SUPPLEMENTAL METHODS

Bile-duct ligation (BDL) animal groups

For the BDL-induced fibrosis animal model, we used a set of 32 animals. From these, 5 animals were separated as control animals and 27 were submitted to BDL surgery. On day 3 after BDL, we further randomly separated animals in groups of 5 each for the MLN4924-treatment groups (3d and 7d), and the others were maintained on vehicle treatment (n=17).

In vitro silencing and transfection

Primary hepatocytes and hepatic stellate cells (HSC) were transfected with 100 nM Nedd8 activating enzyme E1 subunit 1 (NAE1) siRNA or 100 nM c-Jun siRNA (both from Qiagen) using Jetprime reagent (Polyplus-Transfection, Strasbourg, France). Controls were transfected with an unrelated siRNA (Qiagen). Protein knockdown was confirmed by protein or mRNA expression levels.

In vitro drug treatments

Following plating and overnight incubation, mouse hepatocytes were stimulated with 50 µM deoxycholic bile acid (DCA) (Sigma-Aldrich, St. Louis, USA) and after 30 min treated up to 4h with 3 µM MLN4924. Mouse isolated Kupffer cells (KC) were plated overnight and on the next morning stimulated with 200 ng/ml lipopolysaccharide (LPS). After 1 hour, cells were treated with 3 µM MLN4924 for 24h. Primary mouse hepatic stellate cells (HSC) grown on uncoated plastic plates were treated with 3 µM MLN4924 for 4h. LX-2 cells were treated with 3 µM MLN4924 for 24h and 8 ng/ml TGFβ (Peprotech, Rocky Hill, USA), starting 1h before MLN4924-treatment.

Protein Isolation & Western Blotting

Extraction of total protein from cultured cells has been previously described (1). Briefly, 10 to 20 mg of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto membranes. Antibodies used for western Blotting are described in Supplemental Table 2.

RNA Isolation and Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA). One to two µg of total RNA was treated with DNAse (Invitrogen) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen). Then, qPCR was performed using iQ™ SYBR® Green Supermix (BioRad, Hercules, USA) using the CFX Connect™ RT-PCR Detection System (BioRad). Expression levels were normalized to the average level of either GAPDH or 18S mRNA in each sample**.** *Sequence of primers used for RT-PCR analysis can be provided upon demand.*

Immunohistochemistry

Paraffin embedded liver samples were sectioned, dewaxed and hydrated. Immunohistochemistry was performed as previously described (2). All procedures were done according to standard protocols with EnVision+ System HRP (Dako, Denmark). Finally, samples were incubated with Vector Vip substrate (Vectorlabs, Burlingame, USA) for color development. Images were taken with a 10X or 20X objective from an AXIO Imager A1 microscope (Carl Zeiss AG, Jena, Germany). Quantification of staining intensity, average sum of intensities and stained area percentage of each sample were calculated using FRIDA software (FRamework for Image Dataset

Analysis) http://bui3.win.ad.jhu.edu/frida/. The conditions for each antibody are detailed in Supplemental Table 3.

Immunofluorescence

Paraffin embedded liver samples were sectioned, dewaxed and hydrated. Samples were unmasked 15 minutes with proteinase K (Roche, Basel, Switzerland) at room temperature and then incubated firstly with Nedd8 overnight at 4ºC followed by either mouse Cy3-conjugated anti-aSMA (1h, RT), Albumin (1h, RT), F4/80 (, 37ºC), or Desmin antibodies. Finally, sections were incubated for 30 min at room temperature with Cy3-conjugated secondary antibody. Sections were counterstained with DAPI. Images were captured using an Immunofluorescence Microscope (Axioimager D1, Zeiss) (Antibody conditions and references detailed in Supplemental Table 3).

Standard staining

H&E and Sirius red staining were performed with standard reactives from Sigma-Aldrich.

Liver Metabolomics analysis

Bile acids profiles were analyzed as previously described (3). Briefly, UPLC/time-of-flight mass spectrometry (TOF)-MS based platforms analyzing methanol liver extracts were combined. Data obtained with the UPLC®-MS were processed with the TargetLynx application manager for MassLynx (Waters Corp., Millford, USA) as detailed previously (3). Intra- and interbatch normalization followed the procedure

described (3). All the calculations were performed with R v2.13.0 (R Development Core Team, 2010).

TUNEL assay

TUNEL assay was performed on frozen liver sections using the in situ cell death detection kit (Roche) according to manufacturer's instructions.

Determination of IL6, TNFa and TGFb

Interleukin-6 (IL6) and tumor necrosis factor alpha (TNFa) cytokine levels from liver extracts and cell media were determined by using the DuoSet ELISA Development Kit according to the manufacturer's instructions (R&D Systems, Minnesota, USA). For the transforming growth factor beta (TGFb) tissue measurement, samples were previously submitted to an acid activation as described earlier (4).

Caspase 3 activity measurement in cells

Caspase 3 activity was measured as previously described (5).

Supplemental Table 2. List of antibodies and their conditions used for Western blot.

Antibody	Supplier	Western Blot Dilution	Incubation Solution	Catalog N.
b-Actin	Sigma-Aldrich	1/5000	TBS 0,1%Tween 5% Milk	A-5316
pJNK	Invitrogen	1/1000	TBS 0,1%Tween 5% Milk	446826
JNK	Cell Signaling Technology	1/1000	TBS 0,1%Tween 5% Milk	9252
c-Jun	Cell Signaling Technology	1/1000	TBS 0,1%Tween 5% Milk	9165S
GAPDH	Abcam	1/5000	TBS 0,1%Tween 5% Milk	ab8245
NAE1	Abgent	1/1000	TBS 0,1%Tween 5% Milk	AP13067c
Nedd8	Abcam	1/1000	TBS 0,1%Tween 5% Milk	ab81264
p53	Santa Cruz Biotechnology	1/1000	TBS 0,1%Tween 5% Milk	SC-126
TNF-R1	Santa Cruz Biotechnology	1/1000	TBS 0,1%Tween 5% Milk	sc-8436
Tubulin	Cell Signaling Technology	1/5000	TBS 0,1%Tween 5% Milk	2146
V5	Invitrogen	1/500	TBS 0,1%Tween 5% Milk	377500

Supplemental Table 3. List of antibodies used for Immunohistochemistry.

Antibody	Supplier	Dilution	Source	Catalog N.
Albumin	Abcam	1:100	Sheep	Ab8940-1
Cleaved Caspase 3	Cell Signaling	1:50	Rabbit	9661
F4/80	BioRad	1:50	Rat	NCA497bb
NEDD8	Cell Signaling Technology	1:300	Rabbit	2745
NAE1	Abgent	1:100	Rabbit	API3067C
aSMA	Invitrogen	1:50	Mouse	180106
Desmin	Dako	1:50	Mouse	M0760

Supplemental References

1. Embade N, Fernández-Ramos D, Varela-Rey M, Beraza N, Sini M, Gutiérrez de Juan V, et al. Murine double minute 2 regulates Hu antigen R stability in human liver and colon cancer through NEDDylation. Hepatol. Baltim. Md. 2012;55:1237–1248.

2. Martínez-López N, García-Rodríguez JL, Varela-Rey M, Gutiérrez V, Fernández-Ramos D, Beraza N, et al. Hepatoma cells from mice deficient in glycine N-methyltransferase have increased RAS signaling and activation of liver kinase B1. Gastroenterology. 2012;143:787–798.e1–13.

3. Barr J, Caballería J, Martínez-Arranz I, Domínguez-Díez A, Alonso C, Muntané J, et al. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. J. Proteome Res. 2012;11:2521–2532.

4. Khan SA, Joyce J, Tsuda T. Quantification of active and total transforming growth factor-β levels in serum and solid organ tissues by bioassay. BMC Res. Notes. 2012;5:636.

5. Barbier-Torres L, Delgado TC, García-Rodríguez JL, Zubiete-Franco I, Fernández-Ramos D, Buqué X, et al. Stabilization of LKB1 and Akt by neddylation regulates energy metabolism in liver cancer. Oncotarget. 2015;6:2509–2523.

Supplemental Figure 1. Schematic representation of experimental procedures. Time scheme of the bile-duct ligation (BDL) mouse model with MLN4924-treatment starting at **(A)** 3 and **(B)** 7 days after BDL. **(C)** Time scheme of CCl_4-administration and MLN4924 treatment.

Supplemental Figure 2. Fibrosis evaluation of human patients. Sirius red staining of livers from healthy and non-alcoholic fatty liver disease (NAFLD) patients used in figure 1 and suppl. table 1. Both peri-sinusoidal and peri-portal regions are shown. Graphs represent immunohistochemistry quantification (n=13 control and n=15 NAFLD). **p<0.01 is indicated.

Supplemental Figure 3. Neddylation is altered in liver disease regardless of etiology. Nedd8 and NAE1 staining of control and fibrotic livers from either hepatitis B infection **(A)** or alcohol abuse **(B)**. Graphs represent immunohistochemistry quantification (n=5 control, n=7 hepatitis B infection and n=6 alcohol abuse). *p<0.05 and ***p<0.001 *vs.* healthy liver are indicated.

Supplemental Figure 4. Bile-duct ligation (BDL) and tetrachloride (CCl_4) mouse models immunohistochemical liver characterization. (A) Hematoxylin/eosin (H&E) staining of BDL livers together with immunohistochemical of global neddylation, Nedd8-activating enzyme E1 subunit 1 (Nae1) and inflammation marker F4/80 at 3 days after BDL. **(B)** Sirius red and alpha smooth muscle actin (αSMA) stainings at 7 days after BDL**. (C).** Hematoxylin/eosin (H&E) staining together with global neddylation, Nedd8-activating enzyme E1 subunit 1 (Nae1), inflammation marker F4/80, sirius red, and alpha smooth muscle actin (αSMA) stainings at 2 weeks after initiating CCl_4 chronic administration. Graphical representations are shown to the right (n=5 control, n=5 BDL 3d and n=5 BDL 7d; n=6 control and n=6 CCl_4). *p<0.05 and **p<0.01 are indicated.

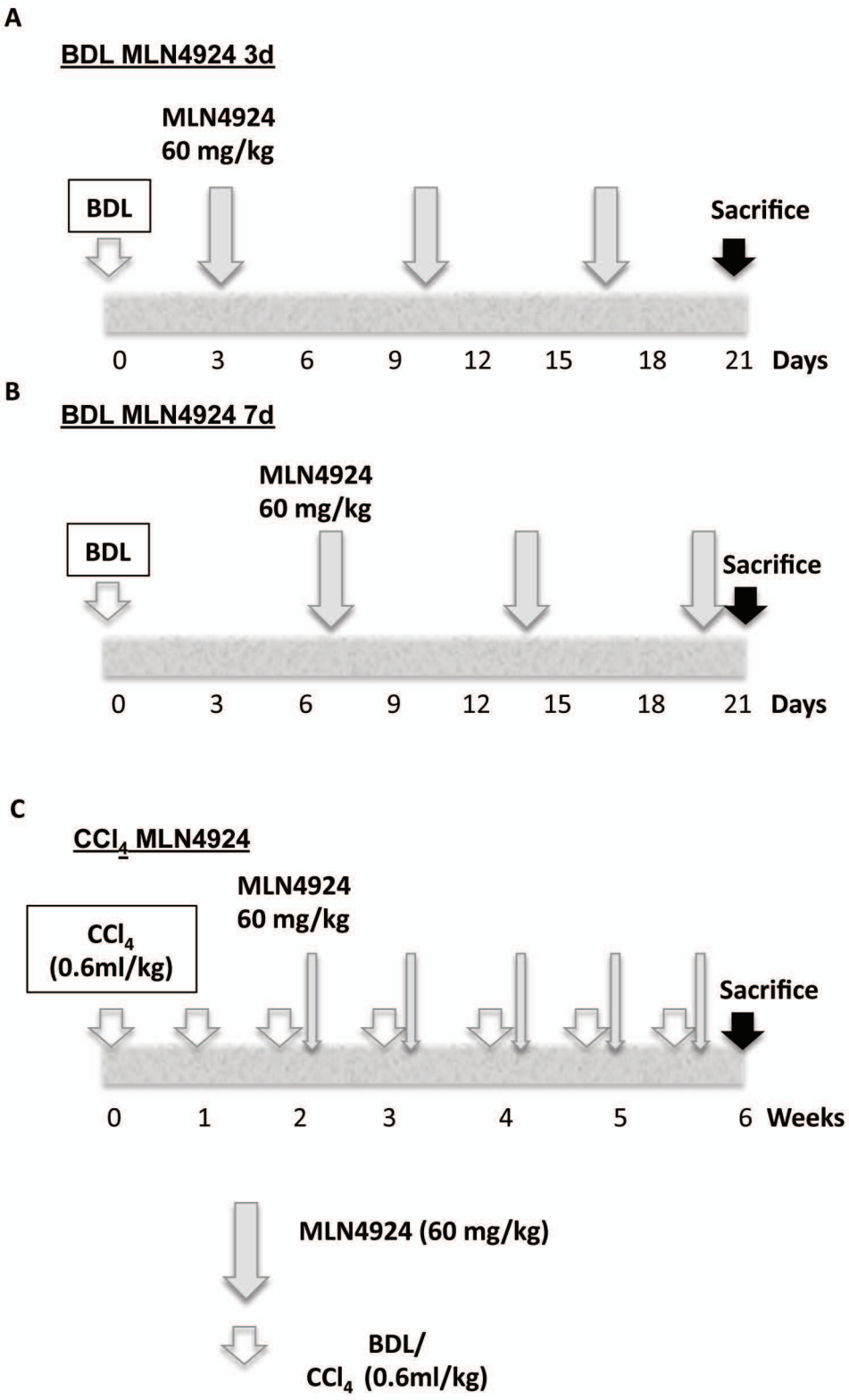
Supplemental Figure 5. Neddylation is not altered at mRNA levels in fibrosis. mRNA levels of *Nedd8* and *Nae1* in **(A)** clinical fibrosis, **(B)** Bile-duct ligation (BDL)-induced and **(C)** Carbon tetrachloride (CCl_4)-induced liver injury in mouse model. Healthy (n=13) and fibrotic liver patients (n=15); Control (n=5) and 21 days BDL (n=10); Control (n=5), 6 weeks CCl_4 (n=8).

Supplemental Figure 6. Individual immunofluorescence staining (Albumin (Alb), alpha smooth muscle actin (aSMA), Desmin and Nedd8) for the merged images shown in Figure 2.

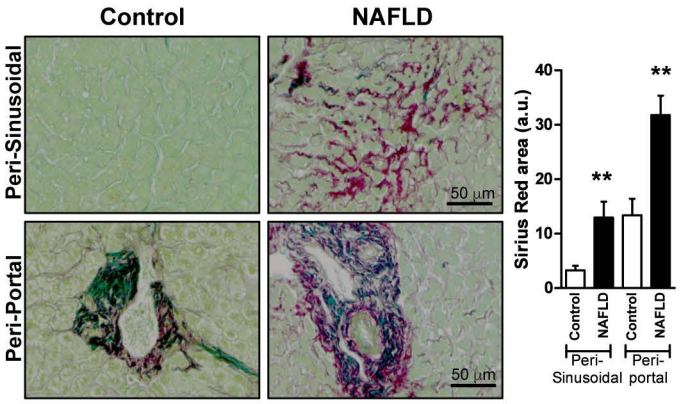
Supplemental Figure 7. Hepatic bile acids concentrations after 21 days of bile-duct ligation (BDL) in MLN4924 or vehicle treated rodents. Student's *t*-test was used to compare groups (n=5 control, n=10 BDL 3d, and n=5 BDL MLN3d). $p<0.05$ vs. control is indicated.

Supplemental Table 1. Characterization of liver fibrosis patients.

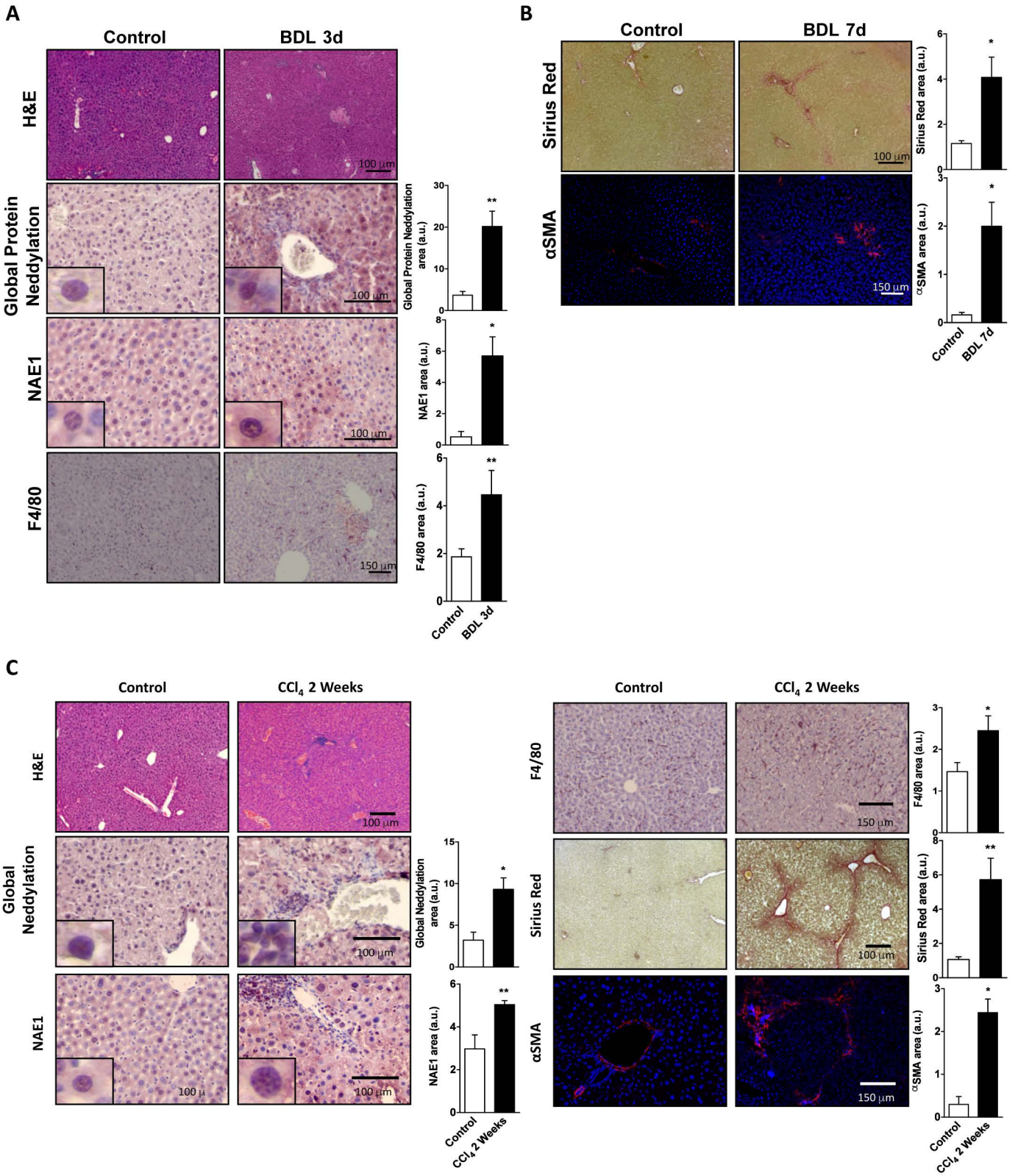
NUMBER OF PACIENTS	**N= 15**
GENDER (FEMALE/MALE)	9/6
MEAN AGE (YEARS)	44 (27-65)
MEAN SERUM TRANSAMINASES	
AST (U/L)	38 (18-87)
ALT (U/L)	38 (14-82)
GGT (U/L)	47 (9-233)
MEAN ALBUMIN (G/DL)	4.2 (3.8-4.5)
MEAN PLATELET COUNT ($X10^9$/L)	263 (127-410)
METABOLIC SYNDROME (%)	47
FIBROSIS	
STAGE 1- PERISINUSOIDAL OR PERIPORTAL	4
STAGE 2- PERISINUSOIDAL AND PORTAL/PERIPORTAL	9
STAGE 3-BRIDGING FIBROSIS	2



Zubiete-Franco *et al.*
Supplemental Figure 1

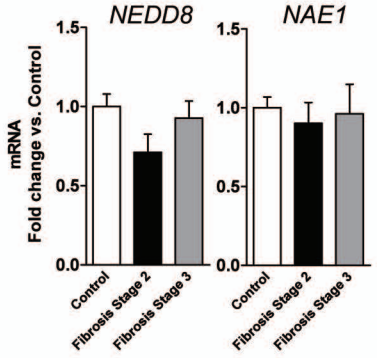


Zubiete-Franco *et al.*
Supplemental Figure 2

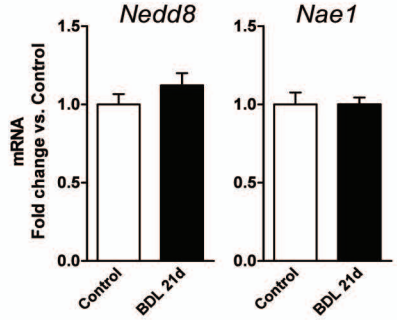


Zubiete-Franco *et al.*
Supplemental Figure 4

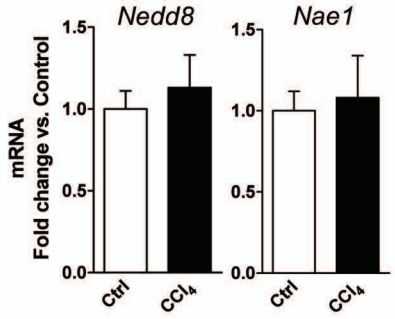
A



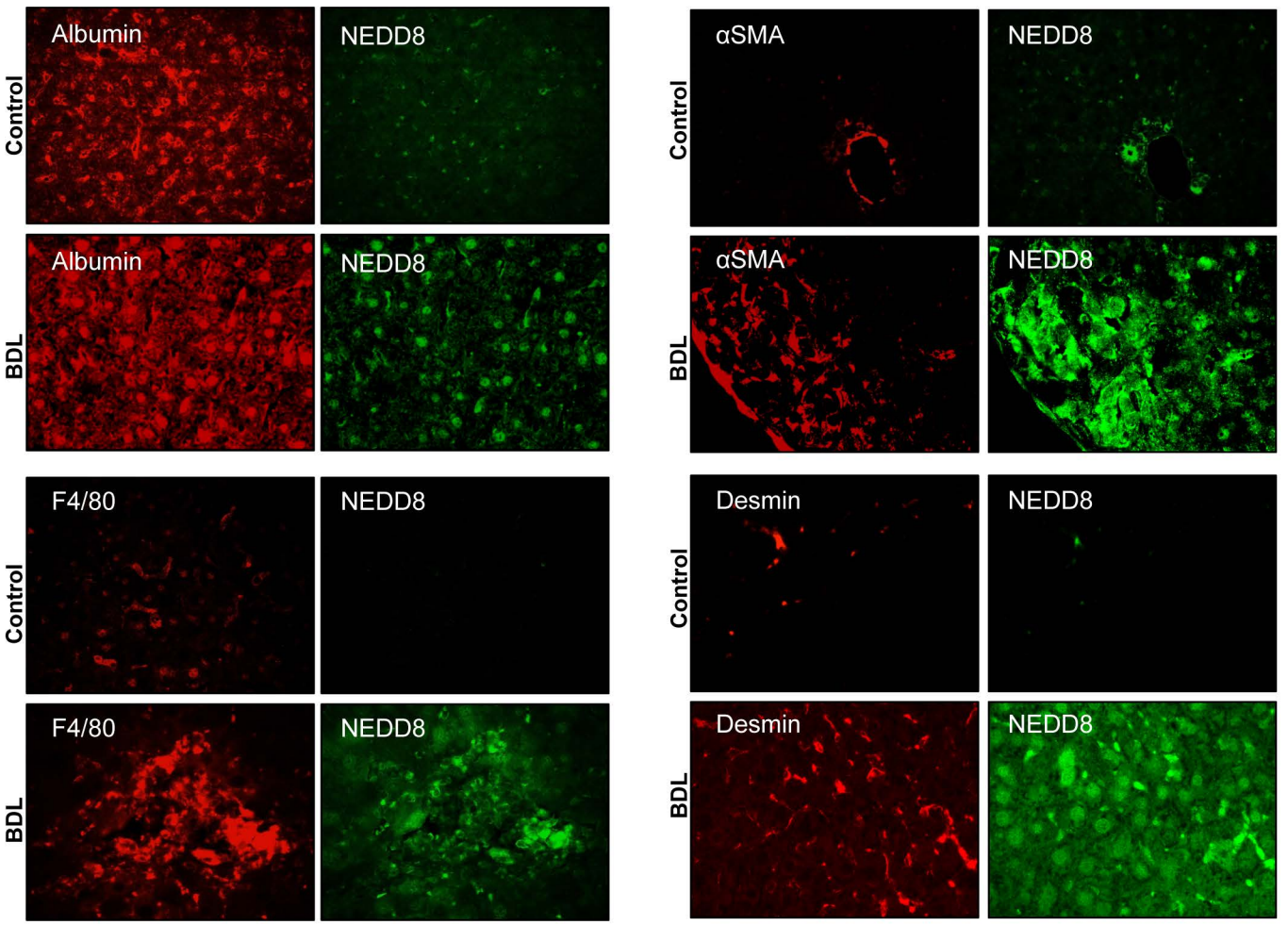
B



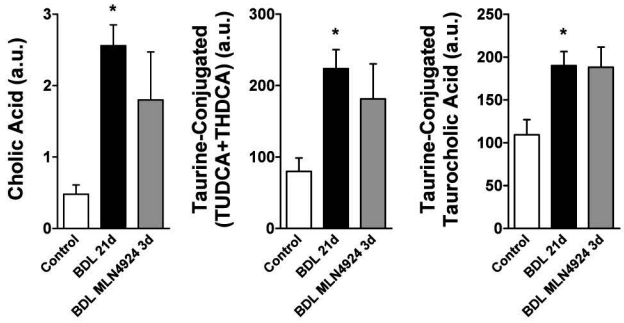
C



Zubiete-Franco *et al.*
Supplemental Figure 5



Zubiete-Franco *et al.*
Supplemental Figure 6



Zubiete-Franco *et al.*
Supplemental Figure 7