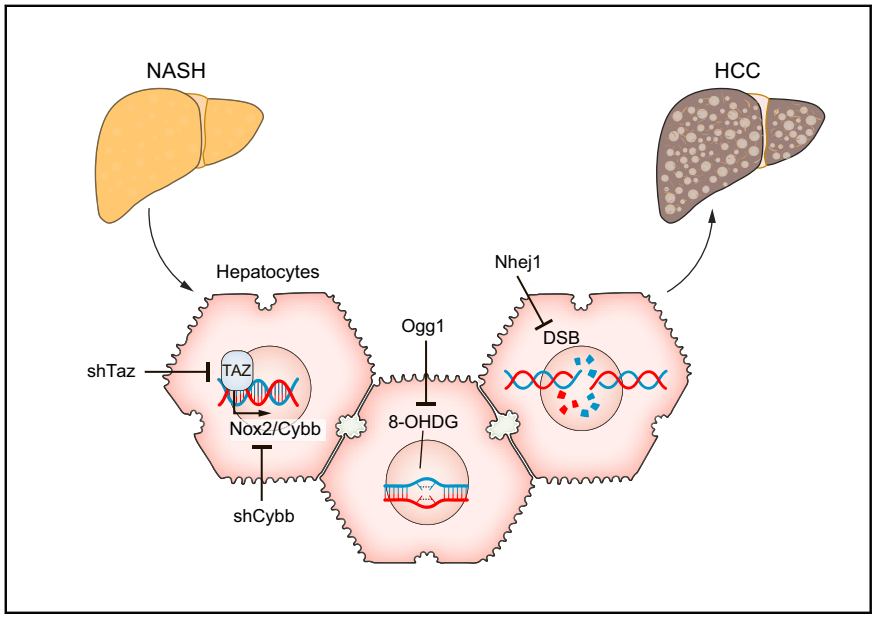
Research Article
Experimental and Translational Hepatology

JOURNAL
OF HEPATOLOGY

TAZ-induced Cybb contributes to liver tumor formation in non-alcoholic steatohepatitis

Graphical abstract



Highlights

- Silencing hepatocyte TAZ in pre-tumor NASH suppresses subsequent HCC.
- *Cybb* is the key TAZ-induced gene in NASH hepatocytes that triggers tumor formation.
- *Cybb* encodes NOX2, which promotes HCC by inducing oxidative DNA damage.
- Silencing hepatocyte Cybb in pre-tumor NASH, or blocking DNA damage, suppresses HCC.
- TAZ, NOX2, oxidative DNA damage are strongly correlated in human NASH-HCC liver.

Authors

Xiaobo Wang, Sharon Zeldin, Hongxue Shi, …, Utpal B. Pajvani, Robert F. Schwabe, Ira Tabas

Correspondence

xw2279@columbia.edu (X. Wang), iat1@columbia.edu (I. Tabas).

Lay summary

Non-alcoholic steatohepatitis (NASH) is emerging as the leading cause of a type of liver cancer called hepatocellular carcinoma (HCC), but molecular events in pre-tumor NASH hepatocytes leading to HCC remain largely unknown. Our study shows that a protein called TAZ in pre-tumor NASH-hepatocytes promotes damage to the DNA of hepatocytes and thereby contributes to eventual HCC. This study reveals a very early event in HCC that is induced in pre-tumor NASH, and the findings suggest that NASH therapies targeting TAZ might also prevent NASH-HCC.

https://doi.org/10.1016/j.jhep.2021.11.031

Research Article
Experimental and Translational Hepatology

JOURNAL OF HEPATOLOGY

TAZ-induced Cybb contributes to liver tumor formation in non-alcoholic steatohepatitis

Xiaobo Wang[1,*], Sharon Zeldin[1], Hongxue Shi[1], Changyu Zhu[1], Yoshinobu Saito[1], Kathleen E. Corey[2,3], Stephanie A. Osganian[2], Helen E. Remotti[4], Elizabeth C. Verna[1], Utpal B. Pajvani[1,5], Robert F. Schwabe[1,5], Ira Tabas[1,4,5,6,*]

[1]*Department of Medicine, Columbia University Irving Medical Center, New York, NY 10032, USA;* [2]*Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114, USA;* [3]*Harvard Medical School, Boston, MA 02115, USA;* [4]*Department of Pathology and Cell Biology, Columbia University Irving Medical Center, New York, NY 10032, USA;* [5]*Institute of Human Nutrition, Columbia University Irving Medical Center, New York, NY 10032, USA;* [6]*Department of Physiology and Cellular Biophysics, Columbia University Irving Medical Center, New York, NY 10032, USA*

Background & Aims: Non-alcoholic steatohepatitis (NASH) is a leading cause of hepatocellular carcinoma (HCC), but mechanisms linking NASH to eventual tumor formation remain poorly understood. Herein, we investigate the role of TAZ/WWTR1, which is induced in hepatocytes in NASH, in the progression of NASH to HCC.

Methods: The roles of hepatocyte TAZ and its downstream targets were investigated in diet-induced and genetic models of NASH-HCC using gene-targeting, adeno-associated virus 8 (AAV8)-H1-mediated gene silencing, or AAV8-TBG-mediated gene expression. The biochemical signature of the newly elucidated pathway was probed in liver specimens from humans with NASH-HCC.

Results: When hepatocyte-TAZ was silenced in mice with pre-tumor NASH using AAV8-H1-shTaz (short-hairpin Taz), subsequent HCC tumor development was suppressed. In this setting, the tumor-suppressing effect of shTaz was not dependent of TAZ silencing in the tumors themselves and could be dissociated from the NASH-suppressing effects of shTaz. The mechanism linking pre-tumor hepatocyte-TAZ to eventual tumor formation involved TAZ-mediated induction of the NOX2-encoding gene *Cybb*, which led to NADPH-mediated oxidative DNA damage. As evidence, DNA damage and tumor formation could be suppressed by treatment of pre-tumor NASH mice with AAV8-H1-shCybb; AAV8-TBG-OGG1, encoding the oxidative DNA-repair enzyme 8-oxoguanine glycosylase; or AAV8-TBG-NHEJ1, encoding the dsDNA repair enzyme non-homologous end-joining factor 1. In surrounding non-tumor tissue from human NASH-HCC livers, there were strong correlations between TAZ, NOX2, and oxidative DNA damage.

Conclusions: TAZ in pre-tumor NASH-hepatocytes, via induction of *Cybb* and NOX2-mediated DNA damage, contributes to subsequent HCC tumor development. These findings illustrate how NASH provides a unique window into the early molecular events that can lead to tumor formation and suggest that NASH therapies targeting TAZ might also prevent NASH-HCC.

Lay summary: Non-alcoholic steatohepatitis (NASH) is emerging as the leading cause of a type of liver cancer called hepatocellular carcinoma (HCC), but molecular events in pre-tumor NASH hepatocytes leading to HCC remain largely unknown. Our study shows that a protein called TAZ in pre-tumor NASH-hepatocytes promotes damage to the DNA of hepatocytes and thereby contributes to eventual HCC. This study reveals a very early event in HCC that is induced in pre-tumor NASH, and the findings suggest that NASH therapies targeting TAZ might also prevent NASH-HCC.

Introduction

Non-alcoholic steatohepatitis (NASH) is emerging as the leading cause of both liver disease[1–3] and hepatocellular carcinoma (HCC).[3,4] NASH-HCC can develop in the absence of cirrhosis,[5–7] suggesting that NASH-mediated signals within hepatocytes may drive carcinogenesis before the carcinogenic effects of cirrhosis are present. Indeed, the prolonged pre-cancer stage of NASH provides a unique opportunity to address a major challenge in cancer, namely, identifying very early changes in non-cancer cells that can subsequently lead to tumor formation. However, the mechanisms of how NASH predisposes to eventual HCC tumor formation remain largely unknown.

To address this challenge, we investigated 3 features that are common to NASH hepatocytes and HCC tumor cells, namely, TAZ/WWTR1, oxidative stress, and DNA damage.[8–11] The gene regulator TAZ/WWTR1 is increased in mouse and human hepatocytes as hepatosteatosis progresses to NASH[8,12–14] and promotes NASH by inducing the secretory protein Indian hedgehog (Ihh).[8] Oxidative stress occurs in NASH hepatocytes and can cause double-stranded DNA breaks and chromosome instability, which, by causing mutations in tumor-suppressor genes, can induce HCC when other "hits" are present.[10,11,15,16] Although TAZ can promote tumor growth and spread, including in liver cancer,[17–19] we hypothesized that it might have an independent role in activating molecular events in pre-tumor NASH hepatocytes

Keywords: nonalcoholic steatohepatitis (NASH); hepatocellular carcinoma (HCC); TAZ/WWTR1; NOX2/Cybb; oxidative DNA damage.
Received 1 July 2021; received in revised form 8 November 2021; accepted 25 November 2021; available online 11 December 2021
* Corresponding authors. Address: Department of Medicine, Columbia University Irving Medical Center, New York, NY 10032, USA.
E-mail addresses: xw2279@columbia.edu (X. Wang), iat1@columbia.edu (I. Tabas).
https://doi.org/10.1016/j.jhep.2021.11.031

that could eventually lead to HCC. We now present evidence that TAZ, by inducing the pro-oxidant gene *Cybb*, promotes oxidative DNA damage in pre-tumor NASH, leading to eventual HCC tumor formation. This conclusion is supported by molecular-genetic causation data in experimental NASH-HCC, and the biochemical signature of this pathway is present in human NASH-HCC.

Materials and methods

Animal studies

Male wild-type C57BL/6J mice (#000664, 9-10 weeks/old), *Cybb*$^{fl/fl}$ mice (#031777), and *Rosa*^{NICD} mice (#008159) were from Jackson Laboratory (Bar Harbor, ME) and allowed to adapt in the animal facility for 1 week prior to random assignment to experimental cohorts. *Wwtr1*$^{fl/fl}$ mice,[20] backcrossed to C57BL/6J, were provided by Dr. Eric Olson (University of Texas Southwestern). The mice were fed a diet containing sugar water (23.1 g fructose/L and 18.9 g glucose/L), palmitate, and 1.25% cholesterol ("NASH diet"; Teklad, TD.160785 PWD), which induces NASH after 16 weeks.[8] All adeno-associated virus 8 (AAV8) vectors were injected by tail vein ($2x10^{11}$ genome copies/mouse) as indicated in the figure legends. For the DMBA model, 50 μl of 0.5% DMBA (7,12-dimethylbenz [a]anthracene, Sigma) in acetone was administered to the dorsal surface on postnatal day 4–5[21]; the NASH diet was begun after weaning (1 month old). Animals were housed in standard cages at 22 °C in a 12-12-hour light-dark cycle in a barrier facility. For mouse HCC, the predetermined endpoint was tumor weight estimated to be <10% of body weight. All animal experiments were performed in accordance with institutional guidelines and regulations and approved by the Institutional Animal Care and Use Committee at Columbia University.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Silencing hepatocyte TAZ in pre-tumor NASH mice suppresses HCC tumor development

We began our investigation with a well-characterized and validated NASH model that uses a diet rich in fructose, palmitate, and cholesterol.[8,14,22–24] In this model, early fibrosis occurs after 16 weeks on diet, but we extended the feeding period to 15 months to look for HCC. All of the mice developed tumors showing features of HCC, including far fewer portal tracts in the tumors than in the surrounding liver, reticulin staining showing expanded hepatocyte cords, and positive glypican-3 staining (Fig. 1A). In another cohort, we administered AAV8-H1-shTaz (short-hairpin Taz) or AAV8-H1-scrambled RNA (Scr) at the 8-month time point, which is before tumors develop, and analyzed the mice at 13 months (Fig. 1B). AAV8-shTaz potently lowers TAZ specifically in hepatocytes,[8] and we documented TAZ silencing in the livers of the 13-month NASH diet-fed mice (Fig. 1C). We found that hepatocyte-TAZ silencing completely prevented tumor development (Fig. 1D). The percentage of Ki67$^+$ HNF4α$^+$ liver cells in non-tumor tissue was also decreased in the shTaz cohort (Fig. 1E). For a second model, we treated newborn mice with the mutagen DMBA and then placed them on the NASH diet from 1-9 months of age. DMBA alone does not cause tumors in this timeframe,[21] but the combination of DMBA and the NASH diet led to the development of numerous tumors (Fig. 1F). DMBA/NASH diet-treated *Wwtr1*$^{fl/fl}$ mice were treated with AAV8-TBG-Cre to delete hepatocyte-TAZ, or AAV8-TBG-LacZ control, at the 5-month time point, which is before tumors form (Fig. 1G,H). Deletion of hepatocyte-TAZ markedly decreased tumor number and size at 10 months (Figs 1I and S1A). The percentage of Ki67$^+$HNF4α$^+$ cells in non-tumor tissue was also decreased by hepatocyte-TAZ deletion (Fig. 1J). As a third model, we activated hepatocyte Notch by treating *Rosa*^{NICD} mice with AAV8-TBG-Cre and then feeding them the NASH diet, which leads to NASH features after 2 months and NASH diet-dependent HCC tumor formation by 3-4 months.[23] We confirmed that tumors formed at 4 months (Fig. 1K) and then used *Rosa*^{NICD} *Wwtr1*$^{fl/fl}$ to test our hypothesis. The experimental group was administered AAV8-TBG-Cre to enable both Notch activation and TAZ deletion in hepatocytes, with Cre-injected *Rosa*^{NICD} mice serving as the intact-TAZ control cohort (Fig. 1L,M). The mice were then fed the NASH diet for 4 months. Deletion of hepatocyte TAZ lowered tumor number and size and the percentage of Ki67$^+$HNF4α$^+$ cells in non-tumor tissue (Figs 1N,O and S1B). Thus, in 3 separate models of NASH diet-dependent HCC, silencing or deleting TAZ in hepatocytes before tumors form suppresses the eventual formation of HCC tumors.

HCC tumor suppression by hepatocyte-TAZ silencing is not dependent of TAZ silencing in tumors

As with other types of HCC,[25,26] TAZ was expressed in human and mouse NASH-HCC tumors (Fig. S1C-S1G), and we found that TAZ deletion using the cre-lox method, *i.e.*, AAV8-TBG-Cre in the DMBA-*Wwtr1*$^{fl/fl}$ and *Rosa*^{NICD} *Wwtr1*$^{fl/fl}$ models, lowered tumor TAZ (Fig. S1H,I). Thus, it was possible that silencing of TAZ in tumor cells was responsible for tumor suppression. In contrast, episomally expressed AAV8-shTaz becomes diluted as cells divide, resulting in eventual elimination of gene silencing in tumors. Thus, the tumor-preventative effect of AAV8-H1-shTaz in the 13-month NASH-diet model (Fig. 1B-E) suggests a pre-tumor effect. To test this principle in a more robust model, we turned to the Notch-NASH diet model. Three months after Notch activation and the start of the NASH diet, mice were injected with AAV8-H1-shTaz or AAV8-H1-Scr and analyzed 2 months later (Fig. 2A). As designed, shTaz silenced TAZ in surrounding NASH tissue but not in the tumor tissue (Fig. 2B). We found that tumor number and size were decreased by shTaz treatment (Fig. 2C and S1J), as was the percentage of Ki67$^+$HNF4α$^+$ cells in surrounding tissue (Fig. 2D). Note that shTaz did not alter the expression of the Notch downstream gene *Hes1* (Fig. S1K), indicating lack of interference with Notch function itself. These data suggest the TAZ in pre-tumor hepatocytes contributes to molecular events that can eventually lead to tumor formation.

The tumor-suppressing and NASH-suppressing effects of shTaz can be dissociated in experimental NASH-HCC

As expected from our previous work,[8] hepatocyte-TAZ deletion lowered liver inflammation, fibrosis, and cell death and plasma ALT in the models studied in Figs. 1 and 2 (Fig. S2A-T). Thus, the anti-tumor-suppressing effect of hepatocyte-TAZ silencing in our models could be secondary to suppressing the NASH niche, which can in its advanced form contribute to HCC development.[27,28] However, NASH is relatively low-grade in our models, suggesting that the anti-tumor effect of shTaz might be independent of its NASH-suppressing effect. To test this idea, we examined the role of Indian hedgehog (Ihh) in NASH-HCC, as Ihh is the major gene target of TAZ/TEAD responsible for TAZ-induced NASH liver inflammation, fibrosis, and cell death.[8] Using the Notch (NICD)/NASH-diet model, mice were treated with AAV8-H1-shTaz plus either AAV8-TBG-Ihh or control AAV8-TBG-LacZ at the 2-month timepoint and then examined at 4 months

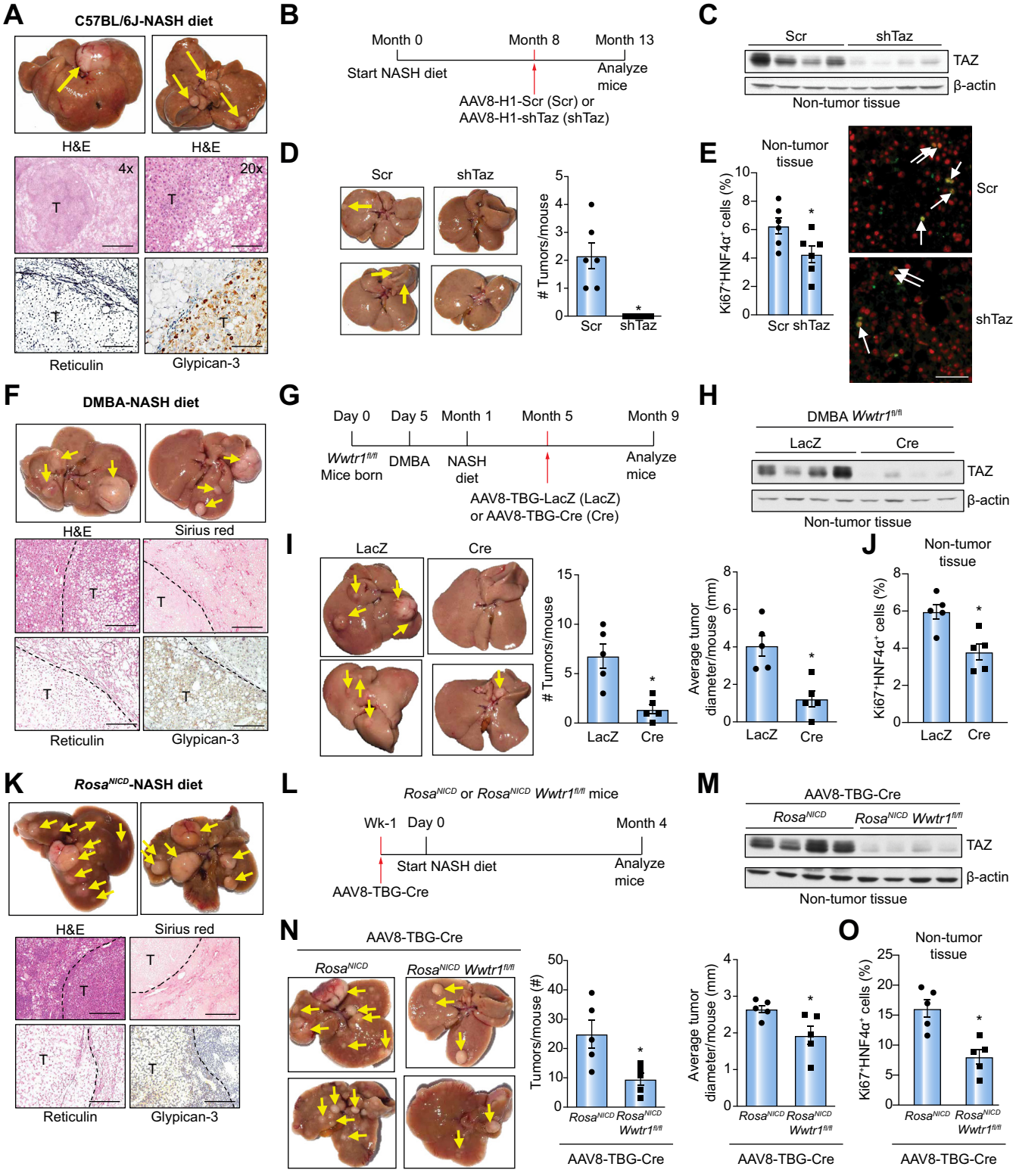


Fig. 1. Silencing hepatocyte TAZ in pre-tumor NASH mice suppresses the development of HCC tumors. (A) Livers (arrows, tumors) and liver sections of mice fed the NASH diet for 15 months. The sections were stained with H&E (imaged at 4X and 20X; bars, 1 mm and 200 μm, respectively) and with reticulin and anti-glypican-3 (bars, 100 μm). (B-E) Mice were fed the NASH diet for 13 months, with AAV8-H1-shTaz (shTaz) or control vector (Scr) administered at 8 months. (B) Experimental scheme. (C) TAZ immunoblot from non-tumor liver tissue. (D) Livers (arrows, tumors) and tumor numbers/mouse. (E) Liver sections from non-tumor areas stained for Ki67 (green) and HNF4α (red) and quantified for the percent $Ki67^+HNF4\alpha^+$ cells (arrows, $Ki67^+HNF4\alpha^+$ cells; bar, 100 μm). For D-E, n = 6 mice/group; means ± SEM; $^*p$ <0.05 by Student's *t* test. (F) Livers (arrows, tumors) and liver sections of mice that were administered DMBA on post-natal day 5; placed on NASH diet at 1 month of age, and analyzed at 9 months. The sections were stained with H&E, Sirius red (bars, 500 μm), reticulin, and anti-glypican-3 (bars, 200 μm). (G-J) $Wwtr1^{fl/fl}$ male mice were administered DMBA on postnatal day 5; placed on the NASH diet at 1 month; injected with AAV8-TBG-LacZ or

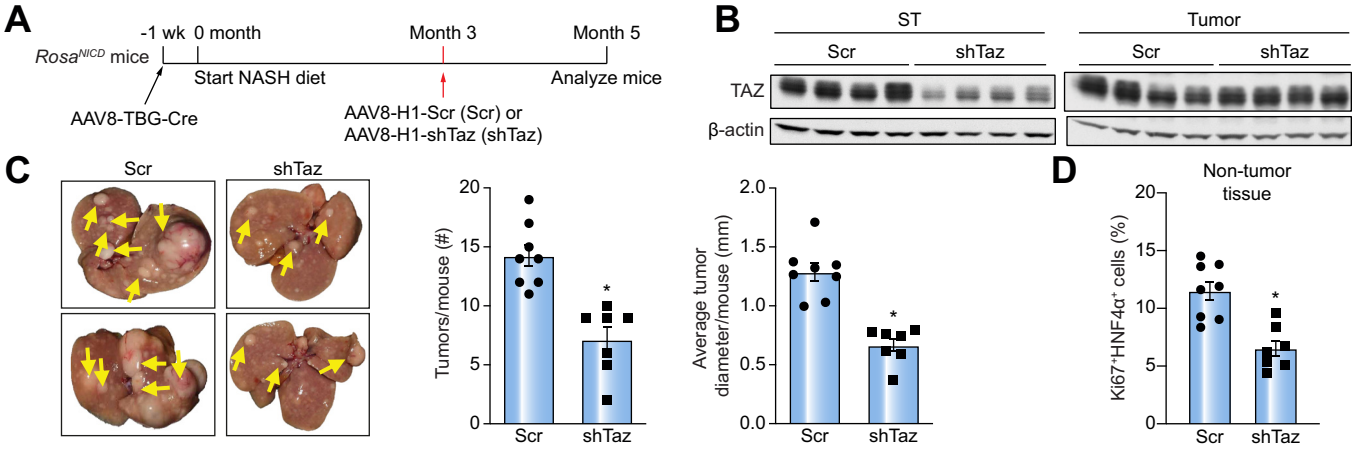


Fig. 2. Suppression of hepatocellular carcinoma formation by hepatocyte-TAZ silencing is not dependent of tumor-TAZ silencing. (A-D) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 3 months later, injected with AAV8-H1-scrambled RNA or AAV8-H1-shTaz. The mice were analyzed at month 5. (A) Experimental scheme. (B) TAZ immunoblot from ST and tumor tissue. (C) Livers (arrows, tumors) and tumor numbers and average diameter. (D) Quantification of the percentage of Ki67^{+}HNF4α^{+} cells in liver sections from non-tumor areas. For C-D, n = 7-8 mice/group; means ± SEM; *p <0.05 by Student's t test. AAV8, adeno-associated virus 8; NASH, non-alcoholic steatohepatitis; Scr, scrambled RNA; shTAZ, short-hairpin TAZ; ST, surrounding tissue; TBG, thyroxine-binding globulin promoter.

(Fig. 3A). In a parallel experiment, we showed that shTaz in this 2-month to 4-month protocol suppressed tumors (Fig. S3A,B), lowered TAZ and Ihh expression only in non-tumor tissue (Fig. S3C), and decreased NASH endpoints without affecting body weight or fasting plasma glucose (Fig. S3D-F). As designed, treatment of TAZ-silenced mice with AAV8-TBG-Ihh increased Ihh in non-tumor-bearing NASH liver but not in the tumors themselves (Fig. 3B), and, consistent with our previous data,[8] Ihh restored NASH features in the TAZ-silenced mice (Fig. 3C-E) without affecting body weight or fasting plasma glucose (Fig. S3G,H). Most importantly, AAV8-TBG-Ihh did not increase tumor number or size in the TAZ-silenced mice (Fig. 3F and S3I). Next, we directly silenced Ihh in this model (Fig. 3G), which resulted in lower Ihh in non-tumor tissue but not tumor tissue (Fig. 3H). As expected, this intervention lowered liver inflammation, fibrosis, and TUNEL^{+} cells in the liver and plasma ALT (Fig. 3I-3K) without affecting body weight or fasting plasma glucose (Fig. S3J,K). Most importantly, shIhh did not lower HCC development (Figs 3L and S3L). These combined data dissociate the tumor-suppressing effect of shTaz from its NASH-suppressing effects in this model. Moreover, while Ihh is a key TAZ gene target that contributes to NASH progression, Ihh does not appear to be involved in TAZ-induced HCC.

TAZ-mediated oxidative DNA damage in pre-tumor NASH is linked to tumor formation

In our search for TAZ-mediated processes in NASH that might contribute to eventual tumor formation, we investigated a key process in HCC, namely, DNA damage.[9,10] First, we found that a marker of double-stranded DNA (dsDNA) damage, γH2AX (phospho-H2AX), was increased in the livers of humans and mice with non-tumor NASH (Fig. S4A,B). Next, we found that treatment with AAV8-H1-shTaz eliminated the increase in γH2AX in NASH mice (Fig. 4A). Although the decrease in the γHA2X signal by shTaz could have resulted from the suppression of hepatocyte proliferation,[10] shTaz did not affect the percentage of Ki67^{+}HNF4α^{+} cells in these non-HCC NASH livers (Fig. S4C). Moreover, this finding shows that TAZ promotes DNA damage in hepatocytes before proliferation occurs. One mechanism of dsDNA damage in HCC is oxidative DNA damage, and there is evidence that this process is relevant to NASH-HCC in humans.[11] Using the livers of mice fed the NASH diet for 8 weeks (steatosis) or 16 weeks (early NASH), we used immunofluorescence microscopy to detect hepatocytes expressing a marker of oxidative DNA damage, 8-oxo-2'-deoxyguanosine (8-OHDG). The percent of 8-OHDG^{+} hepatocytes increased during the period of steatosis-to-NASH progression (Figs 4B and S4D), and the increase at 16 weeks was diminished in mice by AAV8-H1-shTaz treatment (Fig. 4C).

We next sought direct evidence that oxidative DNA damage was involved in NASH-HCC by testing the effect of 8-oxoguanine glycosylase (OGG1), which mediates base excision repair of oxidatively damaged DNA.[29] First, transfection of AML12 cells with Ogg1 prevented cholesterol/palmitate-induced DNA damage as assessed by γH2AX immunoblot (Fig. 4D). Next, we administered AAV8-TBG-Ogg1 or control virus (AAV8-TBG-GFP) 2 months after the start of the NASH diet in Cre-treated *Rosa*^{NICD} mice and then analyzed the mice 1 month later (Fig. 4E). AAV8-TBG-Ogg1 successfully increased liver *Ogg1* mRNA without affecting *Wwtr1* (TAZ); increased OGG1 protein in surrounding tissue but not tumors; and decreased γH2AX1 in surrounding

AAV8-TBG-Cre at 5 months; and analyzed at 9 months. (G) Experimental scheme. (H) TAZ immunoblot from non-tumor liver tissue. (I) Livers (arrows, tumors) and tumor numbers and average diameter. (J) Percent Ki67^{+}HNF4α^{+} cells in non-tumor areas. For I-J, n = 5 mice/group; means ± SEM; *p <0.05 by Student's t test. (K) Livers (arrows, tumors) and liver sections of *Rosa*^{NICD} mice injected with AAV8-TBG-Cre to activate hepatocyte Notch; started on the NASH diet 1 week later; and analyzed after 4 months on diet. The sections were stained with H&E, Sirius red (bars, 500 μm), reticulin, and anti-glypican-3 (bars, 200 μm). (L-O) *Rosa*^{NICD} or *Rosa*^{NICD} *Wwtr1*$^{fl/fl}$ mice were treated with AAV8-TBG-Cre; placed on NASH diet 1 week later; and analyzed 4 months later. (L) Experimental scheme. (M) TAZ immunoblot from non-tumor liver tissue. (N) Livers (arrows, tumors) and tumor numbers and average diameter. (O) Percent Ki67^{+}HNF4α^{+} cells in non-tumor areas. For N-O, n = 5 mice/group; means ± SEM; *p <0.05 by Student's t test. AAV8, adeno-associated virus 8; DMBA, 7,12-dimethylbenz [a]anthracene; NASH, non-alcoholic steatohepatitis; Scr, scrambled RNA; shTAZ, short-hairpin TAZ; T, tumor; TBG, thyroxine-binding globulin promoter.

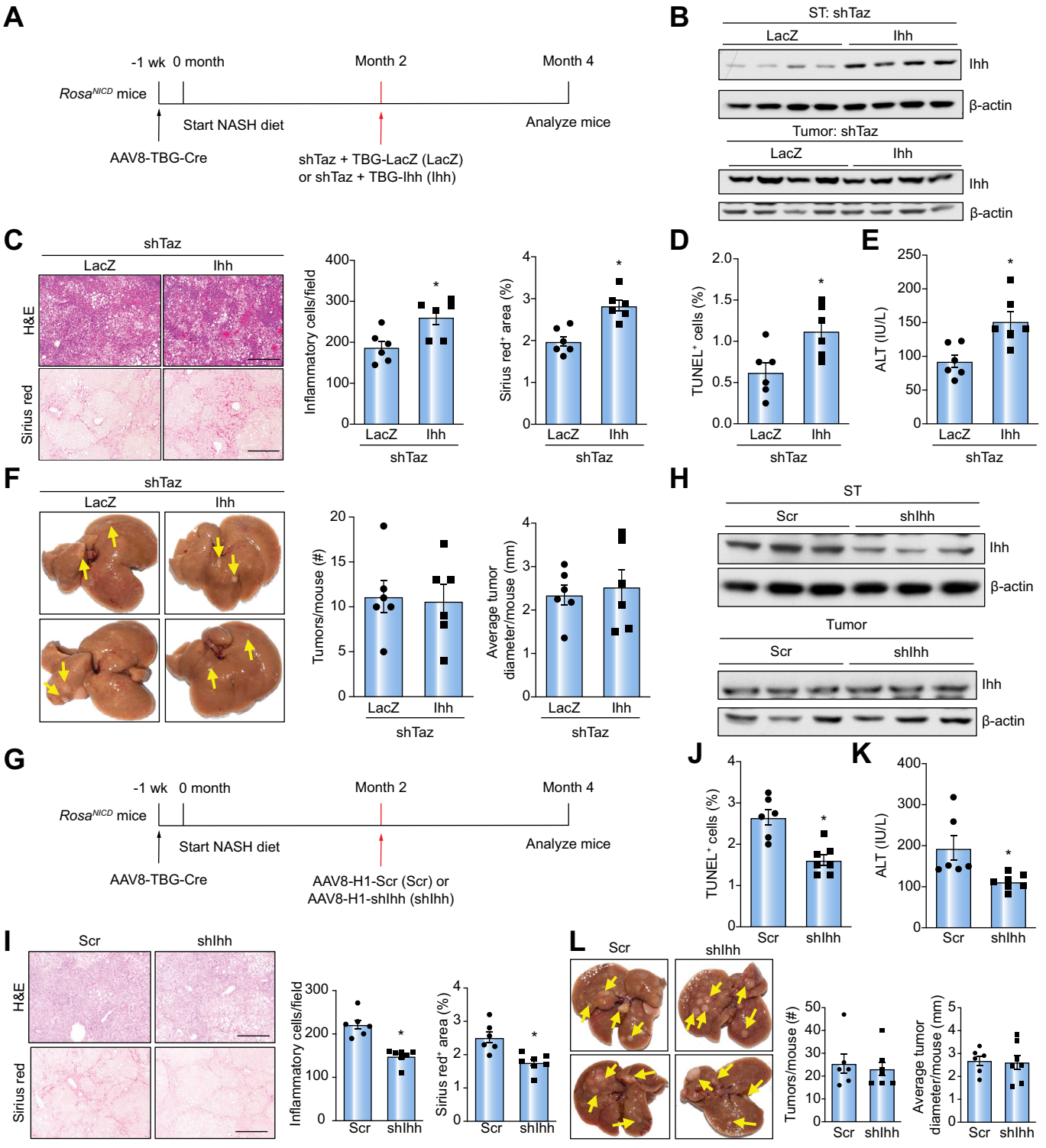


Fig. 3. Tumor-suppressing effect of shTaz in experimental NASH-HCC can be dissociated from its NASH-suppressing effects. (A-F) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 2 months later, injected with AAV8-H1-shTaz and either AAV8-TBG-LacZ or AAV8-TBG-Ihh. The mice were analyzed at month 4. (A) Experimental scheme. (B) Ihh immunoblot from ST and tumor tissue. (C) Liver sections stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (D) Percent TUNEL$^+$ cells from non-tumor areas. (E) Plasma ALT. (F) Livers (arrows, tumors) and tumor numbers and diameter. For C-F, n = 6 mice/group; means ± SEM; $^*p$ <0.05 by Student's *t* test. (G-L) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 2 months later, injected with AAV8-H1-Scr or AAV8-H1-shIhh. The mice were analyzed at month 4. (G) Experimental scheme. (H) Ihh immunoblot from ST and tumor tissue. (I) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (J) Percent TUNEL$^+$ cells from non-tumor areas. (K) Plasma ALT. (L) Livers (arrows, tumors) and tumor numbers and diameter. For I-L, n = 6-7 mice/group; means ± SEM; $^*p$ <0.05 by Student's *t* test. AAV8, adeno-associated virus 8; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; Scr, scrambled RNA; shTAZ, short-hairpin TAZ; ST, surrounding tissue; TBG, thyroxine-binding globulin promoter.

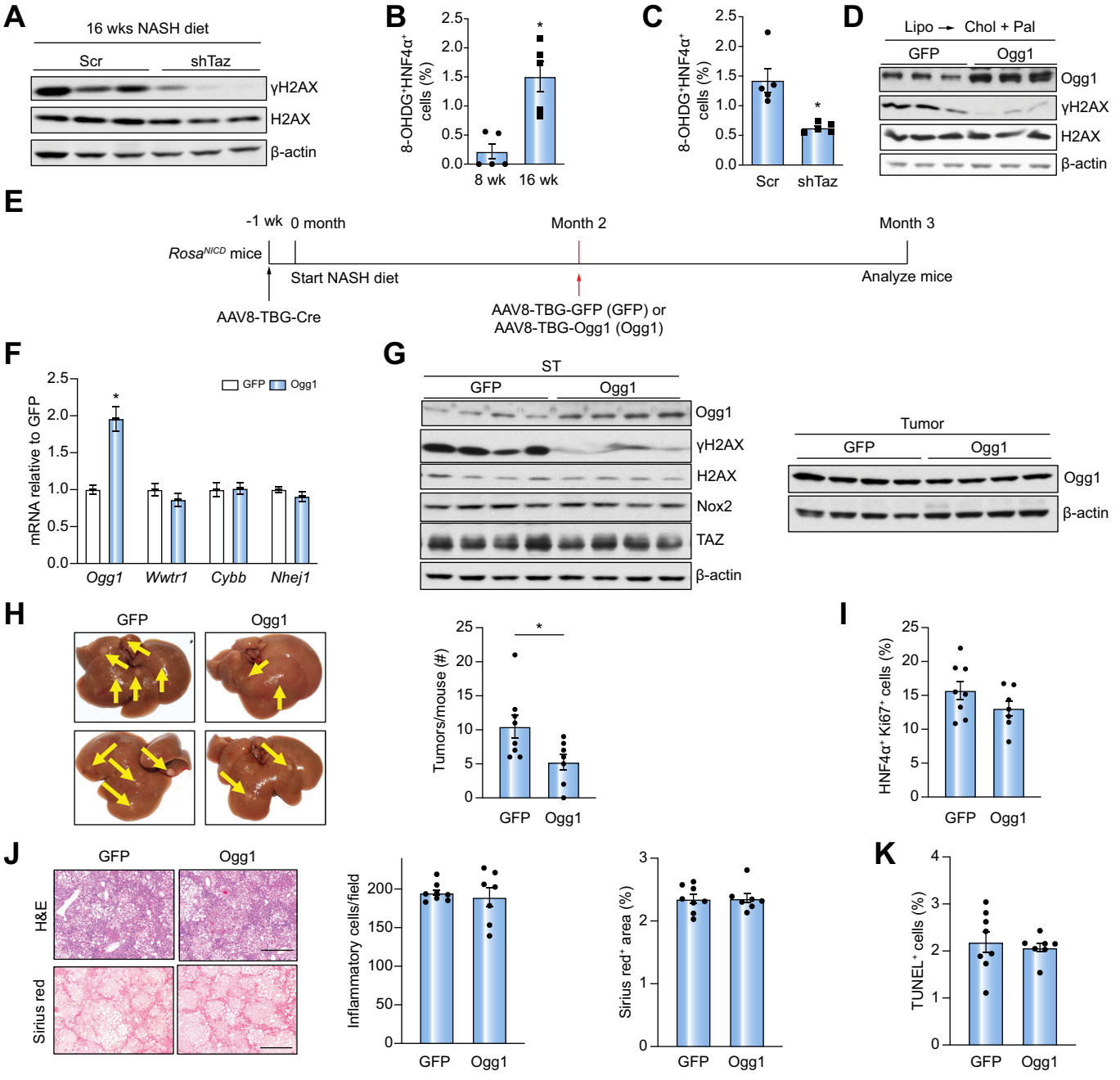


Fig. 4. TAZ promotes hepatocyte oxidative DNA damage in NASH, and its suppression by OGG1 supresses tumor formation in NASH-HCC mice. (A) γH2AX and H2AX immunoblots from livers of mice injected with AAV8-H1-Scr or AAV8-H1-shTaz and fed the NASH diet for 16 weeks. (B) Percent 8-OHDG$^+$ HNF4α$^+$ cells in the livers of mice fed the NASH diet for 8 or 16 weeks (n = 5 mice/group; means ± SEM; $^{**}p$ <0.01 by Student's *t* test). (C) Percent 8-OHDG$^+$ HNF4α$^+$ cells in livers of mice treated like those in panel A (n = 5 mice/group; means ± SEM; $^{**}p$ <0.01 by Student's *t* test). (D) Ogg1, γH2AX, and H2AX immunoblots from AML12 cells transfected with GFP or Ogg1 plasmids and then incubated for 24 h with liposomes to deplete cholesterol (Lipo) and then 16 h with cholesterol-rich liposomes and palmitate (Lipo → Chol + Pal). (E-K) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 2 months later, injected with AAV8-TBG-GFP or AAV8-TBG-Ogg1. The mice were analyzed at month 3. (n = 7-8 mice/group; means ± SEM) (E) Experimental scheme. (F) Liver *Ogg1*, *Wwtr1*, *Cybb* and *Nhej1* mRNA. ($^*p$ <0.05 by two-way ANOVA/Sidak's *post hoc* analysis). (G) Immunoblots of the indicated proteins in the ST and tumor tissue from the livers of the 2 groups of mice. (H) Livers (arrows, tumors) and tumor numbers/mouse. ($^*p$ <0.05 by Student's *t* test) (I) Percent Ki67$^+$HNF4α$^+$ cells in non-tumor tissue. (J) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 500 μm. (K) Percent TUNEL$^+$ cells in non-tumor areas. 8-OHDG, 8-Oxo-2'-deoxyguanosine (8-Oxo-dG); AAV8, adeno-associated virus 8; Chol, cholesterol; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; Pal, palmitate; Scr, scrambled RNA; shTAZ, short-hairpin TAZ; ST, surrounding tissue; TBG, thyroxine-binding globulin promoter; γH2AX, phospho H2AX.

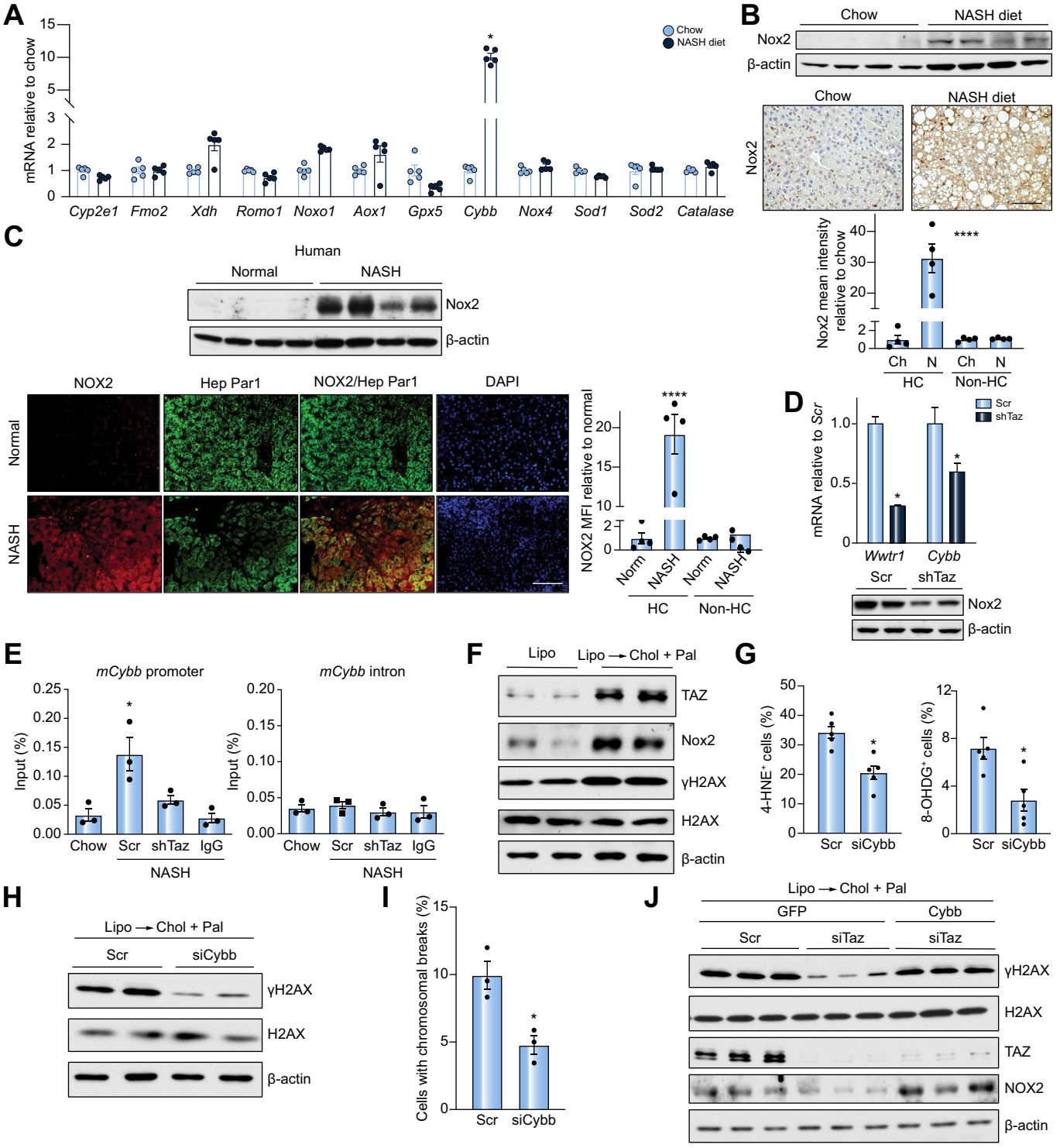


Fig. 5. Cybb is a TAZ gene target that contributes to hepatocyte oxidative DNA damage in NASH. (A) Quantification of pro-oxidant genes in the livers of mice fed chow or the NASH diet for 16 weeks (n = 5 mice/group; means ± SEM; **p* <0.0001 by two-way ANOVA with Sidak's *post hoc* analysis). (B) Immunoblot and immunohistochemical staining of Nox2 in the livers of mice fed chow or NASH diet for 28 weeks. Bar, 100 μm, with quantification of Nox2 MFI in hepatocytes and non-hepatocytes (n = 4 mice/group; means ± SEM; *****p* <0.0001 by two-way ANOVA/Sidak's *post hoc* analysis). (C) Immunoblot of NOX2 and immunofluorescence of NOX2 and Hep Par1 (hepatocytes) in normal human livers or livers from patients with NASH. Bar, 100 μm, with quantification of NOX2 MFI in hepatocytes and non-hepatocytes (n = 4 specimens/group; means ± SEM; *****p* <0.0001 by two-way ANOVA/Sidak's *post hoc* analysis). (D) *Wwtr1* and *Cybb* mRNA and Nox2 immunoblot in the livers of mice injected with AAV8-H1-Scr or AAV8-H1-shTaz and then fed the NASH diet for 16 weeks (n = 5 mice/group; means ± SEM; **p* <0.05 by two-way ANOVA/Sidak's *post hoc* analysis). (E) The livers of chow-fed mice or mice injected with AAV8-H1-Scr or AAV8-H1-shTaz and then fed the NASH diet for 16 weeks were subjected to TAZ ChIP, followed by qPCR of the precipitated DNA for a TAZ/TEAD binding sequence in a *Cybb* promoter or a non-consensus sequence in a *Cybb* intron. IgG served as the antibody control; the data were normalized to the values obtained from input DNA (n = 3 mice/group; means ± SEM; **p* <0.05 by one-way ANOVA/Tukey's *post hoc* analysis). (F) Immunoblots of TAZ, Nox2, γH2AX, and H2AX in AML12 cells incubated for 40 h with

tissue (Fig. 4F,G). Most importantly, OGG1 decreased the number of tumors that developed in these mice (Fig. 4H) without affecting the percentage of Ki67$^+$HNF4α$^+$ cells in the liver (Fig. 4I) or systemic or NASH parameters (Figs 4J-K and S4E-F). These combined data show causative links between TAZ and DNA damage in pre-tumor NASH and eventual tumor formation.

TAZ-induced *Cybb*/NOX2 contributes to oxidative DNA damage in NASH and to NASH-HCC tumor formation

We surveyed 12 mRNAs that encode oxidant-related proteins in pre-tumor NASH *vs.* control livers and found that *Cybb*, which encodes the NOX2 (gp91) subunit of the pro-oxidant protein complex NADPH oxidase, was increased in NASH (Fig. 5A). *Cybb* mRNA was also elevated in both tumor tissue and non-tumor surrounding tissue in the livers of the 13-month NASH-diet HCC model (Fig. S4G). Further, NOX2 protein was increased in mouse and human NASH liver (Fig. 5B,C); in surrounding tissue and tumor tissue of 2 of our mouse NASH-HCC models; and in human NASH-HCC tumors (Fig. S4H-J). We also found strong correlations between TAZ and NOX2, NOX2 and γH2AX, and TAZ and γH2AX and between the percent of 8-OHDG$^+$ cells and NOX2, TAZ, and γH2AX in non-tumor tissue from human NASH-HCC livers (Fig. S4K-N). Immunostaining using a validated anti-NOX2 antibody (Fig. S4O) showed a strong NOX2 signal in hepatocytes in mouse and human NASH livers (Fig. 5B,C). Although NOX2 staining was also seen in liver macrophages, there was a far greater number of NOX2-positive hepatocytes (Fig. S4P). Further, neutrophils were not a major source of NOX2 in human NASH livers, as their numbers were very low (Fig. S4Q). Thus, hepatocytes contributed to most of the NOX2 signal in NASH livers (Fig. 5B,C). Most importantly, silencing hepatocyte TAZ in NASH mice led to a substantial decrease in hepatic *Cybb* and NOX2 expression (Fig. 5D). We next conducted anti-TAZ chromatin immunoprecipitation analysis and showed that TAZ was enriched on a TAZ/TEAD binding sequence in a *Cybb* promoter in liver extracts from NASH diet-fed mice compared with either control liver extracts or liver extracts from NASH diet-fed mice that had been treated with AAV8-H1-shTaz (Fig. 5E).

To document cell-autonomous links among TAZ, *Cybb*/NOX2, and dsDNA damage, we turned to a NASH-relevant model in which AML12 hepatocytes are first depleted of cholesterol using phospholipid liposomes and then loaded with cholesterol using cholesterol-loaded liposomes. This treatment induces TAZ by the same mechanism that occurs in NASH hepatocytes *in vivo*.[14] We also added palmitate to the incubation medium to induce NASH-relevant lipid stress. We found that AML12 cells treated in this manner had increases in TAZ, NOX2, and γH2AX compared with control AML12 cells (Fig. 5F). Further, using a chromosomal spread assay, we observed an increase in chromosomal breaks in the treated cells but not in the control cells (Fig. S4R). Most importantly, small-interfering (si)Cybb lowered 4-hydroxynonenal, a marker of oxidative stress; 8-OHDG; γH2AX; and chromosomal breaks (Fig. 5G-5I). Moreover, siTaz treatment lowered γH2AX and NOX2 by approximately 50%, and genetic restoration of NOX2 in the siTaz-treated hepatocytes abrogated the decrease in γH2AX (Fig. 5J). These combined data show that the increase in hepatocyte TAZ in NASH leads to the induction of *Cybb*, which results in NOX2-mediated oxidative dsDNA damage.

We reasoned that expressing a dsDNA-repair enzyme in hepatocytes in pre-tumor NASH HCC might provide a causal link between DNA damage in NASH and eventual tumor formation. Based on a screen of mRNAs encoding dsDNA-damage repair enzymes, we chose non-homologous end joining factor 1 (NHEJ1; also known as XRCC4-like factor [XLF]). *Nhej1* was uniquely decreased in NASH *vs.* control livers (Fig. S5A). *Nhej1* was also decreased in cholesterol/palmitate-treated *vs.* untreated AML12 cells (Fig. S5B), and transfection of these cells with *Nhej1* lowered γH2AX (Fig. S5C). For the *in vivo* test, the Notch (NICD)/NASH-diet model was transduced with AAV8-TBG-Nhej1 between months 2 and 3 (Fig. S5D). As planned, the vector increased liver *Nhej1* expression without affecting *Wwtr1* (TAZ) or *Cybb*; increased NHEJ1 protein in surrounding tissue but not tumors; and decreased γH2AX1 in non-tumor tissue (Fig. S5E,F). Most importantly, NHEJ1 decreased the number of tumors in these mice (Fig. S5G), without affecting the percentage of Ki67$^+$HNF4α$^+$ cells (Fig. S5H) or systemic or NASH parameters (Fig. S5I-L).

We next tested the role of Cybb in NASH-HCC by treating Notch (NICD)/NASH-diet mice with AAV8-H1-shCybb or AAV8-H1-Scr between months 2-4 (Fig. 6A). NOX2 was decreased in non-tumor tissue but not the tumors, and this was accompanied by a decrease in γH2AX in the non-tumor tissue (Figs 6B and Fig. S6A). Most importantly, shCybb lowered tumor numbers in these mice (Fig. 6C) without affecting systemic or NASH endpoints (Figs 6D-F and S6B,C). Interestingly, neither the average size of the tumors nor the percentage of Ki67$^+$HNF4α$^+$ cells (Fig. 6G and S6D) was altered, suggesting that hepatocyte-Cybb contributed to an early, pre-proliferative molecular process that can eventually lead to new tumor formation.

Finally, we asked whether genetic restoration of Cybb in shTaz-treated mice could restore dsDNA damage and tumor development. Accordingly, Notch (NICD)/NASH-diet mice were treated with AAV8-H1-shTaz plus either AAV8-TBG-Cybb or AAV8-TBG-LacZ control (Fig. 6H). In the AAV8-TBG-Cybb mice, NOX2 and γH2AX were restored in non-tumor liver tissue but not tumors (Fig. 6I and Fig. S6E). Most importantly, NOX2 restoration increased average tumor number to the value that is typically observed in control Notch/NASH-diet mice (Fig. 6J) without affecting systemic or NASH endpoints (Figs 6K-M and S6F,G). Consistent with the shCybb data above, neither average tumor size nor the percentage of Ki67$^+$HNF4α$^+$ cells was affected (Fig. 6N and S6H). When combined with the previous data, these findings suggest NASH-mediated induction of TAZ in hepatocytes, by inducing *Cybb* and NOX2-mediated oxidative DNA damage, promotes an early, pre-

liposomes (Lipo) to deplete cholesterol (Lipo) or for 24 h with liposomes and then 16 h with cholesterol-rich liposomes and palmitate (Lipo → Chol + Pal). (G) Percent 4-HNE$^+$ and 8-OHDG$^+$ cells among scrambled RNA- or siCybb-treated AML12 cells that were incubated for 24 h with liposomes and then 16 h with liposomal-cholesterol and palmitate (n = 5 biological replicates/group; means ± SEM; $^*p$ <0.05 by Student's *t* test). (H) γH2AX and H2AX immunoblots from the AML12 cells in panel G. (I) Chromosome spread assay of the AML12 cells in panel G, with quantification of percent cells with chromosomal breaks (n = 3 biological replicates/group; means ± SEM; $^*p$ <0.05 by Student's *t* test). (J) γH2AX, H2AX, TAZ, and Nox2 immunoblots from AML12 cells transfected with Scr or siTaz and with GFP control or *Cybb*, and then incubated for 24 h with liposomes and then 16 h with liposomal-cholesterol and palmitate. Ch, chow; Chol, cholesterol; HC, hepatocytes; MFI, mean fluorescence intensity; N, NASH diet; NASH, non-alcoholic steatohepatitis; Norm, normal; Pal, palmitate; Scr, scrambled RNA; shTAZ, short-hairpin TAZ; siCybb/siTAZ, small-interfering Cybb/TAZ; γH2AX, phospho H2AX.

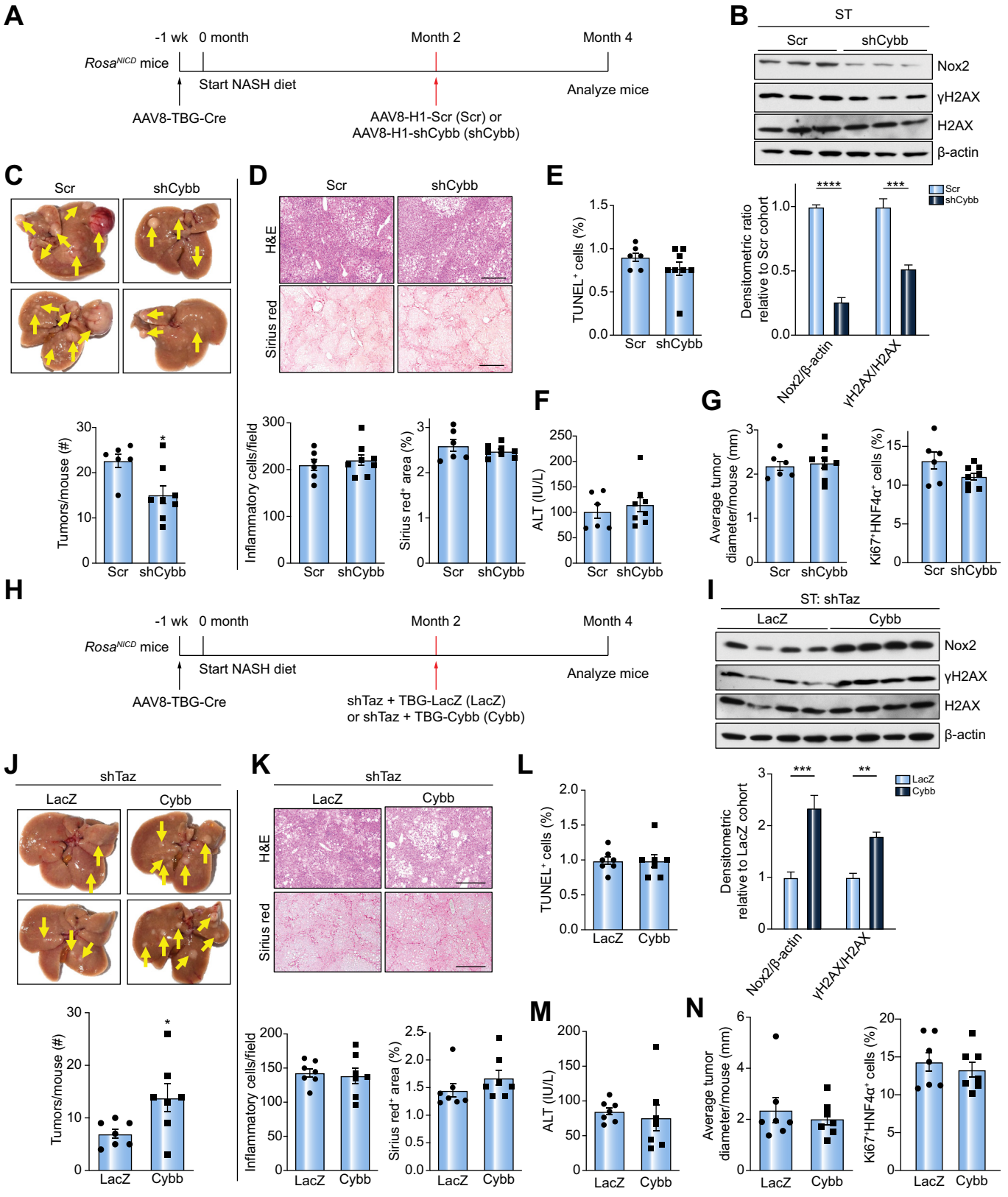


Fig. 6. TAZ-induced Cybb/NOX2 contributes to the development of NASH-HCC tumors. (A-G) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 2 months later, injected with AAV8-H1-Scr or AAV8-H1-shCybb. The mice were analyzed at month 4. (A) Experimental scheme. (B) Nox2, γH2AX, and H2AX immunoblots from ST, with quantification (n = 3; means ± SEM; ***p <0.001, ****p <0.0001 by two-way ANOVA/Sidak's *post hoc* analysis). (C) Livers (arrows, tumors) and tumor numbers/mouse. (D) Liver sections were stained with H&E (upper images) and Sirius red (lower images) and quantified for the number of inflammatory cells and the percent Sirius red-positive area. Bars, 200 μm. (E) Percent TUNEL$^+$ cells in non-tumor areas. (F) Plasma ALT. (G) Average tumor

proliferative process that contributes to eventual HCC tumor formation.

Discussion

In NASH-induced HCC, hepatocytes undergo biological changes over a prolonged period of time prior to the formation of tumors, providing a unique window into the earliest molecular-cellular processes of tumor formation. Herein, we show the importance of a TAZ-*Cybb*-oxidative dsDNA damage pathway. Future studies will be needed to elucidate the molecular-genetic links between dsDNA damage, additional carcinogenic hits, and eventual tumor formation in this setting. Multiple mechanisms are possible for DNA damage, including the inactivation of tumor-suppressor genes.[30] With regard to additional hits, the NASH niche is likely important,[27,28] and TAZ itself may play and addition role to promote hepatocyte proliferation.[17–19]

The pathway described here focuses specifically on NOX-induced oxidative DNA damage. Oxidative stress is a well-known inducer of DNA damage and cancer-causing mutations, and it is associated with NASH-HCC.[10,15] More specifically, the formation of 8-OHDG is linked to epigenetic instability in human HCC[11] and has been identified as a risk factor for HCC in chronic hepatitis C infection.[16] Moreover, NOX2-mediated superoxide generation has been implicated previously in certain non-liver cancers,[31] and several studies have shown correlations between the expression of various NOX proteins and HCC in cell lines, mouse models of HCC, and human HCC liver specimens.[32] However, direct *in vivo* causation studies and mechanistic links to NASH-HCC were previously lacking.

Most therapeutic efforts in HCC focus on arresting tumor growth or promoting tumor regression after the diagnosis of HCC in patients with cirrhosis. However, in the case of NASH, HCC can develop before frank cirrhosis occurs.[5–7] Moreover, pre-tumor NASH requires treament in its own right, *i.e.*, to prevent liver failure. The fact that TAZ is induced in hepatocytes in NASH and contributes to both NASH and HCC provides a strong rationale for TAZ-based therapy in patients with NASH. For example, GalNAc-siTAZ, which is based on a platform currently in human use, can lower hepatocyte-TAZ to its healthy-liver level and block or reverse progression to fibrosis in experimental NASH.[22] Based on the pathway revealed here, we suggest that hepatocyte-targeted siTaz therapy would also block NASH-to-HCC progression.

Abbreviations

8-OHDG, 8-Oxo-2'-deoxyguanosine (8-Oxo-dG); AAV8, adeno-associated virus 8; DMBA, 7,12-dimethylbenz [a]anthracene; dsDNA, double-stranded DNA; HCC, hepatocellular carcinoma; Ihh, Indian hedgehog; NASH, non-alcoholic steatohepatitis; Nhej1, non-homologous end-joining factor; Nox2, NADPH oxidase-2; Ogg1, 8-oxoguanine DNA glycosylase; Scr, scrambled RNA; shRNA, short-hairpin RNA; siRNA, small-interfering RNA; TAZ/Wwtr1, WW domain-containing transcription regulator-1; TBG, thyroxine-binding globulin promoter; γH2AX, phospho H2AX.

Financial support

This work was supported by an American Liver Foundation Liver Scholar Award (to X.W.); NIH grants DK103818 and DK119767 (to U.P.); R01CA190844 and R01CA228483 (to R.F.S.); and R01DK116620 (to R.F.S. and I.T.). Human liver samples were obtained from the Liver Tissue Cell Distribution System (University of Minnesota), which was funded by NIH contract HHSN276201200017C. Samples for histological analysis were prepared in the Molecular Pathology Shared Resource of the Herbert Irving Comprehensive Cancer Center at Columbia University, supported by NIH/NCI grant #P30 CA013696.

Conflict of interest

Dr. Tabas received an academic research grant from Takeda Pharmaceuticals to study the therapeutic potential of silencing TAZ in NASH.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

X.W., Y.S., R.F.S., and I.T. developed the study concept and experimental design. X.W. and S.Z. conducted the in vitro and mouse studies. X.W., S.Z., and H.S. performed the histological analyses. H.R., E.V., K.E.C., and S.A.O. provided human liver specimens and de-identified pathologic and clinical diagnoses for the subjects. C.Z. and U.B.P. provided advice for experiments using the Notch (NICD)/NASH-diet model of HCC, which they created. X.W., S.Z., H.S., Y.S., R.F.S., and I.T. analyzed the data. X.W. and I.T. wrote the manuscript, and all authors read and commented on the text and figures.

Data availability statement

Data are available from the corresponding author upon reasonable request.

Acknowledgements

We thank Dr. Eric Olson (University of Texas Southwestern) for providing the *Wwtr1*$^{fl/fl}$ mice; Drs. Anwesha Dey and Philamer Calses (Genentech) and Dr. Vivette D'Agati (CUIMC) for help with the chromosomal spread assay; and Dr. Ricard Masia (MGH) for advice on mouse HCC tumor characterization.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2021.11.031.

diameter and percent Ki67$^+$HNF4α$^+$ cells in non-tumor areas. For C-G, n = 6-8 mice/group; means ± SEM; $^*p$ <0.05 by Student's *t* test. (H-N) AAV8-TBG-Cre-treated *Rosa*^{NICD} mice were fed the NASH diet and, 2 months later, injected with AAV8-H1-shTaz and either AAV8-TBG-LacZ or AAV8-TBG-Cybb. The mice were analyzed at month 4. (H) Experimental scheme. (I) Nox2, γH2AX, and H2AX immunoblots from ST, with quantification (n = 4; means ± SEM; $^{**}p$ <0.01, $^{***}p$ <0.001 by two-way ANOVA/Sidak's *post hoc* analysis). (J) Livers (arrows, tumors) and tumor numbers/mouse. (K) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (L) Percent TUNEL$^+$ cells in non-tumor areas. (M) Plasma ALT. (N) Average tumor diameter and percent Ki67$^+$HNF4α$^+$ cells. For J-N, n = 7 mice/group; means ± SEM; $^*p$ <0.05 by Student's *t* test. ALT alanine aminotransferase; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; Scr, scrambled RNA; shCybb/TAZ, short-hairpin Cybb/TAZ; ST, surrounding tissue; TBG, thyroxine-binding globulin promoter; γH2AX, phospho H2AX.

References

Author names in bold designate shared co-first authorship

[1] Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol 2013;10:686–690.
[2] Corey KE, Kaplan LM. Obesity and liver disease: the epidemic of the twenty-first century. Clin Liver Dis 2014;18:1–18.
[3] Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. Nat Rev Gastroenterol Hepatol 2013;10:656–665.
[4] Huang DQ, El-Serag HB, Loomba R. Global epidemiology of NAFLD-related HCC: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol 2021;18:223–238.
[5] Mittal S, El-Serag HB, Sada YH, Kanwal F, Duan Z, Temple S, et al. Hepatocellular carcinoma in the absence of cirrhosis in United States veterans is associated with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 2016;14:124–131 e121.
[6] Perumpail RB, Wong RJ, Ahmed A, Harrison SA. Hepatocellular carcinoma in the setting of non-cirrhotic nonalcoholic fatty liver disease and the metabolic syndrome: us experience. Dig Dis Sci 2015;60:3142–3148.
[7] Paradis V, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, et al. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. Hepatology 2009;49:851–859.
[8] Wang X, Zheng Z, Caviglia JM, Corey KE, Herfel TM, Cai B, et al. Hepatocyte TAZ/WWTR1 promotes inflammation and fibrosis in nonalcoholic steatohepatitis. Cell Metab 2016;24:848–862.
[9] Linhart KB, Glassen K, Peccerella T, Waldherr R, Linhart H, Bartsch H, et al. The generation of carcinogenic etheno-DNA adducts in the liver of patients with nonalcoholic fatty liver disease. Hepatobiliary Surg Nutr 2015;4:117–123.
[10] Boege Y, Malehmir M, Healy ME, Bettermann K, Lorentzen A, Vucur M, et al. A dual role of caspase-8 in triggering and sensing proliferation-associated DNA damage, a key determinant of liver cancer development. Cancer Cell 2017;32:342–359.e310.
[11] Kakehashi A, Suzuki S, Ishii N, Okuno T, Kuwae Y, Fujioka M, et al. Accumulation of 8-hydroxydeoxyguanosine, L-arginine and glucose metabolites by liver tumor cells are the important characteristic features of metabolic syndrome and non-alcoholic steatohepatitis-associated hepatocarcinogenesis. Int J Mol Sci 2020;21:7746.
[12] Yang X, Sheng S, Du X, Su W, Tian J, Zhao X. Hepatocyte-specific TAZ deletion downregulates p62/Sqstm1 expression in nonalcoholic steatohepatitis. Biochem Biophys Res Commun 2021;535:60–65.
[13] Khajehahmadi Z, Mohagheghi S, Nikeghbalian S, Geramizadeh B, Khodadadi I, Karimi J, et al. Downregulation of hedgehog ligands in human simple steatosis may protect against nonalcoholic steatohepatitis: is TAZ a crucial regulator? IUBMB Life 2019;71:1382–1390.
[14] Wang X, Cai B, Yang X, Sonubi OO, Zheng Z, Ramakrishnan R, et al. Cholesterol stabilizes TAZ in hepatocytes to promote experimental non-alcoholic steatohepatitis. Cell Metab 2020;31:969–986 e967.
[15] **Pinyol R**, **Torrecilla S**, Wang H, Montironi C, Pique-Gili M, Torres-Martin M, et al. Molecular characterisation of hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol 2021;75:865–878.
[16] Chuma M, Hige S, Nakanishi M, Ogawa K, Natsuizaka M, Yamamoto Y, et al. 8-Hydroxy-2'-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection. J Gastroenterol Hepatol 2008;23:1431–1436.
[17] Hagenbeek TJ, Webster JD, Kljavin NM, Chang MT, Pham T, Lee HJ, et al. The Hippo pathway effector TAZ induces TEAD-dependent liver inflammation and tumors. Sci Signal 2018;11:eaaj1757.
[18] Hayashi H, Higashi T, Yokoyama N, Kaida T, Sakamoto K, Fukushima Y, et al. An imbalance in TAZ and YAP expression in hepatocellular carcinoma confers cancer stem cell-like behaviors contributing to disease progression. Cancer Res 2015;75:4985–4997.
[19] **Wang H**, **Wang J**, Zhang S, Jia J, Liu X, Zhang J, et al. Distinct and overlapping roles of hippo effectors YAP and TAZ during human and mouse hepatocarcinogenesis. Cell Mol Gastroenterol Hepatol 2021;11:1095–1117.
[20] Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, et al. Hippo pathway effector Yap promotes cardiac regeneration. Proc Natl Acad Sci U S A 2013;110:13839–13844.
[21] Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature 2013;499:97–101.
[22] Wang X, Sommerfeld MR, Jahn-Hofmann K, Cai B, Filliol A, Remotti HE, et al. A therapeutic silencing RNA targeting hepatocyte TAZ prevents and reverses fibrosis in nonalcoholic steatohepatitis in mice. Hepatol Commun 2019;3:1221–1234.
[23] Zhu C, Ho YJ, Salomao MA, Dapito DH, Bartolome A, Schwabe RF, et al. Notch activity characterizes a common hepatocellular carcinoma subtype with unique molecular and clinicopathologic features. J Hepatol 2021;74:613–626.
[24] Zhu C, Kim K, Wang X, Bartolome A, Salomao M, Dongiovanni P, et al. Hepatocyte Notch activation induces liver fibrosis in nonalcoholic steatohepatitis. Sci Transl Med 2018;10:eaat0344.
[25] **Moon H**, **Cho K**, Shin S, Kim DY, Han KH, Ro SW. High risk of hepatocellular carcinoma development in fibrotic liver: role of the Hippo-YAP/TAZ signaling pathway. Int J Mol Sci 2019;20:581.
[26] Zhang S, Zhou D. Role of the transcriptional coactivators YAP/TAZ in liver cancer. Curr Opin Cell Biol 2019;61:64–71.
[27] **Anstee QM**, **Reeves HL**, **Kotsiliti E**, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. Nat Rev Gastroenterol Hepatol 2019;16:411–428.
[28] Peiseler M, Tacke F. Inflammatory mechanisms underlying nonalcoholic steatohepatitis and the transition to hepatocellular carcinoma. Cancers (Basel) 2021;13.
[29] Nishimura S. Mammalian Ogg1/Mmh gene plays a major role in repair of the 8-hydroxyguanine lesion in DNA. Prog Nucleic Acid Res Mol Biol 2001;68:107–123.
[30] Schumacher B, Pothof J, Vijg J, Hoeijmakers JHJ. The central role of DNA damage in the ageing process. Nature 2021;592:695–703.
[31] Okada F, Kobayashi M, Tanaka H, Kobayashi T, Tazawa H, Iuchi Y, et al. The role of nicotinamide adenine dinucleotide phosphate oxidase-derived reactive oxygen species in the acquisition of metastatic ability of tumor cells. Am J Pathol 2006;169:294–302.
[32] Choi J, Corder NL, Koduru B, Wang Y. Oxidative stress and hepatic Nox proteins in chronic hepatitis C and hepatocellular carcinoma. Free Radic Biol Med 2014;72:267–284.

TAZ-induced Cybb contributes to liver tumor formation in non-alcoholic steatohepatitis

Xiaobo Wang, Sharon Zeldin, Hongxue Shi, Changyu Zhu, Yoshinobu Saito, Kathleen E. Corey, Stephanie A. Osganian, Helen E. Remotti, Elizabeth C. Verna, Utpal B. Pajvani, Robert F. Schwabe, Ira Tabas

Table of contents

Supplementary materials and methods

Human Liver Specimens

The human liver specimens used in Fig. S1C-D and S4J-N were obtained retrospectively from adult patients with NAFLD and HCC who underwent surgical resection or liver transplantation at Columbia University. Stored FFPE tissues were utilized for all scientific and histological analyses. Phenotypic and pathological characterizations were conducted by an experienced hepatologist (E.C.V.) and pathologist (H.R.). Some of the samples in Fig. S1C also came from the MGH NAFLD Biorepository, which has liver biopsy specimens from individuals undergoing weight loss surgery. Patients gave informed consent at the time of recruitment, and their records were anonymized and de-identified. De-identified human liver specimens used for Figure 5C and S4A were acquired from the Liver Tissue Cell Distribution System at the University of Minnesota. The specimens were collected on the date of liver transplantation and preserved as frozen samples. Phenotypic and pathological characterizations were conducted by medical physicians and pathologists associated with the Liver Tissue Cell Distribution System. The diagnostic information for all samples is included in **Table S1**. All protocols were approved by the Institutional Review Board (IRB) at the Columbia University Irving Medical Center.

Viral Constructs

Adeno-associated virus subtype 8 (AAV8)-shRNA targeting murine *Wwtr1* (TAZ) was made by annealing complementary oligonucleotides (5'-CACCAcagccgaatctcgcaatgaat CTCGAGATTCATTGCGAG ATTCGGCTG-3'), which were then ligated into the self-complementary (sc) AAV8-RSV-GFP-H1 vector as described previously (Lisowski et al., 2014). AAV8-H1-shRNA targeting murine Ihh was made by annealing complementary oligonucleotides (5'-CACCAaccaccttcagtgatgtgcttaTCAAGAGTAAGCACATCA CTGAAGGTGGG-3'), which were then ligated into the scAAV-RSV-GFP-H1 vector as above. AAV8-H1-shRNA targeting murine *Cybb* was made by annealing complementary oligonucleotides (5'-CACCAgaacgaagagtatctcaatttCTCGAGAAATTGAGATACTCTTCGTTC-3'), which were

then ligated into the scAAV-RSV-GFP-H1 vector as above. The resultant constructs were amplified by Vector Biolabs, Malvern, PA. AAV8-TBG-Ihh, AAV8-TBG-Cybb AAV8-TBG-Nhej1 and AAV8-TBG-Ogg1 were from Vector Biolabs. AAV8 containing hepatocyte-specific TBG-Cre recombinase (AAV-TBG-Cre, 107787-AAV8) and the control vectors, AAV8-TBG-LacZ (105534-AAV8) and AAV8-TBG-GFP (105535-AAV8), were purchased from the Addgene. *Cybb, Nhej1,* and *Ogg1* plasmids were from Origene (#MC204867, MC200480, MR227443).

Preparation of Liposomes and Palmitate

DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids, 850345; molecular mass: 677.5) and cholesterol (Sigma, C8667; molecular mass: 386.6) were dissolved in chloroform. Liposomes were made by adding 40 mg of DMPC with or without 80 mg of cholesterol to a glass vessel and then removing the solvent using a stream of nitrogen gas. Ten ml PBS was added and, after mixing, the lipids were subjected to probe sonication on ice for 5 minutes using 10-second on-off intervals. The preparation was then centrifuged at 10,000 x g for 10 minutes, and the supernatant fraction was extruded through a 100-nm polycarbonate filter (Avanti, 610000-1Ea) at room temperature. Each aliquot was stored in glass vials under argon at 4^{o}C and used within 2 weeks. For BSA- conjugated palmitate, 1 volume of 20 mM palmitate (Sigma, # P9767) and 1 volume of fatty acid free BSA (sigma, #A7030) were mixed gently and filtered. The final palmitate concentration was 10 mM, and the ratio of palmitate:BSA was 6.8:1.

Cell Culture and Cell Treatment

AML12 mouse hepatocytes were purchased from ATCC (CRL-2254) and cultured in DMEM/F12 medium (Life Technologies, #11320) with 10% (vol/vol) heat-inactivated FBS (Gibco, #16140-071) and 1X penicillin-streptomycin solution (Corning, 30-002-Cl). For treatment with phospholipid liposomes, 1x10^{5} AML12 cells were plated in 24-well plate and cultured for 24-48 hours until cell confluence was ~90%. A solution containing liposomes (above) was added to the medium at 1:10 volume ratio in a total volume of 500 μl for 8 hrs. For some groups of cells, cholesterol-loaded liposomes were added at

1:10 ratio after removal of the liposome-containing media, and at the same time 400 μM palmitate was added. The cells were harvested after treatment in Laemmle Sample Buffer (Bio-Rad, #1610737) with 2-mercaptoethanol (Bio-Rad, #161-0710) for immunoblotting or in RNA lysis buffer (Qiagen, #79216) for mRNA quantification.

Chromosome Spread Assay

Cells were incubated with 100 ng colcemid per mL at 37˚C for 1.5 hours, rinsed in 1X Trypsin-EDTA, and incubated in 1x Trypsin-EDTA at 37˚C for 5 minutes. The cells were centrifuged at 1,000 rpm for 10 minutes at 4˚C. A pre-warmed 0.8% solution of sodium citrate was added slowly to the pellet, followed by incubation at 37˚C for 20 minutes. The mixture was centrifuged at 1,000 rpm for 10 minutes at 4˚C to remove most of the supernate, and the pellet was resuspended in the remaining supernate, followed by the slow addition of Carnoy's Fixative (75% methanol, 25% acetic acid). After mixing, the suspension was incubated at RT for 10 minutes at 4˚C. This centrifugation-resuspension procedure was repeated two additional times. The final supernate was removed, and the pellet was resuspended in Carnoy's Fixative using a glass Pasteur pipette. Three drops of this suspension were added to a slide that was angled against the wall of a small box. Once dried, the slides were treated with Giemsa stain for 30 minutes and rinsed twice with ddH_2O. After air drying, mounting medium was added and sealed with nail polish. Slides were imaged with a histological microscope using a 100X objective.

siRNA-Mediated Gene Silencing and Transfection

Scrambled RNA control and oligo-targeting siRNAs were transfected into AML12 or HepG2 cells using Lipofectamine RNAiMAX (Life Technologies) at 40 nM of siRNA in 24-well plates following the manufacturer's instructions. Briefly, 2 X 10^5 cells at 30 - 40% confluence were incubated for 18 hours with 0.5 ml of culture medium containing 1.5 μl Lipofectamine RNAiMAX and 20 pmol siRNA. The siRNA sequences are listed in **Table S2**. The plasmids were transfected into AML12 cells using Lipofectamine® LTX Reagent with PLUS™ Reagent (Life Technologies, #15338100). For each well in a 24-well plate, 2 μl LTX, 0.5 μl PLUS reagent, and 0.5 μg plasmid DNA are used when cells

reached 30-40% confluence. After overnight incubation, the cells were switched back to normal culture medium.

Blood and Plasma Analyses

Fasting blood glucose was measured using a glucose meter (One Touch Ultra, Life-scan) in mice that were fasted for 5 hours, with free access to water. Plasma ALT was assayed using a kit from TECO Diagnostics (#A526-120).

Histopathological Analysis

Inflammatory cells in H&E-stained liver section images were quantified as the number of mononuclear cells per field (20x objective). For other parameters involving various stains, computerized image analysis (ImageJ) was used to quantify the area stained. The same threshold settings were used for all analyses. For all analyses, we quantified 6 randomly chosen fields per section per mouse. Liver fibrosis was assessed by quantifying Picrosirius (Sirius) red-stained area (Polysciences, #24901). Reticulin staining was performed according to the kit instruction (Abcam, ab150684). For immunohistochemistry, paraffin sections were rehydrated and subjected to antigen retrieval by placing in a pressure cooker for 10 mins in Target Retrieval Solution (Dako, S1699). The slides were then treated with 3% hydrogen peroxide for 10 min and then blocked with Serum-Free Protein Block (Dako, X0909) for 30 min. Sections were incubated overnight with primary antibodies **(Table S4)** and then developed with DAB or AP substrate kit (Cell Signaling, #8059 and #76713).

Immunofluorescence Microscopy

Frozen liver sections were subjected to antigen retrieval at 70°C in HistoVT One solution (Nacalai, 06349-64) for 20 min and then blocked with serum. Sections were labeled with primary antibodies **(Table S4)** overnight, followed by incubation with a fluorophore-conjugated secondary antibody for 1 h. The stained sections were mounted with DAPI-containing mounting medium (Life Technologies, P36935) and then viewed by fluorescence microscopy. AML12 or HepG2 cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed using PBS, and stained with antibodies for 2

hours, followed by incubation with a fluorophore-conjugated secondary antibody for 1 hour. The cells were viewed by fluorescence microscopy. TUNEL staining was conducted using a kit from Roche (#12156792910).

Immunoblotting

Liver protein was extracted using RIPA buffer (Thermo, #89900), and the protein concentration was measured by a BCA assay (Thermo, #23227). Proteins were separated by electrophoresis on 4-20% Tris gels (Life technologies, EC60285) and transferred to nitrocellulose membranes (Bio-Rad, #1620115). The membranes were blocked for 30 min at room temperature in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% (wt/vol) nonfat milk and then incubated with primary antibody **(Table S4)** in the same buffer at 4°C overnight, using 1:1000 dilution. The protein bands were detected with horse radish peroxidase-conjugated secondary antibodies and Supersignal West Pico enhanced chemiluminescent solution (Thermo, #34080). Cultured cells were lysed in Laemmle sample buffer (Bio-Rad, #161-0737) containing 5% 2-mercaptoethanol, heated at 100ºC for 5 min, and then electrophoresed and immunoblotted as above.

Quantitative RT-qPCR

Total RNA was extracted from liver tissue or cultured hepatocytes using the RNeasy kit (Qiagen, 74106). The quality and concentration of the RNA was assessed by absorbance at 260 and 280 nm using a Thermo Scientific NanoDrop spectrophotometer. cDNA was synthesized from 1 µg total RNA using oligo (dT) and Superscript II (Invitrogen). qPCR was performed with a 7500 Real time PCR system (Applied Biosystems) using SYBR Green Master Mix (Life Technologies, #4367659). The primer sequences are listed in **Table S3**.

Statistical Analysis

Data that passed the normality test were analyzed using Student's t test for two groups; one-way ANOVA with Tukey's post-hoc analysis for more than two groups; or two-way ANOVA with Sidak's post-hoc analysis for two factors. Correlations were analyzed by

using linear regression. Data that were not normally distributed were analyzed using the nonparametric Mann-Whitney U test, or, for more than two groups, by Kruskal-Wallis with post-hoc analysis by the Dunn test. Power calculations were used to determine adequate n number and to avoid the use of excess mice. The power calculations used (i) a minimum 75% change in the experimental group; (ii) a 20% coefficient of variation; (iii) a p value less than 0.05; and (iv) a power of 0.80.

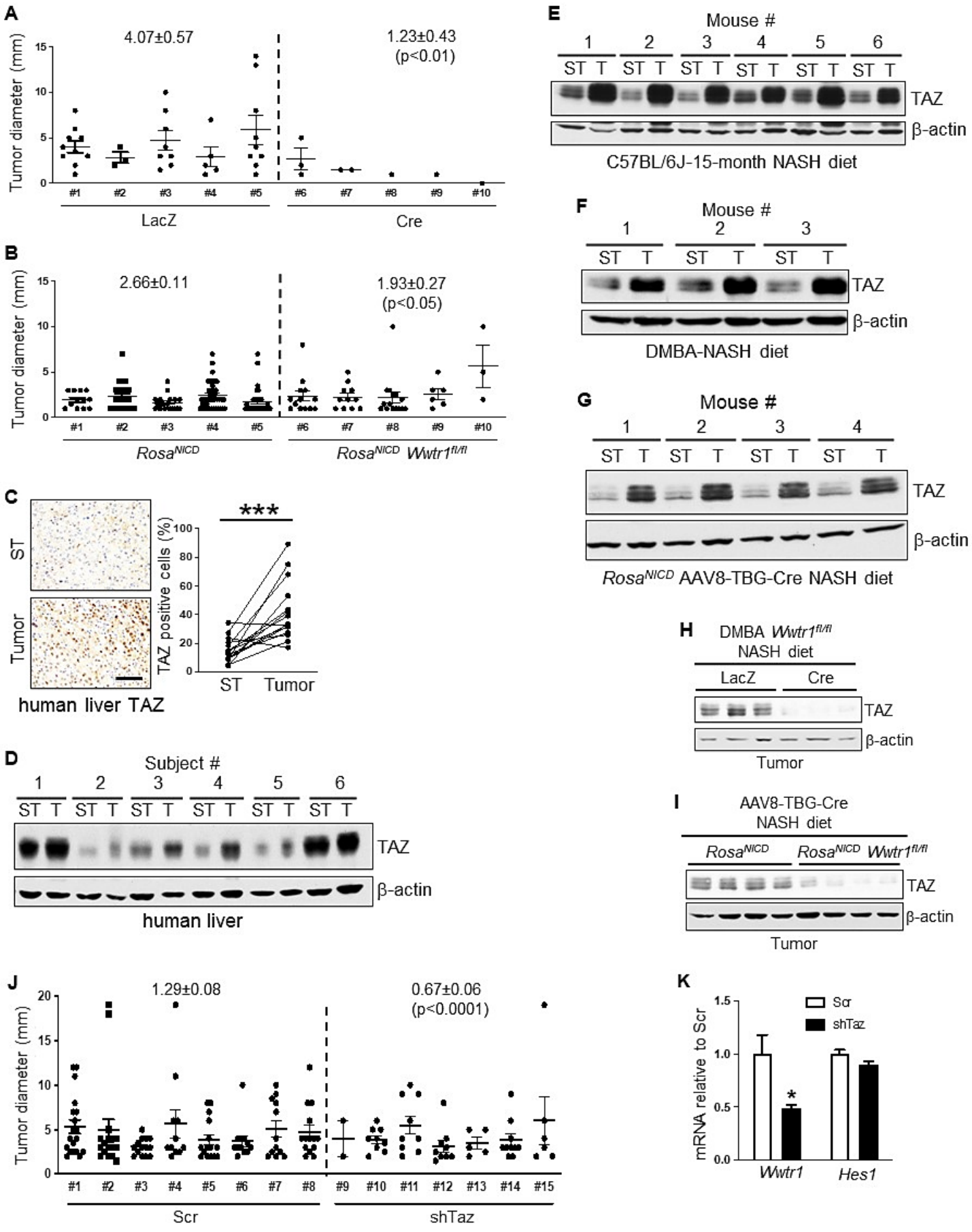

A
Tumor diameter (mm)
4.07±0.57
1.23±0.43
(p<0.01)
LacZ
Cre
B
Tumor diameter (mm)
2.66±0.11
1.93±0.27
(p<0.05)
RosaNICD
RosaNICD Wwtr1fl/fl
C
ST
Tumor
***
TAZ positive cells (%)
human liver TAZ
D
Subject #
ST T
TAZ
β-actin
human liver
E
Mouse #
TAZ
β-actin
C57BL/6J-15-month NASH diet
F
Mouse #
DMBA-NASH diet
G
Mouse #
RosaNICD AAV8-TBG-Cre NASH diet
H
DMBA Wwtr1fl/fl
NASH diet
LacZ
Cre
Tumor
I
AAV8-TBG-Cre
NASH diet
RosaNICD
RosaNICD Wwtr1fl/fl
Tumor
J
Tumor diameter (mm)
1.29±0.08
0.67±0.06
(p<0.0001)
Scr
shTaz
K
mRNA relative to Scr
Scr
shTaz
Wwtr1
Hes1

Fig. S1. TAZ expression in NASH-HCC tumors, Related to Figure 1 and 2. (A) Tumor diameters for the 10 mice in Figure 1I. Means ± SEM are shown for each group, $p<0.01$ vs. LacZ. (B) Tumor diameters for the 10 mice in Figure 1N. Means ± SEM are shown for each group, $p<0.05$ vs. *Rosa*^{NICD}. (C) Sections of livers from subjects with NASH-HCC were assayed for TAZ by immunohistochemistry in surrounding tissue (ST) and tumor tissue; the positive TAZ signal is brown. Scale bar, 200 μm. The data were quantified as the percentage of TAZ^{+} cells relative to total cells (n = 15 paired specimens/group, ***$p < 0.001$ by Student's t test). (D) Immunoblots of TAZ in ST and tumor tissue (T) in the livers of 6 subjects with NASH-HCC. (E) Immunoblots of TAZ in ST and T in the livers of 6 mice fed the NASH diet for 15 months. (F) Immunoblots of TAZ in ST and T in the livers of 3 DMBA-NASH diet-fed mice. (G) Immunoblots of TAZ in ST and T in the livers of 4 NICD-NASH diet-fed mice. (H) Immunoblot of TAZ in tumor tissue in the 2 groups of mice in Figures 1G-J. (I) Immunoblot of TAZ in tumor tissue in the 2 groups of mice in Figures 1L-O. (J) Tumor diameters for the 15 mice in Figure 2C. Means ± SEM are shown for each group, $p<0.0001$ vs. Scr. (K) Relative levels of liver *Wwtr1* and *Hes1* mRNAs in the samples from Figure 2D.

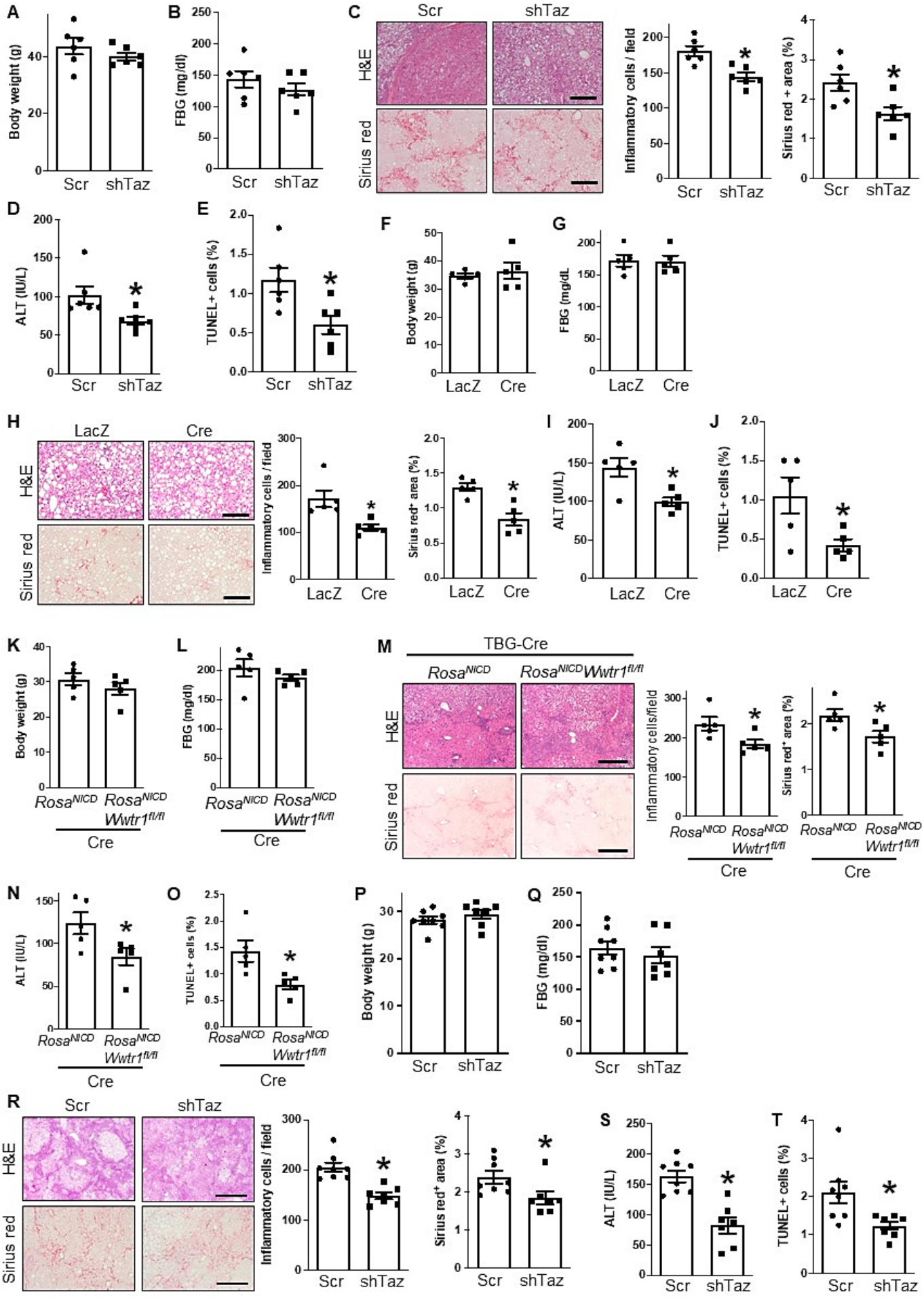

A
Body weight (g)
Scr
shTaz
B
FBG (mg/dl)
C
Scr
shTaz
H&E
Sirius red
Inflammatory cells / field
Sirius red + area (%)
D
ALT (IU/L)
E
TUNEL+ cells (%)
F
Body weight (g)
LacZ
Cre
G
FBG (mg/dL
H
LacZ
Cre
Inflammatory cells / field
Sirius red+ area (%)
I
ALT (IU/L)
J
TUNEL+ cells (%)
K
Body weight (g)
RosaNICD
RosaNICD Wwtr1fl/fl
Cre
L
FBG (mg/dl)
M
TBG-Cre
RosaNICD
RosaNICD Wwtr1fl/fl
Inflammatory cells/field
Sirius red+ area (%)
N
ALT (IU/L)
O
TUNEL+ cells (%)
P
Body weight (g)
Q
FBG (mg/dl)
R
Scr
shTaz
Inflammatory cells / field
Sirius red+ area (%)
S
ALT (IU/L)
T
TUNEL+ cells (%)

Fig. S2. Additional data related to the effects of hepatocyte-Taz silencing on eventual tumor development in NASH mice, Related to Figures 1 and 2. (A-E) The mice in Figures 1B-1E were further characterized as follows (n = 6 mice/group; means ± SEM; *p < 0.05 by Student's t test): (A) Body weight. (B) Fasting blood glucose. (C) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (D) Plasma ALT. (E) Percentage of $TUNEL^+$ cells in liver sections from non-tumor areas. (F-J) The mice in Figure 1G-1J were further characterized as follows (n = 5 mice/group; means ± SEM; *p < 0.05 by Student's t test): (F) Body weight. (G) Fasting blood glucose. (H) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (I) Plasma ALT. (J) Percentage of $TUNEL^+$ cells in liver sections from non-tumor areas. (K-O) The mice in Figure 1L-1O were further characterized as follows (n = 5 mice/group; means ± SEM; *p < 0.05 by Student's t test): (K) Body weight. (L) Fasting blood glucose. (M) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (N) Plasma ALT. (O) Percentage of $TUNEL^+$ cells in liver sections from non-tumor areas. (P-T) The mice in Figure 2A-2D were further characterized as follows (n = 7-8 mice/group; means ± SEM; *p < 0.05 by Student's t test): (P) Body weight. (Q) Fasting blood glucose. (R) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (S) Plasma ALT. (T) Percentage of $TUNEL^+$ cells in liver sections from non-tumor areas.

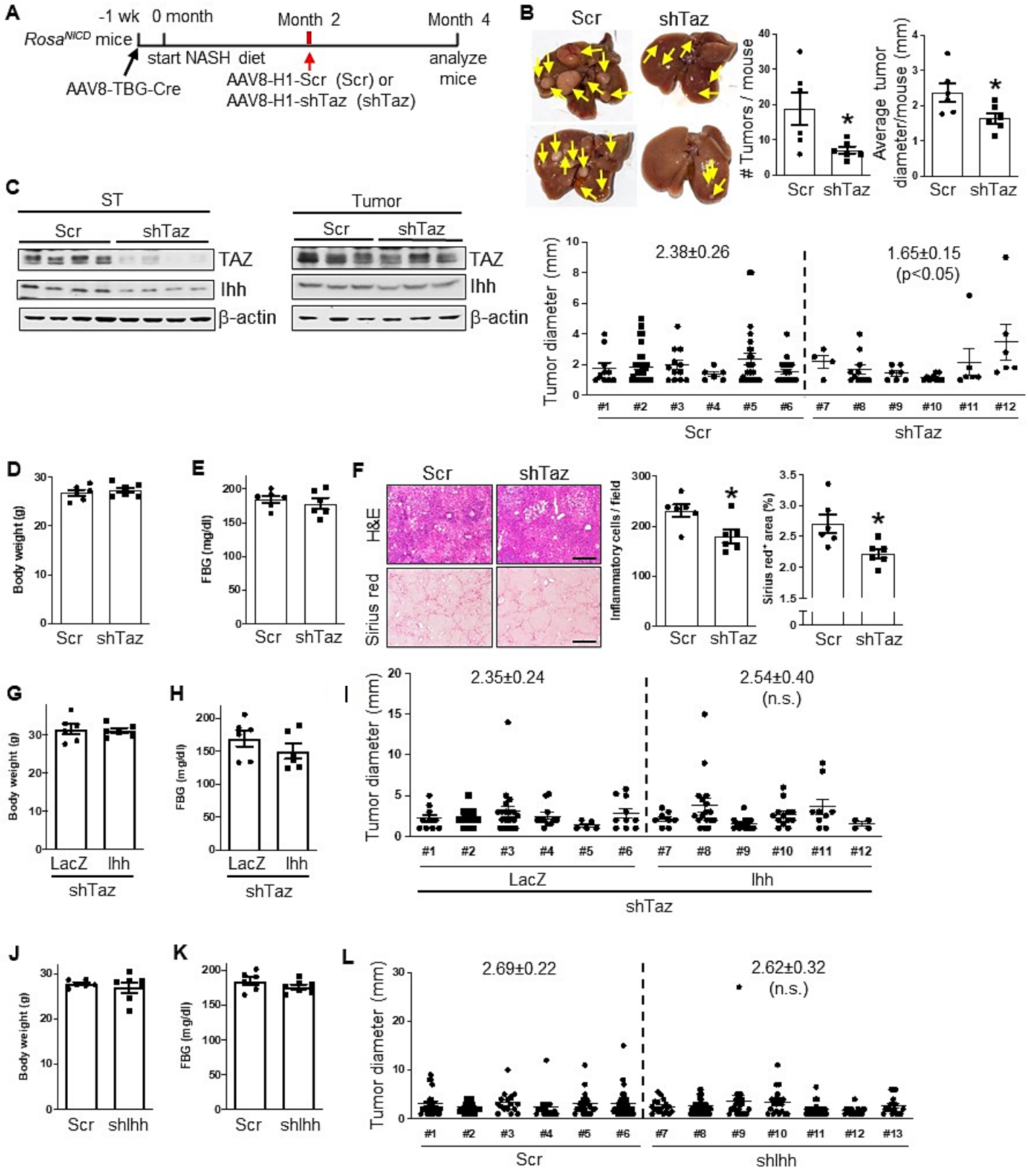

A
-1 wk 0 month
Month 2
Month 4
Rosa^NICD mice
start NASH diet
analyze mice
AAV8-TBG-Cre
AAV8-H1-Scr (Scr) or
AAV8-H1-shTaz (shTaz)
B
Scr
shTaz
Tumors / mouse
Average tumor diameter/mouse (mm)
C
ST
Tumor
Scr
shTaz
TAZ
Ihh
β-actin
Tumor diameter (mm)
2.38±0.26
1.65±0.15
(p<0.05)
D
Body weight (g)
E
FBG (mg/dl)
F
H&E
Sirius red
Inflammatory cells / field
Sirius red+ area (%)
G
LacZ
Ihh
H
I
2.35±0.24
2.54±0.40
(n.s.)
J
shlhh
K
L
2.69±0.22
2.62±0.32
(n.s.)

Fig. S3. Additional data related to dissociation of the tumor-suppressing effect of shTaz in experimental NASH-HCC from its NASH-suppressing effects, Related to Figure 3. (A-F) *Rosa*^{NICD} mice were treated with AAV8-TBG-Cre and then started on the NASH diet 1 week later. After 2 months, the mice were injected with AAV8-H1-scrambled RNA (Scr) or AAV8-H1-shTaz (shTaz), and the mice were analyzed at month 4 (n = 6 mice/group; means ± SEM; *p < 0.05 by Student's t test): (A) The experimental scheme. (B) Photographs of representative livers (arrows indicate tumors) and quantification of tumor numbers and average diameter. The bottom graph shows the tumor diameters for each mouse, with means ± SEM shown for each group, p<0.05 vs. Scr. (C) Immunoblots of TAZ and Ihh in surrounding tumor tissue (ST) and tumor tissue. (D) Body weight. (E) Fasting blood glucose. (F) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (G-H) Body weight and fasting blood glucose of the mice in Figures 3A-3F (n = 6 mice/group; means ± SEM). (I) Tumor diameters for the 12 mice in Figure 3F. Means ± SEM are shown for each group. (J-K) Body weight and fasting blood glucose of the mice in Figures 3G-3L (n = 6-7 mice/group; means ± SEM). (L) Tumor diameters for the 13 mice in Figure 3L.

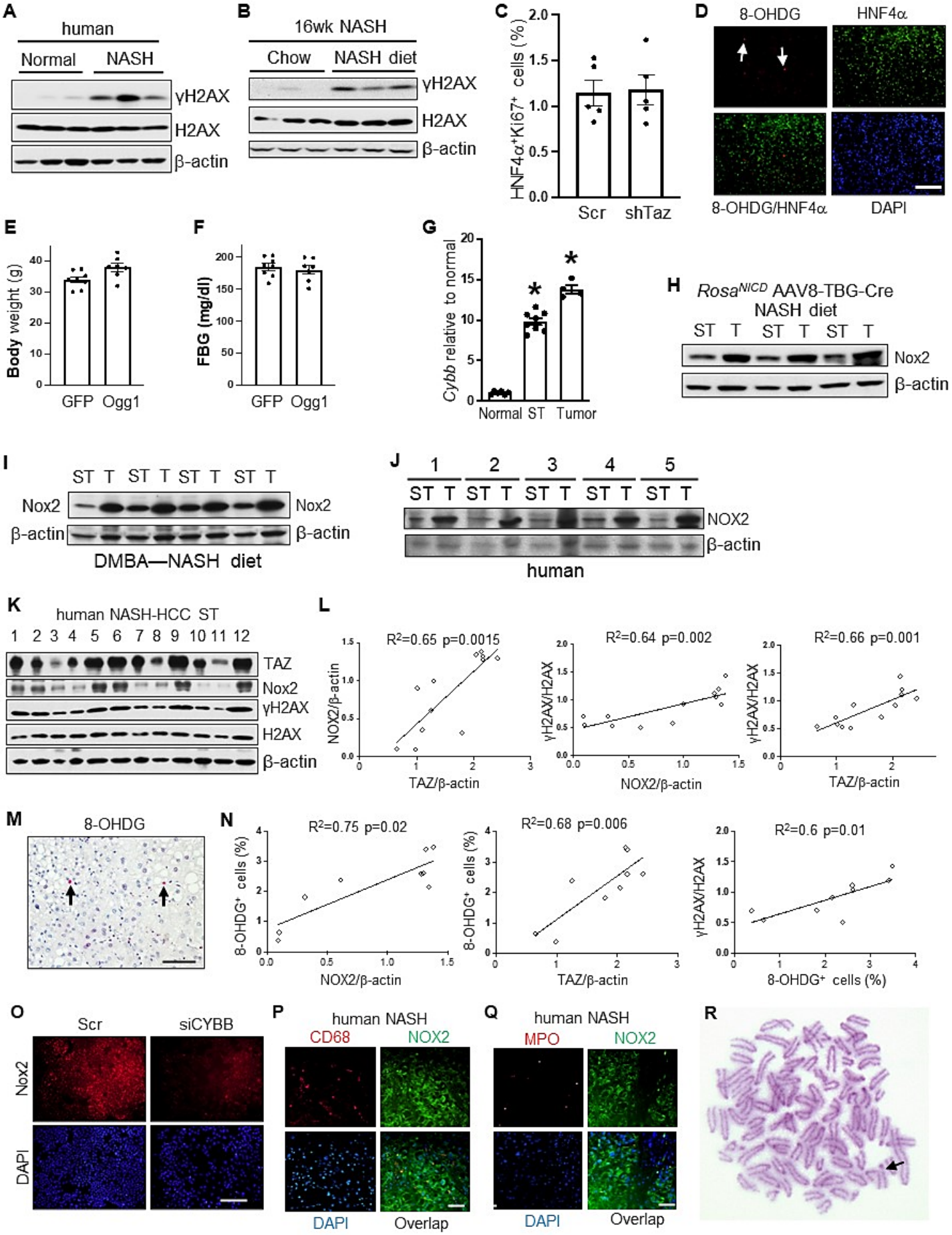

A
human
Normal
NASH
γH2AX
H2AX
β-actin
B
16wk NASH
Chow
NASH diet
γH2AX
H2AX
β-actin
C
HNF4α+Ki67+ cells (%)
Scr
shTaz
D
8-OHDG
HNF4α
8-OHDG/HNF4α
DAPI
E
Body weight (g)
GFP
Ogg1
F
FBG (mg/dl)
GFP
Ogg1
G
Cybb relative to normal
Normal
ST
Tumor
H
Rosa^NICD AAV8-TBG-Cre
NASH diet
ST T ST T ST T
Nox2
β-actin
I
ST T ST T ST T ST T
Nox2
β-actin
DMBA—NASH diet
J
1 2 3 4 5
ST T ST T ST T ST T ST T
NOX2
β-actin
human
K
human NASH-HCC ST
1 2 3 4 5 6 7 8 9 10 11 12
TAZ
Nox2
γH2AX
H2AX
β-actin
L
R²=0.65 p=0.0015
NOX2/β-actin
TAZ/β-actin
R²=0.64 p=0.002
γH2AX/H2AX
NOX2/β-actin
R²=0.66 p=0.001
γH2AX/H2AX
TAZ/β-actin
M
8-OHDG
N
R²=0.75 p=0.02
8-OHDG+ cells (%)
NOX2/β-actin
R²=0.68 p=0.006
8-OHDG+ cells (%)
TAZ/β-actin
R²=0.6 p=0.01
γH2AX/H2AX
8-OHDG+ cells (%)
O
Scr
siCYBB
Nox2
DAPI
P
human NASH
CD68
NOX2
DAPI
Overlap
Q
human NASH
MPO
NOX2
DAPI
Overlap
R

Fig. S4. Additional data related to the role of Cybb/NOX2 in NASH-HCC, Related to Figure 5. (A) Immunoblots of γH2AX and H2AX in normal and NASH human livers. (B) Immunoblots of γH2AX and H2AX in the livers of mice fed chow diet or the NASH diet for 16 weeks. (C) Quantification of percent $Ki67^{+}HNF4\alpha^{+}$ cells in the livers of NASH (non-HCC) mice injected with AAV8-H1-scrambled RNA (Scr) or AAV8-H1-Taz (shTaz) and then fed the NASH diet for 16 weeks (n = 5 mice/group; means ± SEM). (D) 8-OHDG and HNF4α immunofluorescence staining liver fed the NASH diet for 16 weeks. Bar, 200 μm. (E-F) Body weight and fasting blood glucose of the mice in Figures 4E-K (n = 7-8 mice/group; means ± SEM). (G) *Cybb* mRNA in normal mouse liver and in surrounding tumor tissue (ST) and tumor tissue of livers from mice fed the NASH diet for 13 months (n = 4-8 mice/group; means ± SEM; *p < 0.05 by one-way ANOVA with Tukey's post-hoc analysis). (H) ST and T in the livers of the AAV8-TBG-Cre-treated $Rosa^{NICD}$ mice depicted in Figure 1K were immunoblotted for Nox2. (I) ST and T in the livers of the control DMBA-NASH diet model depicted in Figure 1F mice were immunoblotted for Nox2. (J) NOX2 immunoblot in ST and T tissues in the livers of humans with NASH-HCC. (K) Immunoblots of TAZ, NOX2, γH2AX and H2AX in the surrounding tissue of livers of 12 subjects with NASH-HCC. (L) Correlations, with linear regression analyses, of NOX2 and TAZ protein (left, R^2=0.65, n=12, p<0.01); γH2AX and NOX2 protein (middle, R^2=0.64, n=12, p<0.01); and γH2AX and TAZ protein (right, R^2=0.66, n=12, p<0.01). (M) Representative IHC image used to quantify 8-OHDG in ST liver sections (arrows depict examples of $8\text{-}OHDG^{+}$ cells). Scale bar, 100 μm. (N) Correlations, with linear regression analyses, of $8\text{-}OHDG^{+}$ cells with NOX2 protein (left, R^2=0.75, n=9, p<0.05); TAZ protein (middle, R^2=0.68, n=9, p<0.01); and γH2AX protein (R^2=0.6, n=9, p<0.05). (O) As evidence that the anti-NOX2 antibody used in Figure 5C is specific, *CYBB* silencing in human HepG2 liver cells eliminated NOX2 immunofluorescence (red). Scr, control RNA. Bar, 200 μm. (P) Immunofluorescence of CD68 (macrophages) and NOX2 in human NASH liver sections. Bar, 100 μm. (Q) Immunofluorescence of MPO (neutrophils) and NOX2 in human NASH liver sections. Bar, 100 μm. (R) Related to Figure 5I, representative image used to quantify chromosomal breaks in AML12 cells incubated for 24 h with liposomes and then 16 h with liposomal-cholesterol and palmitate. Arrow, example of a chromosome break.

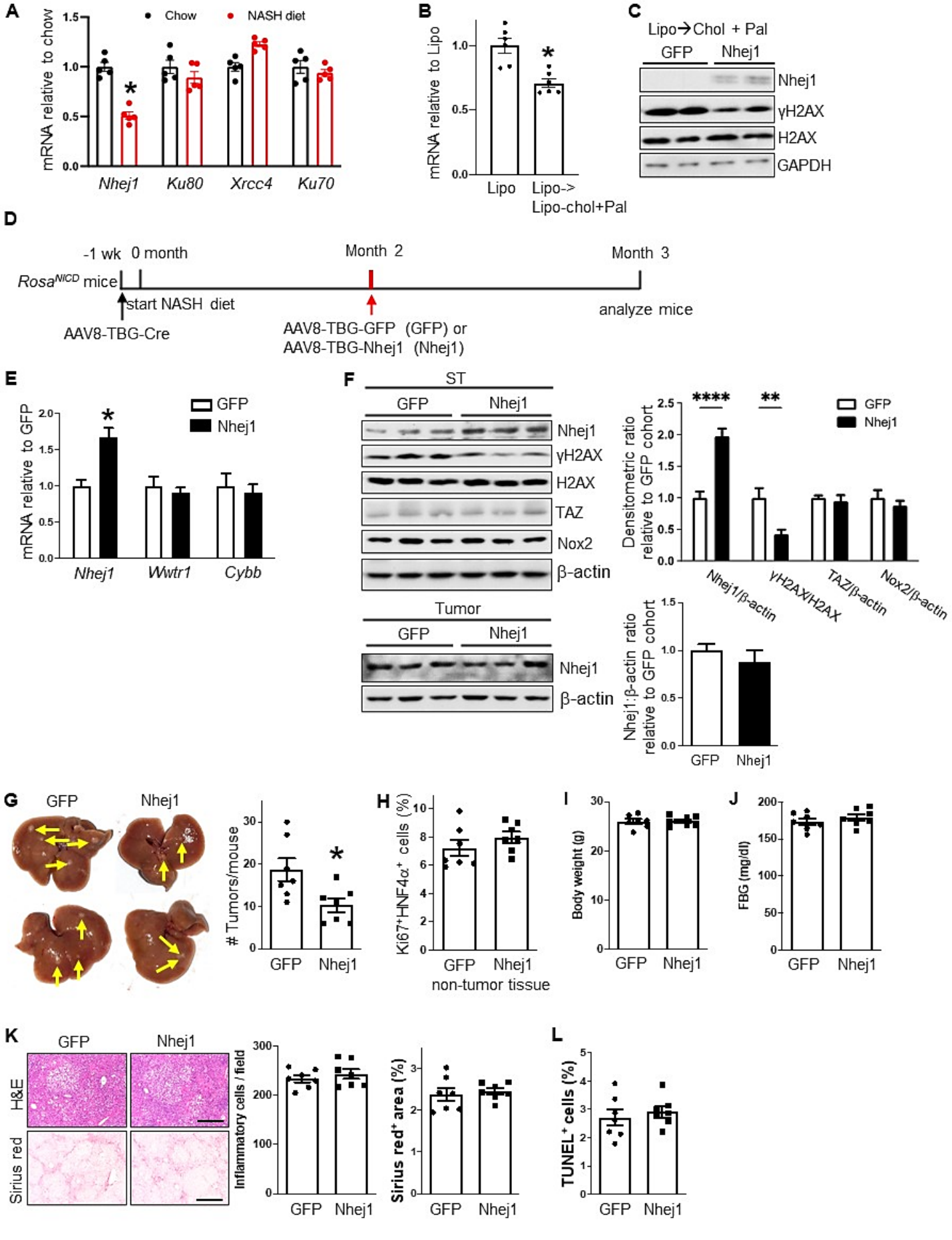

A
Chow
NASH diet
mRNA relative to chow
Nhej1
Ku80
Xrcc4
Ku70
B
mRNA relative to Lipo
Lipo
Lipo-> Lipo-chol+Pal
C
Lipo→Chol + Pal
GFP
Nhej1
Nhej1
γH2AX
H2AX
GAPDH
D
-1 wk
0 month
Month 2
Month 3
Rosa^NICD mice
start NASH diet
analyze mice
AAV8-TBG-Cre
AAV8-TBG-GFP (GFP) or
AAV8-TBG-Nhej1 (Nhej1)
E
GFP
Nhej1
mRNA relative to GFP
Nhej1
Wwtr1
Cybb
F
ST
GFP
Nhej1
Nhej1
γH2AX
H2AX
TAZ
Nox2
β-actin
Tumor
GFP
Nhej1
Nhej1
β-actin
Densitometric ratio relative to GFP cohort
****
**
GFP
Nhej1
Nhej1/β-actin
γH2AX/H2AX
TAZ/β-actin
Nox2/β-actin
Nhej1:β-actin ratio relative to GFP cohort
GFP
Nhej1
G
GFP
Nhej1
Tumors/mouse
GFP
Nhej1
H
Ki67+HNF4α+ cells (%)
GFP
Nhej1
non-tumor tissue
I
Body weight (g)
GFP
Nhej1
J
FBG (mg/dl)
GFP
Nhej1
K
GFP
Nhej1
H&E
Sirius red
Inflammatory cells / field
GFP
Nhej1
Sirius red+ area (%)
GFP
Nhej1
L
TUNEL+ cells (%)
GFP
Nhej1

Fig. S5. NHEJ1 is lower in NASH, and genetic restoration of NHEJ lowers tumors in NASH-HCC, Related to Figures 4-6. (A) Relative levels of *Nhej1*, *Ku80*, *Xrcc4*, and *Ku70* mRNAs in the livers of mice fed chow diet or the NASH diet for 16 weeks (n = 5 mice/group; means ± SEM; *p < 0.05 by two-way ANOVA with Sidak's post-hoc analysis). (B) Relative level of *Nhej1* mRNA in AML12 cells that were incubated for 40 h with liposomes (Lipo) to deplete cholesterol (Lipo) or for 24 h with liposomes and then 16 h with cholesterol-rich liposomes and palmitate (Lipo → Chol + Pal) (n = 6 biological replicates; means ± SEM; *p < 0.05 by Student's t test). (C) The Lipo → Chol + Pal cells in panel B were transfected with GFP or Nhej1 plasmids and immunoblotted for Nhej1, γH2AX, and H2AX. (D-L) *Rosa*^{NICD} mice were treated with AAV8-TBG-Cre and then started on the NASH diet 1 week later. After 2 months, the mice were injected with AAV8-TBG-GFP (GFP) or AAV8-TBG-Nhej1 (Nhej1), and the mice were analyzed at month 3. (D) The experimental scheme. (E) Relative levels of liver *Nhej1*, *Wwtr1*, and *Cybb* mRNAs. (F) Immunoblots and quantification data of the indicated proteins in the surrounding tumor tissue (ST) and tumor tissue from the livers of the two groups of mice (n = 3; means ± SEM; **p < 0.01, ****p<0.0001 by two-way ANOVA with Sidak's post-hoc analysis). (G) Photographs of representative livers (arrows indicate tumors) and quantification of tumor numbers per mouse. (H) Liver sections were quantified for the percentage of Ki67^{+}HNF4α^{+} cells in non-tumor tissue. (I) Body weight. (J) Fasting blood glucose. (K) Liver sections were stained with H&E (upper images) and Sirius red (lower images), with quantification of inflammatory cells and percent Sirius red-positive area. Bars, 200 μm. (L) Quantification of the percentage of TUNEL^{+} cells in liver sections from non-tumor areas. For E and G-L, n = 7 mice/group; means ± SEM; *p < 0.05 by Student's t test.

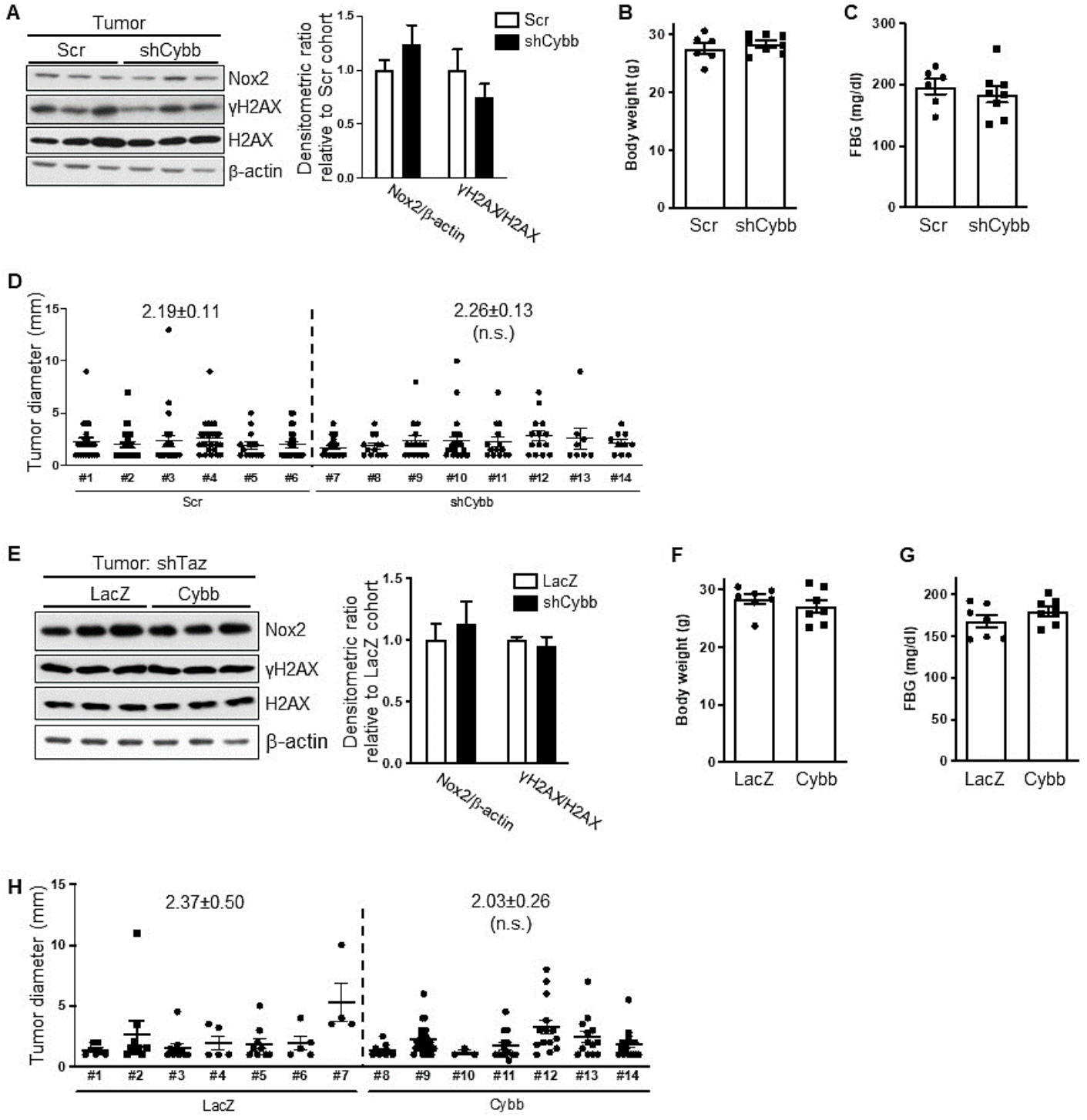

A
Tumor
Scr
shCybb
Nox2
γH2AX
H2AX
β-actin
Densitometric ratio relative to Scr cohort
Scr
shCybb
Nox2/β-actin
γH2AX/H2AX
B
Body weight (g)
Scr
shCybb
C
FBG (mg/dl)
Scr
shCybb
D
Tumor diameter (mm)
2.19±0.11
2.26±0.13
(n.s.)
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13
#14
Scr
shCybb
E
Tumor: shTaz
LacZ
Cybb
Nox2
γH2AX
H2AX
β-actin
Densitometric ratio relative to LacZ cohort
LacZ
shCybb
Nox2/β-actin
γH2AX/H2AX
F
Body weight (g)
LacZ
Cybb
G
FBG (mg/dl)
LacZ
Cybb
H
Tumor diameter (mm)
2.37±0.50
2.03±0.26
(n.s.)
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13
#14
LacZ
Cybb

Fig. S6. Additional data related to the non-tumor-promoting effect of Cybb in tumor (relative to ST) in experimental NASH-HCC, Related to Figure 6. (A) Immunoblots of Nox2, γH2AX, and H2AX in tumor tissue in Figures 6A-6G and quantification data (n = 3; means ± SEM). (B-C) Body weight and fasting blood glucose of the mice in Figures 6A-6G (n = 6-8 mice/group; means ± SEM). (D) Tumor diameters for the 14 mice in Figure 6G. Means ± SEM are shown for each group, n.s., not significant. (E) Immunoblots of Nox2, γH2AX, and H2AX in tumor tissue in Figures 6H-6N and quantification data (n = 3; means ± SEM). (F-G) Body weight and fasting blood glucose of the mice in Figures 6H-6N (n = 6-7 mice/group; means ± SEM). (H) Tumor diameters for the 14 mice in Figure 6N. Means ± SEM are shown for each group, n.s., not significant.

Table S1 (related to Figures S1C-D, 5C, S4A, S4J-S4N and S4P-S4Q). Pathology of liver specimens from humans with NAFLD/NASH and HCC or NASH

ID*	Age	Gender	Pathological diagnoses
HCC001	71	male	Cirrhosis with steatosis, with some areas showing evidence of ablation without residual HCC and other areas showing moderately to well differentiated HCC
HCC002	59	male	Cirrhosis with steatosis, HCC present
HCC003	59	male	Cirrhosis with NASH, no viable tumor cells seen
HCC005	71	male	NAFLD patient with cirrhosis and multifocal HCC
107841	74	male	Non-neoplastic liver: NASH with mild steatosis, mild activity, pericellular stage 1 fibrosis. HCC well differentiated, 5.0 cm (pT1b)
107916	68	male	Non-neoplastic liver: NAFLD with mild steatosis, stage 2 fibrosis. HCC moderately differentiated,5.4cm with multiple satellite nodules, large vein invasion (pT2)
108105	56	male	Non-neoplastic liver: No steatosis or steatohepatitis, stage 4 fibrosis (cirrhosis). HCC well differentiated, 9 cm (pT1b)
108663	88	male	Non-neoplastic liver: NAFLD with mild steatosis, stage 4 fibrosis (cirrhosis) HCC poorly differentiated, 4.5 cm and multifocal (pT2)
110317	80	female	Non-neoplastic liver: NASH with moderate steatosis, mild activity, stage 2 fibrosis. HCC moderately differentiated, 9.4 cm (pT1b)
110382	60	male	Non-neoplastic liver: NAFLD with mild steatosis, inactive, stage 4 fibrosis (cirrhosis) HCC well differentiated, 4.3cm (pT1b)
119327	80	male	Non-neoplastic liver: NASH with mild steatosis, mild activity, stage 3 fibrosis. HCC well differentiated, 5.1 cm (pT1b)
124182	77	male	Non-neoplastic liver: NAFLD with mild steatosis, stage 3 fibrosis. HCC well differentiated, 18.5 cm with large vein invasion (pT4)
126076	76	male	Non-neoplastic liver: NAFLD with mild steatosis, stage 0 fibrosis.

			HCC well differentiated, 3.4 cm (pT1a)
127458	70	male	Non-neoplastic liver: NAFLD with mild steatosis, stage 0 fibrosis. HCC poorly differentiated, 7.0 cm with large vein invasion (pT4)
128627	67	male	Non-neoplastic liver: NASH with mild steatosis, mild activity, stage 4 fibrosis (cirrhosis) HCC poorly differentiated, 8.0 cm with satellite nodules (pT3)
128036	68	male	Non-neoplastic liver: NASH with minimal steatosis, moderate activity, stage 4 fibrosis (cirrhosis) HCC moderately differentiated, 5.9 cm and 2.1 cm (pT3)
NASH1	68	male	Non-alcoholic steatohepatitis; cirrhosis, mixed macronodular and micronodular type. Minimal inflammatory activity, with minimal or no ongoing steatosis. No evidence of hepatocyte dysplasia or malignancy.
NASH2	56	female	Advanced chronic liver disease (stage 4: "cirrhosis"). Marked fibrosis throughout the entire liver. The residual hepatocellular parenchyma shows abundant well-formed Mallory hyaline, consistent with advanced chronic liver disease from steatohepatitis.
NASH3	53	male	NASH. Cirrhosis. Mild steatosis and focal active steatohepatitis. Focal Mallory hyaline is identified.
NASH4	67	female	Cirrhosis, NASH. Active steatohepatitis, grade 2 of 3.

*Source of the specimens is indicated by shading color: blue = MGH; tan = Columbia; white = Liver Tissue Cell Distribution System. See Human Liver Specimens section of Methods.

Table S2 (related to multiple figures). siRNA and shRNA sequences used in this study.

Target Gene	siRNA or shRNA	Sense Sequences (5' to 3')
Wwtr1 (TAZ)	siRNA	ACA UGG ACG AGA UGG AUA CAG GUG A
Wwtr1 (TAZ)	shRNA	CACCAcagccgaatctcgcaatgaatCTCGAGATTCATTGCGAG ATTCGGCTG
Ihh	shRNA	CACCAaccaccttcagtgatgtgcttaTCAAGAGTAAGCACATCACTGAAGGTGGG
Cybb	siRNA	GUU CAA GGU CAG UUU AUU GAA UGA A
Cybb	shRNA	CACCAgaacgaagagtatctcaatttCTCGAGAAATTGAGATACTCTTCGTTC

Table S3 (Related to multiple figures). Primers used for qPCR.

Primers	Organism	5'- Sequence -3'
Hprt F	mouse	TCAGTCAACGGGGGACATAAA
Hprt R	mouse	GGGGCTGTACTGCTTAACCAG
Taz (*Wwtr1*) F	mouse	CATGGCGGAAAAAGATCCTCC
Taz (*Wwtr1*) R	mouse	GTCGGTCACGTCATAGGACTG
Cyp2e1 F	mouse	TCACTGGACATCAACTGCC
Cyp2e1 R	mouse	TGGTCTCTGTTCCTGCAAAG
Fmo2 F	mouse	AGTGGCCTAATCTCTCTGAAGT
Fmo2 R	mouse	CATCGGGAAGTCACTGAAACAG
Xdh F	mouse	ATGACGAGGACAACGGTAGAT
Xdh R	mouse	TCATACTTGGAGATCATCACGGT
Romo1 F	mouse	TTCGACCGCGTGAAGATGG
Romo1 R	mouse	CCCGCATTCCGATCCTGAG
Noxo1 F	mouse	GCTCCATTGCTGACACGTC
Noxo1 R	mouse	AGGTTTGGGTACAAAGAAGCC
Aox1 F	mouse	GAGGAAGAATCTCCGACTCACA
Aox1 R	mouse	TGGTGACTGCTGTACCATGTAG
Gpx5 F	mouse	TCTAGCCAGCTATGTGCAGAC
Gpx5 R	mouse	TCCTTCCCATTAAGAGACAGAGC
Cybb F	mouse	TGTGGTTGGGGCTGAATGTC
Cybb R	mouse	CTGAGAAAGGAGAGCAGATTTCG
Nox4 F	mouse	GAAGGGGTTAAACACCTCTGC
Nox4 R	mouse	ATGCTCTGCTTAAACACAATCCT
Sod1 F	mouse	AACCAGTTGTGTTGTCAGGAC
Sod1 R	mouse	CCACCATGTTTCTTAGAGTGAGG
Sod2 F	mouse	CAGACCTGCCTTACGACTATGG
Sod2 R	mouse	CTCGGTGGCGTTGAGATTGTT
Cat F	mouse	CTGTGTGAGAACATTGCCGGCCA
Cat R	mouse	TGTACTTGTCCAGAAGAGCCTGGA
Nhej1 F	mouse	GGTTACAACTTGCGGAGAACT
Nhej1 R	mouse	GTCCACCTGTTCATGCCACA
Ku80 F	mouse	ATGGCGTGGTCGGTAAATAAG
Ku80 R	mouse	CCTGTCGTTGGACAAACATAGTC
Xrcc4 F	mouse	CTTGCTTCTGAACCCAACGTA
Xrcc4 R	mouse	TGGCCGTCAGTAAGTGTAATAAC
Ku70 F	mouse	AGAAGCACTTCCGAGACACG
Ku70 R	mouse	TCGTCTTCATTGGTGAACAGC
Ogg1 F	mouse	CTGCCTAGCAGCATGAGACAT
Ogg1 R	mouse	CAGTGTCCATACTTGATCTGCC
Hes1 F	mouse	CCAGCCAGTGTCAACACGA
Hes1 R	mouse	AATGCCGGGAGCTATCTTTCT

Cybb promoter F	mouse	CTCCATGCATTACCTAGACAGG
Cybb promoter R	mouse	AGAGTGAATCCAACTAGCAAAGA
Cybb non-specific F	mouse	GGGAGGAAAGAGAGTATGGAAAG
Cybb non-specific R	mouse	GAGGTGCAAATCAGGAAGTAGA

Hprt, hypoxanthine guanine phosphoribosyl transferase; *Taz* (*Wwtr*1), WW domain containing transcription regulator 1; *Cyp2e1*, cytochrome P450, family 2, subfamily e, polypeptide 1; *Fmo2*, flavin containing monooxygenase 2; *Xdh*, xanthine dehydrogenase; *Romo1*, reactive oxygen species modulator 1; *Noxo1*, NADPH oxidase organizer 1; *Aox1*, aldehyde oxidase 1; *Gpx5*, glutathione peroxidase 5; *Cybb*, cytochrome b-245, beta polypeptide; *Nox4*, NADPH oxidase 4; *Sod1*, superoxide dismutase 1, soluble; *Sod2*, superoxide dismutase 2, mitochondrial; *Cat*, catalase; *Nhej1*, Non-Homologous End Joining Factor 1; *Ku80*, X-ray repair complementing defective repair in Chinese hamster cells 5; *Xrcc4, X-ray repair complementing defective repair in Chinese hamster cells 4*; *Ku70,* X-ray repair complementing defective repair in Chinese hamster cells 6; *Ogg1*, 8-oxoguanine DNA glycosylase; *Hes1*, hes family bHLH transcription factor 1; *Cybb promoter*: specific TAZ/TEAD binding area in promoter region of Cybb gene; *Cybb* non-specific: non-specific TAZ/TEAD binding site in mouse Cybb gene intron 6.

Table S4 (Related to multiple figures). Antibodies used for Immunoblots, IF, and IHC.

Antibodies	Source	Identifier; Proper Citation
TAZ (Immunoblot)	Cell Signaling	#8418; RRID: AB_10950494
TAZ (IP)	Cell Signaling	#70148; RRID: AB_2799776
GAPDH (Immunoblot)	Cell Signaling	#3683; RRID: AB_1642205
β-Actin (Immunoblot)	Cell Signaling	#5125; RRID: AB_1903890
γH2AX (Immunoblot)	Cell Signaling	#9718; RRID: AB_2118009
H2AX (Immunoblot)	Cell Signaling	#7631; RRID: AB_10860771
Glypican 3 (IHC)	Sigma	SAB2108511;
Nox2 (IF, IHC, Immunoblot)	Proteintech	19013-1-AP; RRID: AB_2833044
TAZ (IHC)	Sigma	HPA007415; RRID: AB_1080602
Ki67 (IF)	Thermo Fisher	14-5698-82; RRID: AB_10854564
Ki67 (IF)	R & D	AF7649; RRID: AB_2687500
8-OHDG (IF, IHC)	Millipore	AB5830; RRID: AB_92060
Ogg1 (Immunoblot)	Proteintech	15125; RRID: AB_2156780
Nhej1 (Immunoblot)	Cell Signaling	#2854; RRID: AB_2152954
HNF4α (IF)	Cell Signaling	#3113; RRID: AB_2295208
CD68 (IF)	Dako	M0814; RRID: AB_2314148
Hep Par-1 (IF)	Abcam	ab234028;
4-HNE (IF)	Millipore	AB5605; RRID: AB_569332
Ihh (Immunoblot)	Proteintech	13388; RRID: AB_2248725
MPO (IF)	R & D	BAF3667; RRID: AB_2146326

JOURNAL
OF HEPATOLOGY

Journal of Hepatology

CTAT methods

Tables for a "Complete, Transparent, Accurate and Timely account" (CTAT) are now mandatory for all revised submissions. The aim is to enhance the reproducibility of methods.

- Only include the parts relevant to your study
- Refer to the CTAT in the main text as 'Supplementary CTAT Table'
- Do not add subheadings
- Add as many rows as needed to include all information
- Only include one item per row

If the CTAT form is not relevant to your study, please outline the reasons why:

1.1 Antibodies

Name	Citation	Supplier	Cat no.	Clone no.
TAZ	RRID: AB_10950494	Cell Signaling	#8418	D24E4
TAZ	RRID: AB_2799776	Cell Signaling	#70148	D3I6D
GAPDH	RRID: AB_1642205	Cell Signaling	#3683	14C10
β-Actin	RRID: AB_1903890	Cell Signaling	#5125	13E5
γH2AX	RRID: AB_2118009	Cell Signaling	#9718	20E3
H2AX	RRID: AB_10860771	Cell Signaling	#7631	D17A3
Glypican 3		Sigma	SAB2108511	N/A
Nox2	RRID: AB_2833044	Proteintech	19013-1-AP	N/A
TAZ	RRID: AB_1080602	Sigma	HPA007415	N/A
Ki67	RRID: AB_10854564	Thermofisher	14-5698-82	N/A
Ki67	RRID: AB_2687500	R & D	AF7649	N/A
8-OHDG	RRID: AB_92060	Millipore	AB5830	N/A
Ogg1	RRID: AB_2156780	Proteintech	15125	N/A
Nhej1	RRID: AB_2152954	Cell Signaling	#2854	N/A
HNF4α	RRID: AB_2295208	Cell Signaling	#3113	C11F12
CD68	RRID: AB_2314148	Dako	M0814	KP1
Hep Par-1		Abcam	ab234028	N/A
4-HNE	RRID: AB_569332	Millipore	AB5605	N/A
Ihh	RRID: AB_2248725	Proteintech	13388	N/A
MPO	RRID: AB_2146326	R & D	BAF3667	N/A

1.2 Cell lines

Name	Citation	Supplier	Cat no.	Passage no.	Authentication test method
AML12 cells		ATCC	CRL-2254	5-7	
HepG2 cells		ATCC	HB-8065	4-6	

1.3 Organisms

Name	Citation	Supplier	Strain	Sex	Age	Overall n number
C57BL/6J		The Jackson Laboratory	000664	Male	10-12wks	58

Wwtr1$^{fl/fl}$	PMID: 23918388	Laboratory of Dr. Eric Olson, UT-Southwestern		Male	10-12wks	10
Rosa^{NICD}		The Jackson Laboratory	008159	Male	10-12wks	114

1.4 Sequence based reagents

Name	Sequence	Supplier
Hprt F	TCAGTCAACGGGGGACATAAA	IDTDNA
Hprt R	GGGGCTGTACTGCTTAACCAG	IDTDNA
Taz (*Wwtr1*) F	CATGGCGGAAAAAGATCCTCC	IDTDNA
Taz (*Wwtr1*) R	GTCGGTCACGTCATAGGACTG	IDTDNA
Cyp2e1 F	TCACTGGACATCAACTGCC	IDTDNA
Cyp2e1 R	TGGTCTCTGTTCCTGCAAAG	IDTDNA
Fmo2 F	AGTGGCCTAATCTCTCTGAAGT	IDTDNA
Fmo2 R	CATCGGGAAGTCACTGAAACAG	IDTDNA
Xdh F	ATGACGAGGACAACGGTAGAT	IDTDNA
Xdh R	TCATACTTGGAGATCATCACGGT	IDTDNA
Romo1 F	TTCGACCGCGTGAAGATGG	IDTDNA
Romo1 R	CCCGCATTCCGATCCTGAG	IDTDNA
Noxo1 F	GCTCCATTGCTGACACGTC	IDTDNA
Noxo1 R	AGGTTTGGGTACAAAGAAGCC	IDTDNA
Aox1 F	GAGGAAGAATCTCCGACTCACA	IDTDNA
Aox1 R	TGGTGACTGCTGTACCATGTAG	IDTDNA
Gpx5 F	TCTAGCCAGCTATGTGCAGAC	IDTDNA
Gpx5 R	TCCTTCCCATTAAGAGACAGAGC	IDTDNA
Cybb F	TGTGGTTGGGGCTGAATGTC	IDTDNA
Cybb R	CTGAGAAAGGAGAGCAGATTTCG	IDTDNA
Nox4 F	GAAGGGGTTAAACACCTCTGC	IDTDNA
Nox4 R	ATGCTCTGCTTAAACACAATCCT	IDTDNA
Sod1 F	AACCAGTTGTGTTGTCAGGAC	IDTDNA
Sod1 R	CCACCATGTTTCTTAGAGTGAGG	IDTDNA
Sod2 F	CAGACCTGCCTTACGACTATGG	IDTDNA
Sod2 R	CTCGGTGGCGTTGAGATTGTT	IDTDNA
Cat F	CTGTGTGAGAACATTGCCGGCCA	IDTDNA
Cat R	TGTACTTGTCCAGAAGAGCCTGGA	IDTDNA
Nhej1 F	GGTTACAACTTGCGGAGAACT	IDTDNA
Nhej1 R	GTCCACCTGTTCATGCCACA	IDTDNA
Ku80 F	ATGGCGTGGTCGGTAAATAAG	IDTDNA
Ku80 R	CCTGTCGTTGGACAAACATAGTC	IDTDNA
Xrcc4 F	CTTGCTTCTGAACCCAACGTA	IDTDNA
Xrcc4 R	TGGCCGTCAGTAAGTGTAATAAC	IDTDNA
Ku70 F	AGAAGCACTTCCGAGACACG	IDTDNA
Ku70 R	TCGTCTTCATTGGTGAACAGC	IDTDNA
Ogg1 F	CTGCCTAGCAGCATGAGACAT	IDTDNA
Ogg1 R	CAGTGTCCATACTTGATCTGCC	IDTDNA
Hes1 F	CCAGCCAGTGTCAACACGA	IDTDNA
Hes1 R	AATGCCGGGAGCTATCTTTCT	IDTDNA
Cybb promoter F	CTCCATGCATTACCTAGACAGG	IDTDNA
Cybb promoter R	AGAGTGAATCCAACTAGCAAAGA	IDTDNA
Cybb non-specific F	GGGAGGAAAGAGAGTATGGAAAG	IDTDNA

Cybb non-specific R	GAGGTGCAAATCAGGAAGTAGA	IDTDNA

1.5 Biological samples

Description	Source	Identifier
Human NASH-HCC samples	MGH Hospital	
Human NASH-HCC samples	Columbia University	
Human NASH samples	Liver Tissue Cell Distribution System	

1.6 Deposited data

Name of repository	Identifier	Link

1.7 Software

Software name	Manufacturer	Version
ImageJ2	NIH	2.3.0
PRISM	GraphPad Software	Version 8

1.8 Other (e.g. drugs, proteins, vectors etc.)

AAV8-TBG-LacZ virus	Addgene	105534-AAV8
AAV8-TBG-Cre virus	Addgene	107787-AAV8
AAV8-TBG-GFP virus	Addgene	105535-AAV8
AAV8-TBG-Ogg1 virus	Vector Biolabs	Customized
AAV8-TBG-Nhej1 virus	Vector Biolabs	Customized
AAV8-TBG-Cybb virus	Vector Biolabs	Customized
AAV8-TBG-Ihh virus	Vector Biolabs	Customized
AAV8-H1-Scr virus	Vector Biolabs	Customized
AAV8-H1-shCybb virus	Vector Biolabs	Customized
AAV8-H1-shIhh virus	Vector Biolabs	Customized
GFP plasmid	Lonza	pmaxGFP
Cybb plasmid	Origene	MC204867
Nhej1 plasmid	Origene	MC200480
Ogg1 plasmid	Origene	MR227443

1.9 Please provide the details of the corresponding methods author for the manuscript:

Ira Tabas, M.D., Ph.D.
630 West 168th Street
New York, NY 10032
Office: Room PH 8-East 105F
Laboratory: Rooms PH 9-405-406

Office Tel: 212-305-9430
Laboratory Tel: 212-305-5669
FAX: 212-305-4834
E-mail: iat1@columbia.edu

2.0 Please confirm for randomised controlled trials all versions of the clinical protocol are included in the submission. These will be published online as supplementary information.

N/A

ICMJE DISCLOSURE FORM

Date: 12/2/2021

Your Name: Kathleen Corey

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	☒ **None**	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☐ **None**	
		NIBR - Grant	
		BMS- Grant	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☐ **None**	
		Novo Nordisk	
		Theratechnologies	
		BMS	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☐ **None**	
		Indiana University	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☐ **None**	
		AASLD Guidelines Commitee	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/6/2021

Your Name: Changyu Zhu

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/9/2021

Your Name: Helen Remotti

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/8/2021

Your Name: Utpal Pajvani

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/6/2021

Your Name: Hongxue Shi

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/11/2021

Your Name: Stephanie A. Osganian

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/29/2021

Your Name: Elizabeth Verna

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☐ **None**	
		NIH	
		Salix	Investigator Initiated Research Grant
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☐ **None**	
		Councilor at Large on the Board of the American Society of Transplantation	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/6/2021

Your Name: Xiaobo Wang

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	☒ None	
		American Liver Foundation- Liver Scholar Award	Research grant
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
		U.S. Patent Application PCT/US2017028109, 16/094,111 (US), 17786458.4 (Europe) "Therapeutic Targets Involved in the Progression of Nonalcoholic Steatohepatitis (NASH)."	
		U.S. Patent Application PCT/US2018/0066886 "Therapeutic Targets for NASH-Induced Hepatocellular Carcinoma"	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/6/2021

Your Name: Ira Tabas

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): JHEPAT-D-21-01415

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	☐ **None**	
		NIH grant **R01DK116620**	research grant
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☐ **None**	
		Academic research grant from Takeda Pharmaceuticals to study the therapeutic potential of silencing TAZ in NASH.	Nothing in this grant related to hepatocellular carcinoma, which is the topic of this article. Payments made to Columbia University for research.
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☐ **None**	
		U.S. Patent Application PCT/US2017028109, 16/094,111 (US), 17786458.4 (Europe) "Therapeutic Targets Involved in the Progression of Nonalcoholic Steatohepatitis (NASH)."	
		U.S. Patent Application PCT/US2018/0066886 "Therapeutic Targets for NASH-Induced Hepatocellular Carcinoma"	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/9/2021

Your Name: Robert Schwabe

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/8/2021

Your Name: Sharon Zeldin

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): JHEPAT-D-21-01415R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: 11/18/2021

Your Name: Yoshinobu Daito

Manuscript Title: TAZ-Induced Cybb in Hepatocytes in Non-Alcoholic Steatohepatitis Contributes to Eventual Liver Tumor Formation

Manuscript Number (if known): **JHEPAT-D-21-01415**

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	☒ None	
			Click the tab key to add additional rows.
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	☒ **None**	
3	Royalties or licenses	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
4	Consulting fees	☒ **None**	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	☒ **None**	
6	Payment for expert testimony	☒ **None**	
7	Support for attending meetings and/or travel	☒ **None**	
8	Patents planned, issued or pending	☒ **None**	
9	Participation on a Data Safety Monitoring Board or Advisory Board	☒ **None**	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	☒ **None**	

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
11	Stock or stock options	☒ **None**	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	☒ **None**	
13	Other financial or non-financial interests	☒ **None**	

Please place an "X" next to the following statement to indicate your agreement:

☒ I certify that I have answered every question and have not altered the wording of any of the questions on this form.