EGF/STAT1 signals to maintain ECM1 expression in hepatic homeostasis are disrupted by IFNγ/NRF2 in chronic liver disease

Yujia Li[1], Frederik Link[1], Weiguo Fan[2], Zeribe C. Nwosu[1,3], Weronika Pioronska[1], Kerry Gould[1], Christoph Meyer[1], Ye Yao[1], Seddik Hammad[1], Rilu Feng[1,4], Hui Liu[5], Chen Shao[5], Bing Sun[6], Huiguo Ding[7], Roman Liebe[8], Matthias P. A. Ebert[1,9,10], Hong-Lei Weng[1], Peter ten Dijke[11], Steven Dooley[1*], and Sai Wang[1*]

[1] Department of Medicine II, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany; [2] Gastroenterology and Hepatology, Stanford University, 300 Pasteur Dr, and VA Palo Alto, CA, USA; [3] Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA; [4] Department of Endocrinology and Metabolism, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China; [5] Department of Pathology, Beijing You'an Hospital, Affiliated with Capital Medical University, Beijing, China; [6] State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences, Shanghai, China; [7] Department of Gastroenterology and Hepatology, Beijing You'an Hospital, Affiliated with Capital Medical University, Beijing, China; [8] Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke-University, Magdeburg, Germany; [9] Molecular Medicine Partnership Unit, European Molecular Biology Laboratory, Heidelberg, Germany; [10] DKFZ-Hector Cancer Institute at the University Medical Center, Mannheim, Germany; [11] Oncode Institute and Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, Netherlands.

Corresponding author:

Prof. Dr. rer. nat. Steven Dooley

Department of Medicine II, Section Molecular Hepatology, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University
Theodor-Kutzer-Ufer 1-3
68167 Mannheim, Germany
Email: steven.dooley@medma.uni-heidelberg.de
Tel: +49-621-383-3768
Dr. sc. hum. Sai Wang
Department of Medicine II, Section Molecular Hepatology, University Medical Center Mannheim, Medical Faculty Mannheim, Heidelberg University
Theodor-Kutzer-Ufer 1-3
68167 Mannheim, Germany
Email: sai.wang@medma.uni-heidelberg.de
Tel: +49-621-383-5595

Keywords: ECM1, EGF, STAT1, IFNγ, NRF2, fibrosis, chronic liver disease
Number of figures and tables: 7 figures
Conflict of interest: The authors have declared that there is no conflict of interest.

Financial support:
This study was supported by the Federal Ministry of Education and Research (BMBF) Programs [Liver Systems Medicine (LiSyM), grant number PTJ-031L0043 and LiSyM-HCC, grant number PTJ-031L0257A; to SD], Deutsche Forschungsgemeinschaft (DFG) [grant number DO 373/20-1 SD; to SW], [grant number DO 373/19-1 SD; to YL].

Author's contributions:
Conception, design, and hypothesis: Steven Dooley, Sai Wang
In vitro experiments: Yujia Li, Frederik Link, Weronika Pioronska, Ye Yao, Rilu Feng, Kerry Gould

In vivo experiments: Yujia Li, Christoph Meyer, Seddik Hammad

Bioinformatics analysis: Yujia Li, Sai Wang, Zeribe C. Nwosu

Patients sampling and pathological experiments: Yujia Li, Frederik Link, Hong-Lei Weng, Hui Liu, Chen Shao

Drafting the article: Yujia Li, Sai Wang, Frederik Link, and Steven Dooley

Data discussion, reviewing and editing the article critically: Yujia Li, Sai Wang, and Steven Dooley, Frederik Link, Peter ten Dijke, Zeribe C. Nwosu, Roman Liebe, Weiguo Fan, Christoph Meyer, Hong-Lei Weng, Matthias P. A. Ebert, Bing Sun, Huiguo Ding

Acknowledgement:

We acknowledge the support of the LIMa Live Cell Imaging at Microscopy Core Facility Platform Mannheim (CFPM), the ENCODE Consortium and the Michael Snyder laboratory (Stanford) for generating the dataset.

Abbreviations:

ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1
BDL	Bile duct ligation
CCl_4	Carbon tetrachloride
ChIP	Chromatin immunoprecipitation
CLD	Chronic liver disease
CYP1B1	cytochrome P450 family 1 subfamily B member 1
ECM1	Extracellular matrix protein 1
Ecm1-KO	*Ecm1* knockout
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GEO	Gene Expression Omnibus
H_2O_2	Hydrogen peroxide
HBV	Hepatitis-B-virus
HCC	Hepatocellular carcinoma
HPHs	Human primary hepatocytes
HSCs	Hepatic stellate cells

IFNγ	Interferon gamma
i.p.	Intraperitoneal
KEAP1	Kelch-like ECH-associated protein 1
LTGF-β	Latent TGF-β
MELD	Model for End-Stage Liver Disease
MMP	Matrix metalloproteinase
MPHs	Mouse primary hepatocytes
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NQO1	NAD(P)H:quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
OPZ	Oltipraz
qRT-PCR	Quantitative real-time PCR
ROS	Reactive oxygen species
siRNA	Small interfering RNA
S727	Serine727
TSP-1	Thrombospondin 1
TSS	Transcription start site
Y701	Tyrosine701

Abstract

Objective In healthy livers, latent transforming growth factor-β (LTGF-β) is stored in the extracellular matrix and kept quiescent by extracellular matrix protein 1 (ECM1). Upon damage, ECM1 is downregulated in hepatocytes, facilitating LTGF-β activation and hepatic fibrosis. This study investigates the underlying molecular mechanisms by which ECM1 expression in the liver is controlled under patho-physiological conditions.

Design *In silico* promoter analysis was used to predict pathways that regulate *Ecm1* transcription. Functional assays were performed in AML12 cells, mouse and human primary hepatocytes (MPHs, HPHs), and in liver tissue of mice and patients.

Results In healthy liver, EGF/Egfr signaling maintains Ecm1 expression through phosphorylation of Stat1 at S727, which promotes its binding to the *Ecm1* gene promoter to enhance gene transcription. During liver inflammation, accumulated IFNγ interferes with EGF signaling by downregulating Egfr expression and by disrupting EGF/Egfr/Stat1-mediated *Ecm1* promoter binding. Mechanistically, IFNγ induces Stat1 phosphorylation at position Y701, which is competing with the ability of p-Stat1 S727 to bind to the *Ecm1* gene promoter. Additionally, IFNγ induces Nrf2 nuclear translocation and repressive binding to the *Ecm1* gene promoter, thus further reducing Ecm1 expression. Importantly, patients suffering from liver cirrhosis who lack nuclear NRF2 expression consistently maintain higher levels of ECM1, inferring a better prognosis.

Conclusion ECM1 expression in healthy livers is controlled by EGF/EGFR/STAT1 signaling. Upon liver injury, ECM1 expression is repressed by accumulating IFNγ/NRF2, leading to increased LTGF-β activation and the onset of hepatic fibrosis.

236 words

Introduction

Extracellular matrix protein 1 (ECM1) is a secreted 85-kDa glycoprotein [1] primarily found in the epidermis and dermis [2], where it functions to maintain the integrity and homeostasis of the skin [3], as well as regulating endochondral bone formation [4] and promoting the proliferation of endothelial cells [5]. Dysfunction of ECM1 is associated with two distinct skin disorders [6], namely, lipoid proteinosis, a rare autosomal-recessive genodermatosis and lichen sclerosus, a common and acquired inflammatory disease, primarily affecting the skin. ECM1 is also involved in the development of cancer and its levels are elevated in most malignant epithelial and metastatic tumours [7,8], including breast cancer, colon cancer, ovarian cancer, and melanoma. In contrast, we found that ECM1 is essential to maintain liver architecture and function, and its loss is associated with the progression of liver disease [9-12].

In the liver, ECM1 is produced mainly by hepatocytes and quiescent hepatic stellate cells (HSCs) [9,13]. We identified ECM1 as gatekeeper of liver homeostasis by interacting with and inhibiting αv integrin-, thrombospondin 1 (TSP-1)-, a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1)-, and matrix metalloproteinase (MMP)-mediated latent TGF-β (LTGF-β) activation [9,14,15]. Upon liver damage or injury, ECM1 expression is significantly decreased, leading to LTGF-β activation, upregulated TGF-β signaling, HSC activation and fibrosis. *Ecm1*-KO mice display a severely disturbed liver phenotype and die between ages of 8 to 12 weeks [9]. Several tested mouse models of liver disease e.g., carbon tetrachloride (CCl_4)-, non-alcoholic steatohepatitis (NASH)-, and bile duct ligation (BDL)-associated liver injury consistently display Ecm1 downregulation, suggesting a common initiating mechanism of liver fibrogenesis [9]. A progressive loss of ECM1 with disease severity is also present in patients with liver fibrosis/cirrhosis, e.g., viral, due to an infection with the hepatitis-B virus (HBV), or alcohol related [9], and this also correlates with poorer prognosis in hepatocellular carcinoma (HCC) patients [11,12]. These data suggest that in contrast to most studied cancers, loss of ECM1 is required for disease progression and HCC development, indicating that in the liver, ECM1 plays a protective role. It is therefore

of great interest to explore mechanistic details of ECM1 expression regulation in the liver context.

Our research investigated the (pathophysiological down)-regulation of ECM1 expression in the liver, which may provide a basis for therapeutic manipulation, since a rescue of the ECM1 loss in liver is a promising treatment aim to improve the prognosis of chronic liver disease (CLD).

Results

EGF and HGF promote ECM1 expression in hepatocytes

As reported previously [9], hepatic ECM1 expression undergoes a significant downregulation in response to liver injury. To gain a comprehensive understanding of its modulation in liver pathology, we analyzed the *ECM1* mRNA expression in liver tissues obtained from patients with CLD sourced from the Gene Expression Omnibus (GEO) online database. It revealed a consistent reduction in *ECM1* expression across various cohorts of CLD patients, demonstrating a correlation with disease progression **(Figure 1A)**. Specifically, this decline was evident in patients with non-alcoholic fatty liver disease (NAFLD, n=15) and non-alcoholic steatohepatitis (NASH, n=16), in comparison to healthy individuals with normal weight (n=14) and obesity (n=12) [16]. Furthermore, *ECM1* expression was notably diminished in alcoholic cirrhosis patients (n=67) relative to those with non-severe alcoholic hepatitis (n=13) and alcoholic steatosis (n=6) [17]. In addition, the observed a significant decrease in *ECM1* mRNA expression extended to tumor tissues obtained from HBV-associated HCC patients (n=21) compared to non-neoplastic liver tissues [18] **(Figure 1A)**. This consistent pattern underscores the potential role of ECM1 in the pathogenesis and progression of various liver diseases.

To get a broader understanding of signaling pathways and transcription factors potentially implicated in the regulation of *Ecm1* transcription, we performed an *in silico* analysis of the *Ecm1* gene (NCBI Gene ID: 13601) promoter (-2000bp ~ +200bp relative to transcription start site (TSS)) using PROMO (https://alggen.lsi.upc.es/cgi-

bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), GeneCards (ECM1 Gene - GeneCards | ECM1 Protein | ECM1 Antibody) and JASPAR (https://jaspar.genereg.net/), and identified multiple binding sites for candidate transcription factors, including, among others, Fos, Jun, cMyc, NF-κB and Stat1 **(Suppl. Fig. 1)**. NF-κB is mainly regulated by inflammatory cell signaling induced by TNF-α and LPS. Fos, Jun, cMyc, and Stat1 are downstream transcription factors of growth factors, especially EGF and HGF [19,20]. Additionally, Stat1 is a major downstream mediator of the interferon family. As these signaling molecules all are having prominent roles in liver physiology and pathophysiology, they are promising candidates as regulators of hepatic Ecm1 expression.

Next, we tested previously described pathways for their impact on *Ecm1* mRNA expression levels. TNF-α or LPS treatment did not impact on *Ecm1* mRNA expression in MPHs **(Suppl. Fig. 2)**; EGF and HGF treatment consistently induces Ecm1/ECM1 expression both on mRNA and protein levels in AML12, mouse primary hepatocytes (MPHs), and human primary hepatocytes (HPHs) **(Figure 1B, C; Suppl. Fig. 3A, B)**. Given that the ECM1 expression upregulation by EGF was substantially greater than that induced by HGF, our emphasis was directed towards investigating the regulation of EGF-induced ECM1.

EGF-Egfr signaling contributes to physiological Ecm1 expression in liver homeostasis

Given the crucial role of the Egfr in the EGF signaling pathway [21], we applied erlotinib, a selective Egfr inhibitor in EGF-treated AML12 cells and MPHs. qRT-PCR and Western blotting analyses confirm that erlotinib substantially inhibits EGF-induced Egfr phosphorylation and Ecm1 expression in both cellular systems **(Figure 1D; Suppl. Fig. 3C)**. In line, *Egfr* knockdown with siRNA as well suppresses Egfr expression/phosphorylation levels, and Ecm1 expression in MPHs upon treatment with EGF **(Figure 1E)**. To confirm our finding *in vivo*, we injected WT mice intraperitoneally (i.p.) with erlotinib (40mg/kg/day, for 2 days) and analysed the liver

tissue 48hrs later. qRT-PCR, Western blotting, and immunofluorescence analyses show a significant decrease in Ecm1 expression in the erlotinib-treated group compared to the control group **(Figure 1F-G)**.

These results suggest that EGF-Egfr maintains Ecm1 expression in healthy livers.

EGF induces Ecm1 expression through Stat1

To elucidate potential downstream components of the EGF-Egfr signaling pathway that regulate Ecm1 expression, we tested several transcription factors as predicted from the *in silico* promoter study, such as Fos, Jun, cMyc, and Stat1, together with its major downstream Erk signaling, by depleting their availability with siRNA interference experiments. Knockdown of Fos, Jun, or cMyc, Erk1 and Erk2 did not interfere with EGF-induced Ecm1 expression **(Suppl. Fig. 4),** whereas qRT-PCR and immunoblot data revealed that *Stat1* silencing by siRNA markedly suppresses EGF-induced Ecm1 expression **(Figure 2A; Suppl. Fig. 5A)**. This suggested Stat1 as a promising candidate. In line, 1hr EGF treatment of MPHs induces phosphorylation of Stat1 at the S727 phosphorylation site, which remains stable for at least 24hrs **(Figure 2B)**. As Stat1 is also an EGF-Egfr downstream target [22], its total expression and phosphorylation at S727 were measured in EGF-treated MPHs with or without siRNA targeting *Egfr*. Western blotting showed EGF-induced total expression and phosphorylation of Stat1 to be dependent on Egfr **(Figure 2C)**. Notably, Egfr inactivation also inhibited the phosphorylation of Stat1 at S727 site in the mice treated with erlotinib **(Figure 2D)**.

Next, we studied whether exogenous Stat1 overexpression is sufficient to upregulate Ecm1. Therefore, we transiently transfected AML12 cells with eGFP-Stat1-WT pcDNA 3.1 and eGFP-pcDNA 3.1 plasmids, respectively. Western blotting revealed that overexpression of Stat1-WT increased total Stat1 expression, Stat1 phosphorylation at position S727 and Ecm1 expression **(Figure 2E)**. These data consistently demonstrate that Stat1 is required for EGF-Egfr-mediated signal transduction towards Ecm1 expression in hepatocytes.

EGF induces Stat1 binding to the *Ecm1* promoter for its transcriptional activation

To further investigate the regulatory role of STAT1 with regard to the *ECM1* gene, its peak in binding to the promotor of *ECM1* was analyzed using publicly available STAT1 ChIP-seq data from the ENCODE website (cell line: GM12878, accession number: ENCFF011BMN), showing that there is a STAT1-binding site in the proximal promoter region of *ECM1* **(Figure 2F)**. We functionally confirmed the binding of Stat1 to the Stat1 predicted binding motif (ATGGCAGGAAA, -61 ~ -51bp to *Ecm1* TSS) located in the *Ecm1* gene promoter region between -157bp and +118bp in AML12 cells and MPHs with chromatin immunoprecipitation (ChIP) qRT-PCR experiments. Constitutive binding activity is significantly increased by EGF treatment, leading to a rise in Ecm1 expression in MPHs and AML12 cells. The PCR-amplified products of the ChIP PCR results were visualized on 2% agarose gels **(Figure 2G, H; Suppl. Fig. 5B, C)**.

These results indicate that EGF induces binding of Stat1 to the proximal promoter region (typically within 250bp upstream of the TSS) of the *Ecm1* gene, thereby activating its transcription.

IFNγ interferes with EGF-Egfr-mediated Ecm1 expression

IFNγ is a strong modulator of Stat1 signaling [23] and is induced in inflammation phases of CLD [24]. This made it a promising upstream candidate of Ecm1 expression regulation. Surprisingly, treatment of MPHs with IFNγ decreases Ecm1 expression on mRNA and protein level **(Figure 3A)**. Moreover, IFNγ abrogates EGF-induced Ecm1 expression, and in addition reduces Egfr expression, as determined by qRT-PCR and immunoblotting analyses **(Figure 3A)**. Although both, EGF and IFNγ activate the Stat1 pathway, the outcome in the hepatocytes is distinct between them. A more detailed biochemical analysis shows that EGF and IFNγ, both phosphorylate Stat1 at position S727. The difference is that IFNγ requires phosphorylation at position Y701 prior to the S727 phosphorylation event [25], whereas this is not the case for EGF, which is able via the Egfr to directly phosphorylate Stat1 at position S727 [26]. In detail, immunoblot

analyses of lysates from MPHs that we treated with EGF or IFNγ for different time intervals display that IFNγ induces phosphorylation of Stat1 at position Y701 within 5min, which is followed by phosphorylation of position S727 at between 10 to 30min. In contrast, EGF-mediated S727 phosphorylation of Stat1 arises rapidly, whereas phosphorylation at position Y701 does not take place **(Figure 3B)**. Furthermore, EGF transiently induces Egfr phosphorylation at position Y1068 within 5min, therewith initiating its downstream signaling towards Stat1. Moreover, IFNγ seems to inhibit total Egfr expression at an early time point and is not able to activate Egfr **(Figure 3B)**, which represents an additional mechanism to interfere with the distinct EGF downstream branch. ChIP qRT-PCR experiments indicate that IFNγ treatment does not increase Stat1 binding to the *Ecm1* gene promoter in MPHs **(Figure 3C)**. This suggests that unlike Stat1 phosphorylated at S727 alone, Stat1 phosphorylated at both sites, Y701 and S727 cannot bind to the *Ecm1* gene promoter to induce its transcription. Obviously, this mechanistic difference is critical for the downstream consequences of signal transduction in hepatocytes that are integrating at Ecm1 expression. Moreover, the addition of IFNγ significantly reduced EGF-induced binding of Stat1 to the *Ecm1* gene promoter as evidenced by the fold enrichment of ChIP assay **(Figure 3D)**.

Next, we administered IFNγ to mice via i.p. injection (400μg/kg/day, for 4 days). Expression of Ecm1 and Egfr are significantly reduced in the IFNγ-treated group compared to the control group, as determined by qRT-PCR and immunoblotting **(Figure 3E)**. The decrease in Ecm1 expression was further confirmed by immunofluorescence **(Figure 3F)**.

In conclusion, these results imply that IFNγ disrupts Ecm1 homeostasis maintained by EGF-Egfr signaling through inhibiting Egfr expression, and blocks binding of p-Stat1 S727 to the *Ecm1* gene promoter by inducing additional phosphorylation of Stat1 at the Y701 site.

IFNγ induces nuclear accumulation of Nrf2

We also analyzed whether there are additional transcription factors induced by IFNγ, besides Stat1, that may bind to the *Ecm1* gene promoter and negatively regulate its transcription. In the context of liver disease, IFNγ promotes inflammation and induces ROS [27,28]. In such setting, Nrf2 is activated in response to oxidative stress to provide a compensating anti-oxidative signal [29]. The *in silico* promoter analysis predicted binding sites for Nrf2 at positions -610 ~ -596bp (predicted binding motif antisense GGACATGACTCAGAA) of the *Ecm1* gene. Activation of Nrf2 can be documented by its nuclear accumulation [30]. We therefore treated EGF-incubated MPHs with IFNγ and prepared nuclear and cytoplasmic lysates for immunoblot analysis. In this experiment, the Nrf2 signal in the nuclear fraction is strongly induced upon IFNγ treatment **(Figure 4A)**. IFNγ dependent Nrf2 nuclear accumulation in MPHs was additionally confirmed in an independent experiment with immunofluorescence staining for Nrf2 **(Figure 4B)**. We next injected mice with IFNγ, and analyzed the liver tissue for mRNA expression of *Nox4* (Nadph oxidase) and *Nrf2*, which both are significantly elevated in the IFNγ treatment group as compared to controls **(Figure 4C)**. No differences for total Nrf2 protein are evident based on immunoblots **(Suppl. Fig. 6)**, however, immunofluorescence in tissue sections demonstrate nuclear accumulation of Nrf2 in the livers of IFNγ-treated mice **(Figure 4D)**, indicating that the Nrf2 pathway is directly activated in the hepatocytes of healthy mice by IFNγ injection through nuclear accumulation.

These results suggest that IFNγ may activate Nrf2 in hepatocytes. Given its potential to bind the *Ecm1* promoter, Nrf2 is a candidate regulator for its expression modulation.

Nrf2 inhibits Ecm1 expression through negative regulatory binding to its promoter

Given that Nrf2 is activated by ROS [29], we treated MPHs with hydrogen peroxide (H_2O_2) to directly induce oxidative stress. With this experiment, we demonstrate an alternative route for ROS-mediated Nrf2 activation with the same effect on Ecm1 mRNA and protein expression downregulation **(Figure 4E)**. To further confirm the

inhibitory effect of Nrf2 on Ecm1 expression, we used a specific agonist of Nrf2, Oltipraz (OPZ), and analyzed how it impacts Ecm1 expression. The data show that OPZ, as expected, promotes Nrf2 expression on mRNA and protein levels **(Figure 4F)** and induces its nuclear accumulation in MPHs **(Figure 4G)**. Importantly, Ecm1 expression is significantly downregulated upon OPZ treatment in MPHs **(Figure 4H)**. To functionally proof induced Nrf2 binding to the *Ecm1* gene promoter, we performed ChIP qRT-PCR for the region -867bp to -588bp of *Ecm1* gene promoter, comprising the above mentioned predicted Nrf2 binding sites, in OPZ-treated MPHs, showing that Nrf2 induced by OPZ in MPHs binds directly to the *Ecm1* gene promoter **(Figure 4I)**. Amplification products were visualized on a 2% agarose gel **(Figure 4J)**.

To summarize, ROS/IFNγ-induced Nrf2 is translocated into the nucleus, directly binds to the *Ecm1* gene promoter and negatively regulates its transcription, thereby suppressing Ecm1 expression as a response to cellular stress.

Oxidative stress abrogates EGF/Egfr-maintained Ecm1 expression

In settings of hepatic fibrosis or cirrhosis, there are phases where increased levels of EGF, IFNγ, and oxidative stress can be found in parallel [24,31,32]. To estimate the crosstalk of the different signals in hepatocytes, we treated MPHs with EGF and H_2O_2 or OPZ in combination. H_2O_2 inhibits EGF-induced Y1068 phosphorylation of Egfr, S727 phosphorylation of Stat1, total Stat1 and Ecm1 expression **(Figure 5A)**. Similarly, Nrf2 activation by OPZ incubation is blunting EGF-mediated Ecm1 expression **(Figure 5B)**. These results point to predominance of ROS/Nff2 over EGF in regulating Ecm1 expression in hepatocytes, which explains why Ecm1 is reduced in the microenvironment of CLD despite the presence of elevated EGF levels.

Next, we extracted mRNA expression data of the target genes involved in the Ecm1 expression regulation network from a mouse model of hyperactivated Nrf2. Specifically, liver-specific autophagy adaptor p62/Sqstm1 (p62)-KO mice were injected with/without adenovirus p62 and were analyzed 7 days after injection. In these mice, p62 expression sequesters inhibitory Kelch-like ECH-associated protein 1

(KEAP1) from Nrf2, which results in activation and subsequent nuclear accumulation of activated Nrf2 [33]. The GEO dataset GSE134188 [33] demonstrates that the pro-inflammatory signals (*Ifng*, *Ifngr*, *Tnf*, *Nfkb*, *Il1*, *Il6*, et.al), *Stat1*, and *Nrf2* (*Nfe2l2*) were notably increased, while *Egfr*, and *Ecm1* were decreased **(Figure 5C)**. In consequence, TGF-β signaling is hyperactivated, here documented by upregulated expression of *Tgfbr1*, *Ctgf*, and *Pmepa1*, which results in HSC activation (upregulated *Acta2*), and fibrogenesis, as evident from upregulated expression of fibrogenic genes (*Col1a1*, *Col1a2*, *Col3a1, Fsp1*) **(Figure 5D)** and genes related to extracellular matrix remodelling (*Timp1*, *Integrins*, *Adamts*, *Thbs*, *Mmps*) **(Figure 5E)**.

Activated IFNγ/NRF2 and decreased ECM1 expression aggravate the progression of fibrotic liver diseases

To further verify the relevance of reduced ECM1 expression and its link to activated NRF2 signaling in human patients with liver diseases and different stages of fibrosis, we performed IF or IHC staining for ECM1 and NRF2 in liver tissue sections from patients with early stages of liver fibrosis (F1-F2 fibrosis) and such with more severe disease stages of F3-F4 fibrosis. ECM1 expression decreased at the F3-F4 stage compared to F1-F2 patients, whereas nuclear NRF2 increased considerably with disease progression **(Figure 6A)**. A quantification of positive staining implicated a strong negative correlation (correlation coefficient -0.835) between the expression of NRF2 and ECM1 **(Figure 6B)**. In addition, a characteristic of poorer clinical data associated with high NRF2 accumulation was identified in HCC patients with cirrhotic tumor-surrounds. IHC analysis of non-tumorous tumor-surrounding tissue from HCC patients shows that 35 out of 76 (46%) patients without cirrhosis were NRF2-negative, while 22 of 41 patients (50%) with cirrhosis were negative for nuclear NRF2. It is worth noticing that the cirrhosis associated HCC patients with negative nuclear NRF2 staining present with lower Model for End-Stage Liver Disease (MELD) scores, as compared to those with nuclear NRF2 expression, thus having a better clinical outcome **(Figure 6C, D).**

This was further confirmed by analysing the GEO dataset GSE49541 [34,35] that comprises mRNA expression data of liver tissue from NAFLD patients with mild (n=40) or advanced (n=32) fibrosis. The mRNA expression of *IFNGR1*, *STAT1*, *NRF2* (*NFE2L2*) target genes, including antioxidant enzymes cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) and NAD(P)H:quinone oxidoreductase 1 (*NQO1*) are increased with disease severity, while mRNA levels of *ECM1* are decreased, leading to enhanced TGF-β signaling (*TGFBR1, SMAD2, CTGF, PMEPA1*) [15] which is supported by the presence of upregulated HSC activation marker *ACTA2* (encoding α-SMA) and fibrogenic gene expression, including collagens, such as *ACTN1*, *FSP1*, *COL1A1*, *COL1A2*, and *COL3A1* in the patients with more advanced fibrosis **(Figure 6E)**.

Taken together, IFNγ reduces ECM1 expression in patients with fibrotic liver diseases by (1) EGFR expression inhibition, (2) Y701 phosphorylation of STAT1, (3) NRF2 nuclear accumulation, potentially contributing to a worse clinical outcome.

Discussion

This study investigated how ECM1 expression is regulated under various pathophysiological conditions faced by a diseased liver. In previous research [9], our group confirmed that ECM1 plays a role as a gatekeeper of liver homeostasis and its downregulation is critical following liver damage and injury leading to massive and spontaneous activation of LTGF-β, subsequent activation of HSCs, onset and progression of liver fibrosis. However, the regulatory mechanisms underlying a sustained ECM1 expression in healthy liver and its loss in diseased livers remained largely unknown. Our study illustrated that (1) in healthy hepatocytes, the EGF/EGFR/STAT1 signaling pathway maintains ECM1 expression. But, (2) upon liver injury, IFNγ accumulates in the liver and abrogates the expression of ECM1 through blocking EGFR expression and (3) promoting NRF2 nuclear translocation thus causing the latter to bind to and negatively regulate the *Ecm1* gene promoter. These findings are summarized in the schematic presentation (**Figure 7)**.

EGF-induced ECM1 expression was not achieved through its canonical downstream signaling pathway **(Suppl. Fig. 4)**, which prompted us to explore the possibility of other transcription factors. It was proposed that a plethora of transcription factors may contribute to *Ecm1* transcription. Among these candidates, this study focused on STAT1, as firstly, loss-of-function experiments confirmed that STAT1 was required for *Ecm1* transcription in healthy hepatocytes; and secondly, *in vitro*, our ChIP assay demonstrated that STAT1 possessed binding sites on the promoter of the *Ecm1* gene. Given the importance of STAT1 in the network of signaling pathways regulated by both EGF and IFNγ, we assumed that they would share a similar mechanism for regulating ECM1 expression. Interestingly, EGF and IFNγ play opposing roles in regulating ECM1 in MPHs: EGF promotes, whereas IFNγ inhibits ECM1 expression. Why is the regulation of ECM1 expression so different despite the fact they both exploit STAT1 signaling? Based on three lines of evidence, we elucidated that IFNγ and EGF-induced STAT1 Ser727 phosphorylation are mechanistically independent and distinct, thus causing ECM1 expression to be affected in opposing directions: (1) Previous investigations have revealed that Tyr701 phosphorylation of STAT1 is necessary for IFNγ-induced STAT1 Ser727 phosphorylation [25,36]. However, during upon induction of cellular stress, Ser727 phosphorylation induced by the p38 mitogen-activated protein kinase (MAPK) becomes independent of Tyr701 phosphorylation indicating mechanistically independent events with various biological consequences [37,38]. (2) Similar to p38 MAPK, in JB6 Cl 41 cells, EGF induced Ser727 phosphorylation alone without causing Tyr701 phosphorylation [26], which is consistent with our results showing a stronger Ser727 phosphorylation and no obvious Tyr701 phosphorylation after stimulation of MPHs with EGF **(Figure 3B)**. (3) ChIP assays revealed that EGF treatment enhanced STAT1 binding to the *Ecm1* promoter in hepatocytes **(Figure 2G, H; Suppl. Fig. 5B, C)**, whereas IFNγ treatment did not increase the binding **(Figure 3C)**, but instead impeded the EGF-promoted binding **(Figure 3D)**. In addition, concerning the different impacts of IFNγ and EGF on ECM1 expression in hepatocytes,

we also need to realize the multipotent effects of STAT1 signaling, which may be regulated by EGF and involve essential physiological functions beyond IFNγ.

IFNγ, mainly produced by activated T cells and natural killer cells, is an anti-viral, pro-inflammatory and anti-tumorigenic cytokine, and has been reported to have anti-fibrotic properties in hepatic fibrosis associated with chronic HBV infections [39]. One trial showed that IFNγ treatment for nine months improves fibrosis scores in patients, possibly through antagonizing TGF-β signaling [39]. Later experiments revealed that the anti-TGF-β effect is caused by the upregulation of SMAD7 in activated HSCs via IFNγ-induced STAT1 [40]. Other studies demonstrated that IFNγ inhibits the proliferation and activation of HSCs in a STAT1-dependent manner, thereby inhibiting liver fibrosis [41,42]. Nonetheless, IFNγ also induces liver damage, mainly through triggering apoptosis of hepatocytes and hepatic inflammation. Growing evidence suggested that IFNγ treatment *in vitro* inhibits hepatocyte proliferation and inhibits liver regeneration [43], which in part also promotes the development of liver fibrosis. During Con A-induced hepatotoxicity, IFNγ overproduction may result in T cell-dependent liver injuries, such as hepatocellular apoptosis and necrosis [44,45]. Neutralization of IFNγ prevents STAT1 activation and hepatic damage induced by Con A [46,47]. In viral hepatitis, the hepatic lesions can be attributed to T cell-dependent cytotoxicity against virus-infected hepatocytes, where IFNγ, which is typically elevated in patients with chronic viral liver disease [48], plays a crucial role. When IFNγ was administered to HBV transgenic mice that did not develop hepatitis, hepatic lesions with lymphocytes infiltration were observed [49]. Moreover, in a methionine and choline-deficient high-fat (MCDHF) diet mouse model, unlike CCl_4 or dimethylnitrosamine (DMN) injections, or a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, IFNγ was even identified as a pro-fibrotic cytokine, and its deficiency suppressed the activation and infiltration of immune cells and the subsequent inflammatory response, further inhibiting the activation of HSCs, possibly leading to attenuation of liver fibrosis [50]. Such a phenomenon is not a direct result of switching off IFNγ signaling in HSCs. The role of IFNγ in liver damage appears complex and the verdict remains controversial in light of

these studies. In our study, we reported that one of the adverse effects of elevated hepatic IFNγ levels in CLD is the inhibition of ECM1 expression in hepatocytes, leading to the activation of LTGF-β and subsequent liver injury. As hepatocytes are the main producers of ECM1, injured livers/hepatocytes can further influence the steady expression of ECM1, thus forming a vicious circle and resulting in the progression of CLD.

As previously described, ECM1 expression is maintained by EGF-EGFR signaling in healthy livers; hence, the crosstalk between IFNγ and EGF/EGFR signaling prompted further investigations. Controversies remain regarding the modulatory role of IFNγ on EGFR signaling. Some studies reported that IFNγ transactivated EGFR [51,52] and upregulated EGFR expression [53], however others described that IFNγ inhibited EGFR phosphorylation [54] and its mRNA expression [55], possibly because different cell types show different responses even to the same mediators depending on the context [56]. We found that in primary hepatocytes, IFNγ suppresses total EGFR expression and further prevents EGF-induced EGFR activation, thereby disrupting EGF-EGFR-maintained ECM1 expression and thus promotes HSCs activation and the development of hepatic fibrosis, by partially impairing the anti-fibrotic functions of IFNγ.

NRF2, a transcription factor with predicted binding sites on the *Ecm1* gene promoter, is activated by inflammatory mediators such as ROS, fatty acids, nitric oxide and prostaglandins [57,58]. Studies have shown that activation of NRF2 provides a cytoprotective effect, reduces ROS and pro-inflammatory cytokines, thereby alleviating inflammation [57,59]. As in IFNγ-polarized macrophages, NRF2 activity is increased, the antioxidant response depends on NRF2, and knockdown of *Nrf2* decreases hydrogen peroxide clearance [60]. However, excessive activation of NRF2 and a consecutive nuclear accumulation has detrimental effects. In a *Keap1*-null mouse model, no newborns survived after three weeks, possibly due to starvation caused by a hyperkeratotic esophagus and cardia [61]. In addition, another group identified an association between NRF2 and hepatic steatosis, demonstrating that NRF2 in hepatocytes was responsible for regulating PPARγ, and a specific deletion of NRF2 in

hepatocytes reduced the expression of high-fat diet-induced PPARγ and lipid accumulation, thus impairing the progression of non-alcoholic fatty liver disease [62]. Moreover, pancreatic cancer patients with elevated NRF2 levels have a shorter median survival time [63]. In our study, we found that NRF2 is activated by the pro-inflammatory IFNγ and negatively regulates *Ecm1* gene transcription through binding to the *Ecm1* gene promoter, disrupting the healthy extracellular matrix and activating LTGF-β.

As shown in **Suppl. Fig. 3A**, HGF also induces ECM1 upregulation within hepatocytes. Despite the comparable nature of the signaling pathways associated with EGF and HGF, their respective receptors exhibit dissimilarities: EGFR serves as the receptor for EGF, while the receptor for HGF is c-Met [64]. Our data have shown that IFNγ inhibits EGFR, thereby impeding the homeostasis of ECM1 expression as ensured by EGF **(Figure 3A)**. Given that most studies predominantly focus on STAT3 signaling within HGF downstream pathways, limited reports exist on HGF-induced STAT1 activation [64]. The inhibition of ECM1 expression may be attributed to interference with the formation of STAT1-STAT3 heterodimers, suggesting that STAT3, rather than STAT1, serves as the primary downstream target of HGF. The elucidation of how HGF/c-Met upregulates ECM1 expression, whether it relies on STAT1-STAT3 heterodimer formation, and the manner in which IFNγ disrupts HGF/c-Met and STAT3 phosphorylation or heterodimer formation with STAT1 necessitates further investigations.

Taken together, the current study highlights that EGF and IFNγ control the regulation of ECM1 expression in the liver under physiological and pathological conditions. In healthy livers, the EGF/EGFR/STAT1 signaling pathway maintains ECM1 expression; upon liver damage and injury, accumulated IFNγ impedes ECM1 expression through inhibiting EGFR expression and inducing NRF2 nuclear translocation thereby failing to maintain the quiescence of LTGF-β, which contributes to hepatic fibrosis. Regarding the clinical applicability of IFNγ and NRF2 agonist, hepatotoxicity induced by IFNγ and NRF2 over-accumulation should be taken into account. In this context, ECM1 has the potential to be developed as an anti-fibrotic agent, especially in combination with

IFNγ and NRF2 agonist. It will be interesting to further investigate whether ECM1-derived therapies constitute an effective treatment route for CLD.

Materials and Methods

Patients

6 F1-F2 fibrotic liver tissues were collected by biopsy; 16 F3-F4 fibrotic liver tissues were got from patients following liver transplantation; 76 paraneoplastic liver tissues were obtained from HCC patients with or without cirrhosis during surgery at the Beijing You'an Hospital, Affiliated with Capital Medical University. The study protocol was approved by local Ethics Committees (Jing-2015-084, and 2017-584N-MA). Written informed consent was obtained from patients or their representatives.

Animals

Male C57BL/6J mice (8 to 10 weeks old) were purchased from the Janvier Lab. The animals weighed 22-25g at the beginning of the corresponding experiments. All animals were allowed to acclimatize to controlled conditions of temperature (23 ± 2°C), humidity (35 ± 5%) and a 12hrs light–dark cycle in the animal house at the Universitätsmedizin Mannheim for at least 1 week. They were provided with standard laboratory chow and water ad libitum and housed in laboratory cages. The mice were divided randomly into groups (n=3) and were injected with erlotinib or IFNγ. Erlotinib and IFNγ were dissolved in DMSO (41639-500ML, Sigma-Aldrich) and PBS (14190169, ThermoFisher) respectively. For erlotinib injection, mice were administered intraperitoneally with 40mg/kg/day of erlotinib or DMSO for 2 days (once per day). Liver samples were collected 48hrs after the first dose of erlotinib or DMSO. For IFNγ injection, mice received a series of 4 intraperitoneal injections (once per day) of 400μg/kg/day IFNγ or PBS. Liver tissues were collected 96hrs after the first dose of IFNγ or PBS.

All animal protocols were carried out in full accordance with animal care guidelines and were approved by the local animal care committee.

Chemical reagents, primers, antibodies used in this study, and further detailed methodical information are presented in the supplementary materials.

References

1 Mathieu, E., Meheus, L., Raymackers, J. & Merregaert, J. Characterization of the osteogenic stromal cell line MN7: identification of secreted MN7 proteins using two-dimensional polyacrylamide gel electrophoresis, western blotting, and microsequencing. *J Bone Miner Res* **9**, 903-913, doi:10.1002/jbmr.5650090616 (1994).

2 Smits, P. *et al.* Differentiation-dependent alternative splicing and expression of the extracellular matrix protein 1 gene in human keratinocytes. *J Invest Dermatol* **114**, 718-724, doi:10.1046/j.1523-1747.2000.00916.x (2000).

3 Sercu, S. *et al.* ECM1 interacts with fibulin-3 and the beta 3 chain of laminin 332 through its serum albumin subdomain-like 2 domain. *Matrix Biol* **28**, 160-169, doi:10.1016/j.matbio.2009.02.003 (2009).

4 Deckers, M. M. *et al.* Recombinant human extracellular matrix protein 1 inhibits alkaline phosphatase activity and mineralization of mouse embryonic metatarsals in vitro. *Bone* **28**, 14-20, doi:10.1016/s8756-3282(00)00428-2 (2001).

5 Han, Z. *et al.* Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. *FASEB J* **15**, 988-994, doi:10.1096/fj.99-0934com (2001).

6 Chan, I. The role of extracellular matrix protein 1 in human skin. *Clin Exp Dermatol* **29**, 52-56, doi:10.1111/j.1365-2230.2004.01440.x (2004).

7 Wang, L. *et al.* Extracellular matrix protein 1 (ECM1) is over-expressed in malignant epithelial tumors. *Cancer Lett* **200**, 57-67, doi:10.1016/s0304-3835(03)00350-1 (2003).

8 Sercu, S., Zhang, L. & Merregaert, J. The extracellular matrix protein 1: its molecular interaction and implication in tumor progression. *Cancer Invest* **26**, 375-384, doi:10.1080/07357900701788148 (2008).

9 Fan, W. *et al.* ECM1 Prevents Activation of Transforming Growth Factor beta, Hepatic Stellate Cells, and Fibrogenesis in Mice. *Gastroenterology* **157**, 1352-1367 e1313, doi:10.1053/j.gastro.2019.07.036 (2019).

10 Liu, Q. *et al.* ECM1 modified HF-MSCs targeting HSC attenuate liver cirrhosis by inhibiting the TGF-beta/Smad signaling pathway. *Cell Death Discov* **8**, 51, doi:10.1038/s41420-022-00846-4 (2022).

11 Bai, K. H. *et al.* Identification of cancer stem cell characteristics in liver hepatocellular carcinoma by WGCNA analysis of transcriptome stemness index. *Cancer Med* **9**, 4290-4298, doi:10.1002/cam4.3047 (2020).

12 Gao, F. *et al.* Integrated analyses of DNA methylation and hydroxymethylation reveal tumor suppressive roles of ECM1, ATF5, and EOMES in human hepatocellular carcinoma. *Genome Biol* **15**, 533, doi:10.1186/s13059-014-0533-9 (2014).

13 Filliol, A. *et al.* Opposing roles of hepatic stellate cell subpopulations in hepatocarcinogenesis. *Nature* **610**, 356-365, doi:10.1038/s41586-022-05289-6 (2022).

14 Li, Y., Fan, W., Link, F., Wang, S. & Dooley, S. Transforming growth factor beta latency: A mechanism of cytokine storage and signalling regulation in liver homeostasis and disease. *JHEP Rep* **4**, 100397, doi:10.1016/j.jhepr.2021.100397 (2022).

15 Link, F., Li, Y., Zhao, J., et al. Extracellular Matrix Protein 1 Attenuates Hepatic Fibrosis by Inhibiting TSP-, ADAMTS-, and MMP-Mediated Latent TGF-β1 Activation. *bioRxiv preprint 2023.12.12.571289* (2023).

16 Suppli, M. P. *et al.* Hepatic transcriptome signatures in patients with varying degrees of nonalcoholic fatty liver disease compared with healthy normal-weight individuals. *Am J Physiol Gastrointest Liver Physiol* **316**, G462-G472, doi:10.1152/ajpgi.00358.2018 (2019).

17 Trepo, E. *et al.* Combination of Gene Expression Signature and Model for End-Stage Liver Disease Score Predicts Survival of Patients With Severe Alcoholic Hepatitis. *Gastroenterology* **154**, 965-975, doi:10.1053/j.gastro.2017.10.048 (2018).

18 Yoo, S. *et al.* A pilot systematic genomic comparison of recurrence risks of hepatitis B virus-associated hepatocellular carcinoma with low- and high-degree liver fibrosis. *BMC Med* **15**, 214, doi:10.1186/s12916-017-0973-7 (2017).

19 Carpenter, G. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu Rev Biochem* **56**, 881-914, doi:10.1146/annurev.bi.56.070187.004313 (1987).

20 Massague, J. & Pandiella, A. Membrane-anchored growth factors. *Annu Rev Biochem* **62**, 515-541, doi:10.1146/annurev.bi.62.070193.002503 (1993).

21 Zeineldin, R. & Hudson, L. G. Epithelial cell migration in response to epidermal growth factor. *Methods Mol Biol* **327**, 147-158, doi:10.1385/1-59745-012-X:147 (2006).

22 Wang, Z. ErbB Receptors and Cancer. *Methods Mol Biol* **1652**, 3-35, doi:10.1007/978-1-4939-7219-7_1 (2017).

23 Platanias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386, doi:10.1038/nri1604 (2005).

24 Horras, C. J., Lamb, C. L. & Mitchell, K. A. Regulation of hepatocyte fate by interferon-gamma. *Cytokine Growth Factor Rev* **22**, 35-43, doi:10.1016/j.cytogfr.2011.01.001 (2011).

25 Sadzak, I. *et al.* Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. *Proc Natl Acad Sci U S A* **105**, 8944-8949, doi:10.1073/pnas.0801794105 (2008).

26 Zhang, Y., Cho, Y. Y., Petersen, B. L., Zhu, F. & Dong, Z. Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MSK1. *Carcinogenesis* **25**, 1165-1175, doi:10.1093/carcin/bgh115 (2004).

27 Miyawaki, A., Iizuka, Y., Sugino, H. & Watanabe, Y. IL-11 prevents IFN-gamma-induced hepatocyte death through selective downregulation of IFN-gamma/STAT1 signaling and ROS scavenging. *PLoS One* **14**, e0211123, doi:10.1371/journal.pone.0211123 (2019).

28 Lee, H. J. *et al.* The role of STAT1/IRF-1 on synergistic ROS production and loss of mitochondrial transmembrane potential during hepatic cell death induced by LPS/d-GalN. *J Mol Biol* **369**, 967-984, doi:10.1016/j.jmb.2007.03.072 (2007).
29 Jaeschke, H. & Woolbright, B. L. Current strategies to minimize hepatic ischemia-reperfusion injury by targeting reactive oxygen species. *Transplant Rev (Orlando)* **26**, 103-114, doi:10.1016/j.trre.2011.10.006 (2012).
30 Cullinan, S. B. & Diehl, J. A. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* **279**, 20108-20117, doi:10.1074/jbc.M314219200 (2004).
31 Komuves, L. G., Feren, A., Jones, A. L. & Fodor, E. Expression of epidermal growth factor and its receptor in cirrhotic liver disease. *J Histochem Cytochem* **48**, 821-830, doi:10.1177/002215540004800610 (2000).
32 Cichoz-Lach, H. & Michalak, A. Oxidative stress as a crucial factor in liver diseases. *World J Gastroenterol* **20**, 8082-8091, doi:10.3748/wjg.v20.i25.8082 (2014).
33 He, F. *et al.* NRF2 activates growth factor genes and downstream AKT signaling to induce mouse and human hepatomegaly. *J Hepatol* **72**, 1182-1195, doi:10.1016/j.jhep.2020.01.023 (2020).
34 Moylan, C. A. *et al.* Hepatic gene expression profiles differentiate presymptomatic patients with mild versus severe nonalcoholic fatty liver disease. *Hepatology* **59**, 471-482, doi:10.1002/hep.26661 (2014).
35 Murphy, S. K. *et al.* Relationship between methylome and transcriptome in patients with nonalcoholic fatty liver disease. *Gastroenterology* **145**, 1076-1087, doi:10.1053/j.gastro.2013.07.047 (2013).
36 Kovarik, P. *et al.* Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression. *EMBO J* **20**, 91-100, doi:10.1093/emboj/20.1.91 (2001).
37 Kovarik, P. *et al.* Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. *Proc Natl Acad Sci U S A* **96**, 13956-13961, doi:10.1073/pnas.96.24.13956 (1999).
38 Uddin, S. *et al.* The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. *J Biol Chem* **275**, 27634-27640, doi:10.1074/jbc.M003170200 (2000).
39 Weng, H. L. *et al.* Effect of interferon-gamma on hepatic fibrosis in chronic hepatitis B virus infection: a randomized controlled study. *Clin Gastroenterol Hepatol* **3**, 819-828, doi:10.1016/s1542-3565(05)00404-0 (2005).
40 Weng, H., Mertens, P. R., Gressner, A. M. & Dooley, S. IFN-gamma abrogates profibrogenic TGF-beta signaling in liver by targeting expression of inhibitory and receptor Smads. *J Hepatol* **46**, 295-303, doi:10.1016/j.jhep.2006.09.014 (2007).

41 Jeong, W. I., Park, O., Radaeva, S. & Gao, B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. *Hepatology* **44**, 1441-1451, doi:10.1002/hep.21419 (2006).

42 Rockey, D. C., Maher, J. J., Jarnagin, W. R., Gabbiani, G. & Friedman, S. L. Inhibition of rat hepatic lipocyte activation in culture by interferon-gamma. *Hepatology* **16**, 776-784, doi:10.1002/hep.1840160325 (1992).

43 Sun, R. *et al.* STAT1 contributes to dsRNA inhibition of liver regeneration after partial hepatectomy in mice. *Hepatology* **44**, 955-966, doi:10.1002/hep.21344 (2006).

44 Kusters, S., Gantner, F., Kunstle, G. & Tiegs, G. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. *Gastroenterology* **111**, 462-471, doi:10.1053/gast.1996.v111.pm8690213 (1996).

45 Tagawa, Y., Sekikawa, K. & Iwakura, Y. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *J Immunol* **159**, 1418-1428 (1997).

46 Sun, C. *et al.* Spred2 controls the severity of Concanavalin A-induced liver damage by limiting interferon-gamma production by CD4(+) and CD8(+) T cells. *J Adv Res* **35**, 71-86, doi:10.1016/j.jare.2021.03.014 (2022).

47 Mizuhara, H. *et al.* Critical involvement of interferon gamma in the pathogenesis of T-cell activation-associated hepatitis and regulatory mechanisms of interleukin-6 for the manifestations of hepatitis. *Hepatology* **23**, 1608-1615, doi:10.1053/jhep.1996.v23.pm0008675184 (1996).

48 Fukuda, R. *et al.* The expression of IL-2, IL-4 and interferon-gamma (IFN-gamma) mRNA using liver biopsies at different phases of acute exacerbation of chronic hepatitis B. *Clin Exp Immunol* **100**, 446-451, doi:10.1111/j.1365-2249.1995.tb03720.x (1995).

49 Gilles, P. N., Guerrette, D. L., Ulevitch, R. J., Schreiber, R. D. & Chisari, F. V. HBsAg retention sensitizes the hepatocyte to injury by physiological concentrations of interferon-gamma. *Hepatology* **16**, 655-663, doi:10.1002/hep.1840160308 (1992).

50 Luo, X. Y. *et al.* IFN-gamma deficiency attenuates hepatic inflammation and fibrosis in a steatohepatitis model induced by a methionine- and choline-deficient high-fat diet. *Am J Physiol Gastrointest Liver Physiol* **305**, G891-899, doi:10.1152/ajpgi.00193.2013 (2013).

51 Burova, E., Vassilenko, K., Dorosh, V., Gonchar, I. & Nikolsky, N. Interferon gamma-dependent transactivation of epidermal growth factor receptor. *FEBS Lett* **581**, 1475-1480, doi:10.1016/j.febslet.2007.03.002 (2007).

52 Uribe, J. M., McCole, D. F. & Barrett, K. E. Interferon-gamma activates EGF receptor and increases TGF-alpha in T84 cells: implications for chloride secretion. *Am J Physiol Gastrointest Liver Physiol* **283**, G923-931, doi:10.1152/ajpgi.00237.2002 (2002).

53 Hamburger, A. W. & Pinnamaneni, G. D. Increased epidermal growth factor receptor gene expression by gamma-interferon in a human breast carcinoma cell line. *Br J Cancer* **64**, 64-68, doi:10.1038/bjc.1991.240 (1991).

54 Solis, N. V., Swidergall, M., Bruno, V. M., Gaffen, S. L. & Filler, S. G. The Aryl Hydrocarbon Receptor Governs Epithelial Cell Invasion during Oropharyngeal Candidiasis. *mBio* **8**, doi:10.1128/mBio.00025-17 (2017).

55 Midgley, A. C., Morris, G., Phillips, A. O. & Steadman, R. 17beta-estradiol ameliorates age-associated loss of fibroblast function by attenuating IFN-gamma/STAT1-dependent miR-7 upregulation. *Aging Cell* **15**, 531-541, doi:10.1111/acel.12462 (2016).

56 Alberts, B., Johnson A, Lewis J, et al. *Molecular Biology of the Cell*. 5th edn, Vol. Chapter 15 (2008).

57 Saha, S., Buttari, B., Panieri, E., Profumo, E. & Saso, L. An Overview of Nrf2 Signaling Pathway and Its Role in Inflammation. *Molecules* **25**, doi:10.3390/molecules25225474 (2020).

58 Wang, L. & He, C. Nrf2-mediated anti-inflammatory polarization of macrophages as therapeutic targets for osteoarthritis. *Front Immunol* **13**, 967193, doi:10.3389/fimmu.2022.967193 (2022).

59 Panda, H., Wen, H., Suzuki, M. & Yamamoto, M. Multifaceted Roles of the KEAP1-NRF2 System in Cancer and Inflammatory Disease Milieu. *Antioxidants (Basel)* **11**, doi:10.3390/antiox11030538 (2022).

60 Staitieh, B. S. *et al.* Activation of Alveolar Macrophages with Interferon-gamma Promotes Antioxidant Defenses via the Nrf2-ARE Pathway. *J Clin Cell Immunol* **6**, doi:10.4172/2155-9899.1000365 (2015).

61 Wakabayashi, N. *et al.* Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* **35**, 238-245, doi:10.1038/ng1248 (2003).

62 Li, L. *et al.* Hepatocyte-specific Nrf2 deficiency mitigates high-fat diet-induced hepatic steatosis: Involvement of reduced PPARgamma expression. *Redox Biol* **30**, 101412, doi:10.1016/j.redox.2019.101412 (2020).

63 Su, H. *et al.* Collagenolysis-dependent DDR1 signalling dictates pancreatic cancer outcome. *Nature* **610**, 366-372, doi:10.1038/s41586-022-05169-z (2022).

64 Zhao, Y., Ye, W., Wang, Y. D. & Chen, W. D. HGF/c-Met: A Key Promoter in Liver Regeneration. *Front Pharmacol* **13**, 808855, doi:10.3389/fphar.2022.808855 (2022).

Figures and figure legends

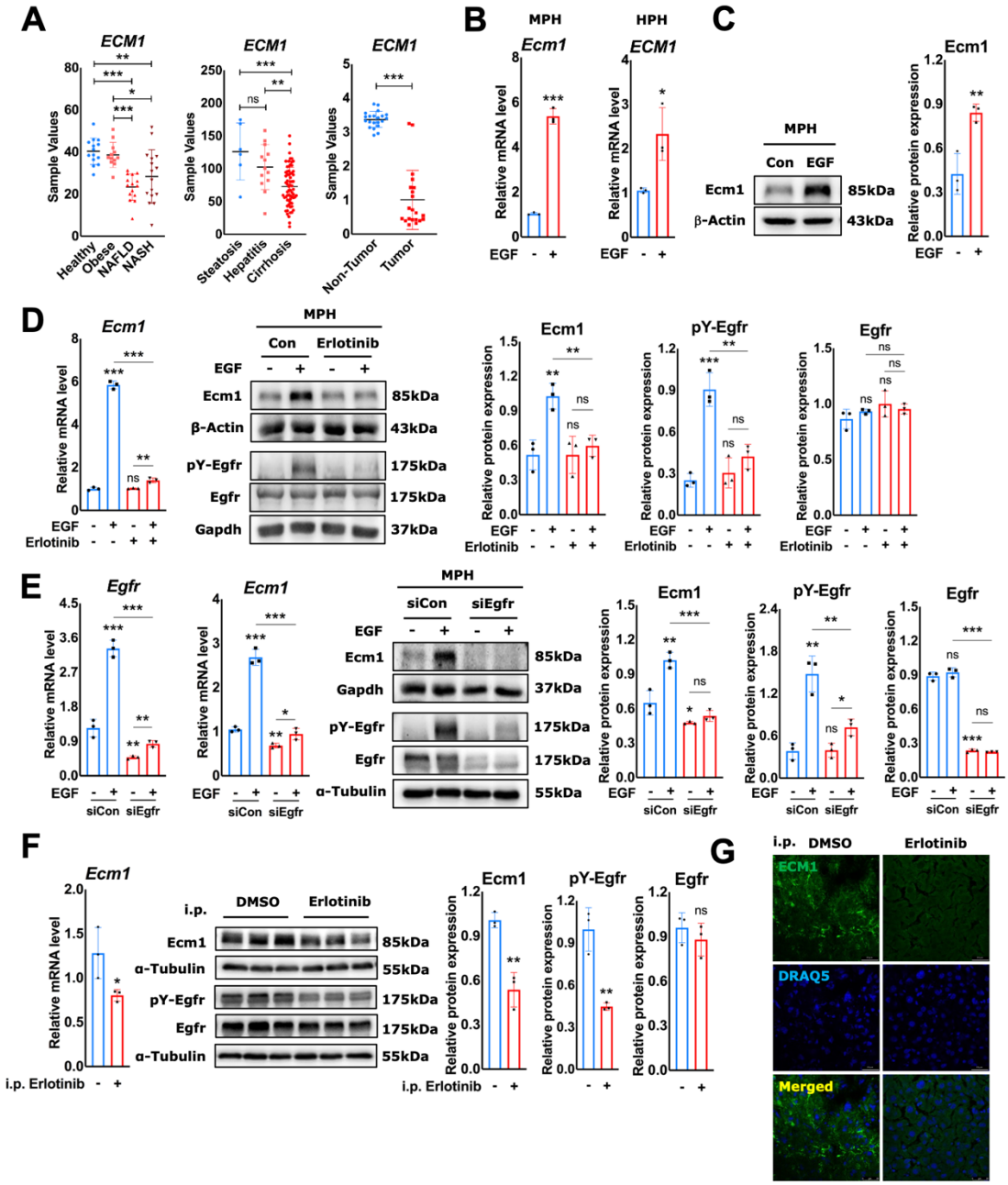

Figure 1. EGF-Egfr signaling maintains Ecm1 expression in hepatocytes of the quiescent liver

(A) mRNA expression data of hepatic *ECM1* in patients with CLD, as shown in the figures extracted from the GEO DataSet GSE126848, GSE103580 and GSE94660, respectively. **(B)** qRT-PCR for *ECM1* mRNA expression in MPHs and HPHs with or without EGF treatment for 24hrs. **(C)** Immunoblotting of Ecm1 protein expression in MPHs, treated with EGF for 24hrs. **(D)** qRT-PCR and immunoblotting showing the effect of erlotinib on Ecm1 expression and Egfr Y1068 phosphorylation in MPHs with or without EGF treatment. **(E)** Effects of *Egfr* knockdown on mRNA and protein expression of Ecm1 and Egfr in EGF-treated MPHs by qRT-PCR and Western blotting. Immunoblots for p-Egfr Y1068 are also shown. **(F)** qRT-PCR and Western blotting for Ecm1 expression in liver tissues from mice treated with DMSO or erlotinib (40mg/kg/day, i.p., for 2 days). Immunoblot data for p-Egfr Y1068 and Egfr are shown. Mice were divided into two groups (n=3), DMSO was administered as a placebo. **(G)** Immunofluorescence staining for Ecm1 expression in liver tissues from DMSO- or erlotinib-treated mice. DRAQ5 fluorescent probe stains DNA. Scale bar, 25μm. The results of qRT-PCR were normalized to *PPIA*. In western blots β-Actin, Gapdh and α-Tubulin are loading controls. Quantification of protein expression was performed by ImageJ (National Institutes of Health, Bethesda, Maryland, USA). *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

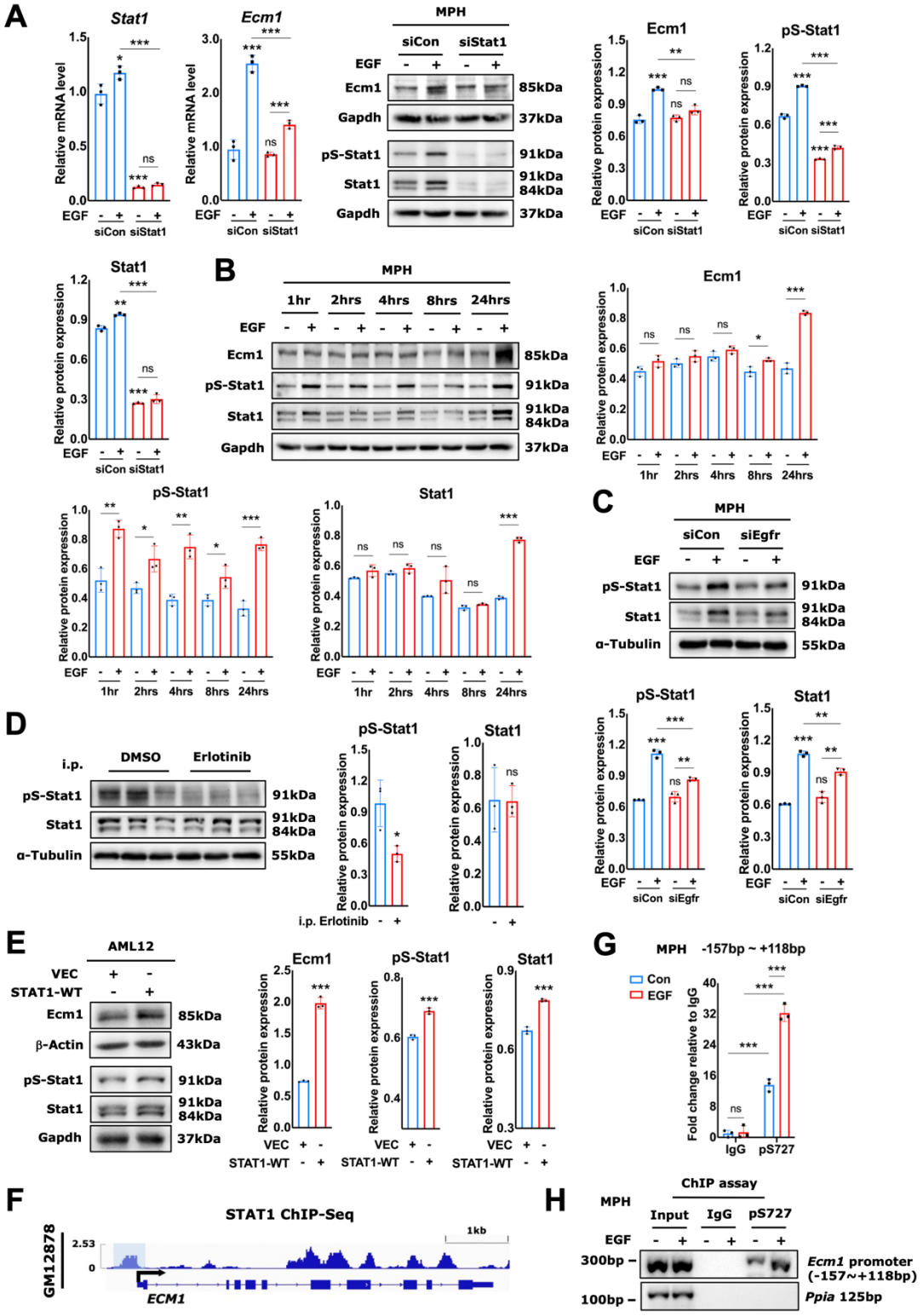
A
Stat1
Ecm1
Relative mRNA level
EGF
siCon siStat1
MPH
siCon siStat1
Ecm1
Gapdh
pS-Stat1
Stat1
85kDa
37kDa
91kDa
91kDa
84kDa
Relative protein expression
B
1hr 2hrs 4hrs 8hrs 24hrs
C
siCon siEgfr
α-Tubulin
55kDa
D
i.p.
DMSO
Erlotinib
i.p. Erlotinib
E
AML12
VEC
STAT1-WT
β-Actin
43kDa
F
STAT1 ChIP-Seq
GM12878
2.53
0
1kb
ECM1
G
MPH -157bp ~ +118bp
Con
EGF
Fold change relative to IgG
IgG
pS727
H
ChIP assay
Input
IgG
pS727
300bp
100bp
Ecm1 promoter
(-157~+118bp)
Ppia 125bp

Figure 2. EGF regulates homeostatic Ecm1 expression in hepatocytes through Stat1

(A) qRT-PCR and Immunoblot data for mRNA and protein expression of *Ecm1* and *Stat1* in EGF-treated MPHs with or without RNAi mediated *Stat1* knockdown. Additionally, p-Stat1 S727 levels are shown. **(B)** Immunoblots showing the effect of EGF on protein levels of Ecm1, p-Stat1 S727 and Stat1 in MPHs at different time points, as indicated. **(C)** Immunoblots for p-Stat1 S727 and Stat1 in EGF-treated MPHs with or without knockdown *Egfr*. **(D)** Immunoblot data for p-Stat1 S727 and Stat1 in liver tissues from mice treated with DMSO or erlotinib (40mg/kg/day, i.p., for 2 days). Mice were divided into two groups (n=3), DMSO was administered as a placebo. **(E)** Immunoblots for protein levels of Ecm1, p-Stat1 S727 and total Stat1 in AML12 cells transfected with expression vectors as indicated for 48hrs. **(F)** Representative readouts of peaks (signal *P*-value) from a STAT1 ChIP-seq analysis at the *ECM1* gene locus in the cell line GM12878. Data is retrieved from ENCODE (ENCFF011BMN). **(G)** ChIP qRT-PCR, showing the binding of S727 phosphorylated Stat1 to the *Ecm1* gene promoter in MPHs, with or without EGF treatment for 24hrs. Relative fold change of immunoprecipitated genomic fragments was compared in EGF-treated cells and an untreated control group. Fragment "-157bp ~ +118bp" is calculated relative to the TSS of the *Ecm1* gene. Rabbit IgG-bound chromatin served as negative control. **(H)** The PCR amplified products of "-157bp ~ +118bp" fragments are shown as bands on a 2% agarose gel. *Ppia* represents non-specific binding. The results of qRT-PCR were normalized to *Ppia*. For western blots Gapdh, α-Tubulin and β-Actin are loading controls. **Figure C and D shared same loading controls as Figure 1E and 1F.** Quantification of protein expression was done by ImageJ (National Institutes of Health, Bethesda, Maryland, USA). *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

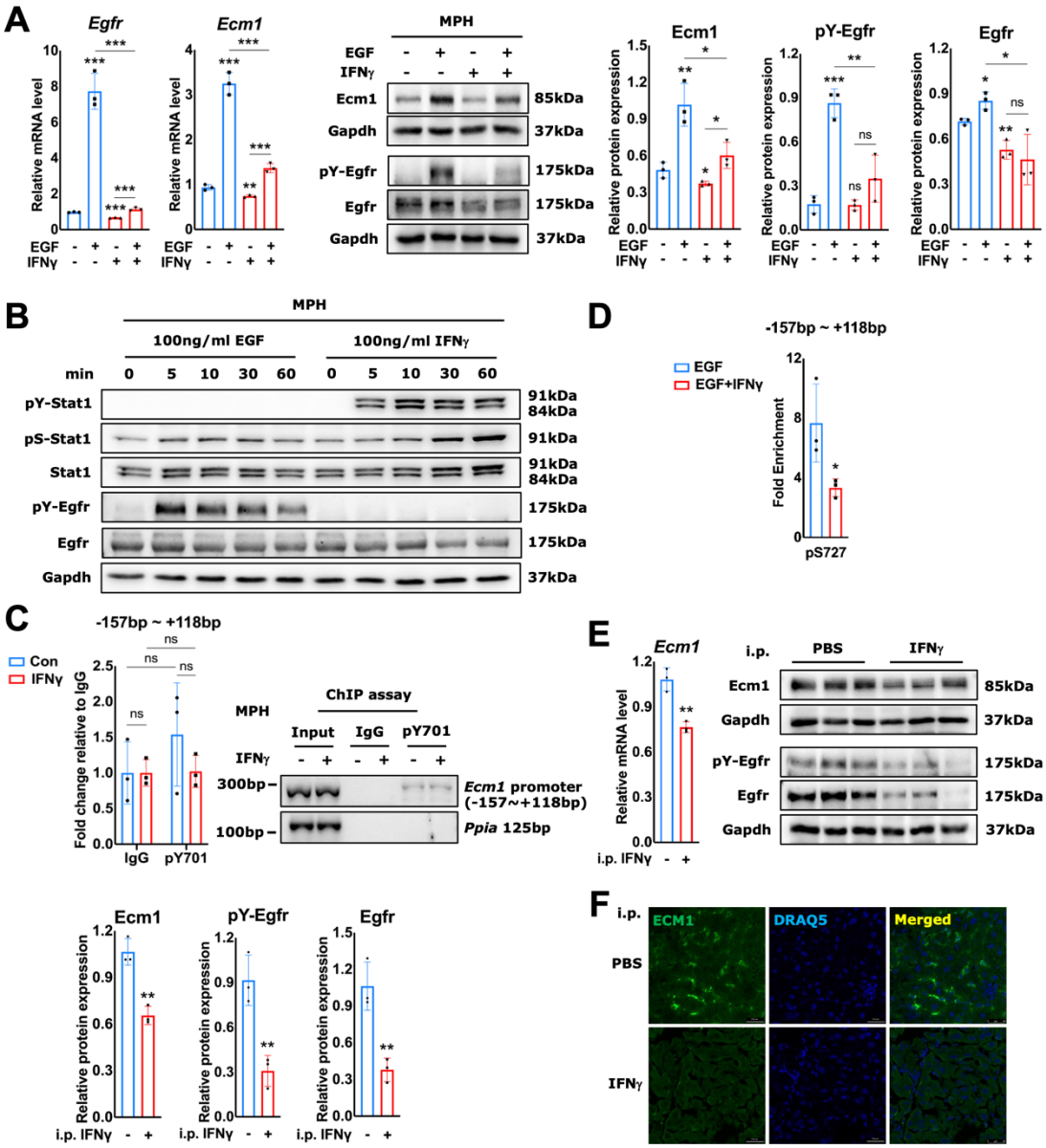
A
Egfr
Ecm1
Relative mRNA level
EGF
IFNγ
MPH
Ecm1
Gapdh
pY-Egfr
Egfr
85kDa
37kDa
175kDa
Relative protein expression
B
MPH
100ng/ml EGF
100ng/ml IFNγ
min
0 5 10 30 60 0 5 10 30 60
pY-Stat1
pS-Stat1
Stat1
pY-Egfr
Egfr
Gapdh
91kDa
84kDa
175kDa
37kDa
C
-157bp ~ +118bp
Con
IFNγ
Fold change relative to IgG
IgG
pY701
ns
ChIP assay
Input
300bp
100bp
Ecm1 promoter (-157~+118bp)
Ppia 125bp
D
-157bp ~ +118bp
EGF
EGF+IFNγ
Fold Enrichment
pS727
E
Ecm1
i.p.
PBS
IFNγ
i.p. IFNγ
F
ECM1
DRAQ5
Merged
PBS
IFNγ

Figure 3. IFNγ abrogates EGF-Egfr-maintained Ecm1 expression

(A) qRT-PCR and immunoblot data showing the impact of IFNγ on Ecm1 and Egfr expression in EGF-treated MPHs. Additionally, p-Egfr Y1086 levels are shown. **(B)** Immunoblot data, showing the effect of EGF or IFNγ on S727 phosphorylation of Stat1, Y701 phosphorylation of Stat1, Stat1 expression, Y1068 phosphorylation of Egfr and Egfr expression in MPHs at 5, 10, 30 and 60 min time points. **(C)** ChIP qRT-PCR data, displaying the effect of 24hrs IFNγ treatment on the binding of p-Stat1 Y701 to the *Ecm1* gene promoter in MPHs. PCR amplified products were separated on a 2% agarose gel. The fragment positions "-157bp ~ +118bp" are calculated relative to the TSS of the *Ecm1* gene. Rabbit IgG-bound chromatin served as negative control. *Ppia* represents non-specific binding. **(D)** Fold enrichment from ChIP qRT-PCR, showing the effect of IFNγ treatment on EGF-induced Stat1 binding to the *Ecm1* gene promoter in MPHs. **(E)** qRT-PCR and immunoblot data, showing Ecm1, Y1068 phosphorylation of Egfr and Egfr expression in liver tissues from mice treated with PBS or IFNγ (400μg/kg/day, i.p., for 4 days). Mice were divided into two groups (n=3), PBS was administered as placebo. **(F)** Immunofluorescence staining for Ecm1 expression in the liver tissues from PBS- or IFNγ-treated mice. DRAQ5 fluorescent probe stains DNA. Scale bar, 25μm. The results of qRT-PCR are normalized to *Ppia*. Gapdh was used as loading control. Quantification of protein expression was done by ImageJ (National Institutes of Health, Bethesda, Maryland, USA). *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

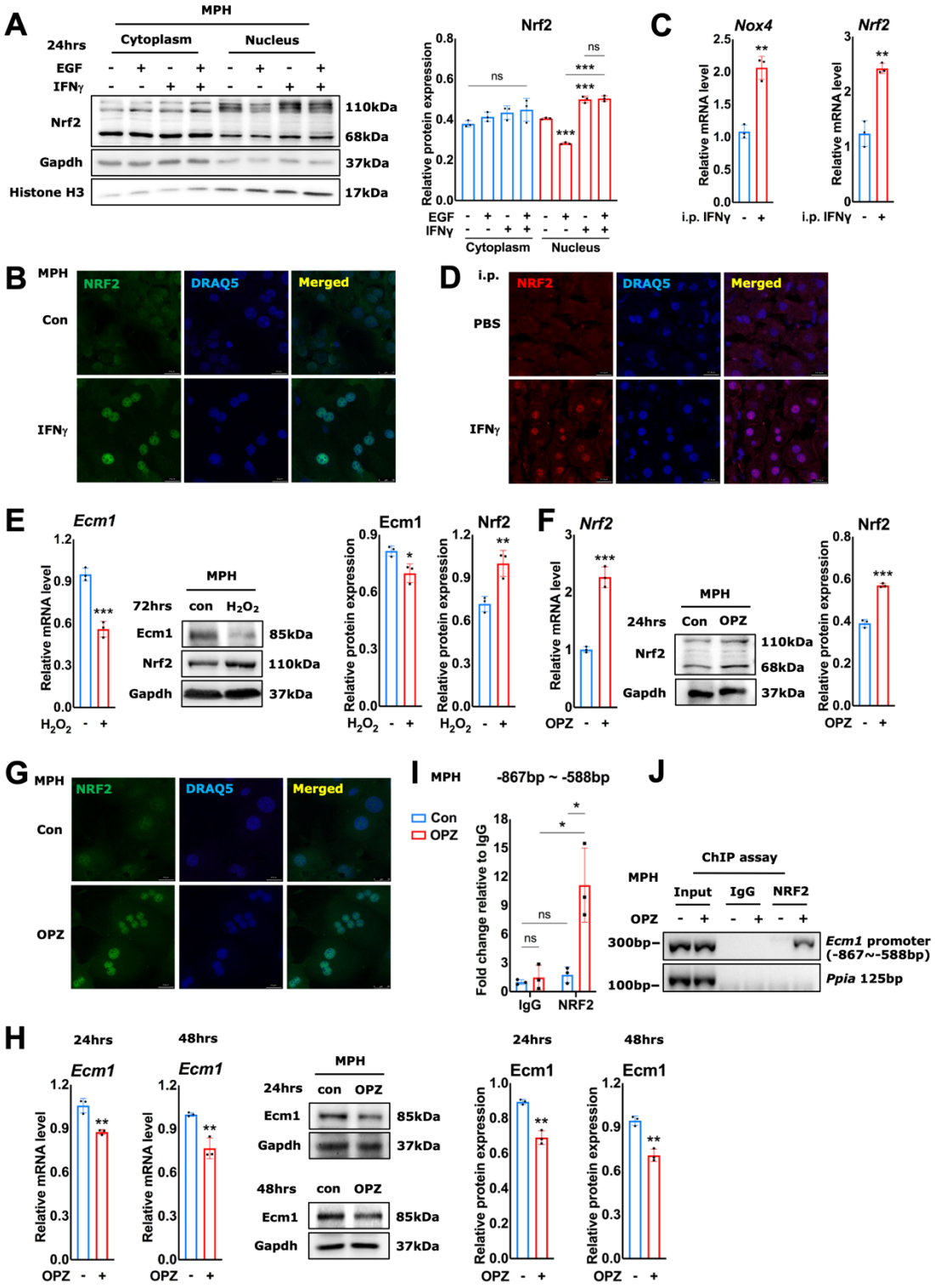
A
MPH
24hrs
Cytoplasm
Nucleus
EGF
IFNγ
Nrf2
Gapdh
Histone H3
110kDa
68kDa
37kDa
17kDa
Nrf2
Relative protein expression
ns
***
EGF
IFNγ
Cytoplasm
Nucleus
C
Nox4
Nrf2
Relative mRNA level
**
i.p. IFNγ
B
MPH
NRF2
DRAQ5
Merged
Con
IFNγ
D
i.p.
PBS
IFNγ
E
Ecm1
Relative mRNA level
***
H2O2
MPH
72hrs
con
H2O2
Ecm1
Nrf2
Gapdh
85kDa
110kDa
37kDa
Relative protein expression
*
**
F
Nrf2
Relative mRNA level
***
OPZ
MPH
24hrs
Con
OPZ
Nrf2
Gapdh
110kDa
68kDa
37kDa
G
MPH
NRF2
DRAQ5
Merged
Con
OPZ
I
MPH
-867bp ~ -588bp
Con
OPZ
Fold change relative to IgG
ns
*
IgG
NRF2
J
ChIP assay
MPH
Input
IgG
NRF2
OPZ
300bp–
100bp–
Ecm1 promoter
(-867~-588bp)
Ppia 125bp
H
24hrs
48hrs
Ecm1
Relative mRNA level
**
OPZ
MPH
con
OPZ
Ecm1
Gapdh
85kDa
37kDa
Relative protein expression

Figure 4. IFNγ inhibits Ecm1 expression through activation of Nrf2

(A) Immunoblot data for cytoplasmic and nuclear localization of Nrf2 in MPHs treated with EGF and/or IFNγ for 24hrs. **(B)** Immunofluorescence staining, showing expression and localization of Nrf2 in MPHs, treated with IFNγ for 24hrs. Scale bar, 25µm. **(C)** qRT-PCR for mRNA expression levels of *Nox4* and *Nrf2* in liver tissue from mice treated with PBS or IFNγ (400µg/kg/day, i.p., for 4 days). **(D)** Immunofluorescence staining for Nrf2 expression in liver tissue from PBS- or IFNγ-treated mice. Scale bar, 12.5µm. **(E)** qRT-PCR and immunoblot data showing the impact of 72hrs H_2O_2 (200µM) treatment on Ecm1 and Nrf2 expression in MPHs. **(F)** qRT-PCR and immunoblot data showing the effect of 24hrs OPZ (50µM) treatment on Nrf2 expression in MPHs. **(G)** Immunofluorescence staining for Nrf2 expression in MPHs, treated with OPZ for 24hrs. Scale bar, 25µm. **(H)** qRT-PCR and immunoblot data, displaying the effect of OPZ treatment on Ecm1 expression in MPHs at the indicated time points. **(I)** ChIP qRT-PCR data, showing the effect of OPZ treatment on binding of Nrf2 to the *Ecm1* gene promoter in MPHs. Relative fold changes of immunoprecipitated genomic fragments in OPZ-treated cells were compared to those of the untreated control group. The fragment positions "-867bp ~ -588bp" were calculated relative to the TSS of the *Ecm1* gene. Rabbit IgG-bound chromatin served as negative control. **(J)** The PCR amplified products of "-867bp ~ -588bp" fragments were separated on a 2% agarose gel. *Ppia* represents non-specific binding. The results of qRT-PCR are normalized to *Ppia*. Gapdh and Histone H3 are loading controls for cytoplasmic and nuclear proteins, respectively. DRAQ5 fluorescent probe stains DNA. Quantification of protein expression was done by ImageJ (National Institutes of Health, Bethesda, Maryland, USA). *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

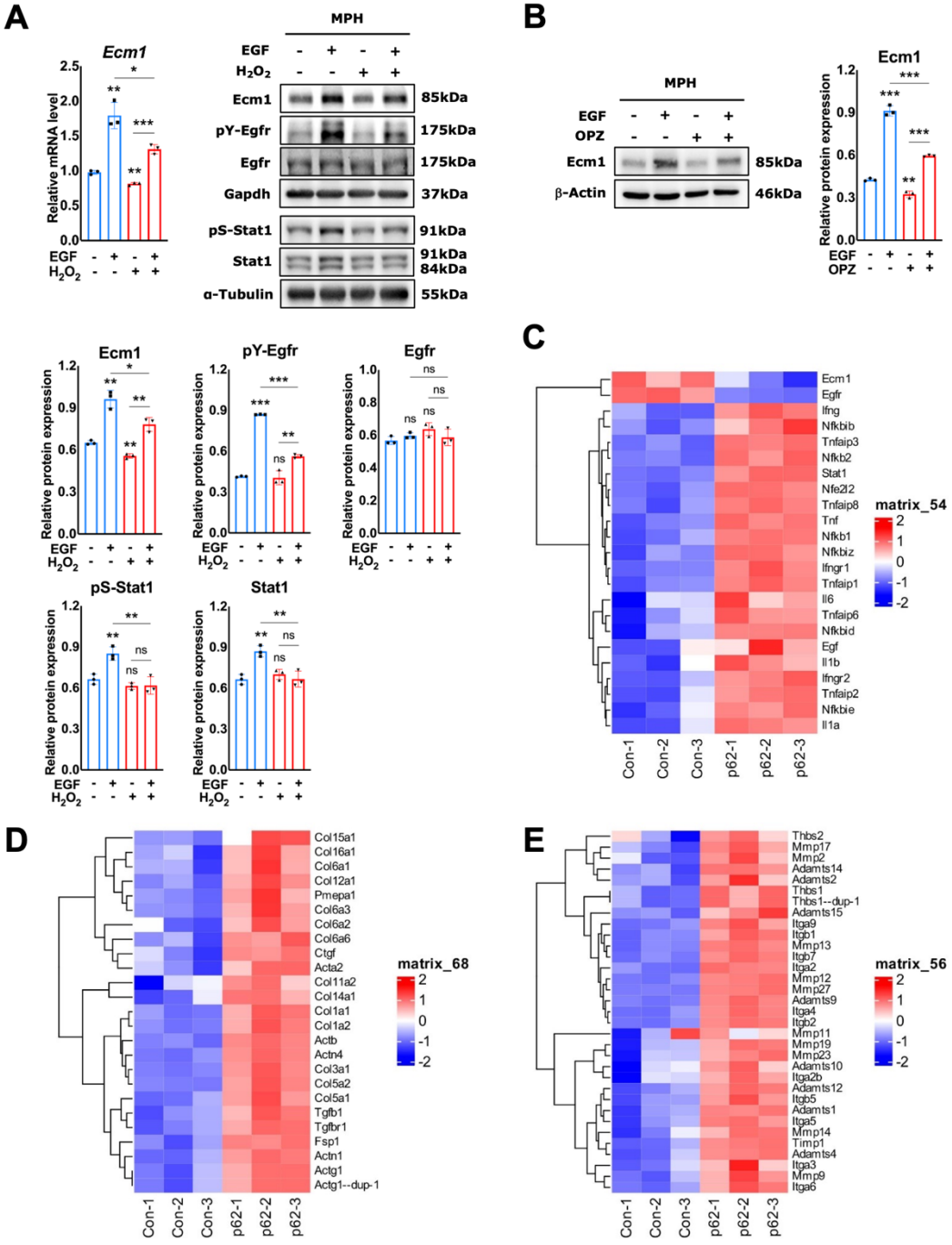
A
Ecm1
Relative mRNA level
EGF
H2O2
MPH
Ecm1
pY-Egfr
Egfr
Gapdh
pS-Stat1
Stat1
α-Tubulin
85kDa
175kDa
175kDa
37kDa
91kDa
91kDa
84kDa
55kDa
Relative protein expression
B
OPZ
β-Actin
46kDa
C
matrix_54
D
matrix_68
E
matrix_56
Con-1
Con-2
Con-3
p62-1
p62-2
p62-3

Figure 5. Oxidative stress abrogates EGF/Egfr-maintained Ecm1 expression

(A) qRT-PCR and immunoblot data, showing the effect of H_2O_2 on Ecm1, Egfr expression and Y1068 phosphorylation of Egfr, Stat1 expression and S727 phosphorylation of Stat1 in EGF-treated MPHs. Cells were treated with H_2O_2 and EGF for 72hrs. **(B)** Immunoblot data, displaying the effect of OPZ treatment on Ecm1 expression in EGF-treated MPHs. Cells were treated with OPZ and EGF for 48hrs. The results of qRT-PCR are normalized to *Ppia*. Gapdh, α-Tubulin and β-Actin are loading controls. Quantification of protein expression was done by ImageJ (National Institutes of Health, Bethesda, Maryland, USA). **(C-E)** mRNA expression data of the target genes, as shown in the figures of liver tissue from p62-KO mice injected with/without adenovirus p62, extracted from the GEO DataSet GSE134188. *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

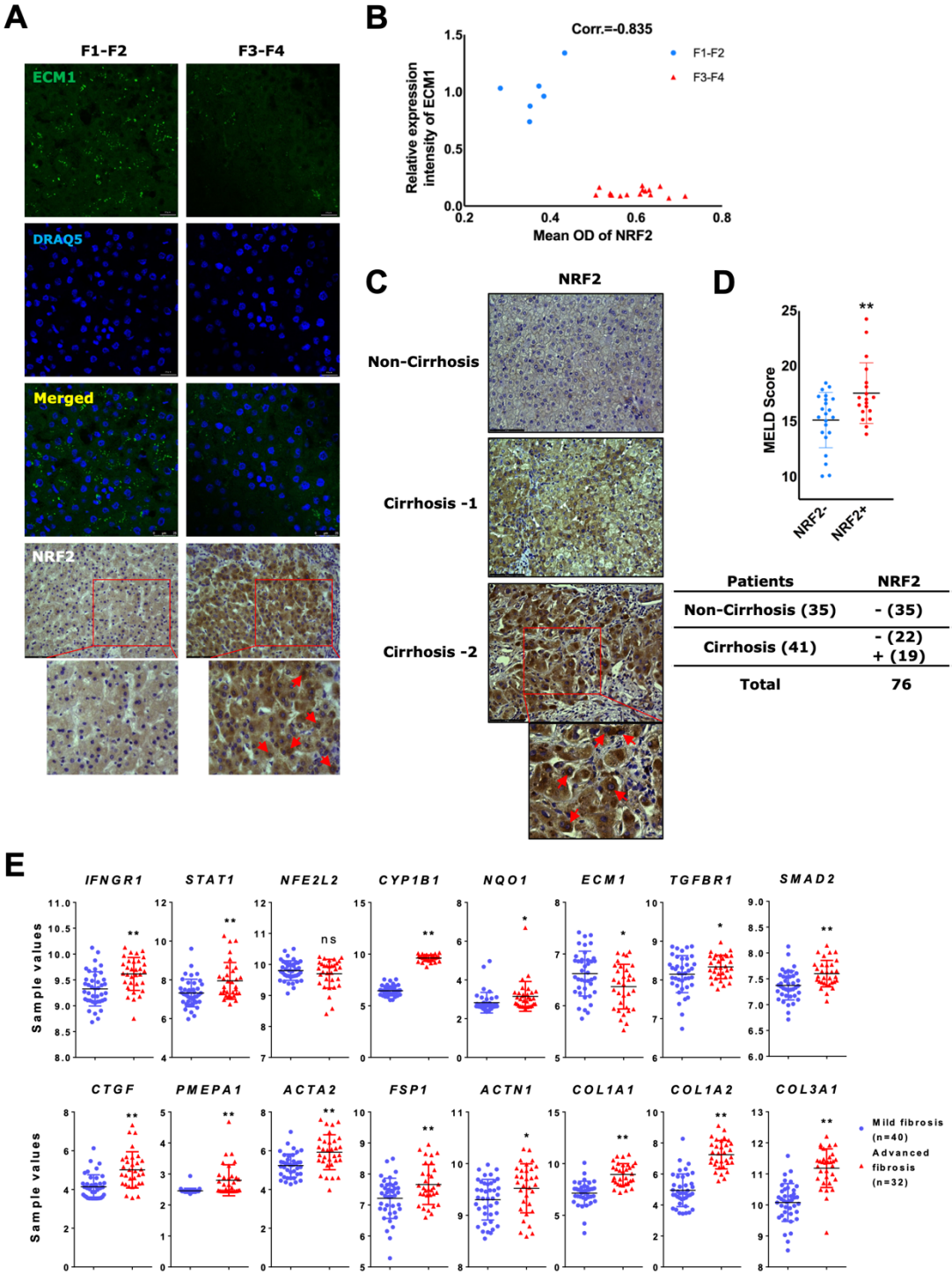
A
F1-F2
F3-F4
ECM1
DRAQ5
Merged
NRF2
B
Corr.=-0.835
F1-F2
F3-F4
Relative expression intensity of ECM1
Mean OD of NRF2
C
NRF2
Non-Cirrhosis
Cirrhosis -1
Cirrhosis -2
D
MELD Score
NRF2-
NRF2+
Patients | NRF2
Non-Cirrhosis (35) | - (35)
Cirrhosis (41) | - (22) + (19)
Total | 76
E
IFNGR1
STAT1
NFE2L2
CYP1B1
NQO1
ECM1
TGFBR1
SMAD2
CTGF
PMEPA1
ACTA2
FSP1
ACTN1
COL1A1
COL1A2
COL3A1
Sample values
ns
Mild fibrosis (n=40)
Advanced fibrosis (n=32)

Figure 6. Activated IFNγ/NRF2 and decreased ECM1 expression aggravate the progression of fibrotic liver diseases

(A) Representative immunofluorescence and immunohistochemistry staining for ECM1 and NRF2 in F1-F2 fibrosis and F3-F4 fibrosis patients. DRAQ5 fluorescent probe stains DNA. Scale bar, IF 25μm, IHC 87μm. **(B)** Correlation analysis of NRF2 and ECM1 expression levels in the liver tissue of patients with F1-F2 or F3-F4 fibrosis. **(C)** Immunohistochemistry staining for NRF2 in the tumor-surrounding tissue from a human HCC patients tissue microarray. Red arrows show positive nuclear expression of NRF2. Scale bar, 87μm. **(D)** Comparative MELD score of cirrhosis associated HCC patients, negative or positive for nuclear NRF2 expression. **(E)** mRNA expression of the target genes, as shown in the figure of liver tissue from patients with F0-F1 or F3-F4 fibrosis, extracted from GEO DataSet GSE49541. *P*-values were calculated by unpaired Student's t test. Bars represent the mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

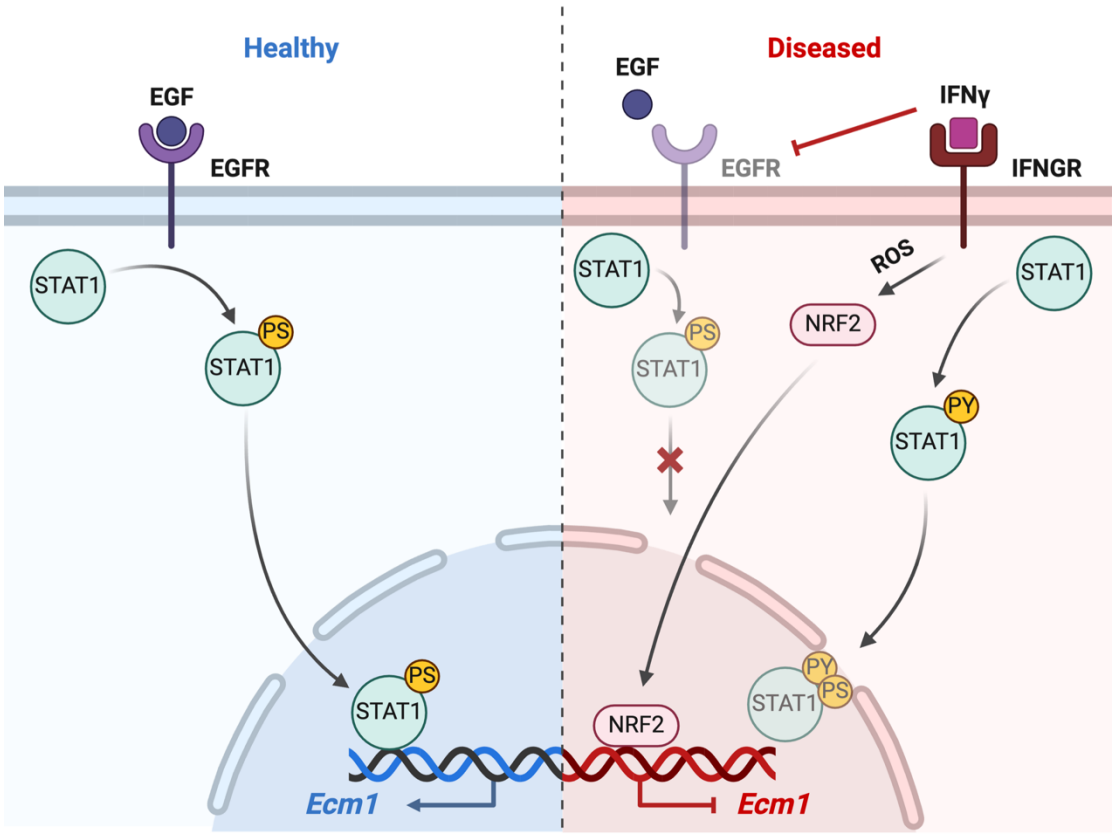
Healthy
Diseased
EGF
EGFR
STAT1
PS
STAT1
Ecm1
EGF
EGFR
IFNγ
IFNGR
STAT1
PS
STAT1
ROS
NRF2
STAT1
PY
STAT1
NRF2
PY
PS
STAT1
Ecm1

Figure 7. Scheme depicting the regulation of ECM1 expression in hepatocytes in healthy and diseased liver

In the physiological state **(left panel)**, ECM1 expression in hepatocytes is maintained by the EGF/EGFR/p-STAT1 S727 pathway. Signal-phosphorylated STAT1 S727 translocates to the nucleus and binds to the *Ecm1* gene promoter to maintain its expression. In damaged hepatocytes of diseased livers **(right panel)**, inflammation driven upregulated IFNγ signals in hepatocytes via the IFNGR and intercepts the EGF signaling pathway (1) through downregulating EGFR expression, (2) through Y701 phosphorylation of STAT1, which inhibits binding of S727-phosphorylated STAT1 to the *Ecm1* gene promoter, and (3) through induction of NRF2 nuclear accumulation, which directly binds to and negatively regulates the *Ecm1* gene promoter. All three mechanisms primary lead to a reduction of ECM1 expression, and secondary to LTGF-β activation, hepatic stellate cell activation and fibrogenesis (Figure created with BioRender.com).