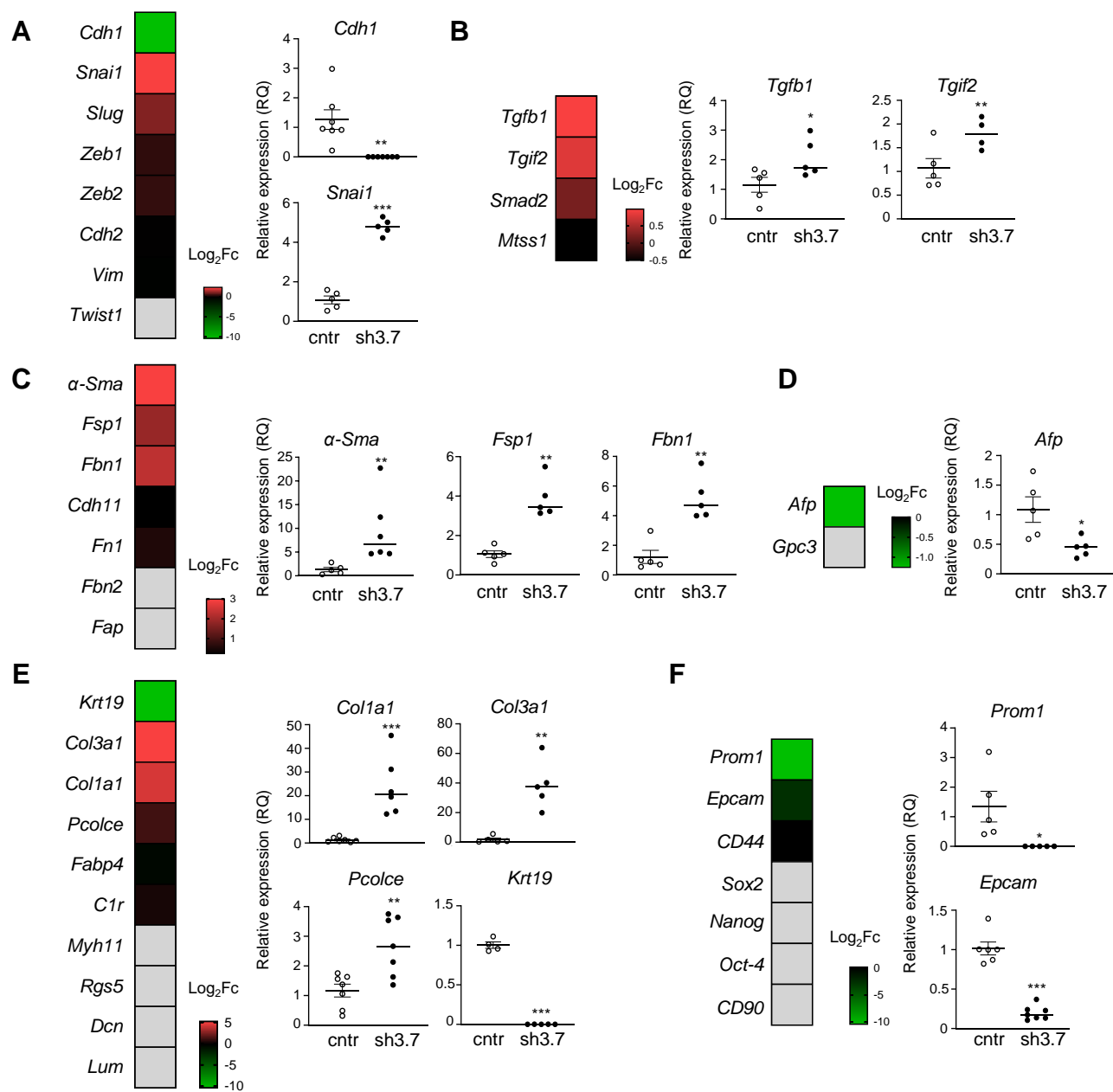


Figure 5

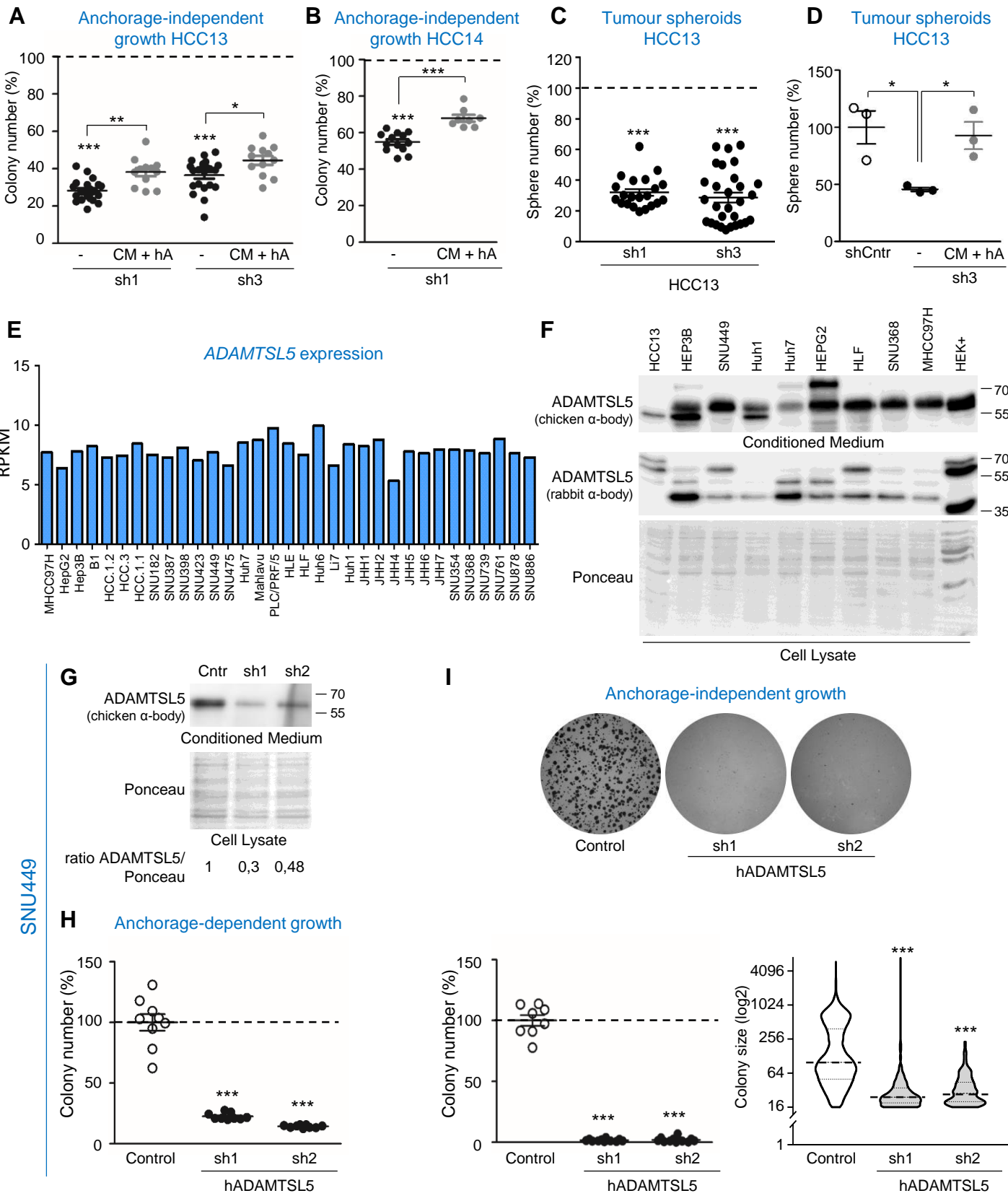


Figure 6

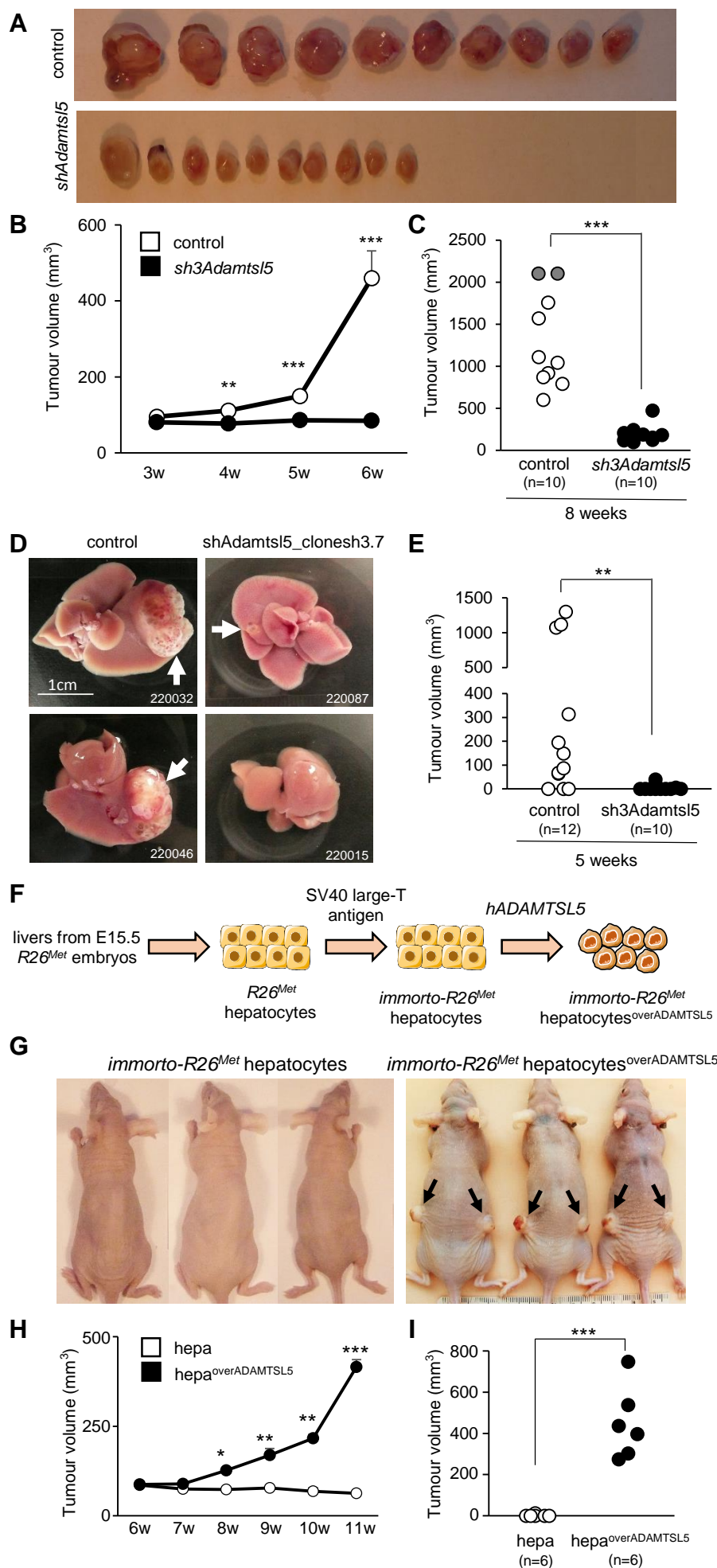


Figure 7

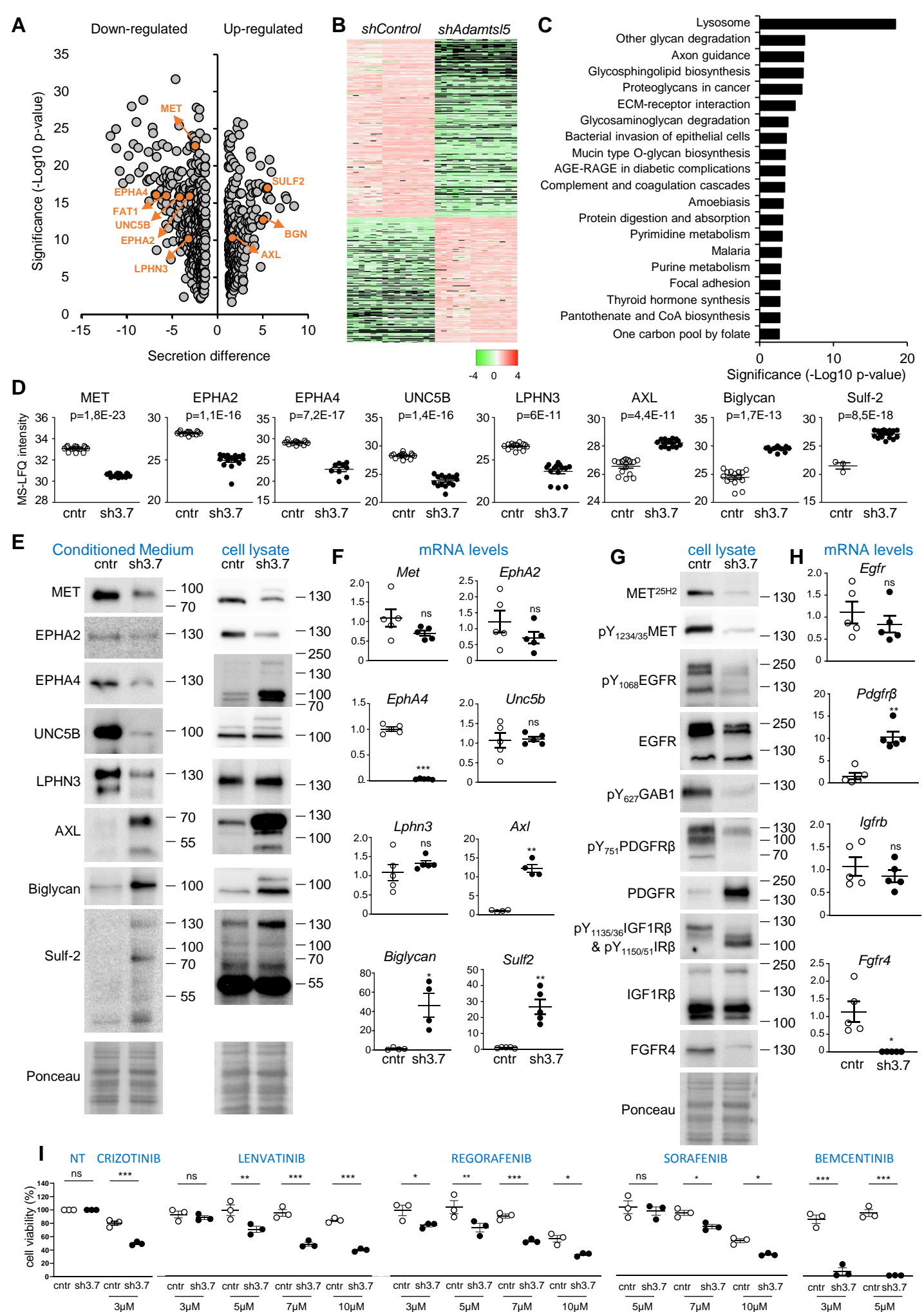
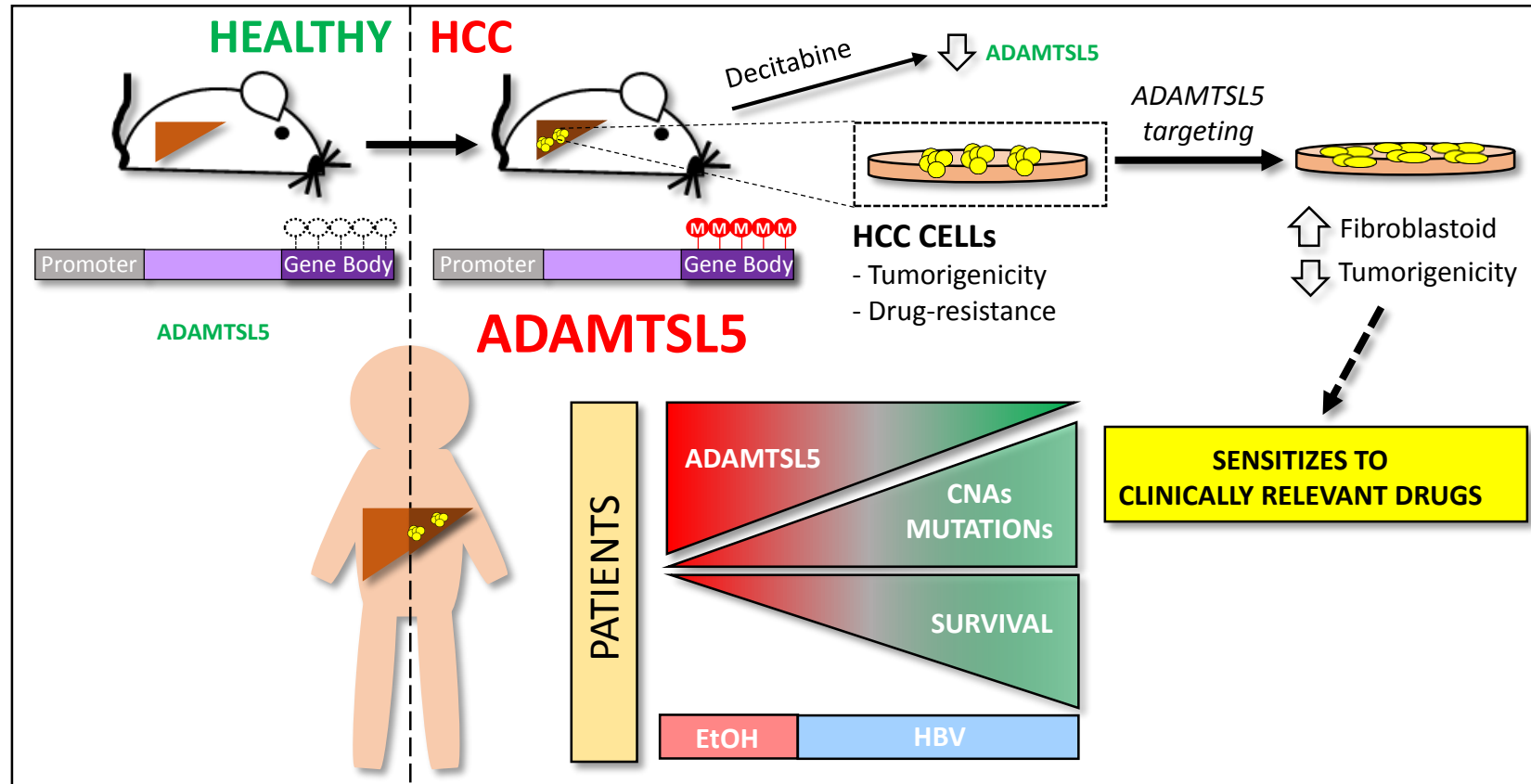


Figure 8



Highlights

- *ADAMTSL5* overexpression in HCC is associated with gene body CGI hypermethylation
- *ADAMTSL5* is strongly expressed in a large fraction of human HCC
- Targeting *ADAMTSL5* diminishes RTK inputs and interferes with tumorigenicity
- *ADAMTSL5* confers tumorigenicity to sensitized, non-transformed liver cells
- Targeting *ADAMTSL5* sensitizes HCC cells to drugs currently used in the clinic