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Activation of the TGFβ/SMAD Transcriptional Pathway Underlies a Novel Tumor Promoting Role of Sulfatase1 in Hepatocellular Carcinoma*

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Abstract

In vitro studies have proposed a tumor suppressor role for Sulfatase1 (SULF1) in hepatocellular carcinoma (HCC), however high expression in human HCC has been associated with poor prognosis. The reason underlying this paradoxical observation remains to be explored. Using a transgenic (Tg) mouse model overexpressing Sulf1 (Sulf1-Tg) we assessed the effects of SULF1 on the diethylnitrosamine (DEN) model of liver carcinogenesis. Sulf1-Tg mice show higher incidence of large and multifocal tumors with DEN injection compared to wild type (WT) mice. Lung metastases were found in 75% of Sulf1-Tg mice but not in WT mice. Immunohistochemistry (IHC), immunoblotting and reporter assays all show a significant activation of the TGFβ/SMAD transcriptional pathway by SULF1 both *in vitro* and *in vivo*. This effect of SULF1 on TGFβ/SMAD pathway is functional; overexpression of SULF1 promotes TGFβ-induced gene expression and epithelial-mesenchymal-transition (EMT), and enhances cell migration/invasiveness. Mechanistic analyses demonstrate that inactivating mutation of the catalytic site of SULF1 impairs the above actions of SULF1 and diminishes the release of TGFβ from the cell surface. And we also show that SULF1expression decreases the interaction between TGF-β1 and its HSPG sequestration receptor TGFβR3. Finally, using gene expression from human HCCs, we show that patients with high SULF1 expression have poorer recurrence-free survival (HR 4.1 (1.9–8.3); p=0.002) compared to patients with low SULF1. We also found strong correlations of SULF1 expression with TGFβ expression and with several TGFβ-related EMT genes in human HCC.

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*__Short Title__: Role of Sulfatase1 in HCC carcinogenesis

CONCLUSION—In summary, our study proposes a novel role of SULF1 in HCC tumor progression through augmentation of the TGFβ pathway, thus defining SULF1 as a potential biomarker for tumor progression and a novel target for drug development for HCC.

Keywords

HCC; TGF beta; tumor progression; SULF1; Heparin Sulfate

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths worldwide, with more than 780,000 new cases and around 745,000 deaths attributed to HCC each year, making it the second most common cause of death from cancer (1). Liver transplantation and resection are potentially curative options for patients with HCC but unfortunately most patients present with advanced stage disease and are not eligible for these surgical therapies. Hence there is an urgent need to decipher molecular pathways involved in HCC progression in order to identify biomarkers for early detection and develop new rational targeted therapeutic strategies.

HSPGs are complex carbohydrate-modified proteins that are present at the cell surface and within the extracellular matrix of all tissues. Previous studies have defined a central role of the cell surface heparan sulfate proteoglycans (HSPGs) in HCC carcinogenesis and tumor progression as they control the downstream effects of various growth factors by modifying their interaction with receptors (2, 3). Cell surface or secreted extracellular matrix endosulfatases (e.g., Sulfatase 1 (SULF1)) modulate the activity of HSPGs by removing sulfate residues from the 6-O position of HS disaccharides. By changing the affinity of HSPGs for signaling ligands for which they serve as extracellular storage sites or as co-receptors, SULF1 can regulate various facets of tumorigenesis (4).

In HCC cells SULF1 transfection was shown to decrease proliferation rate and to sensitize cells to apoptosis, leading to the hypothesis that SULF1 is a tumor suppressor gene (5). Conversely, gene expression analysis of human HCCs showed that high SULF1 expression was associated with poor survival (6). We undertook this study to address these paradoxical findings. To elucidate the role of SULF1 in HCC we asked the following questions 1. Does transgenic overexpression of Sulf1 in mouse liver enhance DEN-induced liver carcinogenesis? 2. Which growth factor pathway is involved in its proposed tumor promoter role? 3. Do the results from mouse experiments translate to human HCC?

MATERIALS AND METHODS

A comprehensive list of all the reagents, kits, antibodies and primers used in this study is found in Supplementary Table 1.

Mouse experiments

To explore the role of Sulf1 in HCC we performed *in vivo* experiments using a hepatocyte-specific Sulf1 transgenic (Tg) mouse model (Supplementary Figure 1A). The Sulf1 Tg mice were not found to have any major phenotypic changes when compared to wild type (WT)

mice and did not show any signs of liver injury or inflammation (Supplementary Figure 1B). We confirmed that overexpression of Sulf1 had functional implications by demonstrating that Sulf1 Tg mice liver tissues had lower levels of sulfated HSPGs compared to WT (Supplementary Figure 1C). Liver carcinogenesis was induced in WT or Sulf1 Tg mice by intraperitoneal injection of the liver carcinogen diethylnitrosamine (DEN).

Transgenic Mouse Microarray Expression

Mouse livers were collected after death from nine non-DEN treated adult Sulf1 Tg and three WT controls and RNA was extracted. Microarray gene expression array analysis of the RNA was used for differential pathway and gene expression analysis.

Human HCC Microarray gene expression analysis

We analyzed microarray data from a previously published study (6, 7). Valid SULF1 expression levels were available from 118 HCC samples. Correlation analysis was performed between SULF1 and genes related to epithelial mesenchymal transition (EMT) (derived from previously published meta-analysis for core EMT signature (8)).

TCGA gene expression analysis

We used mRNA expression and somatic copy number variation (CNV) data generated by The Cancer Genome Atlas (TCGA) from HCC specimens (200 tumors; 50 surrounding normal liver tissues). We downloaded the file containing level 3 normalized RSEM (RNA-Seq by Expectation Maximization) data and the level 3 somatic CNV data from the Firehose run of the Broad Genome Data Analysis Center (9).

Other Materials and Methods

Other materials and methods for immunohistochemistry, quantitative real-time polymerase chain reaction, Western blot analysis, luciferase assay, immunofluorescence, migration assay, invasion assay, ELISA and catalytic mutant generation are described in Supplementary Materials and Methods.

Statistics

SPSS 16 (Chicago, Il) statistical software was used. $P < 0.05$ were considered to be statistically significant. The Kaplan Meier test was used for survival analysis and the Log rank test was used to compare the groups.

RESULTS

Sulfatase1 overexpression in transgenic mice enhances liver tumor progression

To elucidate the role of Sulf1 expression in HCC, transgenic mice overexpressing Sulf1 were cross-bred with WT mice and the progeny mice were injected with a single dose of DEN at day 14 of life. Sulf1 Tg mice (n=25) and WT littermates (n=29) were sacrificed at 8 months of life and tumor burden assessed (Figure 1A). Sulf1 Tg mouse livers were confirmed to significantly overexpress Sulf1 compared to WT animals as assessed by RT PCR and Western immunoblotting (Figure 1B). Significantly higher percentages of Sulf1 Tg

mice developed large tumors and multifocal tumors compared to WT mice (Figure 1C and 1E). Also Sulf1 Tg mice had significantly higher mean liver weight, total body weight and liver/body weight ratio (Figure 1D). The mean number of tumors per mouse in male Sulf1 Tg mice was 3.8 (SD 3.6) compared to 1.6 (SD 2.9) among WT controls (p=0.02). Consistent with the reported literature, female mice (n=27) had a lower HCC burden compared to males (n=27). However, even among female mice, a higher percentage of Sulf1 Tg mice (21%) developed HCC compared to WT mice (8%). Tumors were H&E stained and examined to confirm the diagnosis of HCC (Supplementary Figure 2A and 2B).

Lung tumor nodules were found in 75% of the Tg mice with liver tumors while none of the WT mice had lung metastases (Figure 1F). The metastatic HCCs exhibited large, irregular, dysmorphic, hyperchromatic nuclei. Immunohistochemical staining for Hep Par1 was used to confirm the hepatic origin of metastatic tumor nodules in the lungs (Supplementary Figure 3A and 3B). A few lymphoid aggregates were found in the lungs of both Tg and WT mice (Supplementary Figure 3C), but they were negative for Hep Par1 staining confirming they were not metastatic HCC (Supplementary Figure 3D). Thus, our *in vivo* studies strongly supported a tumor promoter role for Sulf1.

Sulfatase 1 promotes TGFβ pathway activation and EMT in vivo and in vitro

To understand mechanisms underlying the tumor progression noted in Sulf1 Tg mice we evaluated the pathways and biological processes activated in Tg compared to WT mice by microarray gene expression analysis of non-DEN treated liver tissue (Figure 2A and 2B). Multiple cytoskeletal remodeling, immune regulatory and cell adhesion pathways were activated in Sulf1 Tg mice. Correspondingly, biological processes involving cytoskeletal remodeling, cell adhesion and muscle development were up-regulated in Sulf1 Tg mice. While evaluating for processes that could potentially explain the larger tumors and lung metastases observed in Sulf1 Tg mice, we identified that the epithelial mesenchymal transition (EMT) process was preferentially activated in Sulf1 Tg mice ($P=5.3\times10^{-3}$). EMT is a process by which epithelial cells lose their polarity and acquire a mesenchymal phenotype with invasive and migratory capacity and it has been implicated in tumor progression and development of metastases (10). It is well established that TGFβ is an important and potent driver of EMT in cancer. Interestingly, we found that the TGFβ pathway was activated in Sulf1 Tg mice ($P=7.1\times10^{-6}$) (Supplementary Figure 4A). We further confirmed this association by demonstrating that genes involved in the TGFβ pathway and cell adhesion processes, such as SMAD2, SMAD6, N-Cadherin and E-Cadherin were significantly differentially expressed in Sulf1 Tg mice compared to WT mice ($P<0.01$)(Supplementary Figure 4B). These results prompted us to further explore the role of Sulf1 in activating TGFβ-induced EMT.

Protein expression analysis by Western blot of tissue from DEN-treated mice confirmed that Sulf1 expression was higher in both the livers and the tumors of Tg mice than WT mice, but there was no statistical difference in expression between the tumors and surrounding normal liver (Figure 2C). We assessed whether the TGFβ pathway was activated by comparing the expression of phospho SMAD2/3 between peritumoral liver and tumor tissue from DEN injected WT and Sulf1 Tg mice. Immunoblotting showed increased TGFβ1 and increased

phosphorylation of SMAD2/3 in peritumoral liver tissues and also a more marked increase of phospho-SMAD2/3 in tumors of Sulf1 Tg mice when compared to WT mice (Figure 2D). Further, immunohistochemistry confirmed that liver tumors in Sulf1 Tg mice had higher percentage of nuclei with phospho-SMAD2/3 staining and higher intensity of phospho-SMAD2/3 staining than WT tumors (Figure 2E).

We then evaluated the expression of EMT markers in Tg mice. Immunoblotting showed loss of the epithelial marker E-cadherin, and an increase in the mesenchymal markers N-cadherin and vimentin in tumors of Sulf1 Tg mice when compared to tumors of WT mice (Figure 2F). Immunohistochemistry confirmed these findings (Figure 2F). Stromal expression of vimentin and α smooth muscle actin (αSMA) is expected, but we also found cytoplasmic expression of these mesenchymal proteins in tumor cells of Sulf1 Tg mice (demonstrated in higher magnification inserts in Figure 2F) suggesting possible transformation of epithelial cells to the mesenchymal phenotype.

In vitro studies were used to determine whether the findings in mice could be confirmed in HCC cell lines. Two HCC cell lines, Hep3B and PLC/PRF/5, which lack SULF1 expression, were used for the overexpression studies and SNU182 and SNU475, two HCC cell lines that constitutively express high levels of SULF1, were used for shRNA knockdown experiments (Figure 3A). To determine whether SULF1 expression regulates TGFβ pathway activation, we performed immunoblotting for phosphorylation of the transcription factors SMAD2 and SMAD3 in Hep3B and PLC/PRF/5 cell lines after forced expression of SULF1 and treatment with TGFβ1. Figure 3B shows that overexpression of SULF1 increased phosphorylation of both SMAD2 and SMAD3. Conversely, suppression of SULF1 expression in the SNU182 and SNU475 led to the opposite effects (Figure 3B). Immunofluorescence staining of Hep3B and SNU182 cells treated with TGFβ1 was used to confirm these results (Figure 3C, Supplementary Figure 5A).

Next, we performed luciferase assays to assess the activity of the SMAD-responsive SBE reporter in Hep3B and PLC/PRF/5 cells co-transfected with Vector or SULF1 and SBE reporter plasmid and treated with TGFβ1. We demonstrated a greater increase in TGFβ1-induced SBE luciferase activity in SULF1-transfected Hep3B or PLC/PRF/5 cells when compared to Vector transfected cells ($P<0.01$) (Figure 3D and Supplementary Figure 5B). Similarly shRNA mediated suppression of SULF1 expression in SNU182 and SNU475 cells significantly abrogated TGFβ1-induced SBE luciferase activity ($P<0.01$) (Figure 3D and Supplementary Figure 5B).

We used in vitro studies to assess whether the EMT observed in Tg mice could be secondary to TGFβ pathway activation. Immunoblotting of cell extracts from SULF1 or Vector transfected cells treated with TGFβ1 for 48 hours revealed a decrease in the tight junction protein Zona occludens protein 1 (Zo-1), loss of the epithelial marker E-cadherin and increase in the mesenchymal markers N-cadherin, vimentin and αSMA (Figure 4A). Conversely, suppression of SULF1 expression in the SNU182 and SNU475 cell lines led to the opposite changes (Figure 4B). Immunocytochemistry was used to confirm these results (Figure 4C, Supplementary Figure 5C).

Inactivation of the Catalytic domain of Sulfatase1 leads to loss of TGFβ pathway activation

To understand the mechanism behind TGFβ activation by SULF1 we created a mutant with loss of catalytic activity (SULF1 CM) (Supplementary Figure 6A). Initially, flow cytometry was performed with single chain antibodies A04B08, HS4C3 and RB4EA12 which bind to HS chains which have a significant degree of 6-O-sulfation (Supplementary Figure 6B). After expression of SULF1, all three antibodies bound to the Hep3B cell surface to a lower extent compared to Vector, implying that SULF1 expression in HCC cell lines leads to a significant decrease in 6-O-sulfation of HSPGs. We identified two of the five antibodies that were associated with the most significant change (HS4C3 and RB4EA12; $P<0.001$). Next, we transfected Hep3B cells with either Vector, SULF1 or SULF1 CM and performed immunocytochemistry with HS4C3 and RB4EA12 (Figure 5A). We were able to demonstrate a clear decrease in immunofluorescence staining in SULF1 transfected cells compared to Vector controls. In contrast, cells transfected with SULF1 CM did not show decreased immunofluorescence, thus confirming that mutating the catalytic site led to loss of the desulfating action of SULF1.

We then investigated whether this loss of enzymatic action had functional implications. Using the wound healing assay and Boyden's chamber assay, we assessed the influence of SULF1 on cell migration and invasion, respectively. SULF1 transfected cells treated with TGFβ1 showed significant augmentation in migration with almost complete closure of the wound area by 48 hours, a significantly higher closure rate than for Vector transfected cells ($P<0.01$) (Figure 5B and 5C). Also, TGFβ treatment increased the invasiveness of both Vector transfected and SULF1 transfected cells but the effect was statistically significantly higher in SULF1 expressing cells ($P<0.01$) (Figure 5D). Further, the increase in migratory and invasive capacity of HCC cell lines observed in SULF1 transfected cells treated with TGFβ1 was lost when cells were transfected with SULF1 CM (Figure 5B, 5C and 5D). Loss of activation of the TGFβ pathway with CM was also confirmed by the loss of augmentation in phosphorylation of SMAD2/3 and the absence of increase in SBE luciferase activity after TGFβ treatment (Figure 5E and 5F). Taken together, these results demonstrate that the enzymatic activity of SULF1 is essential for TGFβ pathway activation.

SULF1 promotes the release of TGFβ from TGFβR3

We found that TGFβ1 levels were significantly higher in the supernatant media from SULF1 transfected compared to Vector control cells ($P<0.01$), and this increase was not found in cells expressing SULF1 CM (Figure 6A). We thus hypothesized that the desulfating enzymatic action of SULF1 on HSPGs that sequester the ligand, leads to release of TGFβ from the cell surface. Of the three TGFβ cell surface receptors, TGFβR3 is the only HSPG and hence is a likely target of SULF1. We explored therefore the expression of this receptor in mouse and human HCCs and found that TGFβR3 expression was lower in tumor tissue compared to peritumoral liver (Figure 6B and 6C). In the human HCC cell lines used in this study, TGFβR3 expression was confirmed to be relatively uniform with SNU182 showing the highest expression (Figure 6D). We used lysates from SNU182 cells transfected with scr shRNA or SUFL1 shRNA to perform immunoprecipitation (IP) with antibody to SULF1 and subsequently performed Western immunoblotting on the IP eluate for TGFβR3. There was less TGFβR3 in the immunoprecipitate of cells with decreased SULF1 expression,

suggesting that SULF1 and TGFβR3 interact with each other (Figure 6E). We then performed IP with antibody to TGFβR3 and used the resulting eluate for Western blotting for TGFβ1 (Figure 6F). We used whole cell lysate as a positive control (Lane 2 and 3) and IP with non-specific IgG as a negative control (Lane 7 and 8). Decreased SULF1 expression was associated with increased interaction between TGFβR3 and TGFβ1 (Figure 6F, Lane 4 vs Lane 5)), thus supporting the hypothesis that SULF1 releases TGFβ1 from TGFβR3.

Sulfatase 1 expression in human HCC is associated with poor prognosis and EMT

Next, we determined whether the above *in vivo* and *in vitro* findings have translational significance in human HCC. In our microarray data, 24% (n=28) of HCCs were found to have high SULF1 expression, while 76% (n=90) had low expression. We confirmed the SULF1 mRNA overexpression observed in the microarray analysis by immunohistochemistry and Western blot analysis of selected tumor samples (Supplementary Figure 7). High SULF1 expression was associated with significantly poorer overall survival (HR 3.1 (1.8–5.4); p=0.03) and also poorer recurrence free survival (HR 4.1 (1.9–8.3); p=0.002) (Figure 7A and 7B). Lee et al. have described and validated a method for prognostic clustering of HCC based on gene expression profiling (11). We used our microarray expression profile to classify HCC patients into these two prognostic clusters (A and B). We confirmed the prognostic value of this clustering as cluster A was associated with significantly poorer overall survival (HR 5.3 (3.2–8.8); p<0.001). The majority of patients with high SULF1 expression (76 %) belonged to the poor prognosis cluster A, compared to 33% of those with low SULF1 expression (P<.001) (Figure 7C). Next, we examined whether the association between SULF1, TGFβ activation and EMT was present in human HCC. Gene expression correlation analysis confirmed significant correlation in expression of several EMT related genes with SULF1. Five well recognized EMT driver genes significantly correlated with SULF1 expressions are represented in Figure 7D; supplementary Table 2 lists the other genes significantly correlated with SULF1. Tissues from resected HCCs with high and low SULF1 expression were used for immunohistochemistry to confirm TGFβ activation and induction of EMT. High SULF1 expression was confirmed by IHC to be associated with high phospho-SMAD2/3 expression, decreased expression of E-cadherin, and increased expression of vimentin and αSMA (Figure 7E).

To validate the above results we used RNA seq data from the TCGA analysis of human HCC. These data confirmed that SULF1 is significantly over expressed in HCC when compared to surrounding normal tissues (Figure 8A), with a greater than two-fold increase in SULF1 expression in 38% (75/197 of tumors (Figure 8B). Correlation analysis between SULF1 expression and the same EMT gene signature used above confirmed that more than 50% of the genes correlated with SULF1 (Supplementary Table 3). Validation of the same five EMT driver genes (vimentin, SNAI1, COL1A2, TGFβ1, SPARC) which strongly correlated with SULF1 on microarray analysis is shown in Figure 8C. Also, a majority (75%) of the EMT genes correlating with SULF1 in the microarray analysis were also confirmed to correlate with SULF1 in the TCGA analysis (Figure 8D). We also examined the somatic copy number variations (CNV) of the SULF1 gene in the TCGA data and found heterozygous loss in 6%, no change in 43%, gain of one copy in 16% and amplification in

35%. However, we did not find any correlation between SULF1 CNV and mRNA expression (Figure 8E).

The regulation of TGFβ mediated EMT by SULF1 is shared with SULF2

Finally, we explored the role of SULF2, another cell surface enzyme with similar enzymatic actions, in TGFβ mediated EMT. We performed in vitro studies using two cell lines which have high (Huh7) and low expression of SULF2 (Hep3B) (Supplementary Figure 8A and 8B). Using immunoblotting and immunocytochemistry experiments, we demonstrated that SULF2 expression does enhance TGFβ mediated EMT (Supplementary Figure 8C and 8D). We also explored the correlation of EMT-related genes in the human HCC microarray and identified forty-two genes that correlate significantly with SULF2 (Supplementary Table 4); 64% of these genes were also correlated with SULF1 (Supplementary Figure 8E). We confirmed that the Sulf1 Tg mice did not have altered Sulf2 expression when compared to WT mice (Supplementary Figure 8F). Also, the cell lines Hep3B and PLC/PRF5PRF/5 which were used to perform the SULF1 *in vivo* experiments do not express SULF2. Thus the results of this study are unlikely to be confounded by co-expression of SULF2.

DISCUSSION

The strongest evidence for an oncogenic role of SULF1 in our study comes from the observation that Sulf1 transgenic mice developed large and multifocal tumors when compared to WT mice. The presence of lung metastases in the majority of the Sulf1 Tg mice convincingly supports the enhancement of EMT by Sulf1. Our *in vitro* work confirms that SULF1 expression promotes TGFβ pathway activation through phosphorylation of SMAD 2/3 and leads to induction of the EMT phenotype. Thus our results strongly support the novel model that SULF1 expression enhances TGFβ pathway signaling in HCC, thus promoting tumor progression and metastases (Figure 8F).

Our previous data showing an apparent tumor suppressor effect of SULF1 in HCC cell lines (5, 12), has been somewhat paradoxical as data from studies, including this one, show that patients with HCCs over-expressing SULF1 have a poorer overall survival (6, 7). Thus, we postulate that SULF1 could potentially have opposing functional activities. The original canonical cell signaling effects of HSPGs were elucidated by studying their actions as co-receptors for receptor tyrosine kinase (RTK) ligands such as fibroblast growth factor (FGF), heparin binding epidermal growth factor (HB-EGF), and hepatocyte growth factor (HGF). These initial studies in HCC cell lines showed that SULF1 inhibits signaling of RTK ligands leading to decreased cell proliferation and increased apoptosis (5, 13, 14). This is consistent with a model in which desulfation of HSPGs prevents the assembly of the ternary ligand-RTK-HSPG complex required for efficient signaling. We hypothesize that the growth promoting effects of RTK ligands predominate in HCC cell lines that have suppression of SULF1 expression. On the other hand, we propose that HCC tumors that have high levels of SULF1 are primarily driven by signaling pathways such as the TGFβ pathway for which SULF1 expression leads to the release of signaling ligands from sequestration sites in the tumor microenvironment, thus enhancing ligand receptor binding and activation of the signaling pathway.

The catalytic site of SULF1 lies in the amino terminal region of the protein and a conserved cysteine amino acid at this site is essential for sulfatase enzymatic activity (15). We performed insertional mutagenesis at this site to create a catalytic mutant (CM). Using anti-HS antibodies, we demonstrated that mutation of the catalytic site abrogates the 6-O-desulfation of cell surface HSPGs noted in cells overexpressing SULF1. Mutating the catalytic site was also found to be functionally significant as the SULF1-induced augmented activation of the TGFβ pathway, promotion of HCC cell invasiveness and release of TGFβ into the supernatant were all absent in cells expressing SULF1 CM. This supports our hypothesis that the mechanism of SULF1 mediated activation of the TGFβ pathway was through its desulfating activity on cell surface HSPGs, potentially leading to release of TGFβ. We further conjectured that TGFβR3 was the likely target of SULF1 as it is an HSPG that serves both as a co-receptor and as a sequestration site for TGFβ1 (16, 17). It has been proposed to have a tumor suppressor role in several cancers and is usually found to be down regulated in cancer (18–20). Consistent with this, we found lower expression of TGFβR3 in mouse and human HCC tumors. The mechanism for its tumor suppressor action is believed to be its ability to sequester TGβ1 ligand and decrease TGFβ pathway activation (20). We were able to demonstrate by co-IP that SULF1 expression decreased the interaction between TGFβ and TGFβR3 in HCC cell lines, thus supporting our hypothesis that SULF1 releases TGFβ1 bound to TGFβR3 and thus leads to enhanced TGFβ signaling. Future directions of our work include proteomic analysis using HPLC and mass spectrometry to study the specific post translational modifications in the sulfation status of TGFβR3 by SULF1.

The role of SULF2, another sulfatase enzyme, as a tumor promoter has already been established (21–23) and more recently Zheng et al have shown that TGFβ pathway can be activated by SULF2 (24). We explored the role of SULF2 in TGFβ mediated EMT using *in vitro* studies and found that it had similar actions as SULF1. In the context of HCC, this is the first time we are able to demonstrate that SULF1 and SULF2 have similar effects on the TGFβ pathway, contrary to previous data showing their opposing effects on FGF (25), VEGF (26) and HGF pathways (27).

The interaction between SULF1 and TGFβ has not been explored in depth before and this interaction offers a novel mechanism for tumor progression. TGFβ is an established mediator of EMT and increased serum and urine levels of TGFβ1 in patients with HCC have been associated with poor prognosis (28–30). It is possibly in this state of TGFβ abundance in the tumor microenvironment that SULF1 acts to enhance its tumor promoter actions. Our gene expression analysis of human HCC using both microarray data and RNA sequencing data from TCGA confirm strong correlations between SULF1 and several TGFβ related EMT genes. These findings have translational significance as we found high SULF1 expression to be associated with poor prognosis. Thus SULF1 could serve as a potential target for inhibition of TGFβ induced EMT, thus slowing tumor progression and preventing metastases. With new TGF inhibitors entering clinical trials for HCC, SULF1 could also serve as a biomarker to predict response to therapy (31).

In summary, our study proposes a novel role of SULF1 in HCC tumor progression and metastases through augmentation of the TGFβ pathway. Our in vivo and in vitro experiments confirm the role of SULF1 in promoting HCC tumor progression and

invasiveness. Thus SULF1 may potentially serve as a biomarker for tumor progression and also as a novel target for drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HCC	Hepatocellular carcinoma
HSPG	Heparan sulfate proteoglycan
SULF1	Human sulfatase 1
Sulf1	Mouse Sulfatase
TGF-β	Transforming growth factor beta
EMT	Epithelial mesenchymal transition
TGFβR3	Transforming growth factor type III receptor protein
DEN	Diethylnitrosamine
RTK	Receptor tyrosine kinase
HB-EGF	Heparin binding epidermal growth factor
Tg	Transgenic
SNAI1	snail family zinc finger 1
COL1A2	Collagen Type I, Alpha 2
SPARC	Secreted Protein Acidic, Cysteine-Rich
TCGA	The cancer genome atlas

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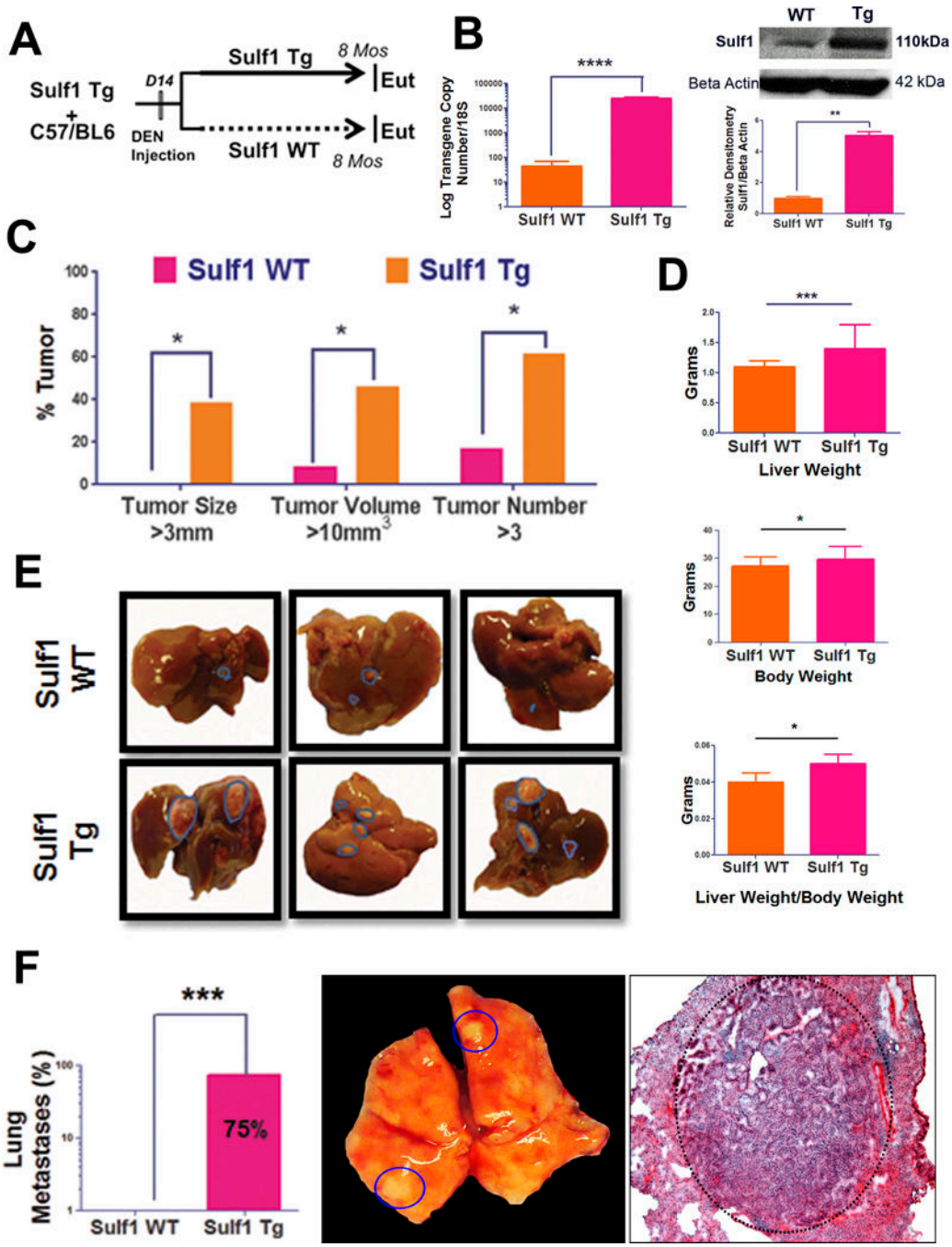

Figure 1. Promotion of DEN-induced carcinogenesis and tumor progression in Sulf1 transgenic (Tg) mice

A. Schematic representation of the mouse experiment shows that Sulf1 Tg mice were cross bred with C57/BL6 and all progeny were given a single intraperitoneal injection of DEN on D 14 of life. All mice were euthanized (Eut) at 8 months. B. Quantitative RT PCR and Western immunoblotting with densitometry demonstrates significantly higher mean expression of Sulf1 in the transgenic mice liver compared to WT mice liver (****P<0.0001). The error bars represent the standard error of mean. C. A higher percentage (39%) of Sulf1 Tg mice had tumors larger than 3 mm in size (longest diameter) while none

of the WT mice had tumors larger than 3 mm in size (*P<0.05). A higher proportion of Sulf1 Tg mice had tumors with volumes greater than $10mm^3$ (46.2%) and multifocal tumors with more than 3 tumors per mouse (61.5%) (*P<0.05). D. Sulf1 Tg mice had significantly higher liver weight (*** P<0.005), higher body weight (*P<0.05) and also higher liver/body weight ratio compared to WT mice (*P<0.05). E. Representative images from livers of WT (top panel) and Sulf1 Tg (bottom panel) mice showing the larger and multifocal tumors in the latter. F. The majority (75%) of Sulf1 Tg mice developed lung metastases while none of the WT mice did (***P<0.001). Gross image of the lungs from Sulf1 Tg mice showing superficial metastatic nodules. H&E staining demonstrates representative tumor nodule in Sulf1 Tg mice at 10× magnification.

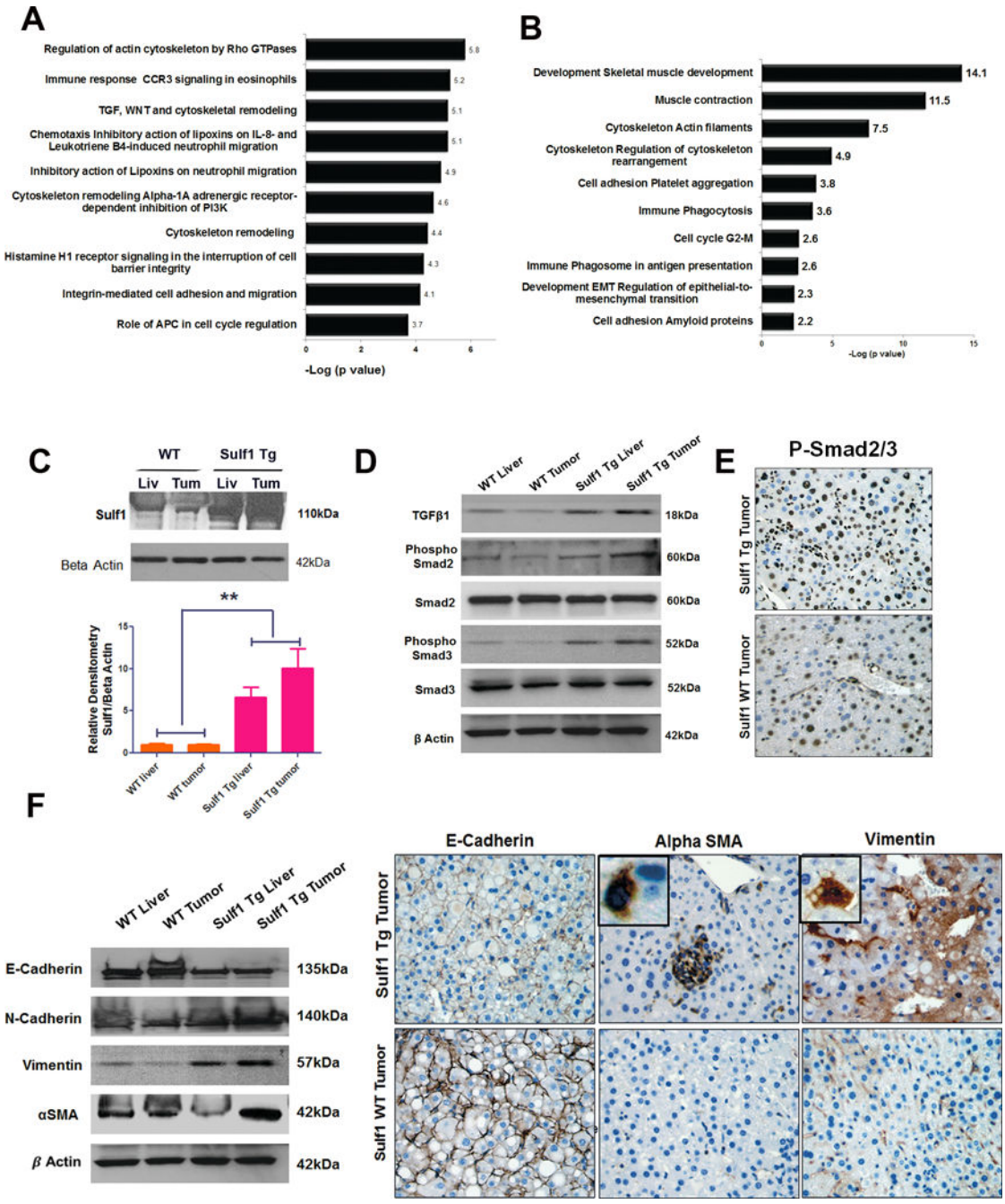

Figure 2. Sulf1 Tg mice demonstrate activation of the TGFβ pathway and induction of the Epithelial Mesenchymal Transition (EMT)

A. The top 10 canonical pathways that were significantly upregulated (P<0.001) in non-DEN treated Sulf1 Tg mouse livers compared to WT mouse liver. B. The top 10 biological processes that were significantly upregulated (P<0.001) in non-DEN treated Sulf1 Tg mouse livers compared to WT mouse livers. C. Western blot results with accompanying densitometry demonstrating higher Sulf1 expression in tumoral and surrounding non-tumoral tissues of Tg mice treated with DEN when compared to WT liver tumoral and surrounding non-tumoral tissues.D. Immunoblotting of protein extracts from WT

peritumoral liver, WT tumor, Tg peritumoral liver and Tg tumor shows increased expression of TGFβ1, phospho-SMAD 2/ 3 in Sulf1 Tg mice indicating TGFβ pathway activation. E. IHC reveals increased P-SMAD 2/3 nuclear staining in Sulf1 Tg mice compared to WT mice. F. Induction of the EMT phenotype in Sulf1 transgenic mice with decrease in E-cadherin and increase in N-cadherin, vimentin and αSMA in Sulf1 Tg mice liver and tumor by immunoblotting. The changes are more pronounced in the Sulf1 transgenic tumor itself than the surrounding liver. Immunohistochemistry (40×) of tumors from Sulf1 Tg (top panel) and WT (bottom panel) confirms decreased expression of E-cadherin and increased expression of αSMA and vimentin in Sulf1 transgenic mice tumors. Inserts in top panel of αSMA and vimentin show higher magnification (100×) of cells demonstrating cytoplasmic expression.

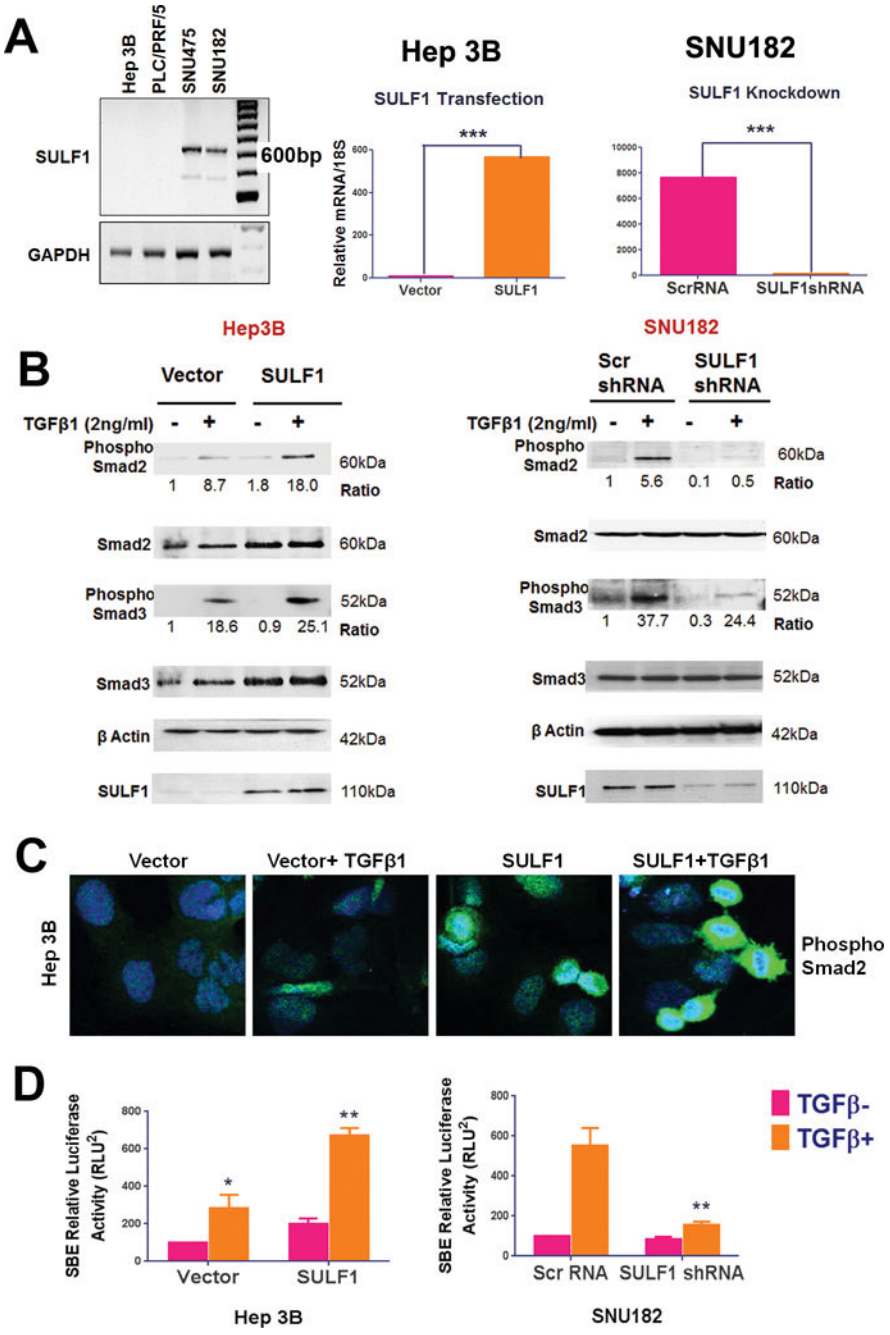

Figure 3. SULF1 expression in HCC cell lines promotes TGFβ pathway activation

A. Qualitative RT-PCR of RNA from 4 cell lines shows lack of SULF1 expression in Hep 3B and PLC/PRF/5 while SNU182 and SNU475 constitutively express SULF1. Quantitative RT-PCR was used to show that transfection of Hep3B cells with SULF1 expressing plasmid led to forced expression of SULF1 (***P<0.001). ShRNA directed against SULF1 was able to successfully knockdown SULF1 expression in SNU182 cells (***P<0.001). B. Immunoblotting of protein extracts from Hep3B cells transfected with SULF1 or Vector that were either serum starved or treated with TGFβ1 (2ng/ml) for 48 hours shows increased ratio of phosphorylated SMAD 2/3 to SMAD 2/3. Knockdown of SULF1 in SNU 182 leads

to decreased phosphorylation of SMAD2 and 3. Results were confirmed in PLC/PRF/5 and SNU475 cells with three repeats each. Mean of relative densitometry values is represented below the blots for phospho-SMAD2 and 3. C. Immunofluorescence staining of Hep3B cells treated with TGFβ1 demonstrates increased phosphorylation of SMAD2 in SULF1 transfected cells D. SBE Luciferase reporter activity demonstrates a significant increase (**P<0.01) in TGFβ1-induced SBE luciferase activity in SULF1-transfected Hep3B cells treated with TGFβ when compared to Vector (*P<0.05). Similarly shRNA mediated suppression of SULF1 expression in SNU182 and led to a significant abrogation (**P<0.01) of SBE luciferase activity.

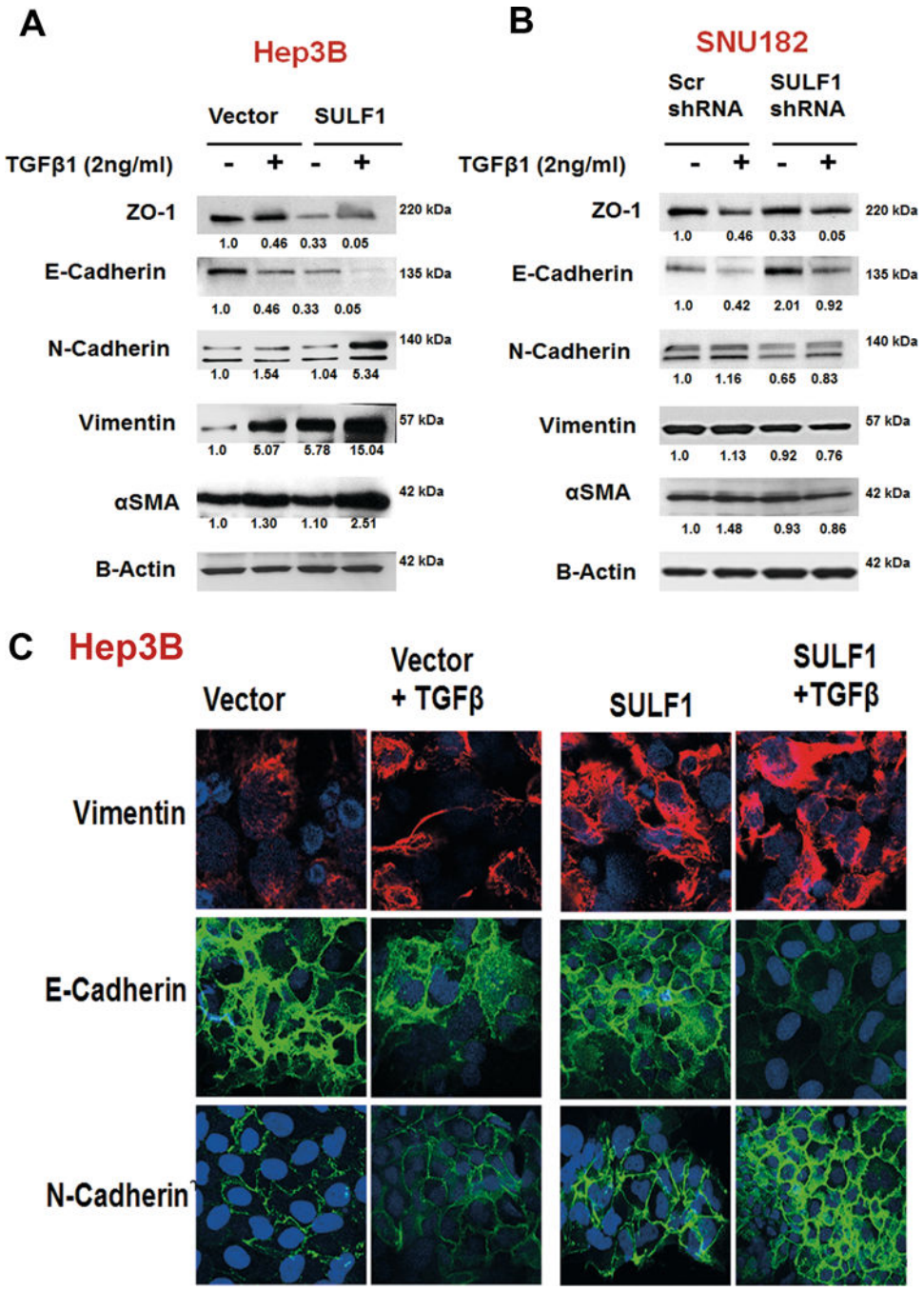

Figure 4. SULF1 expression in HCC cell lines promotes TGFβ induced Epithelial Mesenchymal Transition (EMT)

A. Immunoblotting of protein extracts from Hep3B cells transfected with SULF1 or Vector plasmid that were either serum starved or treated with TGFβ1 (2ng/ml) for 48 hours shows loss of Zona Occludens-1 (ZO-1) and E-cadherin and increased expression of N-cadherin, vimentin and αSMA in SULF1 expressing cells. B. Immunoblotting of protein extracts from SNU182 cells transfected with scr shRNA or SULF1 shRNA that were either serum starved or treated with TGFβ1 (2ng/ml) for 48 hours shows increased expression of ZO-1 and E-cadherin and decreased expression of N-cadherin, vimentin and αSMA when SULF1

expression is down-regulated. C. Immunofluorescence staining of Hep3B cells treated with TGFβ1 demonstrates decreased expression of E-cadherin and increased expression of vimentin and N-cadherin in SULF1 transfected cells treated with TGFβ compared to Vector transfected cells.

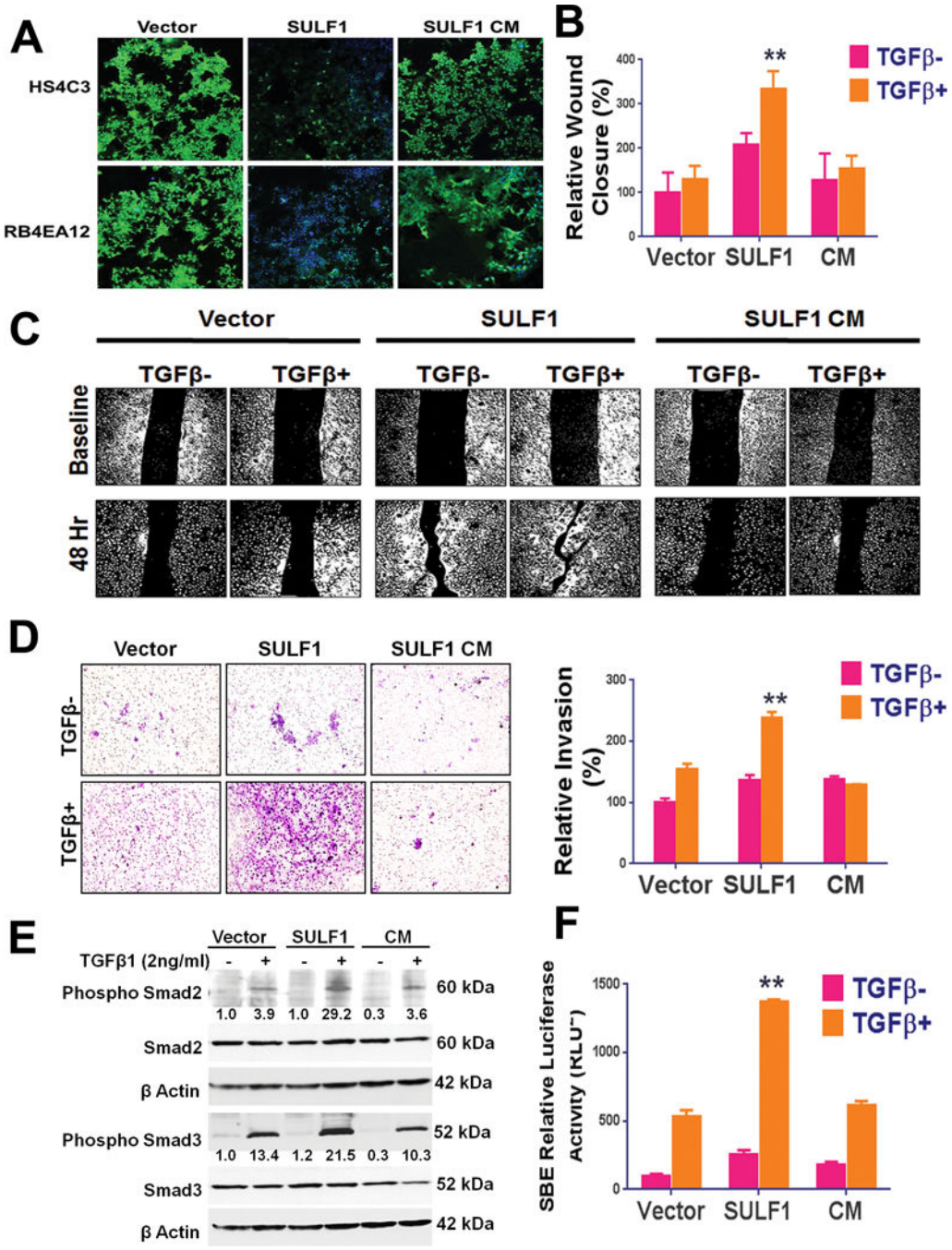

Figure 5. Mutation of the catalytic site of SULF1 abrogates SULF1 mediated activation of the TGFβ pathway

A. Immunofluorescence using single chain variable fragment antibodies HS4C3 and RB4EA12 which recognize 6-O-sulfated HS epitopes show significantly decreased immunofluorescence in cells expressing SULF1 when compared to cells transfected with Vector or Sulf1 catalytic mutant (CM). B and C. Migration assay performed using scratch test. TGFβ1 treatment-enhanced cell migration and wound closure is more pronounced in SULF1 transfected cells (**P<0.01) when compared to Vector or SULF1 CM. Bar graph reports results of migration assay as a percentage of wound closed normalized to the serum

starved Vector. Images shows wound healing over a 48 hour period in the Hep3B cell monolayer. D. Boyden's chamber assay performed to assess cell invasiveness. Vector, SULF1 or SULF1 CM transfected Hep3B cells were plated in the inserts of 24 well plates. Cells in the top layer were either serum starved or treated with TGFβ1 (2 ng/ml). Increased density of invaded cells is noted after TGFβ1 treatment and this effect is more pronounced in SULF1 transfected cells (**P<0.01) when compared to Vector control or SULF1 CM. Bar graph reports results of invasion assay as a percentage of cells invaded normalized to the serum starved Vector. E. Immunoblotting of cell extracts from Hep3B cells transfected with Vector, SULF1 or SULF1 CM either serum starved or treated with TGFβ1. SULF1 transfected cells show higher phosphorylation of SMAD2 and SMAD3 compared to Vector. But this increase was not noted in cells expressing SULF1 CM. The experiment was repeated three times and means of relative densitometry is represented below the blots. D. TGFβ-mediated increase in SBE Luciferase activity is augmented by SULF1 expression (**P<.01) and this increase is abolished when the plasmid carrying a mutation at the catalytic site (CM) is transfected. All experiments were repeated three times and the mean is presented in the bars; the error bars represent the standard deviation.

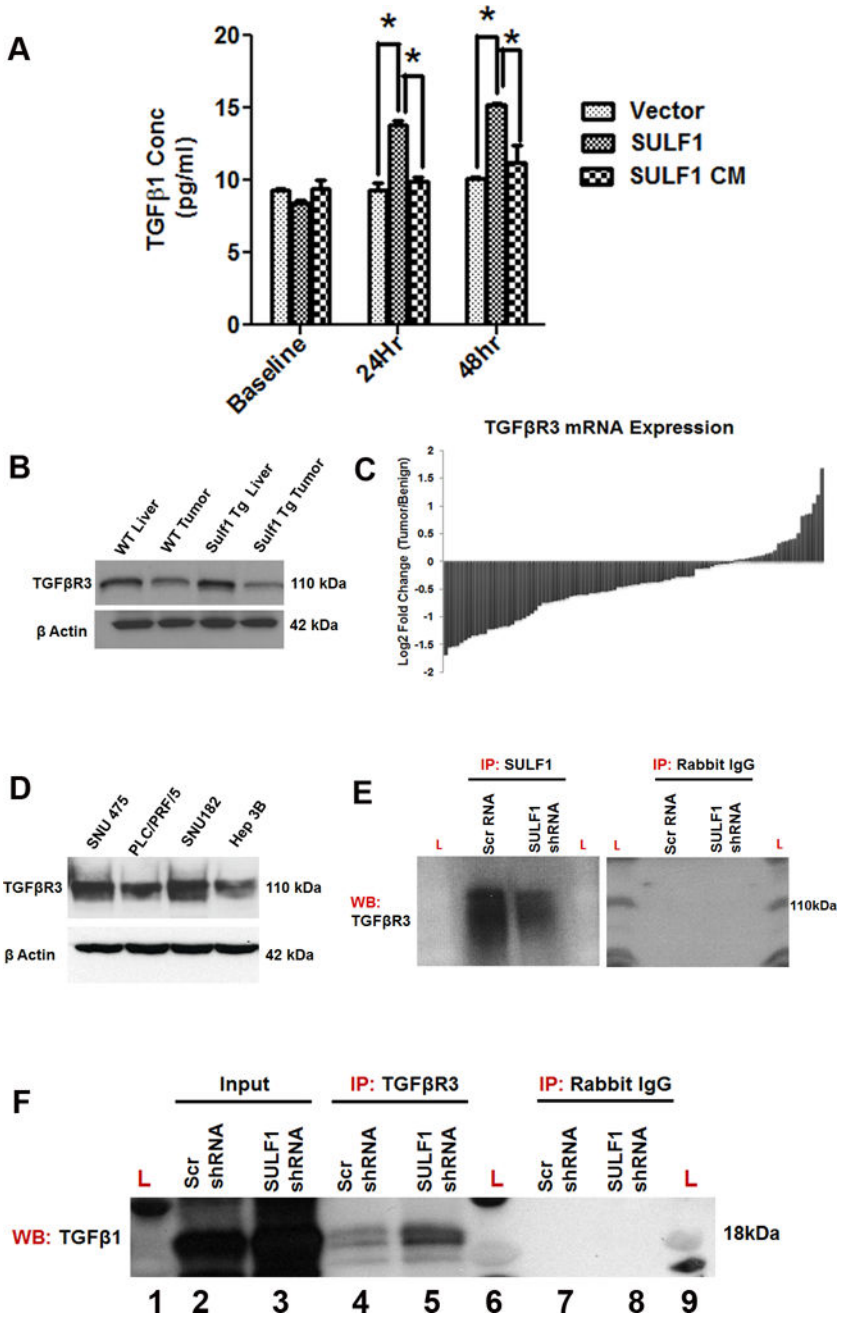

Figure 6. SULF1 releases TGFβ by modifying the sulfation status of cell surface HSPGs

A. TGFβ ELISA assay. Hep3B cells were transfected with Vector, SULF1 or SULF1 CM and serum starved. Conditioned medium from the transfected cells were collected at baseline, 24 and 48 hours and ELISA was performed for TGFβ1 levels. TGFβ1 levels were significantly higher in SULF1 transfected cells compared to Vector control or SULF1 CM (*P<.05). B. Immunoblotting of protein extracts from mice liver and tumor reveals similar expression in TGFβR3 between WT mice and Sulf1 Tg mice livers. And the expression of TGFRβ3 is uniformly lower in both WT tumor and SULF1 Tg tumor. C. Gene expression analysis of TGFβR3 mRNA in human HCC using microarray data shows decreased

expression of TGFRβ3 in a majority of patients. D. Immunoblotting confirms expression of TGFβR3 in all 4 cell lines (SNU475, PLC/PRF/5, SNU182 and Hep3B) used in this study. E. Cell lysates from SNU182 cells transfected with scr shRNA or Sulf1 shRNA were used for co-immunoprecipitation using anti-SULF1 antibody. Incubation with nonspecific IgG was used as negative control. Western immunoblotting was performed using antibodies against TGFβR3. Knockdown to SULF1 leads to decreased pull down of TGFβR3. F. Lysates from SNU182 cells which were transfected with scr shRNA or SUFL1 shRNA were used to perform IP with TGFβR3 and the resulting eluate was used for Western blotting with TGFβ1. We used whole cell lysate as positive control (Lane 2 and 3) and IP with non-specific IgG as negative control (Lane 7 and 8). And we found that lysates from cells with decreased SULF1 expression showed increased interaction between TGFβR3 and its ligand TGFβ1 (Lane 4 vs Lane 5). Lanes 1, 6 and 9 marked as L show the molecular weight marker ladder.

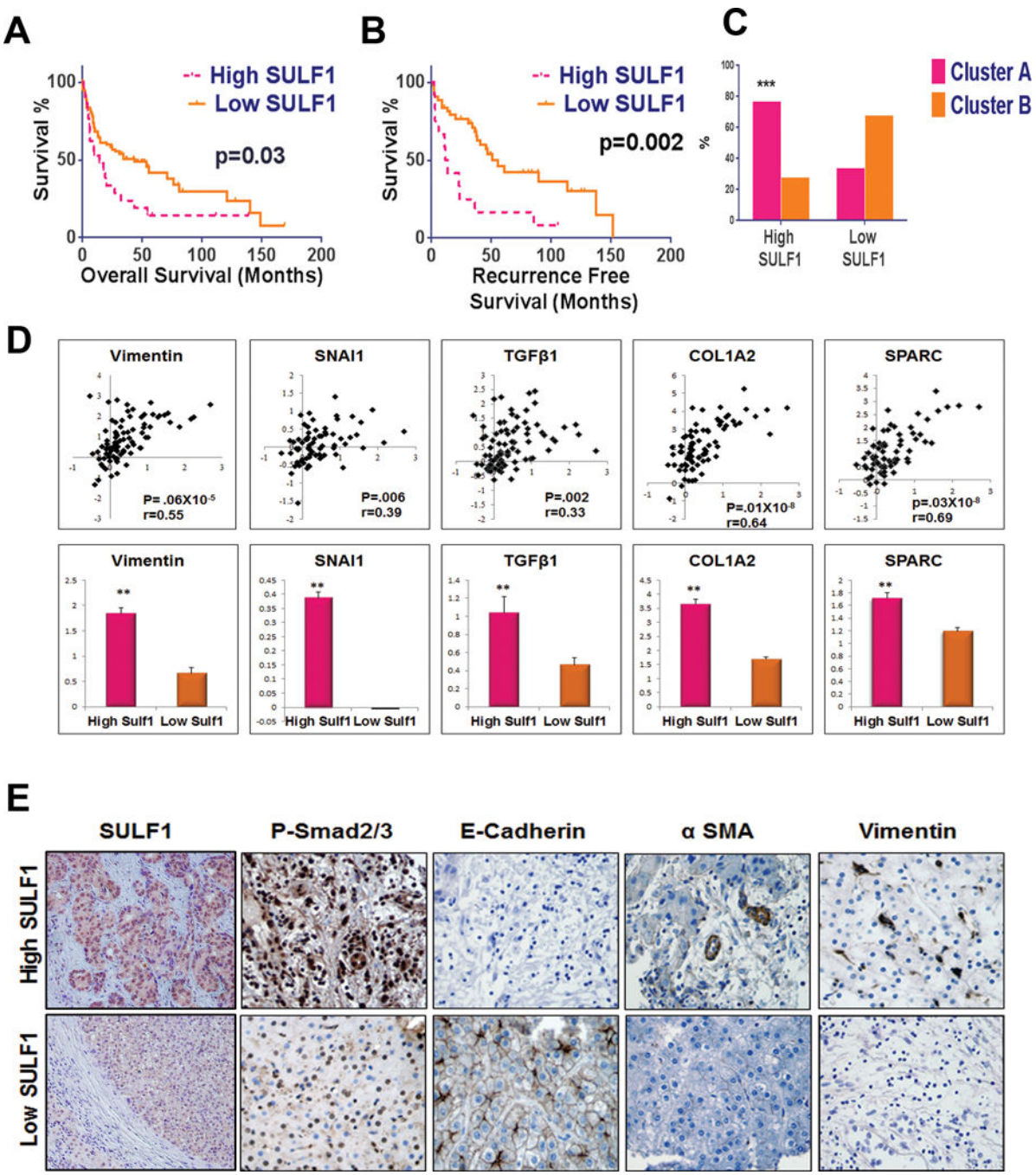

Figure 7. SULF1 over expression in human HCC is associated with poor prognosis and correlates with EMT related genes

A. Kaplan Meier curves demonstrate poorer overall survival in HCCs with high SULF1 expression B. Recurrence free survival is also poorer in HCCs with high SULF1 expression C. Classification of HCC into prognostic clusters with the help of previously validated gene signatures demonstrates that high SULF1 expression is strongly associated with the poor prognosis cluster A (*** P<0.001). D. Top panel of scatter plots shows five well recognized EMT related genes highly correlated with SULF1 expression: vimentin, SNAI1 (Snail family zinc finger 1); TGFβ1; COL1A2 (Collagen, Type I, Alpha 2) and SPARC (Secreted

Protein, Acidic, Cysteine-Rich). Bottom panel confirms these genes are overexpressed in HCC with high SULF1 expression compared to HCC with low SULF1 expression (**P<0.001). E. Immunohistochemistry of HCC from resected tissue. Top row is HCC over-expressing SULF1 and bottom row is HCC with low SULF1 expression. IHC reveals higher p-SMAD2/3, lower E-cadherin, higher αSMA and higher vimentin expression in SULF1 high tumors.

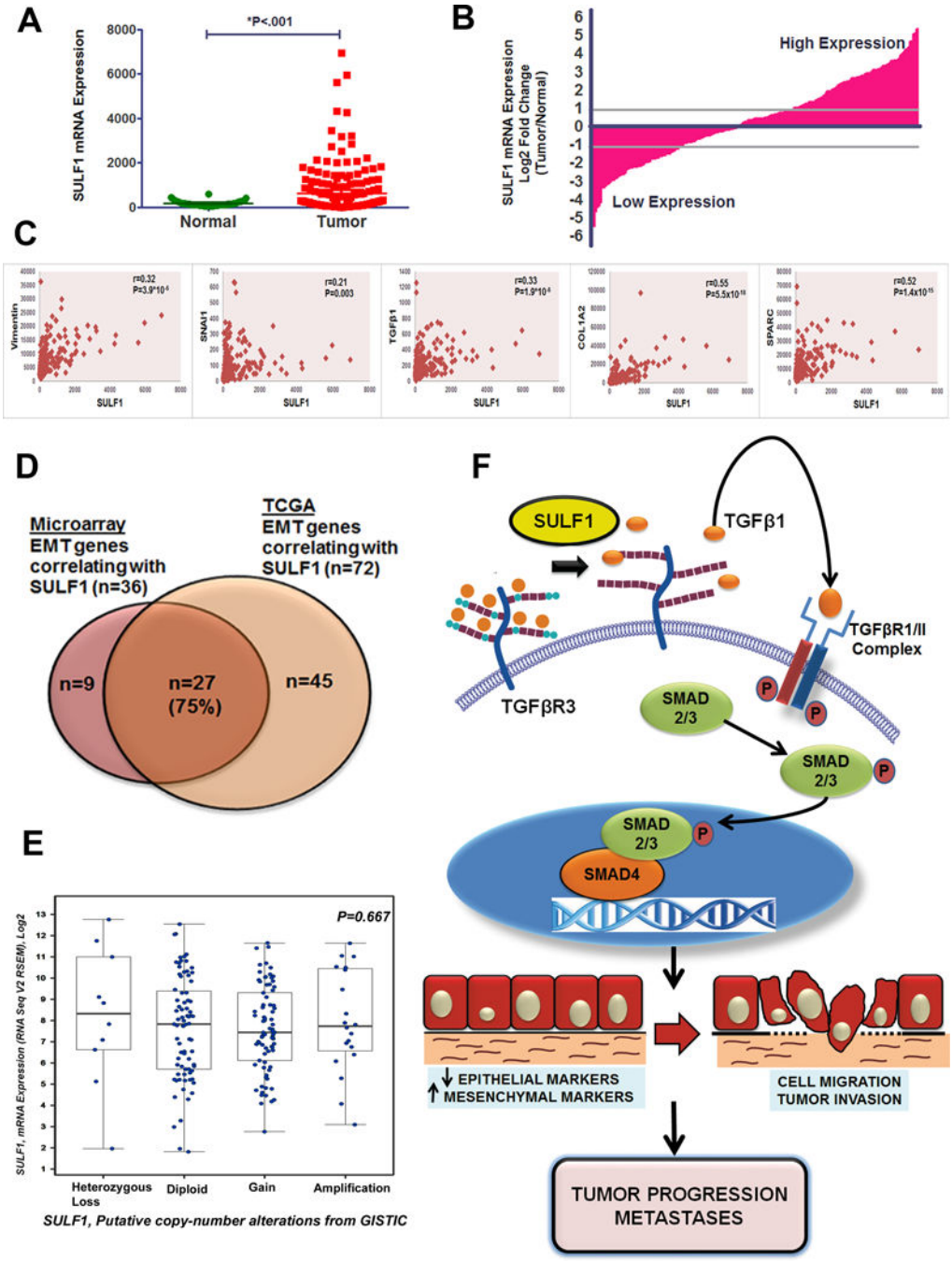

Figure 8. Gene expression analysis of human HCC from The Cancer Genome Atlas (TCGA) confirms correlation of SULF1 with EMT related genes

A. SULF1 expression is significantly higher in human HCC when compared to surrounding normal liver (P<0.001). B. Waterfall plot demonstrating the distribution of SULF1 expression in the TCGA data. SULF1 is overexpressed around 40% of patients with HCC. C. Validation of five well recognized EMT related genes highly correlated with SULF1 expression: vimentin; SNAI1 (Snail family zinc finger 1); TGFβ1; COL1A2 (Collagen, Type I, Alpha 2) and SPARC (Secreted Protein, Acidic, Cysteine-Rich) D. Venn diagram showing that a majority (75%) of the EMT genes from the microarray analysis correlating

with SULF1 were also confirmed to correlate with SULF1 in the TCGA analysis. E. Correlation analysis showing lack of significant correlation between SULF1 mRNA expression (x-axis) and SULF1 copy number variation (y-axis). F. Cartoon depicts our hypothesis. SULF1 desulfates cell surface HSPG (TGFβR3) and releases TGFβ ligand. TGFβ then binds to TGFβRI/RII complex and activates its canonical signaling via phosphorylation of SMAD2/3. Phosphorylated SMAD2/3 translocates to the nucleus, forms a complex with SMAD4 and activates transcriptional factors which promote epithelial mesenchymal transition of HCC cells. This eventually leads to tumor progression and metastases.