SUPPLEMENTARY MATERIAL

"Selective targeting of lysyl oxidase like 2 (LOXL2) suppresses hepatic fibrosis progression and accelerates its reversal" by Ikenaga et al.

Supplementary Material and Methods

Thioacetamide (TAA)-induced model of liver fibrosis progression and reversal

Eight-week-old male C57Bl/6J mice (Jackson Labs, Bar Harbor, ME) received repeated TAA (50-400mg/kg TAA, i/p) injections 3 times/week for 6-12 weeks. In fibrosis reversal studies, TAA administration was ceased after 6 weeks and animals were allowed to recover for an additional 2-12 weeks.

Hepatic collagen content measurement

Hepatic collagen content was measured biochemically via the relative hydroxyproline content (μg/100mg liver) in 250-300 mg liver samples from 2 different lobes (representing >10% of the liver). Total hydroxyproline (mg/whole liver) was calculated based on individual liver weights and the corresponding relative hydroxyproline content

Fibrotic matrix stability assessment

Fibrotic matrix stability was assessed biochemically ex vivo by complete collagen fractionation through serial extractions. Five hundred mg of snap-frozen tissue from two liver lobes was homogenized and a series of overnight extractions (1:20, w:v) under increasingly harsh conditions were performed to obtain the following collagen-containing fractions: acetic acid-soluble (non-crosslinked collagens and pro-collagens); pepsin-soluble (fibrillar, mature and moderately cross-linked collagens); and insoluble (the remaining highly cross-linked collagens). Collagen content in each fraction was quantified via hydroxyproline determination after complete acidic hydrolysis and expressed as percentage of hydroxyproline recovered in all fractions.

Morphometry of fibrotic septa

Architecture of fibrotic septa was assessed by morphometric measurements using the Axiovision Rel 4.6 software (Carl Zeiss, Oberkochen, Germany) using a hemocytometer grid to precisely calibrate the scale. Briefly, pictures of complete fibrotic septa were taken at 200x magnification using a Zeiss Axioskop (Carl Zeiss) and thickness of septa and number of fibrils were recorded at outer boundaries of the middle third. At least 10 fibrotic septa randomly selected from a right and left liver lobes of 4 individual mice/group were assessed.

Immunohistochemistry, immunofluorescence and immunoblotting

Connective tissue stain (Sirius Red) and immunohistochemistry/immunofluorescence were performed in formalin-fixed paraffin-embedded liver sections or snap-frozen liver pieces, as described previously. Morphometric analysis was performed by counting positive cells in >7 random portal tracts of at least four

individual mice/group at 200x magnification. Detailed information about antibodies used is summarized in **Suppl. Table 1**.

Quantitative reverse transcriptional-polymerase chain reaction (qRT-PCR)

Relative mRNA levels were quantified in total liver RNA by RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) with the TaqMan and SYBR Green methodology. Sequences of primers and probes are summarized in **Suppl. Table 2**.

Isolation of primary hepatic stellate cells (HSC)

Primary HSC were isolated from 4-5week-old Mdr2-/- mice. Liver was perfused in situ through the portal vein with 0.5 mg/ml type IV collagenase and 0.25 mg/ml DNAse I in DMEM for 8 min, followed by post-digestion with 1 mg/ml Pronase E (MERCK, Darmstadt, Germany), 0.5 mg/ml collagenase and 0.25 mg/ml DNase I at 37°C for 20 min, and a two-step centrifugation through an 11 and 13% density gradient of Nycodenz (PROGEN, Heidelberg, Germany) at 1,400g for 20 min.

Isolation and culture of primary EpCAM(+) cells

Purification and culture of EpCAM(+) progenitor cells was performed from 4-5 weeks old Mdr2-/- as recently described[1]. The non-parenchymal cell pellet was immediately resuspended and separated into EpCAM(+) and EpCAM(-) cell fractions using EpCAM mAB-conjugated beads (MACS, Miltenyi Biotec, San Diego, CA). The ability of EpCAM(+) cells to spontaneously differentiate into both hepatocyte and cholangiocyte lineages (bipotency) was analyzed by colony formation assay as described by Dorrell et al[2], using a differentiation medium (10%FBS/DMEM, 10 ng/ml HGF, 10 ng/ml EGF, 100 nM dexamethasone and 1x ITS (Life Technologies, Carlsbad, CA). HSC supernatant was diluted with differentiation medium at 1:5 and treated with AB0023 (30μg/ml) or isotype control IgG (CTRL, 30μg/ml) starting at 48h after plating and changed every 3 days thereafter. Colonies were stained with K19+, HNF4α, documented with Celigo S (Nexcelom Bioscience, Lawrence, MA) and quantified with Image J (NIH, Betheseda, MD). Differentiation into functional hepatocyte was assessed by uptake of Dil-Ac-LDL (Alfa Aesar, Ward Hill, MA) and albumin secretion into supernatants via ELISA (Assaypro, St. Charles, MO).

Collagen gel contraction assay

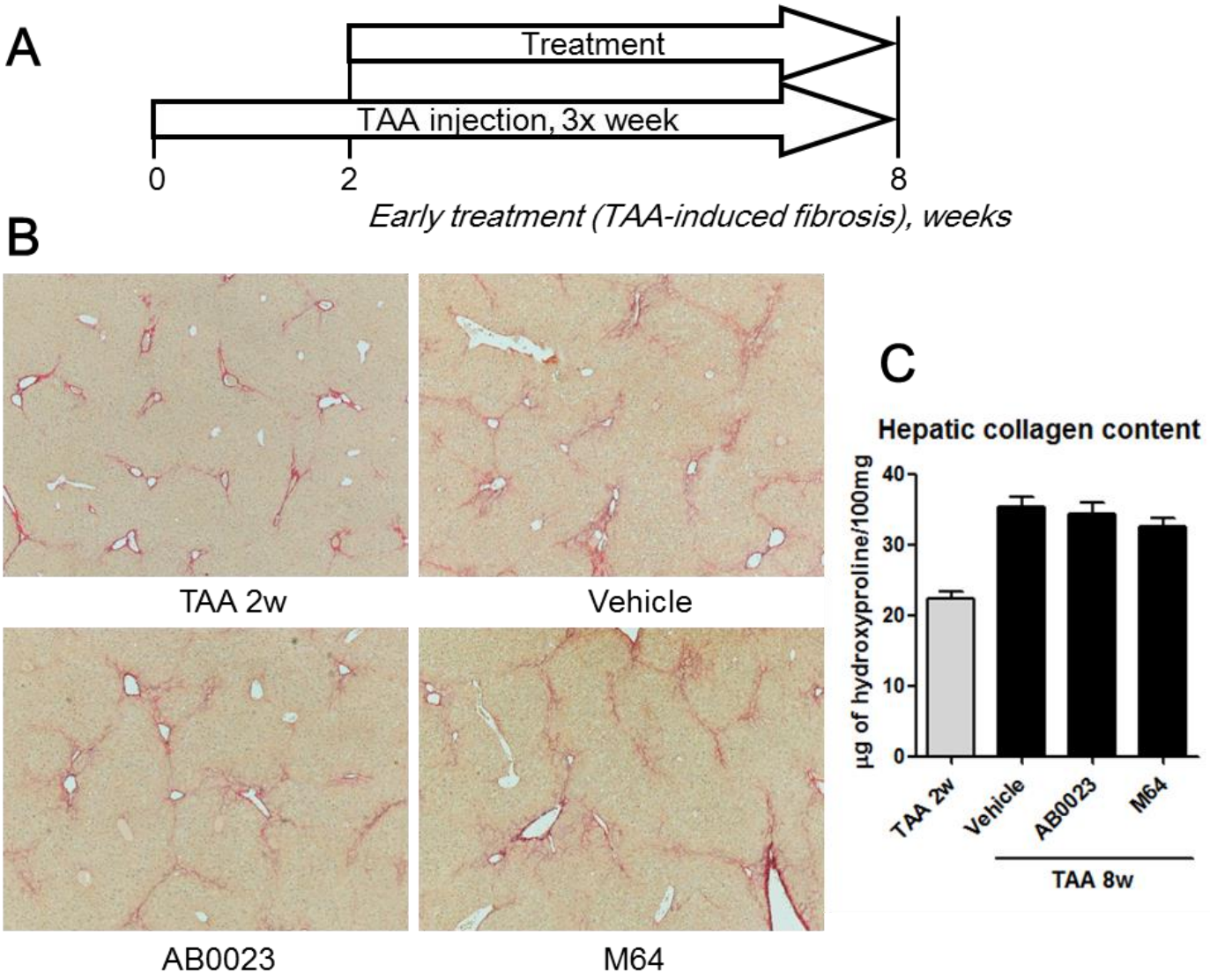
$1x10^4$ of cells were populated in 500 μl of 1 mg/ml collagen gels (BD Biosciences, Bedford MA) and placed on a 24-well dish in triplicate for each group. Once solidified, gels were detached from the walls of the dish and incubated in 10%FBS/DMEM or conditioned media from the immature ductal 603B cells in the presence of vehicle, isotype control antibody, AB0023 (10μg/ml) or M64 (10μg/ml). Media and treatments were changed every 3 days and collagen gel area was measured by Image J (NIH, Betheseda, MD).

Chromogenic In Situ Hybridization (CISH)

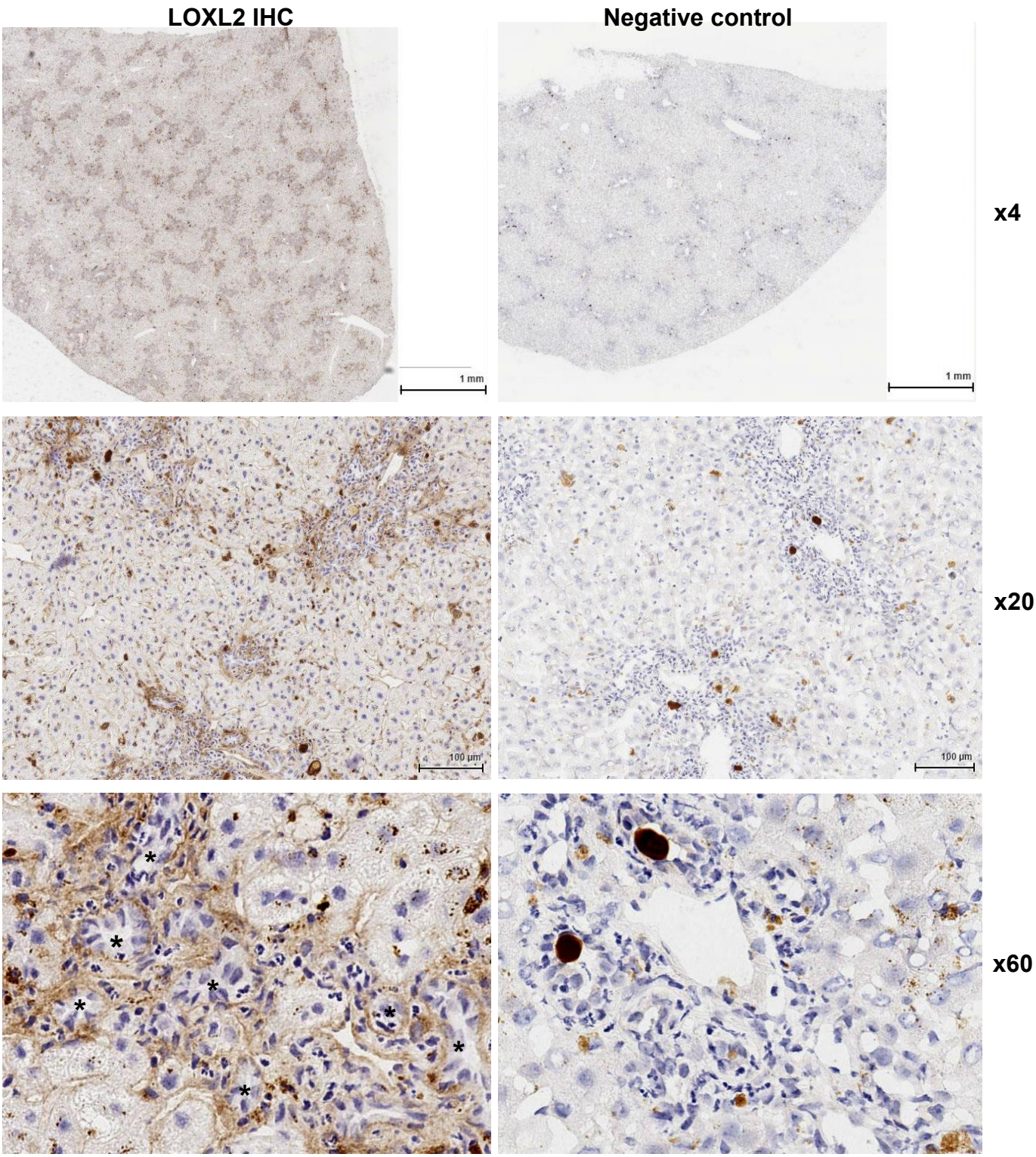
CISH was performed as decribed[3]. Briefly, paraffin-embedded liver sections (5 μm) were processed for RNA in situ detection using the RNAscope 2-plex Detection Kit (Chromogenic) at Advanced Cell Diagnostics (Newark,

CA). RNAscope probe used was LOXL2 (Cat. No. 311341; NM_002318.2, region 2303-3489). Samples were counterstained with Gill's Hematoxylin.

Supplementary Figures and Tables



Supplementary Figure 1. (A) Liver fibrosis was induced in C57Bl/6J mice with repeated TAA injections for 8 weeks (intermediate stage of fibrosis), and treated starting from week 2 with anti-LOXL2 mAb (AB0023, 30mg/kg), vehicle or anti-LOX mAb (M64, 30mg/kg) i/p once a week. (B) Connective tissue staining reveals that early intervention with AB0023 did not affect the extent of liver fibrosis as compared with placebo or M64 treatment (Sirius Red, 50x). (C) Hepatic collagen content as determined biochemically via hydroxyproline. (n=5-10/group). Data are expressed as means±SEM.



Supplementary Figure 2. Immunohistochemistry for LOXL2 in DDC-induced model of biliary fibrosis. LOXL2 immunostaining (left panel) is observed along fibrotic septa and around proliferating bile ducts (marked by asterisk) after 4 weeks of DDC feeding. Primary antibody was substituted with isotype IgG as negative controls (right panel). Note brownish deposits, often inside the ducts, which are unspecific artifacts due to DDC-feeding. Representative images are shown at increasing magnification as indicated.

Supplementary Table 1. Antibodies used for immunostaining and immunoblotting.

Application	Specificity	Catalog No.	Vendor	Dilution
IHC	α-SMA	ab5694	Abcam	1/200
	Collagen I	Ab21285	Abcam	1/1000
	Ki-67	M7249	Dako	1/50
	p-CK	Z0622	Dako	1/500
	TROMA-III(K19)	N/A	Hybridoma Bank, Iowa University	1/200
IF	LOXL2	ab96233	Abcam	1/100
	EpCAM	118202	Biolegend	1/1000
	TROMA-III(K19)	N/A	Hybridoma Bank, Iowa University	1/500
	HNF4α	Sc-8789	Santa Cruz	1/200
Western blot	α-SMA	M0851	Dako	1/1,000
	β-actin	Ab49900	Abcam	1/25,000

IHC, immunohistochemistry; IF, immunofluorescence; α-SMA, alpha-smooth muscle actin; p-CK, pan-cytokeratin; LOXL2, lysyl oxidase-like 2; HNF, hepatocyte nuclear factor

Supplementary Table 2. Primers and probes used in quantitative RT-PCR.

Target gene	5′-Primer	TaqMan probe	3′-Primer
β2MG	CTGATACATACGCCTGCAGAGTTAA	GACCGTCTACTGGGATCGAGACATGTG	ATGAATCTTCAGAGCATCATGAT
procollagen α1(I)	TCCGGCTCCTGCTCCTCTTA	TTCTTGGCCATGCGTCAGGAGGG	GTATGCAGCTGACTTCAGGGATGT
TGFβ1	AGAGGTCACCCGCGTGCTAA	ACCGCAACAACGCCATCTATGAGAAAACCA	TCCCGAATGTCTGACGTATTGA
TGFβ2	GTCCAGCCGGCGGAA	CGCTTTGGATGCTGCCTACTGCTTTAGAAAT	GCGAAGGCAGCAATTATCCT
integrin β6	GCAGAACGCTCTAAGGCCAA	TGGCAAACGGGAACCAATCCTCTGT	AAAGTGCTGGTGGAACCTCG
MMP-2	CCGAGGACTATGACCGGGATAA	TCTGCCCCGAGACCGCTATGTCCA	CTTGTTGCCCAGGAAAGTGAAG
MMP-3	GATGAACGATGGACAGAGGATG	TGGTACCAACCTATTCCTGGTTGCTGC	AGGGAGTGGCCAAGTTCATG
MMP-8	CAGGGAGAAGCAGACATCAACA	TGCTTTCGTCTCAAGAGACCATGGTGAC	GATTCCATTGGGTCCATCAAA
MMP-9	CAGGATAAACTGTATGGCTTCTG	CTACCCGAGTGGACGCGACCGT	GCCGAGTTGCCCCCA
MMP-12	GCAGTGCCCCAGAGGTCA	AGATCCTGTAAGTGAGGTACCGCTTCATCCA	TCACGCTTCATGTCCGGA
MMP-13	GGAAGACCCTCTTCTTCTCT	TCTGGTTAACATCATCATAACTCCACACGT	TCATAGACAGCATCTACTTTGTT
TIMP-1	TCCTCTTGTTGCTATCACTGATAGCTT	TTCTGCAACTCGGACCTGGTCATAAGG	CGCTGGTATAAGGTGGTCTCGTT
LOXL2	Life technologies, Assay Id: Rn01466080_m1		
LOX	Life technologies, Assay Id: Rn01491829_m1		
SOX9	Life technologies, Assay Id: Mm00448840_m1		
HNF4α	Life technologies, Assay Id: Mm01247712_m1		

Supplementary Table 3. Body, liver, spleen weights, and hepatic collagen content in C57Bl6 mice with delayed treatment of TAA-induced liver fibrosis. Liver fibrosis was pre-established with repeated TAA injections for 6 weeks (TAA 6w), followed by 6 weeks of treatment with anti-LOXL2 antibody (AB0023, 30mg/kg), vehicle or anti-LOX antibody (M64, 30mg/kg) in parallel with continued TAA injection (6 to 12 weeks on TAA, see Fig. 1B). Data are expressed as means±SEM. *, $p<0.05$ compared to the vehicle-treated group, ANOVA with Dunnett's post-test. CTRL, healthy controls.

Group	Body weight, g	Relative liver weight, %	Relative spleen weight, %	Relative HYP, µg/100mg	Total HYP, µg/liver
CTRL, n=5	28.68 ± 1.09	5.27 ± 0.21	0.31 ± 0.01	21.48 ± 0.77	326.8 ± 27.72
TAA 6w, n=13	21.18 ± 0.43	6.34 ± 0.06	0.42 ± 0.01	40.91 ± 0.94	550.5 ± 19.93
Vehicle, n=13	23.35 ± 0.16	7.39 ± 0.08	0.48 ± 0.01	48.27 ± 0.98	831.9 ± 15.63
AB0023, n=14	22.74 ± 0.41	7.18 ± 0.10	0.46 ± 0.01	47.35 ± 0.75	772.0 ± 18.55*
M64, n=15	22.99 ± 0.44	7.15 ± 0.11	0.47 ± 0.01	50.22 ± 0.81	821.9 ± 11.30

Supplementary Table 4. Body, liver, spleen weights, and hepatic collagen content in C57Bl6 mice with early treatment of TAA-induced liver fibrosis. After repeated TAA injections for 2 weeks (TAA 2w), a 6 week treatment with anti-LOXL2 mAb (AB0023, 30mg/kg), vehicle or anti-LOX mAb (M64, 30mg/kg) was conducted in parallel with continued TAA injections (weeks 3-8 on TAA, see Suppl. Fig.1A). Data are expressed as means±SEM. *, $p<0.05$ compared to the vehicle-treated group, ANOVA with Dunnett's post-test.

Group	Body weight, g	Relative liver weight, %	Relative spleen weight, %	Relative HYP, µg/100mg	Total HYP, µg/liver
TAA 2w, n=5	22.64 ± 0.62	5.01 ± 0.09	0.35 ± 0.01	22.43 ± 0.87	257.0 ± 11.86
Vehicle, n=10	24.59 ± 0.73	6.44 ± 0.08	0.42 ± 0.01	35.27 ± 1.47	559.5 ± 32.67
AB0023, n=9	24.68 ± 0.42	6.48 ± 0.09	0.43 ± 0.01	34.31 ± 1.62	546.5 ± 21.78
M64, n=8	23.84 ± 0.47	6.30 ± 0.09	0.43 ± 0.02	32.44 ± 1.25	484.7 ± 14.12

Supplementary Table 5. Body, liver, spleen weights, and hepatic collagen content in C57Bl6 mice with pharmacological reversal of pre-established TAA-induced liver fibrosis. Fibrosis was induced by TAA injections for 6 weeks, followed by treatment with anti-LOXL2 mAb (AB0023, 30mg/kg), vehicle or anti-LOX mAb (M64, 30mg/kg) for 4 weeks or 12 weeks (see Fig. 2A). Data are expressed as means±SEM. *, $p<0.05$ compared to the vehicle-treated group, ANOVA with Dunnett's post-test. PF, peak of TAA-induced fibrosis.

Group		Body weight, g	Relative liver weight, %	Relative spleen weight, %	Relative HYP, µg/100mg	Total HYP, µg/liver
PF (TAA 6w), n=17		21.86 ± 0.36	6.36 ± 0.15	0.41 ± 0.01	43.96 ± 1.36	559.1 ± 19.30
4 weeks	Vehicle, n=16	27.82 ± 0.37	5.78 ± 0.14	0.40 ± 0.01	46.61 ± 1.01	718.4 ± 16.22
	AB0023, n=15	26.59 ± 0.69	5.99 ± 0.10	0.39 ± 0.01	41.37 ± 1.43*	632.6 ± 27.35*
	M64, n=15	26.55 ± 0.49	5.59 ± 0.19	0.42 ± 0.01	41.82 ± 0.92*	590.1 ± 14.39*
12 weeks	Vehicle, n=8	29.26 ± 0.73	5.21 ± 0.08	0.33 ± 0.01	39.54 ± 1.48	605.9 ± 37.81
	AB0023, n=8	31.44 ± 0.39*	5.06 ± 0.26	0.33 ± 0.01	40.79 ± 1.88	644.8 ± 41.39
	M64, n=8	29.85 ± 0.52	5.27 ± 0.06	0.34 ± 0.01	39.18 ± 1.64	618.1 ± 32.58

Supplementary Table 6. Body, liver, spleen weights, and hepatic collagen content in BALB/c.*Mdr2-/-* mice treated with anti-LOXL2 antibody (AB0023, 30mg/kg) and anti-LOX antibody (M64, 30mg/kg) for 4 weeks starting from 4 weeks of age (Mdr2-/- 4w). See also Fig. 3B. Data are expressed as means±SEM. *, $p<0.05$ compared to the vehicle-treated group, ANOVA with Dunnett's post-test. WT, wildtype (BALB/c) healthy controls.

Group	Body weight, g	Relative liver weight, %	Relative spleen weight, %	Relative HYP, µg/100mg	Total HYP, µg/liver
WT, n=10	22.45 ± 0.30	5.77 ± 0.15	0.38 ± 0.01	15.64 ± 0.83	200.3 ± 6.90
Mdr2-/- 4w, n=8	19.13 ± 0.59	7.13 ± 2.12	0.87 ± 0.17	26.40 ± 3.54	358.2 ± 65.52
CTRL, n=15	20.94 ± 0.96	8.74 ± 0.23	0.68 ± 0.03	70.29 ± 3.76	1248 ± 46.85
AB0023, n=10	22.73 ± 0.97	8.56 ± 0.12	0.76 ± 0.03	54.05 ± 2.67*	1045 ± 57.72*
M64, n=9	21.41 ± 0.59	8.74 ± 0.11	0.82 ± 0.06	71.30 ± 4.40	1326 ± 74.81

Supplementary Table 7. Body, liver, spleen weights, and hepatic collagen content in DDC-fed C57Bl6 mice treated with anti-LOXL2 antibody (AB0023, 30mg/kg), vehicle or anti-LOX antibody (M64, 30mg/kg) for 4 weeks (week 1-4 on DDC). Data are expressed as means±SEM. *, $p<0.05$ compared to vehicle-treated group, ANOVA with Dunnett's post-test. CTRL, healthy controls.

Group	Body weight, g	Relative liver weight, %	Relative spleen weight, %	Relative HYP, µg/100mg	Total HYP, µg/liver
CTRL, n=5	28.68 ± 1.09	5.36 ± 0.05	0.28 ± 0.02	19.62 ± 0.46	275.1 ± 15.78
DDC+Vehicle, n=10	27.82 ± 0.37	8.74 ± 0.57	0.43 ± 0.03	45.88 ± 1.98	678.0 ± 36.75
DDC+AB0023, n=9	26.59 ± 0.69	7.88 ± 0.40	0.45 ± 0.02	44.54 ± 2.39	522.5 ± 44.93*
DDC+M64, n=10	26.55 ± 0.49	8.20 ± 0.61	0.42 ± 0.02	43.43 ± 3.78	585.8 ± 43.22

References

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[3] Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MT, Pitot HC: Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell. Dev. Biol. 1986, 22:201–211.