Eupalinolide B induces ferroptosis in activated HSCs to attenuate hepatic fibrosis via targeting peroxiredoxin 1

Supplementary Material

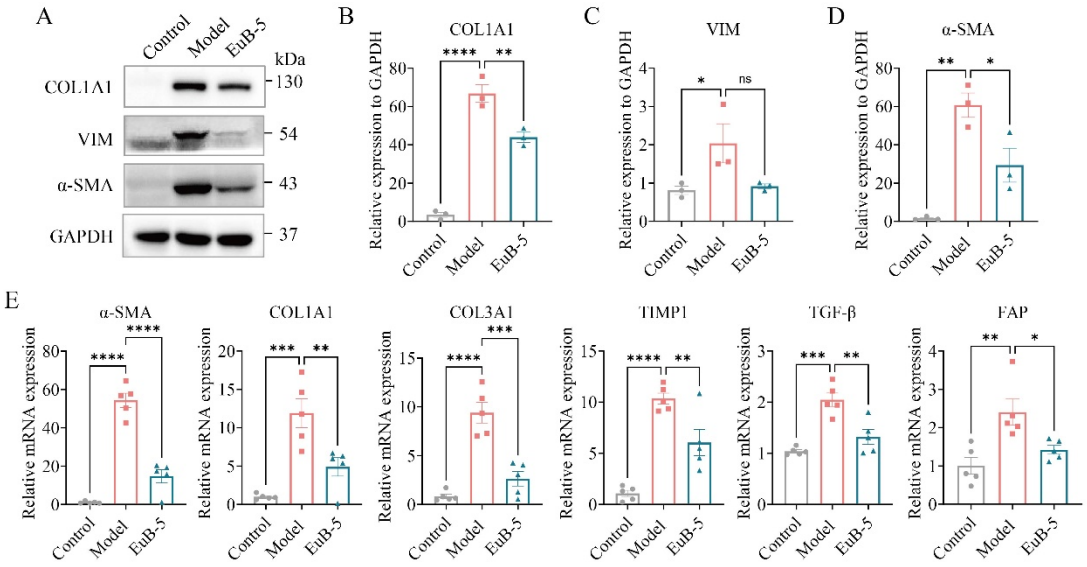


Figure S1. EuB markedly reduced expression levels of fibrosis-associated markers in liver tissues. (A-D) Western blotting assay of the levels of COL1A1, VIM and a-SMA proteins in mice (n = 3). (E) The mRNA expression levels of fibrosis-associated markers (n = 5). Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs*. Model.

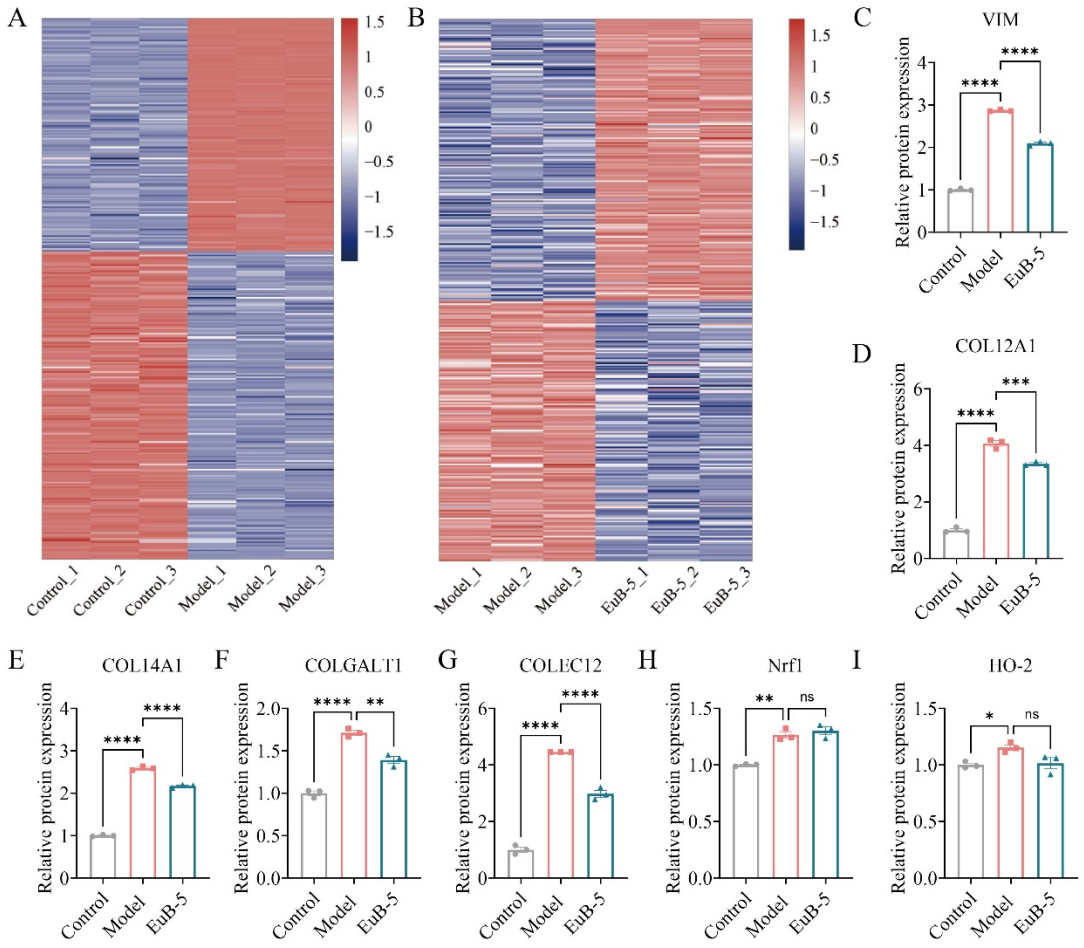


Figure S2. (A-B) Heatmap of hierarchical clustering analysis for the overlapping differential proteins. (C-G) Protein expression levels of hepatic fibrosis-related markers by proteomic analysis (n = 3). (H-I) Protein expression levels of oxidative stress-related molecules (n = 3). Data are presented as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs.* Model group. COL12A1, Collagen type XII alpha 1; COL14A1, Collagen type XIV alpha 1; COLGALT1, Collagen beta (1-O) galactosyltransferase 1; COLEC12, Collectin subfamily member 12; Nrf1, Nuclear factor erythroid 2-related factor 1; HO-2, Heme oxygenase 2.

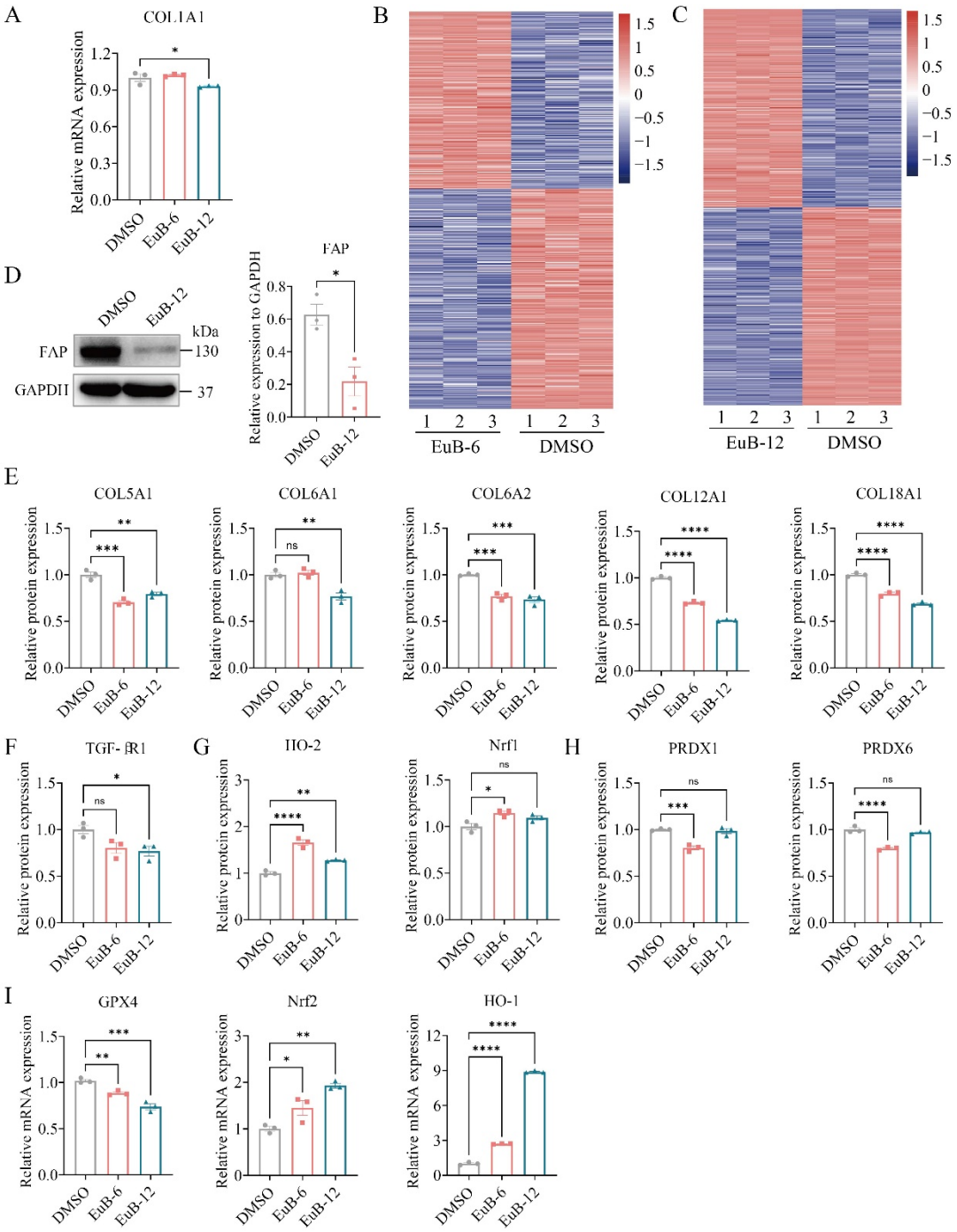
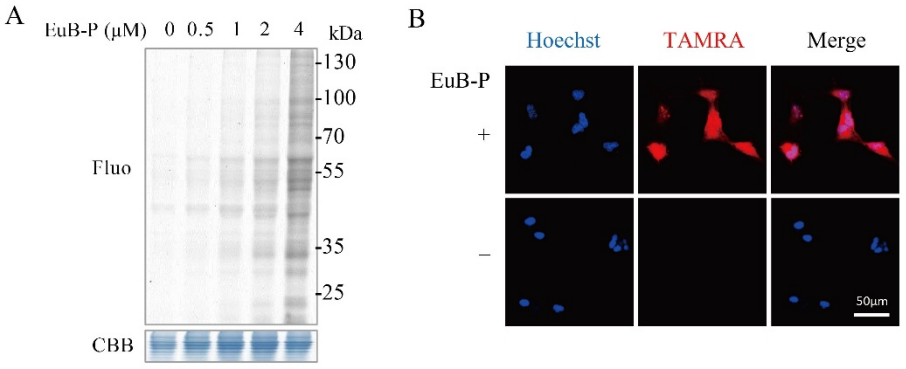


Figure S3. EuB suppresses pro-fibrotic factor expression via activating ROS signaling and inducing ferroptosis in activated HSCs. (A) The mRNA expression levels of f COL1A1 in EuB-treated LX2 cell line (n = 3). (B-C) Heatmap of hierarchical clustering analysis for the overlapping DEPs. (D) Western blotting of FAP protein in EuB-treated activated HSCs (n = 3). (E-F) Protein expression levels of hepatic fibrosis-related markers by proteomic analysis (n = 3). (G-H) Protein expression levels of ferroptosis- and oxidative stress-related molecules (n = 3). (I) The mRNA expression levels of ferroptosis- and oxidative stress-associated markers in EuB-treated activated-HSCs (n = 3). Mean

± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs*. DMSO. COL5A1, Collagen type V alpha 1; COL6A1, Collagen type VI alpha 1; COL6A2, Collagen type VI alpha 2; COL18A1, Collagen type XVIII alpha 1; TGF-βR1, Transforming growth factor-beta receptor 1; PRDX6, Peroxiredoxin 6; Nrf2, Nuclear factor erythroid 2-related factor 2.



C

Protein	TMT tag ratio	Coverage [%]	# Peptides	# PSMs	Sum PEP Score
RPL13A	2.59	46	12	24	39.09
PRDX1	2.90	31	7	16	24.27
RAB10	2.61	26	5	14	27.80
GPX8	6.83	23	5	11	19.00
RAB5C	3.22	22	4	8	18.43
REEP5	6.69	21	6	25	41.67
RABAC1	3.66	20	3	5	8.15
ARF4	2.51	20	4	12	20.44
PGRMC1	2.57	19	4	7	9.10
JAGN1	2.61	13	2	6	9.44
BAX	3.68	12	2	4	8.19
BID	3.17	12	2	5	9.91
THEM6	2.61	9	2	5	9.08
SEC22B	2.53	9	2	4	12.18
CBX3	3.89	8	1	4	9.96
MXRA7	3.07	5	1	3	3.01

Figure S4. (A) EuB-P labeling of potential target proteins in LX2 cells. (B) Imaging of EuB-P in LX2 cells to visualize its intracellular distribution. Scale bar = 50 μm. (C) Proteomic quantification data for potential target proteins with a TMT tag ratio > 2.5.

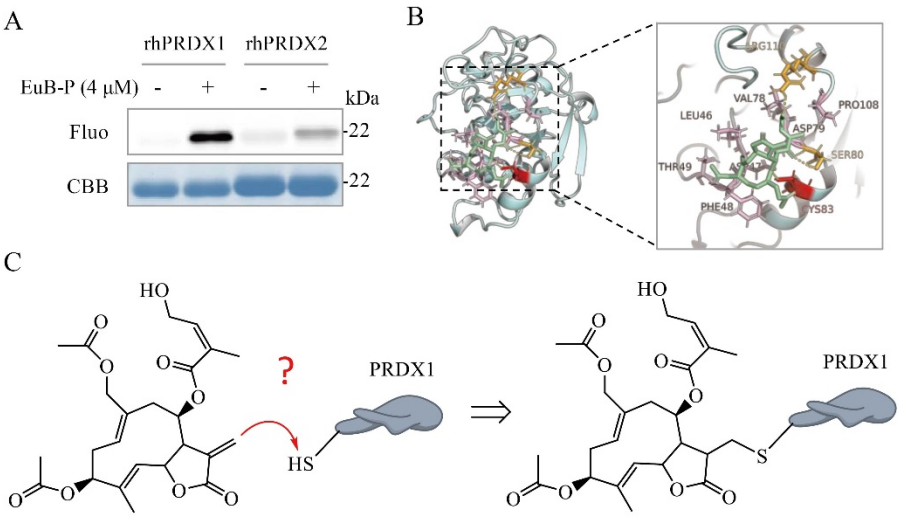


Figure S5. EuB binds directly to the cysteine residues of PRDX1. (A) The labeling of rhPRDX1 and rhPRDX2 by EuB-P. (B) Molecular docking model illustrating the direct interaction between EuB and the Cys83 of PRDX1; pale cyan represents the protein, pale green represents the ligand, light pink represents near-ligand residues, bright orange represents binding sites, and red represents Cys residues. (C) A possible interaction diagram between EuB and PRDX1.

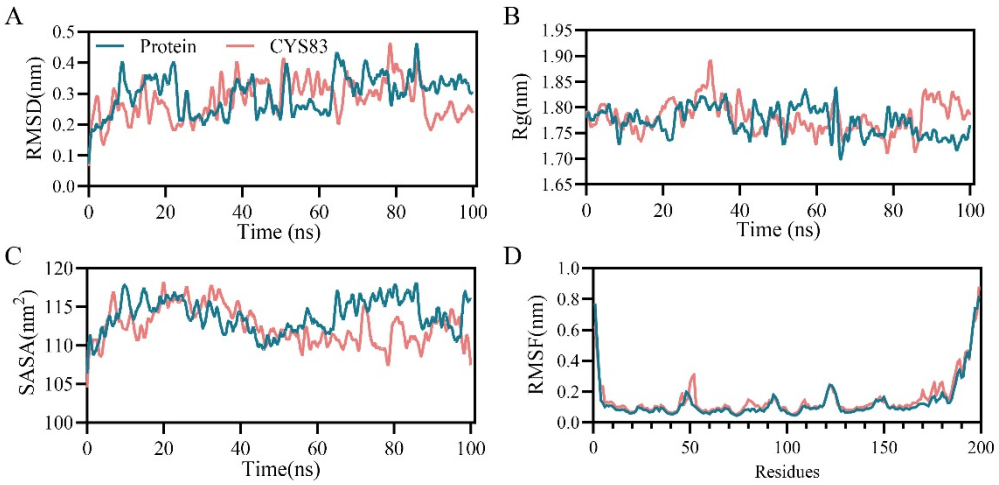


Figure 6S. Molecular docking simulation of EuB binding to PRDX1. (A) RMSD Curve of complex (red line) and ligand (green line). (B) Rg curve of EuB-PRDX1 complex and ligand. (C) SASA curve of EuB-PRDX1 complex and ligand. (D) RMSF curve of EuB-PRDX1 complex and ligand.

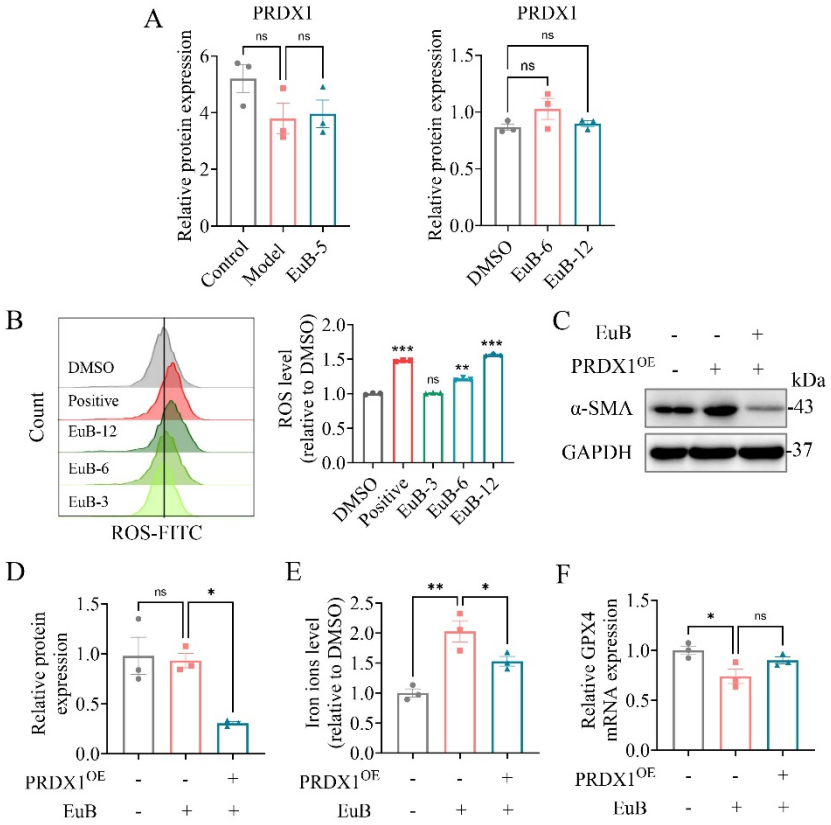


Figure S7. EuB induces ROS accumulation and inhibits HSC activation. (A) Quantitative analysis of relative PRDX1 protein expression (n = 3). (B) Flow cytometry analysis of intracellular ROS levels in activated HSCs treated with EuB, quantitative analysis of relative ROS levels (n = 3). (C) Western blot detection of α-SMA in HSCs under different treatments: EuB alone, or EuB combined with PRDX1 overexpression ($PRDX1^{OE}$). (D) Quantitative analysis of relative α-SMA protein expression (n = 3). (E-F) Effects of $PRDX1^{OE}$ on EuB-mediated changes in cellular iron ions level and GPX4 expression in activated HSCs (n = 3). Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs.* DMSO or EuB group.

Table S1. All primers used for qPCR.

H-ACTA2 Forward	CTATGAGGGCTATGCCTTGCC
H-ACTA2 Reverse	GCTCAGCAGTAGTAACGAAGGA
H-COL1A1 Forward	GTGCGATGACGTGATCTGTGA
H-COL1A1 Reverse	CGGTGGTTTCTTGGTCGGT
H-COL3A1 Forward	TTGAAGGAGGATGTTCCCATCT
*H-COL3A1*Reverse	ACAGACACATATTTGGCATGGTT
H-TGF-β Forward	CTAATGGTGGAAACCCACAACG
H-TGF-β Reverse	TATCGCCAGGAATTGTTGCTG
H-MMP9 Forward	AGACCTGGGCAGATTCCAAAC
H-MMP9 Reverse	CGGCAAGTCTTCCGAGTAGT
H-TIMP1 Forward	ACCACCTTATACCAGCGTTATGA
H-TIMP1 Reverse	GGTGTAGACGAACCGGATGTC
H-GPX4 Forward	GAGGCAAGACCGAAGTAAACTAC
H-GPX4 Reverse	CCGAACTGGTTACACGGGAA
H-HMOX1 Forward	AAGACTGCGTTCCTGCTCAAC
H-HMOX1 Reverse	AAAGCCCTACAGCAACTGTCG
H-NRF2 Forward	TCAGCGACGGAAAGAGTATGA
H-NRF2 Reverse	CCACTGGTTTCTGACTGGATGT
H-Actin Forward	ATCACCATTGGCAATGAGCG
H-Actin Reverse	TTGAAGGTAGTTTCGTGGAT
M-ACTA2 Forward	GTCCCAGACATCAGGGAGTAA
M-ACTA2 Reverse	TCGGATACTTCAGCGTCAGGA
M-COL1A1 Forward	TGGTCCCAAAGGTTCTCCTGGT
M-COL1A1 Reverse	TTAGGTCCAGGGAATCCCATCACA
M-COL3A1 Forward	CTGTAACATGGAAACTGGGGAAA
M-COL3A1 Reverse	CCATAGCTGAACTGAAAACCACC
M-MMP9 Forward	TGACTACGATAAGGACGGCAAA

M-MMP9 Reverse	AGATGAACGGGAACACACAGG
M-TIMP1 Forward	GCAAAGAGCTTTCTCAAAGACC
M-TIMP1 Reverse	AGGGATAGATAAACAGGGAAACACT
M-GPX4 Forward	GATGGAGCCCATTCCTGAACC
M-GPX4 Reverse	CCCTGTACTTATCCAGGCAGA
M-HO-1 Forward	AAGCCGAGAATGCTGAGTTCA
M-HO-1 Reverse	GCCGTGTAGATATGGTACAAGGA
M-NRF2 Forward	CTTTAGTCAGCGACAGAAGGAC
M-NRF2 Reverse	AGGCATCTTGTTTGGGAATGTG
M-GPX4 Forward	CCCACTGTGGAAATGGATGA
M-GPX4 Reverse	ACGCAGCCGTTCTTATCAA
M-GAPDH Forward	TGTGTCCGTCGTGGATCTGA
M-GAPDH Reverse	CCTGCTTCACCACCTTCTTGA