Supplementary Materials and Methods

RT-PCR

Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed with 500 ng RNA using oligo (dT) primer and SuperScript III. Real-time PCR was performed in a volume of 20 µl using Sybr Green Master Mix and the 7500 Real-Time PCR System (Applied Biosystems). Primer sequences are shown in Supplementary Table 1.

Immunoprecipitation and Western Blotting

For immunoprecipitation cells were lysed with Pierce IP buffer (Thermo Fisher, 87788) containing protease inhibitors and precleared with the Protein G Dynabeads (Thermo Fisher, 10003D). Rabbit EZH2 antibody (Cell Signaling, #5246), UBR4 (Bethyl Labs, #A302-279A) or isotype control (Cell Signaling, #3900) was used to immunoprecipitate protein of interest.

For nuclear fractionation, liver tissue were lysed in Buffer A (5mM PIPES, 85mM KCl, 20mM Tris HCl pH 8.1, 0.5% NP-40) containing protease and phosphatase inhibitors. After 30 minutes of incubation at 4^{O} C, lysate was centrifuged at 14,000g for 15 minutes. The pellet was resuspended in Buffer B (50mM Tris HCl pH 8.1, 10mM EDTA, 1% SDS) containing protease and phosphatase inhibitors. After 30 minutes at 4^{O} C, the lysates were sonicated for 15 sec and centrifuged at 14,000g for 15 mins.

5-20µg of protein was loaded onto 4-20% Tris-Glycine gels, electrophoresed, and transferred onto nitrocellulose membranes (Scientific Laboratory Supplies) for blotting. The membrane was blocked, incubated with primary antibodies (Supplementary Table 2), rinsed with TBST, and incubated with HRP-conjuagted secondary antibodies. After washing, the membrane was exposed to luminol (Santa Cruz Biotechnology) and developed. Image J was used for densitometry analysis.

Mass Spectrometry

H69 cells grown to 80% confluency were serum starved overnight. They were treated with vehicle or TGFβ for 6 hours. Cells were then lysed for immunoprecipitation. Rabbit EZH2 antibody or isotype control were cross linked to Protein G Dynabeads using BS3 cross linker (Thermo Fisher, 21580). The precleared lysates were incubated with the crosslinked antibodies overnight at 4^{O}C. After magnetic separation, beads were washed 3 times with the IP lysis buffer, and the complex eluted with SDS sample buffer. The complex separated on 4-20% gel was stained with Coommassie Blue and sent to the Mayo Clinic Mass Spectrometry Core for analysis.

Each gel lane was cut into 6 sections. Proteins were destained in acetonitrile/50mM Tris pH 8.1, reduced with 50mM TCEP/50mM Tris pH8.1, alkylated with 25mM iodoacetamide/50mM Tris pH 8.1. Proteins were then digested in-situ with 0.2µg trypsin (Promega Corporation, Madison WI) in 25 mM Tris pH 8.1/0.0002% Zwittergent 3-16, at 37^{O}C overnight, followed by peptide extraction with 2% trifluoroacetic acid and acetonitrile. Extractions were dried and stored at -20°C.

Dried trypsin digested samples were suspended in sample buffer (0.2% formic acid/0.1% TFA/0.002% zwittergent 3-16). A portion of which was analyzed by nano-flow liquid chromatography electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) using a Thermo Ultimate 3000 RSLCnano HPLC system coupled to a Thermo Scientific Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatography was performed using A solvent (98% water/2% acetonitrile/0.2% formic acid) and B solvent (80 % acetonitrile/10% isopropanol/10% water/2% formic acid), over a 2% to 45% B gradient for 60 minutes separating the peptides using a custom packed MagicC8 trap (Optimize Technologies, Oregon City, OR) and an in-house column packed with Agilent Poroshell 120 EC C18 (Agilent, Santa Clara, CA). Q-Exactive mass spectrometer was set to acquire an ms1 survey scans from 350-1600 m/z at resolution 70,000 (at 200 m/z) with an AGC target of 3e6 ions. Survey scans were followed by HCD MS/MS scans on the top 15 ions at resolution 17,500 with an AGC target of 2e5 ions. Dynamic exclusion placed selected ions on an exclusion list for 40 seconds.

Instrument data files were database searched using Mascot (Matrix Science, London, UK) and X!Tandem (The GPM, the gpm.org) using a Uniprot human database (2017_01 release) based on

taxonomy 9606 with 156,773 entries, including a decoy reverse database. Searches were set up to assume a trypsin digestion with a parent ion tolerance of 10 ppm and fragment ion mass tolerance of 0.02 Da. Oxidation of methionine and iodoacetamide derivative of cysteine were specified as variable modifications. Scaffold (Proteome Software Inc., Portland OR) was used to combine, validate, and generate reports. Peptide and protein identifications were accepted if they were established at greater than 95.0% probability as specified by the Peptide and Protein Prophet algorithms (1, 2).

A protein spectral count was used as a guide for abundance. By considering the number of spectra for any given protein and the ratio of the spectra between two samples, a list of potential binding partners could be identified for the immunoprecipitation (IP). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD012978 and 10.6019/PXD012978".

EnrichR evaluation (http://amp.pharm.mssm.edu/Enrichr/) of protein enrichment was analyzed using the Fisher exact test, as described elsewhere (22, 23).

Adenoviral Transfection

Epitope-tagged (6XHis-Xpress) EZH2α was generated as recombinant adenovirus by the Gene Transfer Vector Core at the University of Iowa. Empty vector (pacAD5 CMV) was used as the experimental control as described elsewhere (16). H69 cells were infected with adenovirus (810 pfu/mL) after 4-6 h starvation. 24 hours later, cells were treated with TGFβ (R&D Systems #240-B) (10 ng/mL) for another 24h.

Cycloheximide Treatment

H69 cells were grown to 80% confluency. Cells were then serum starved and cycloheximide (CHX) was added to the cells at 40μg/ml for the indicated time points in the presence of vehicle or TGFβ (10ng/ml). The cells were lysed and subjected to SDS-PAGE and immunoblotting with EZH2 antibody.

Immunohistochemistry

Paraffin embedded tissue sections were antigen unmasked with citrate. After quenching of endogenous peroxidase, sections were blocked, and incubated with antibody against TGFβ, EZH2, H3K27me3, or CTGF overnight @ 4^0C. The remaining steps were carried out using an immunoperoxidase detection Kit (Vector Laboratories). Image J software was utilized to quantify the positive stained areas.

Conditioned Media Treatment

H69 cells were serum starved at 80% confluence for 4-6 hours. The cells were then treated for 12 hours with Vehicle or TGFβ (10ng/ml) in the presence of either DMSO or MG-132 (5uM). The cells were then washed with PBS and incubated with serum-free media for 24 hours. The media was collected, centrifuged to remove cell debris and used as conditioned media (CM). HSCs or RGF-1 cells at 90% confluence were serum starved overnight. The cells were then incubated with CM as following: 1) DMSO-Vehicle 2) DMSO-TGFβ 3) MG132-Veh 4) MG132- TGFβ 5) DMSO-Vehicle + RGD peptide (40nM) 6) DMSO-TGFβ + RGD peptide (40nM). 24 hours later HSC or RGF-1 cells were collected and lysed for immunoblotting with collagen and alpha-SMA antibody.

Hydroxyproline Assay

Hepatic hydroxyproline levels were quantified using a colorimetric assay. Frozen liver tissues (50–100 mg) were hydrolyzed in 6 N hydrochloric acid at 100°C for 18 hours. Samples then were dried using a speed vacuum overnight. The precipitates were suspended in distilled water and transferred to 0.22-μm–filter centrifuge tubes. 5 μL of the filtered samples were incubated with 50 μL of chloramine-T and distilled water for 20 minutes. Then, 50 μL of Elrich–perchloric acid was added and incubated for 15 minutes at 65°C and 20 minutes at room temperature. The absorbance was read at 561 nm using a spectrophotometer, and the values were normalized by the weight of the liver tissue used for the assay.

Mouse Serum Analysis

Serum ALT, ALP, and total bilirubin were measured using a commercially available veterinary chemistry analyzer (VetScan2, Abaxis, Union City, CA).

Laser capture Microdissection of Bile Ducts and RNA-isolation

Frozen liver sections at 6-7 µm were placed on prechilled clean slides, stained with Histogene staining solution (Thermo Fisher Scientific, # KIT0401), dehydrated with ethanol, and placed on a Veritas microdissection system (Arcturus, Molecular Devices, Sunnyvale, CA). Under light microscopy (40× magnification), bile ducts were visualized. An infrared laser was used to microdissect the delineated regions onto CapSure Macro LCM caps. Total RNA was isolated from the CapSure LCM caps after LCM using the Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific, # KIT0204) according to the vendor instructions. Expression of FN1 and EZH2 was measured using RT-PCR.

Immunofluorescence

Frozen liver sections were fixed with methanol and blocked with 10% FBS. The sections were then incubated with FN (BD Biosciences #610078, 1:500), CK19 (Abcam #ab 52625, 1:200) antibodies diluted in 1% BSA overnight at 4^0C. The sections were washed and incubated with fluorochrome-coupled secondary antibodies (Life Technologies # A21206 and #A10037) diluted in PBS for 1 hour at RT. Slides were washed, incubated with DAPI for 5 mins, and then mounted for imaging.

Supplementary Tables

Supplementary Table 1: RT-PCR primers sequences

Gene	Forward	Reverse
hCTGF	ACCAATGACAACGCCTCC	TTGGAGATTTTGGGAGTACGG
hIGFBP3	CAGAGCACAGATACCCAGAAC	AGCACATTGAGGAACTTCAGG

hTIMP1	TTCTGCAATTCCGACCTCG	TCATAACGCTGGTATAAGGTGG
hCol4A2	CTCACCCTTCCCTAGCAAAAG	TCCTCTGATCTCCATCTCCG
hCol5A1	CGGAACCTTGACGAGAACTAC	TCTCCCTTTTGGCCTTTCTC
hTGFb1	GCCTTTCCTGCTTCTCATGG	TCCTTGCGGAAGTCAATGTAC
hACTA2	AATGCAGAAGGAGATCACGG	TCCTGTTTGCTGATCCACATC
hFN1	GATAAATCAACAGTGGGAGC	CCCAGATCATGGAGTCTTTA
hVEGFA	AGTCCAACATCACCATGCAG	TTCCCTTTCCTCGAACTGATTT
hPDGFb	ATGATCTCCAACGCCTGC	TCAGCAATGGTCAGGGAAC
hEZH2	CCACAGTGTTACCAGCATTTG	ACTGTTATTGGGAAGCCGTC
hSUZ12	CAAGAAAAGAGCAACATGGGAG	AGAGGTTTGGCAATAGGAGC
mFN1	CTTTGGCAGTGGTCATTTCAG	ATTCTCCCTTTCCATTCCCG
mEZH2	TCCCGTTAAAGACCCTGAATG	TGAAAGTGCCATCCTGATCC
mCollagen1A1	CATAAAGGGTCATCGTGGCT	TTGAGTCCGTCTTTGCCAG
mSMA	GTGAAGAGGAAGACAGCACAG	GCCCATTCCAACCATTACTCC
mMCP-1/CCl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
mIL1-β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
mCD68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
mF4/80	ATGGACAAACCAACTTTCAAGGC	GCAGACTGAGTTAGGACCACAA

Supplementary Table 2: Primary Antibodies

Antibody	Supplier/ Catalog No.
EZH2	Cell Signaling Technology #5246S
FN	BD Biosciences #610078
GAPDH	Invitrogen #AM4300
HSC70	Santa Cruz #sc-7298

myc	Cell Signaling Technology #2276
SUZ12	Cell Signaling Technology #3737
HA	Roche #11583816001
UBR4	Bethyl Labs #A302-279A
CTGF	Santa Cruz #sc-14939
Collagen 1	Southern Biotech # 1310-01
Alpha-SMA	Abcam #ab5694
CK19	Abcam #ab52625

Supplementary Table 3: Patient Demographics

De-Identified Number	Age	Stage
10008530	34	S4
10008532	46	S4
10008534	32	S4
10008535	32	S4
10008536	Unknown	Normal
10008537	Unknown	Normal
10008538	Unknown	Normal
10008541	Unknown	Normal

Supplementary Figure Legends

Supplementary Figure 1: Mesenchymal markers in cholangiocytes with TGFβ treatment

A. RPKM values for mesenchymal markers CDH2 (N-Cadherin), VIM (Vimentin), SNAI2 (Snail Family Transcriptional Repressor 2) from RNA-seq data. * $p<0.01$, ** $p<0.001$. All error bars are SEM, n=3.

B. RT-PCR analysis for mesenchymal markers CDH2, VIM, and SNAI-2 on H69 cells treated with vehicle or TGFβ. * $p<0.01$, ** $p<0.001$. All error bars are SEM, n=3.

Supplementary Figure 2: Identification of H3K27me3 on the FN promoter

A. ChIP was performed on about 2.2 Kb region (from the TSS) of the FN promoter using walking primers. The region about 1.5 Kb from the TSS which showed the highest occupancy under vehicle condition and reduced with TGFβ was used for further analysis.

Supplementary Figure 3: Cholangiocyte conditioned media supports myofibroblast activation

A. Immunoblot for Collagen1 and alpha-SMA on hHSC incubated with CM from H69 cholangiocytes treated with vehicle or TGFβ in the presence of DMSO or MG-132. RGD peptide at 40nM was added to CM from the DMSO group. The right panel shows the quantification of the blots. * $p<0.01$, ** $p<0.001$, *** $p<0.0001$. All error bars are SEM, n=3.
B. Immunoblot for Collagen1 and alpha-SMA on RGF-1 cells incubated with CM from H69 cholangiocytes treated with vehicle or TGFβ in the presence of DMSO or MG-132. RGD peptide at 40nM was added to CM from DMSO group. The right panel shows the quantification of the blots. All error bars are SEM, n=3. No statistical differences to report.

Supplementary Figure 4: Cholangiocyte selective knockout of EZH2 in mouse livers.

A. RFP and EZH2 double immunostaining on mouse livers. Merged image on the right shows deletion of EZH2 in RFP positive bile ducts.
B. Immunohistochemistry on mouse livers showing overlap in RFP and CK19 staining.

Supplementary Figure 5: Cholangiocyte selective knockout of EZH2 in mouse livers.

A. Immunofluorescence for FN (red), CK19 (green), and DAPI (blue) shows increased FN deposition in KRT19-Cre/$EZH2^{fl/fl}$ mouse livers with BDL surgery compared to $EZH2^{fl/fl}$ with BDL or Sham surgery.

B. Liver enzymes (AST, ALT, Total Bilirubin) from serum of $EZH2^{fl/fl}$ and KRT19-Cre/$EZH2^{fl/fl}$ animals with sham or BDL surgery. *p<0.01, **p<0.001, ***p<0.0001. All error bars are SEM, n=4-5 animals per group.

C. mRNA analysis for inflammatory markers on liver tissue from $EZH2^{fl/fl}$ and KRT19-Cre/$EZH2^{fl/fl}$ animals with sham or BDL surgery. *p<0.01, **p<0.001. All error bars are SEM, n=4-5 animals per group.

D. Immunohistochemistry staining for CTGF shows increased CTGF levels in KRT19-Cre/$EZH2^{fl/fl}$ mouse livers with BDL surgery compared to $EZH2^{fl/fl}$ with BDL or Sham surgery.

Supplementary Figure 6: Biliary fibrosis in Mdr2-/- mice is reduced with Bortezomib treatment

A. Sirius red staining on Mdr2-/- mice tissue shows progressive fibrosis as the mice age.

B. Serum ALT, ALP, and total Bilirubin levels in Mdr2-/- mice treated with vehicle or Bortezomib. **p<0.001, n=4-5 animals per group.

C. Hydroxyproline analysis shows reduced collagen in liver tissue of Mdr2-/- mice treated with Bortezomib. *p<0.01, n=4-5 animals per group.

Supplementary Figure 7: PSC patient livers have reduced EZH2 and H3K27me3.

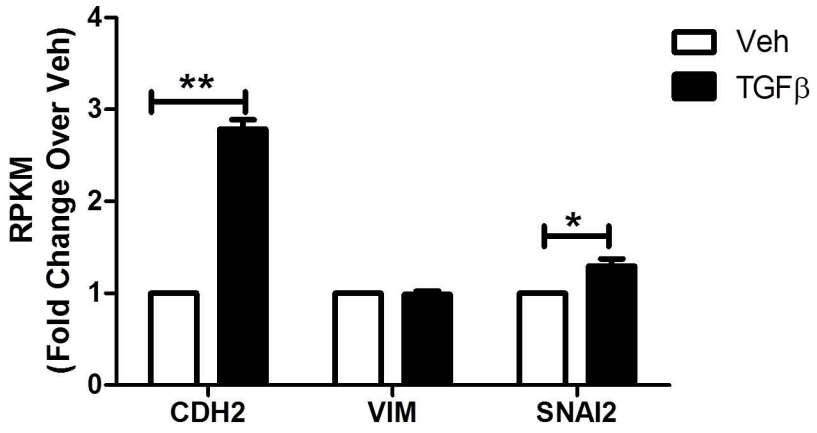
A. Immunohistochemistry for TGFβ on healthy control and PSC patient liver explant tissue (left). Quantification for % positive area shows a 2.5-fold increase in TGFβ in PSC compared to control liver tissue. **p<0.001. All error bars are SEM, n=3.

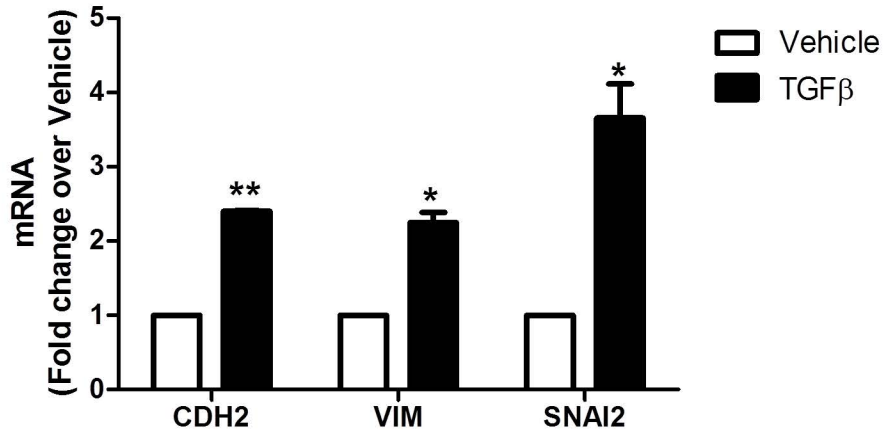
B. Immunohistochemistry for total EZH2 (left) and quantification on the right show a 3-fold decrease in EZH2 levels in PSC livers compared to control. **p<0.001. All error bars are SEM, n=3.

C. Immunohistochemistry for the H3K27me3 mark catalyzed by EZH2 (left) and quantification on the right show a 2-fold decrease in livers of PSC patient compared to controls. ***p<0.0001. All error bars are SEM, n=3.

Supplementary References

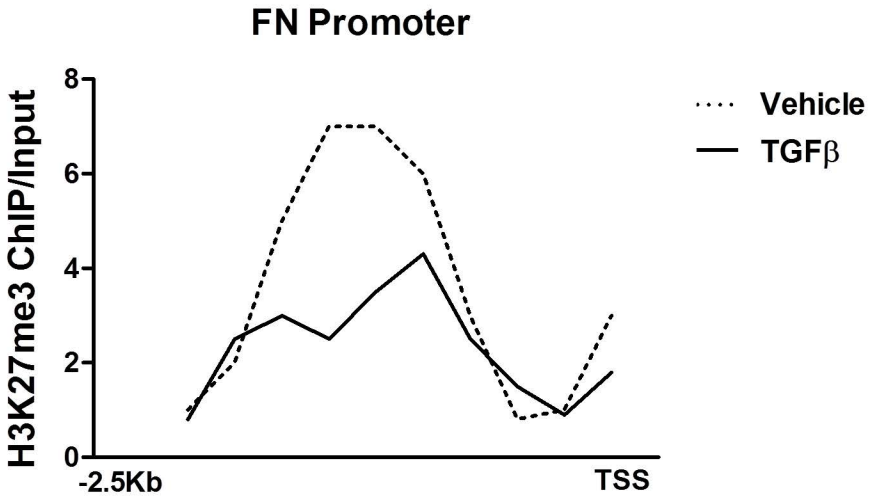
1. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 2002;74:5383-5392.
2. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 2003;75:4646-4658.



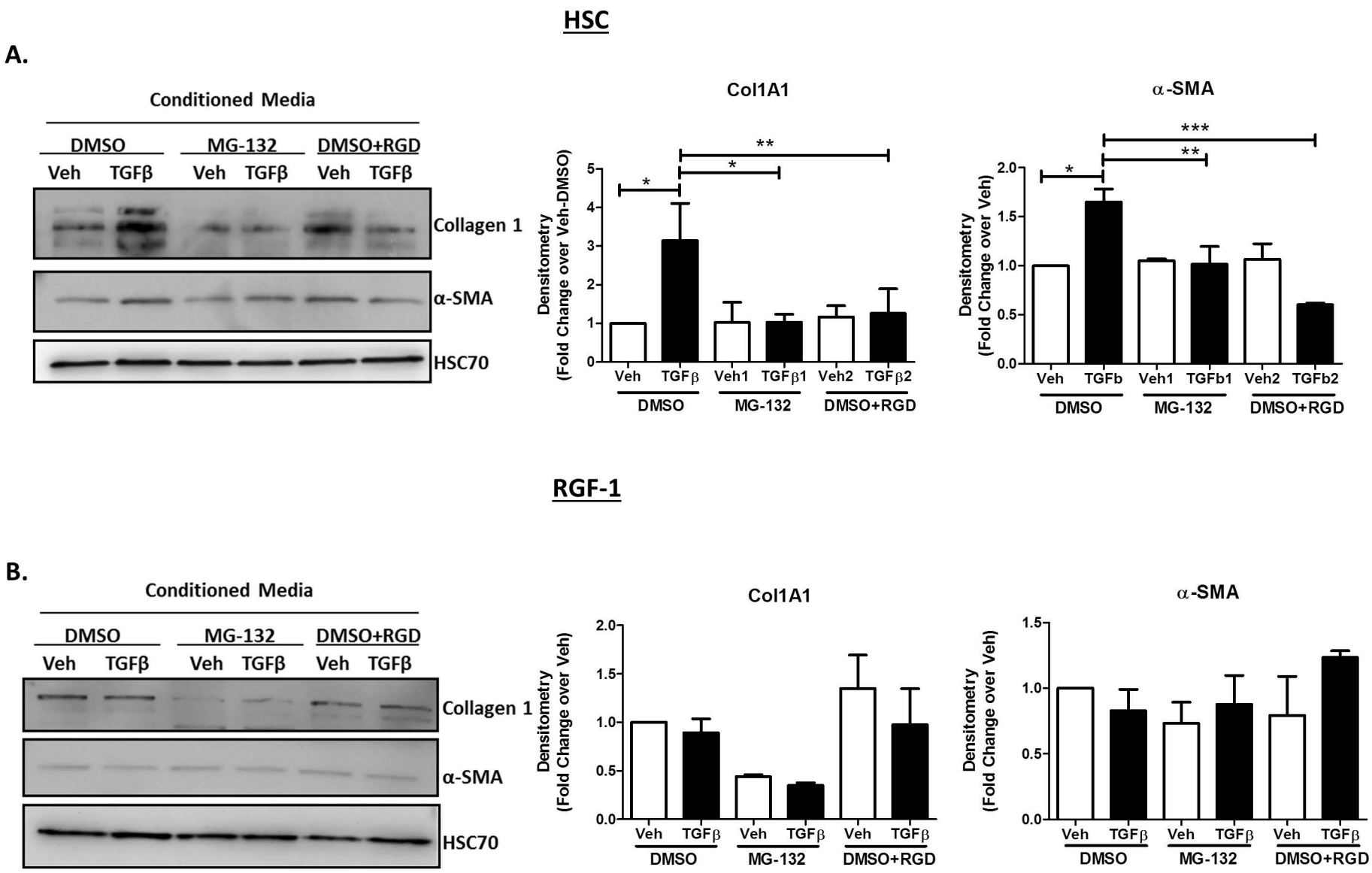


Supplemental Figure 1

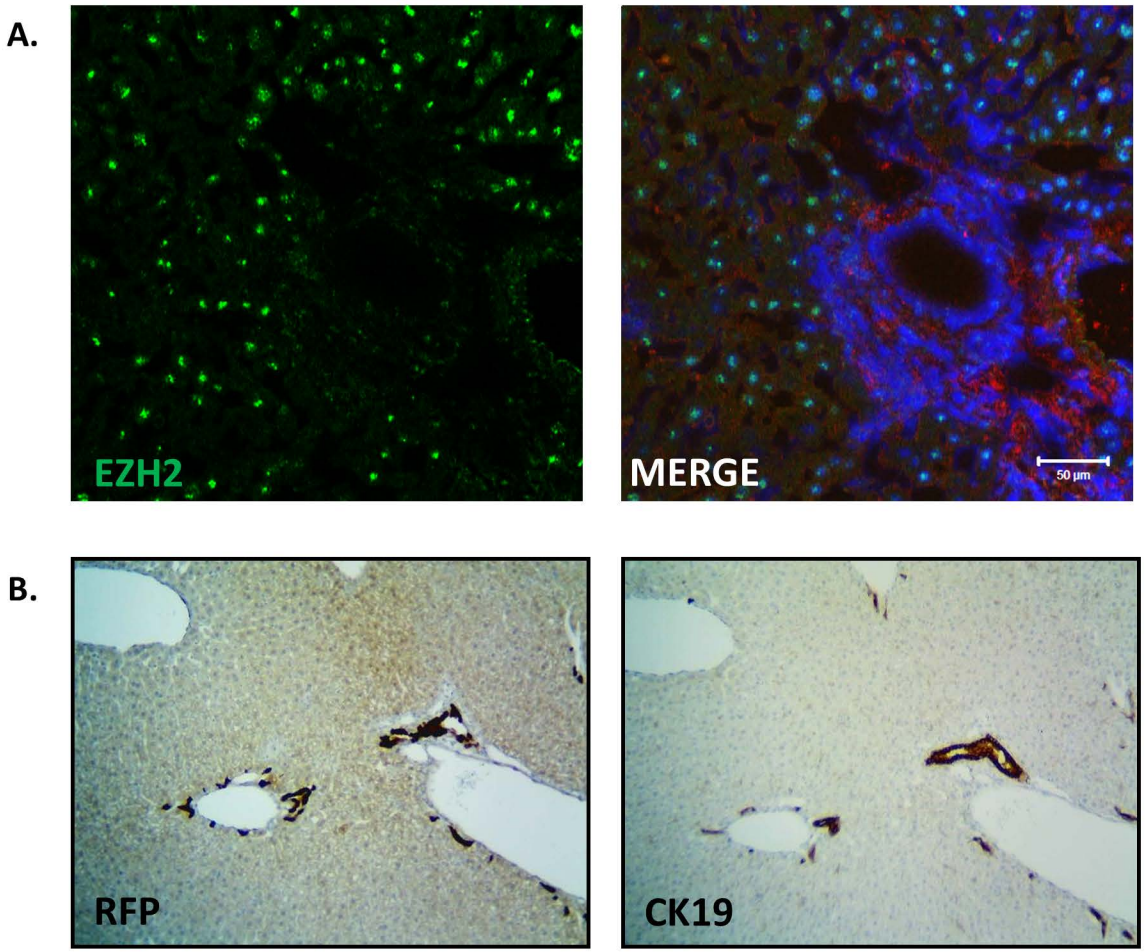
A.



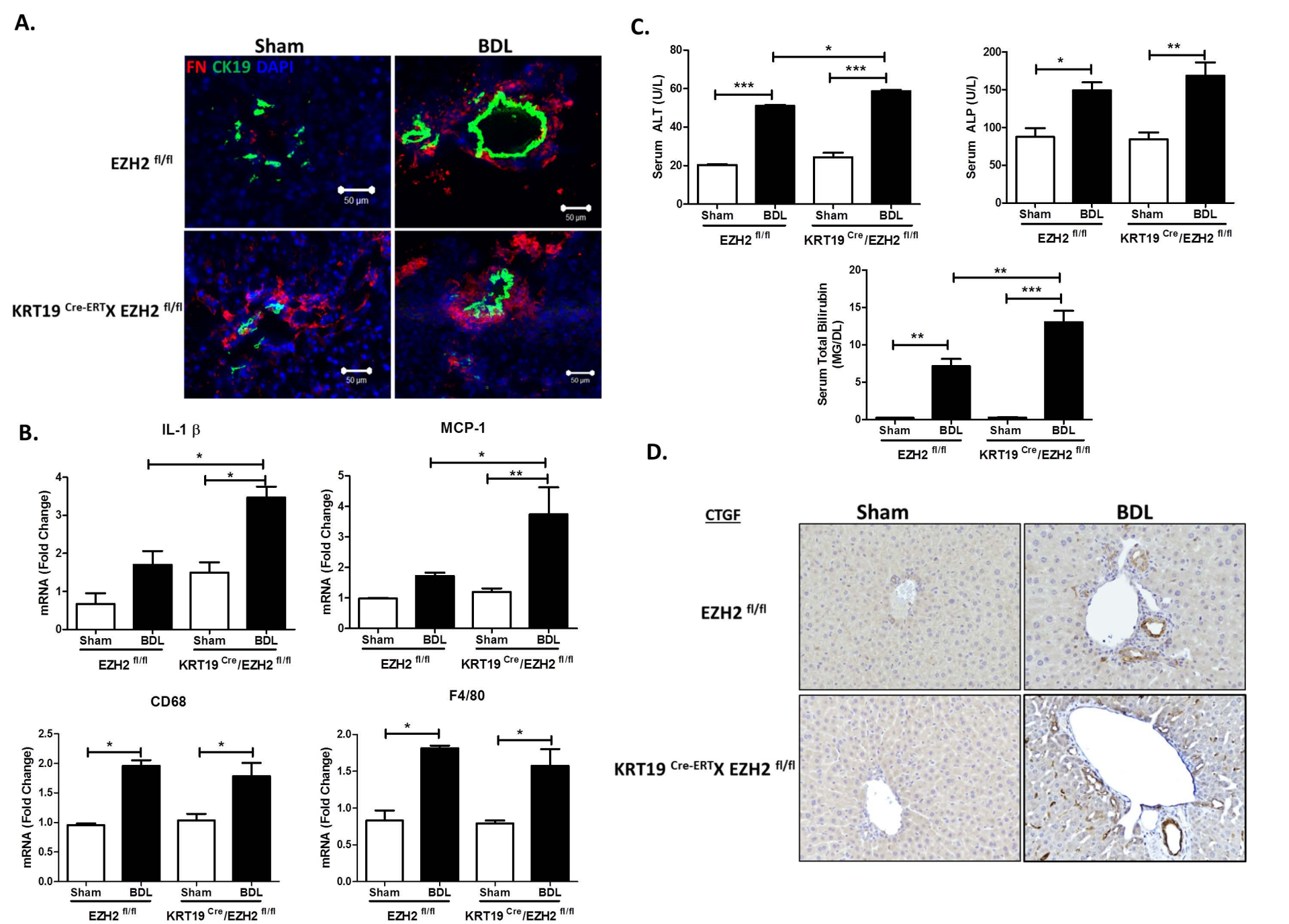
Supplemental Figure 2



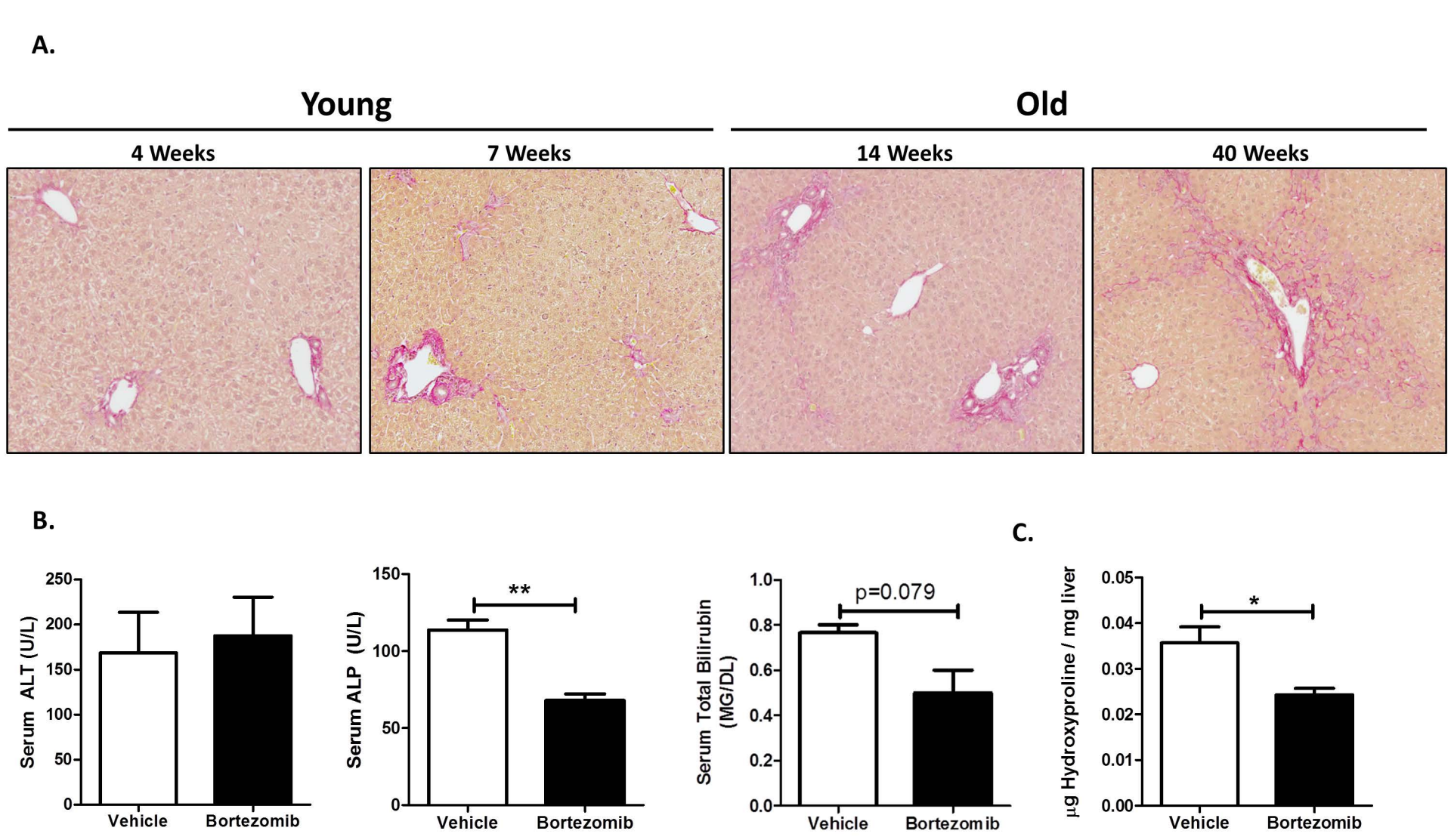
Supplemental Figure 3



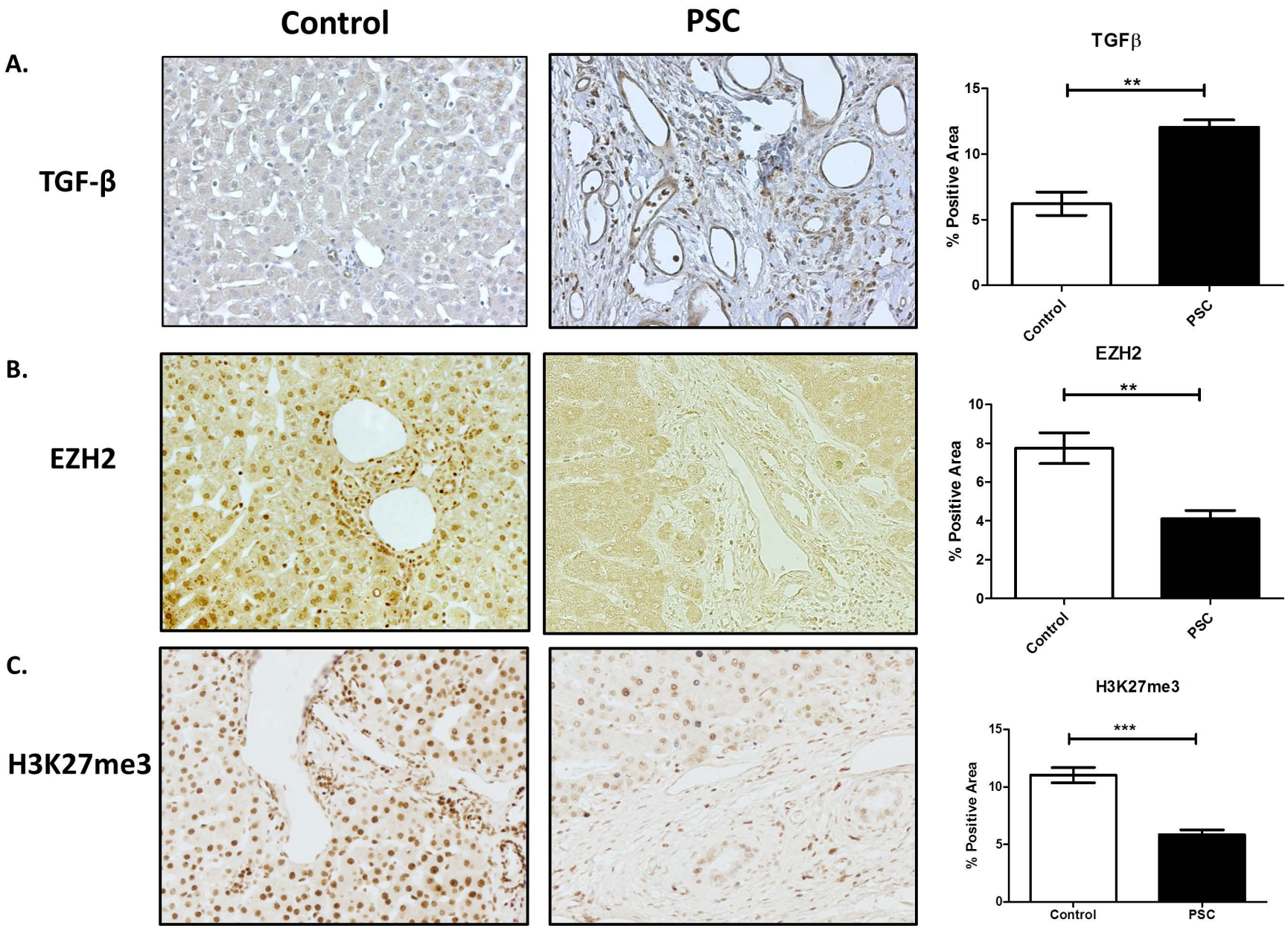
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7