Loss of Gab1 adaptor protein in hepatocytes aggravates experimental liver fibrosis in mice

Takashi Kizu1#, Yuichi Yoshida1#, Kunimaro Furuta1, Satoshi Ogura1, Mayumi Egawa1, Norihiro Chatani1, Mina Hamano1, Takayo Takemura1, Hisao Ezaki1, Yoshihiro Kamada1, Keigo Nishida2†, Yoshikazu Nakaoka3, Shinichi Kiso1, and Tetsuo Takehara1$  

1Department of Gastroenterology and Hepatology, Osaka University, Graduate School of Medicine 2-2 Yamadaoka, Suita, Osaka, Japan, 2Laboratory for Homeostatic Network, RCAI, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Yokohama, Kanagawa, Japan, 3Department of Cardiovascular Medicine, Osaka University, Graduate School of Medicine 2-2 Yamadaoka, Suita, Osaka, Japan  

# Takashi Kizu and Yuichi Yoshida contributed equally to this work.  
† Present address: Laboratory of Immune Regulation, Faculty of Pharmaceutical Sciences, Suzuka, University of Medical Science, Suzuka, Mie, Japan  
$Address correspondence to Tetsuo Takehara, takehara@gh.med.osaka-u.ac.jp  

Short Running Head: Role of hepatocyte Gab1 during liver fibrosis
Abbreviations: ALP, alkaline phosphatase; α-SMA, alpha-smooth muscle actin; BDL, bile duct ligation; CCL5, chemokine (CC motif) ligand 5; ECM, extracellular matrix; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; RTK, receptor tyrosine kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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Abstract

Grb2-associated binder 1 (Gab1) adaptor protein amplifies signals downstream of a broad range of growth factors/receptor tyrosine kinases. Although these signals are implicated in liver fibrogenesis, the role of Gab1 remains unclear. To elucidate the role of Gab1, liver fibrosis was examined in hepatocyte-specific Gab1-conditional knockout (Gab1CKO) mice upon bile duct ligation (BDL). Gab1CKO mice developed exacerbated liver fibrosis with activation of hepatic myofibroblasts after BDL compared with control mice. The anti-fibrotic role of hepatocyte Gab1 was further confirmed by another well-established mouse model of liver fibrosis using chronic injections of carbon tetrachloride. After BDL, Gab1CKO mice also displayed exacerbated liver injury, decreased hepatocyte proliferation, and enhanced liver inflammation. Furthermore, cDNA microarray analysis was used to investigate the potential molecular mechanisms of the Gab1-mediated signal in liver fibrosis, and the fibrosis-promoting factor chemokine (C-C motif) ligand 5 (Ccl5) was identified as up-regulated in the livers of Gab1CKO mice following BDL. Interestingly, in vitro studies using primary hepatocytes isolated from control and Gab1CKO mice revealed that the loss of Gab1 resulted in increased hepatocyte CCL5 synthesis upon lipopolysaccharide stimulation. Finally, pharmacological antagonism of CCL5 reduced BDL-induced liver fibrosis in
Gab1CKO mice. In conclusion, our results demonstrate that hepatocyte Gab1 is required for liver fibrosis and that hepatocyte CCL5 could be an important contributor to this process. Thus, we present a novel anti-fibrotic function of hepatocyte Gab1 in liver fibrogenesis.

**Keywords:** bile duct ligation; CCL5; cholestasis; Gab1; liver fibrosis
Introduction

Liver fibrosis is a major medical issue worldwide because it can progress to liver cirrhosis accompanied by severe architectural distortion and eventually cause significant mortality and morbidity, including liver failure and hepatocellular carcinoma (3, 9, 32). During the progression of liver fibrosis, various growth factors are involved in liver fibrogenesis and fibrolysis (14, 28, 37, 40, 43, 44). To date, several studies have demonstrated protective roles of hepatocyte growth factor (HGF) against liver fibrogenesis (43, 44). In contrast to HGF, the effects of epidermal growth factor (EGF) family members on liver fibrosis have been shown to differ from one another in a mouse model of liver fibrosis (14, 28, 40). We and others recently showed that deletion of heparin-binding EGF (HB-EGF) enhances liver fibrosis (14, 40). In contrast, deletion of amphiregulin, another member of the EGF family, has been reported to improve hepatic fibrogenesis (28). Although these growth factors are implicated in liver fibrosis, the downstream signaling in hepatocytes remains poorly understood.

Grb2-associated binder 1 (Gab1) is a scaffolding adaptor protein that contains a PH domain and potential binding sites for SH2 and SH3 domains (12, 24, 27, 39). Gab1 is tyrosine-phosphorylated through the activation of receptor tyrosine kinases (RTKs) in response to various growth factors (12, 24, 27, 45). We previously demonstrated that conventional Gab1 knock out (KO) mice display embryonic death with developmental impairment of the heart, placenta and skin (15). These phenotypes of conventional Gab1 KO mice are similar to those observed in mice lacking signals of HGF/c-Met (5, 31) or EGF family/EGF receptor (EGFR) (21, 22, 42). These findings indicate that Gab1 amplifies the in vivo downstream signaling of a broad range of
growth factors/RTKs. It could be hypothesized that because these various growth
factors/RTKs are implicated in liver fibrosis, hepatocyte Gab1 might have a possible
role in regulating liver fibrosis. However, until now, the role of hepatocyte Gab1 during
liver fibrosis has remained unclear.

Here, we investigated the role of hepatocyte Gab1 during liver fibrosis using a
genetic approach and identified the molecular mechanisms by which Gab1 affects liver
fibrosis using mouse models.
Material and Methods

Mice. We previously described a generation of mice carrying a Gab1 gene with 2 loxP sequences flanking exon 2 (Gab1\textsuperscript{floxflo}x) C57BL/6 mice (25). Hepatocyte-specific Gab1 conditional knock-out (Gab1CKO) mice were generated by crossing Gab1\textsuperscript{floxflo}x C57BL/6 mice with albumin promoter-driven Cre recombinase (Alb-Cre) transgenic C57BL/6 mice (Jackson Labs). All the animal experiments were approval by the Animal Care and Use Committee of Osaka University Medical School and were conducted according to institutional guidelines.

Polymerase chain reaction analysis for genotyping. Genomic DNA was extracted from mouse tails and subjected to polymerase chain reaction (PCR) to genotype the Gab1\textsuperscript{flo}x and Cre transgene. The primers used for the PCR of genomic DNA from mouse tails were as follows: Gab1\textsuperscript{flo}x: 5’- GCGCCTTCTTTGCATCACCTCATCT-3’ and 5’-GGTAAAGCAGGTCTAGGTGGCTGACAGTCT -3’; Cre: 5’-GGTAAAGCAGGTCTAGGTGGCTGACAGTCT -3’; Cre: 5’-GCGGCATGGCTAGGTGGCTGACAGTCT -3’ and 5’-CGTTCACCAGGCATCAACGTCTT -3’.

Bile duct ligation. Control and Gab1CKO mice were subjected to bile duct ligation (BDL) or sham laparotomy (Sham) as described previously (10). Briefly, after mid-line laparotomy, the common bile duct was mobilized and ligated twice with 5-0 silk sutures.
and dissected between the ligatures in the BDL group. The operation was performed in
the Sham group as in the BDL group, but without the ligation. At 5 or 10 days after
BDL, the animals were sacrificed for further analysis.

**Carbon tetrachloride-induced liver fibrosis model.** For the toxin-induced liver fibrosis
model, we used chronic administration of carbon tetrachloride (CCl₄) (34). Control and
Gab1CKO mice were injected intraperitoneally with CCl₄ (0.5 ml/kg bw) or oil twice a
week for 6 weeks and sacrificed at 24 hours after final injection for further analysis.

**Determination of liver hydroxyproline content.** Wet liver samples (200 mg) were
lyophilized at -40°C for 48 hours. Freeze-dried liver samples were hydrolyzed in 6N
HCl at 100°C for 6 hours. After filtration using a 0.22-μm membrane, 300 μl of the
filtered solution was mixed with 5 μl of 1% phenolphthalein and 290–310 μl of 6N
NaOH until the indicator turned brown in color (pH 7–8). Forty milliliters of this
solution was mixed with 25 ml of Chloramine-T solution containing 0.56 mM
Chloramin-T (Sigma-Aldrich, Saint Louis, MO) and 10% 2-propanol in Chloramin-T
buffer (50 g/l citric acid-monohydrate, 12 g/l acetic acid, 120 g/l sodium
acetate-trihydrate and 34 g/l sodium hydroxide). After allowing this solution to stand for
20 min at room temperature, 150 μl of Ehrlich’s solution (2 g
p-dimethylaminobenzaldehyde, 3 ml of 70% perchloric acid, and 9 ml of 2-propanol)
was added and the solution was incubated for 20 min at 65°C and another 5 min at room temperature. Absorbance was measured at 570 nm. The results are expressed as micrograms of hydroxyproline per gram of wet liver.

_Histological analysis._ Liver tissues were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned (4-μm thick) and stained with hematoxylin and eosin (H&E). The number of areas of oncogenic necrosis was counted per view field at 100× magnification. Hepatocyte apoptosis was analyzed using a transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining kit (Millipore, Molsheim, France) according to the manufacturer’s protocol. The number of TUNEL-positive hepatocytes per view field at 200× magnification was counted. To determine the proliferation rates of hepatocytes, liver sections were stained with anti-Ki67 antibody (1:1000; DAKO). The number of Ki67-positive hepatocytes per view field at 200× magnification was counted. To assess intrahepatic macrophage accumulation, liver sections were stained with anti-CD68 antibody (1:100, Serotec, Oxford, UK). Liver fibrosis was evaluated by measuring hepatic collagen deposition with picrosirius red staining. The fibrosis area stained by picrosirius red per view field at 200× magnification was quantified using image analysis software (Photoshop; Adobe Systems, San Jose, CA). Activation of hepatic stellate cells was assessed by
immunohistochemical staining with anti-α-SMA antibody (1:200; Abcam, Cambridge, MA). To evaluate the expression of CCL5 protein, liver sections were stained with anti-CCL5 antibody (1:500; BD, Heidelberg, Germany). For immunohistochemical staining, antibody-antigen complexes were detected with a VECTASTAIN ABC kit (Vector Lab, Burlingame, CA) according to the manufacturer’s protocol. A minimum of three field views per tissue per mouse was counted for each analysis.

Isolation and culture of mouse primary hepatocytes and non-parenchymal cells.

Hepatocytes and non-parenchymal cells (NPCs) were isolated from control and GabCKO mice by two-step pronase-collagenase perfusion of mouse liver as previously described (20). Briefly, after inserting a cannula into the portal vein and cutting the inferior vena cava, the mouse liver was perfused with pre-perfusion solution for 5 min to remove mouse blood and protect hepatocytes. For digestion of mouse liver, the tissue was perfused with 53% pronase solution and 0.27% collagenase solution for 1 min and 4 min, respectively. All solutions were warmed to 37°C before use and perfused at a flow rate of 4 ml/min. The perfused mouse liver was transferred into Dulbecco’s modified Eagle medium containing 10% fetal calf serum and minced to release the hepatocytes. The suspension was filtered through a 100-μm membrane and washed three times with centrifugation at 50× g for 1 min at 4°C. After aspiration of the
supernatant, the cell pellet was suspended in William’s medium E with 10% fetal calf serum. Hepatocytes with viability of 90% or higher, as determined by trypan blue exclusion, were used for experiments. Hepatocytes (25,000 cells/cm²) were seeded on collagen-coated culture plates in William’s medium E with 10% fetal calf serum, 2 mM L-glutamine, $10^{-7}$ M insulin, $10^{-7}$ M dexamethasone, 100 U/ml penicillin, and 100 U/ml streptomycin. Hepatocytes were allowed to attach to plates overnight, and prior to treatment the cell culture media was changed to an insulin-free media. Isolated hepatocytes were stimulated with hepatocyte growth factor (HGF; Sigma-Aldrich) for 15 min or with 50 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich) for 24 hours, and then hepatocytes or the supernatant were harvested for further analysis.

**Western blot analysis.** Mouse liver samples or cells were homogenized or scraped off in RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, phosphatase inhibitor mixture, and complete protease inhibitor mixture (Roche Applied Science, Basel, Switzerland). Western blot analysis was performed as described previously (40). The following antibodies were used for immunodetection: anti-phospho-Met (Tyr 1234), anti-Met, anti-phospho-ERK1/2 (Thr 202/Tyr 204), anti-ERK1/2, anti-phospho-Gab1 (Tyr 627), and anti-Gab1, purchased from Cell Signaling Technology (Danvers, MA), and
anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), purchased from Trevigen Inc. (Gaithersburg, MD).

**Real-time reverse-transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted using a QIAshredder and an RNA-easy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Quantitative real-time RT–PCR was carried out using the QuantiFast SYBRGreen RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The Quantitect gene assay kit (Qiagen, Hilden, Germany) was used for analysis of mouse *Col1a1, Col1a2, Acta2, TGF-β1, IL-6, Ccl2, CD68, Ccl5,* and *GAPDH.* The primers 5’-AAACGATCATGCCAAGAAGC-3’ and 5’-TAGAAAATCCGGTCGATGGT-3’ were used to amplify and quantify *Gab1* mRNA. The level of gene expression for each sample was normalized to *GAPDH* mRNA expression using the comparative cycle threshold (Ct) method.

**Microarray analysis.** Gene expression in the mouse livers following BDL was analyzed using an Affymetrix Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA). Control and Gab1CKO mice were subjected to BDL or sham laparotomy and sacrificed. Total RNA was extracted from the livers of control and Gab1CKO mice using a QIAshredder and an RNA-easy Mini kit (Qiagen, Hilden, Germany) according to the
manufacturer’s protocol. Total RNA samples from livers of three mice per experimental group were pooled and used for microarray analysis. Samples were hybridized on Affymetrix GeneChip Mouse Genome 430 2.0 arrays in the RIKEN Research Center for Allergy and Immunology. To identify the genes involved in liver fibrosis in Gab1CKO mice, we used a heatmap indicating the top 20 of the 787 genes and selected the genes that had greater than two-fold induction in livers of Gab1CKO mice after BDL compared with control mice after BDL.

**Transcript profiling.** The microarray data can be accessed at RCAI RefDIC (URL: [http://refdic.rcai.riken.jp/welcome.cgi](http://refdic.rcai.riken.jp/welcome.cgi)) under the following accession numbers: RSM10654, RSM10655, RSM10656, and RSM10657.

**Enzyme-linked immunosorbent assay (ELISA).** The expression levels of CCL5 in liver tissue and cultured supernatant were measured using a Mouse CCL5/RANTES Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol.

**Detection of NF-κB activity.** Murine normal hepatocyte BNL.CL2 cells (ATCC, Manassas, VA) were transfected with 10 nM of either siGab1 or siControl using LipofectamineTM RNAiMAX reagent (Invitrogen, Carlsbad, CA) according to the
manufacturer’s instructions for reverse transfection. After 24 hours of incubation, these
cells were stimulated with 50 ng/ml of LPS (Sigma-Aldrich) for 60 min, and then
nuclear protein was extracted to measure NF-κB activity. NF-κB activity was assessed
by the p65 DNA binding activity using a TransAM™ NF-κB Kit (Active Motif,
Carlsbad, CA) according to the manufacturer’s protocol.

**CCL5 antagonist treatment.** Control and Gab1CKO mice were subjected to BDL and
treated daily until sacrifice with intraperitoneal injections of either phosphate-buffered
saline (PBS) or CCL5-antagonist (10 μg/mouse; Bachem, Philadelphia, PA), a selective
antagonist for CCL5 receptors (29). The mice were divided into groups of eight for each
treatment (PBS-treated control, CCL5-antagonist-treated control, PBS-treated
Gab1CKO, and CCL5-antagonist-treated Gab1CKO). Animals were sacrificed 10 days
after the BDL procedure.

**Statistical analysis.** Values for all measurements are presented as the mean ± standard
error of the mean (SEM). We used either the Student’s t test or Tukey HSD test to
determine statistical significance and considered p values of <0.05 as statistically
significant.
Results

*Generation of hepatocyte-specific Gab1 conditional knock out mice.* Because the targeted disruption of mouse Gab1 results in embryonic death (15), we generated hepatocyte-specific Gab1CKO mice by crossing Gab1\textsuperscript{fl/fl} C57BL/6 (control) mice with hepatocyte-specific albumin promoter-driven Cre recombinase transgenic C57BL/6 mice (Fig. 1A). Western blot analysis showed that the expression level of Gab1 was dramatically decreased in the liver of Gab1CKO mice but not in other organs (Fig. 1B). We also demonstrated that the deficiency of Gab1 was specific to hepatocytes and was not observed in NPCs (Fig. 1C). Furthermore, we showed that the HGF-induced tyrosine phosphorylation of Gab1 was lost in cultured hepatocytes from Gab1CKO mice (Fig. 1D). The functional effect of this deletion was confirmed by demonstrating that HGF-induced activation of ERK, a downstream target of Gab1 (39), was exclusively attenuated in Gab1CKO hepatocytes (Fig. 1D).

*Involvement of Gab1 during liver fibrosis after BDL.* To investigate the role of Gab1 during liver fibrosis, we first examined the phosphorylation of Gab1 in the livers after BDL. Western blot analysis showed that Gab1 was tyrosine phosphorylated in the livers...
of control mice but not that of Gab1CKO mice after BDL (Fig. 2). These results indicated the possible involvement of Gab1 during liver fibrosis.

**Hepatocyte Gab1 is required for the protection against liver fibrosis progression after BDL.** To elucidate the role of Gab1 in liver fibrosis, we performed BDL or a sham operation (Sham) in control and Gab1CKO mice. Ten days after BDL, Gab1CKO mice displayed a significant increase in the areas of liver fibrosis between portal tracts compared with control mice (Figs. 3A and B). Gab1CKO mice also showed a significant increase in liver fibrosis compared with control mice 14 days after BDL (data not shown). The enhanced liver fibrosis in the Gab1CKO mice was confirmed by significantly increased levels of hepatic hydroxyproline in the livers of Gab1CKO mice compared with control mice (Fig. 3C). Furthermore, the mRNA expression levels of profibrogenic markers, such as *Col1α1, Col1α2, TGF-β1*, and *Acta2*, were significantly increased in the livers of Gab1CKO mice compared with control mice (Fig. 3D). Consistent with the *Acta2* mRNA result, immunohistochemical analysis confirmed that Gab1CKO mice exhibited a clear increase in α-SMA-positive areas. This result indicates enhanced activation of hepatic myofibroblasts, which are major producers of fibrotic extracellular matrix (ECM; Fig. 3E). These results indicate that hepatocyte Gab1 has a protective role against liver fibrosis progression.
Enhanced liver fibrosis in Gab1CKO mice after the chronic administration of carbon tetrachloride. To further confirm the role of Gab1 in liver fibrosis using another well-established mouse model of liver fibrosis, we chronically administered carbon tetrachloride (CCl₄) to control and Gab1CKO mice. After 6 weeks of CCl₄ administration, Gab1CKO mice showed significant increases in Sirius red-positive areas and hepatic hydroxyproline content compared with WT mice (Figure 4A-C). Quantitative real-time RT-PCR analysis also demonstrated a significant increase in the mRNA expression level of profibrogenic markers, such as Col1α₁, Col1α₂, TGF-β, and Acta2 in KO mice compared with WT mice (Figure 4D). Furthermore, KO mice exhibited enhanced activation of hepatic myofibroblasts, as demonstrated by an increase in α-SMA-positive areas (Figure 4E). These data confirmed the anti-fibrotic role of hepatocyte Gab1 in liver fibrosis.

Gab1CKO mice showed exacerbation of liver injury, impaired hepatocyte proliferation, and enhanced liver inflammation after BDL. Because oncotic necrosis and apoptosis are characteristic features of hepatocellular injury following BDL (10), we evaluated liver injury by quantifying oncotic necrotic areas and hepatocyte apoptosis in liver sections. Five days after BDL, Gab1CKO mice showed a significant increase in the number of oncotic necrotic areas and apoptotic hepatocytes compared with control
mice (Figs. 5A-C). The enhanced liver injury observed in Gab1CKO mice was confirmed by increased levels of serum alanine transaminase (ALT; Fig. 5D). Furthermore, we demonstrated that cholestasis was unchanged between control and Gab1CKO mice, as evidenced by the serum levels of total bilirubin and alkaline phosphatase (ALP; Fig. 5D). These data indicate that loss of Gab1 in hepatocytes affected sensitivity of liver injury induced by cholestasis. We also assessed hepatocyte proliferation in control and Gab1CKO mice following BDL because surviving hepatocytes undergo a complementary proliferative response during cholestasis (10). Staining of liver sections with Ki67 indicated a decreased number of proliferating hepatocytes in Gab1CKO mice compared with control mice (Fig. 5E). These data suggested that hepatic loss of Gab1 resulted in enhanced liver injury with decreased complementary hepatocyte proliferation during cholestasis.

Macrophages are known to play a major role in liver inflammation during the progression of liver fibrosis (35). We therefore examined macrophage infiltration in the livers of control and Gab1CKO mice by immunohistochemical staining for CD68, which is a surface marker for macrophages. After BDL, Gab1CKO mice exhibited a clear increase in the number of CD68-positive cells in the liver compared with control mice (Fig. 5F). Quantitative real-time RT-PCR analysis also confirmed a significant
increase in the hepatic mRNA expression levels of *CD68* in Gab1CKO mice (Fig. 5G).

Furthermore, the mRNA expression levels of the pro-inflammatory cytokine *IL-6* and the pro-inflammatory chemokine *Ccl2* were also significantly higher in the livers of Gab1CKO mice (Fig. 5G). These data indicated that the hepatocyte-specific deletion of Gab1 led to an enhancement of liver inflammation during cholestasis.

**Microarray analysis identifies up-regulation of CCL5 gene in the livers of Gab1CKO mice during fibrosis after BDL.** To investigate Gab1-mediated downstream signaling during the progression of liver fibrosis, we next performed cDNA microarray analysis using total RNA from the livers of control and Gab1CKO mice 10 days after BDL. cDNA microarray analysis revealed the induction of 787 genes in the livers of Gab1CKO mice compared with control mice after BDL. Among these genes, *Ccl5* (also known as RANTES) was identified as strongly up-regulated only in the livers of Gab1CKO mice after BDL (Fig. 6A). We focused on CCL5 because it has been shown to have fibrosis-promoting activity (4). Further validation by quantitative real-time RT-PCR demonstrated an approximate 5-fold increase in *Ccl5* mRNA levels in Gab1CKO mice when compared with control mice 10 days after BDL (Fig. 6B). To identify the hepatic cells in which CCL5 was up-regulated in Gab1CKO mice, we isolated hepatocytes and NPCs by two-step pronase collagenase-perfusion of the livers.
of control and Gab1CKO mice 10 days after BDL. Ccl5 gene expression was significantly higher in the hepatocytes of Gab1CKO mice than in the hepatocytes of control mice (Fig. 6C). Conversely, Ccl5 gene expression in NPCs did not significantly differ between control and Gab1CKO mice (Fig. 6D).

**Loss of Gab1 up-regulates synthesis of CCL5 protein in hepatocytes during liver fibrosis after BDL.** Staining of liver sections with CCL5 revealed increased CCL5 protein expression in the hepatocytes of Gab1CKO mice compared with control mice (Fig. 7A). Quantification of the immunohistochemical staining confirmed that the number of CCL5-positive hepatocytes in Gab1CKO mice was significantly higher than that in control mice (Fig. 7B). In contrast, the number of CCL5-positive cells in NPCs did not differ between the two groups after BDL (Fig. 7C). To assess the CCL5 protein expression level in a more quantitative manner, we measured the concentration of hepatic CCL5 by ELISA and confirmed that CCL5 expression was significantly higher in the livers of Gab1CKO mice than in those of control mice 10 days post-BDL (Fig. 7D). These findings suggest that hepatic deletion of Gab1 up-regulates protein synthesis of CCL5 in hepatocytes at the fibrosis stage after BDL.
Loss of Gab1 in hepatocytes enhances NF-κB activation and CCL5 synthesis in hepatocytes in vitro. To confirm the in vivo data showing CCL5 up-regulation in the livers of Gab1CKO mice after BDL, we performed in vitro experiments using primary mouse hepatocytes isolated from control and Gab1CKO mice. Because it has been reported that LPS is involved in the progression of liver fibrosis (35), we stimulated primary hepatocytes from control and Gab1CKO mice with LPS for 24 hours and analyzed CCL5 gene and protein expression. Quantitative real-time RT-PCR demonstrated significantly higher Ccl5 mRNA expression in Gab1CKO hepatocytes than in control hepatocytes (Fig. 8A). Likewise, CCL5 protein expression in the culture supernatants of Gab1CKO hepatocytes was significantly increased compared with control hepatocyte culture supernatants (Fig. 8B). Because CCL5 gene expression is regulated by transcription factors, including NF-κB (26), we further examined the role of Gab1 on LPS-induced NF-κB activation using a normal murine hepatocyte cell line, BNL.CL2, in vitro. Consistent with the in vivo data, siRNA-mediated depletion of Gab1 enhanced LPS-induced NF-κB activation in BNL.CL2 cells (Figs. 8C and D). These data indicate that loss of Gab1 stimulates NF-κB signaling and subsequent CCL5 synthesis from hepatocytes in vitro.
Pharmacological antagonism of CCL5 reduces liver fibrosis after BDL in Gab1CKO mice. To clarify the functional role of CCL5 over-expression, control and Gab1CKO mice were subjected to daily intraperitoneal injections of a pharmacological CCL5 antagonist (29) or PBS after BDL. Ten days after BDL, the daily administration of the CCL5 antagonist had significantly improved liver fibrosis in Gab1CKO mice, as demonstrated by measurements of Sirius red-positive areas, compared with the PBS-treatment (Figs. 9A and B). This improvement in liver fibrosis in the Gab1CKO mice upon daily administration of the CCL5 antagonist was confirmed by the measurement of hepatic hydroxyproline content (Fig. 9C). These data indicate that hepatic over-expression of CCL5 in Gab1CKO mice functioned to exacerbate liver fibrosis.
Discussion

To our knowledge, the present study is the first to reveal that hepatocyte Gab1 plays a crucial role in liver fibrosis. In this study, we demonstrated that the hepatocyte-specific deletion of Gab1 resulted in enhanced liver fibrosis in a mouse model of cholestasis. We also observed similarly enhanced liver fibrosis in hepatocyte-specific Gab1-KO mice in a model of liver fibrosis induced using the chronic administration of the toxin carbon tetrachloride (Fig. 4A-E), suggesting that Gab1 plays an anti-fibrotic role in hepatocytes irrespective of the etiology of the chronic liver disease.

Gab1 belongs to the Gab/DOS (Daughter of Sevenless) family of adaptor molecules (12, 24, 27), and the Drosophila homolog of Gab1, DOS, has been shown to act downstream of several RTKs, such as Sevenless, Torso, and the EGF receptors (30). Like DOS, Gab1 has also been shown to be tyrosine phosphorylated in response to growth factors/RTKs and is able to amplify signals downstream (25, 27, 45). In this study, we demonstrated that Gab1 was tyrosine phosphorylated in response to the induction of liver fibrosis (Fig. 2). Indeed, we observed that BDL induced the gene expression of several ligands for RTKs in our model, including HGF and EGF family
members (data not shown). These findings indicate that growth factor/RTK/Gab1-mediated signals are involved in the progression of liver fibrosis.

To investigate the potential molecular mechanisms of the Gab1-mediated regulation of liver fibrosis in hepatocytes, we performed cDNA microarray analysis using total RNA obtained from the livers of control and Gab1CKO mice 10 days after BDL. We found that several genes, including Gbp2(11), Fam26f(8), Igtp(41), Ly6a(13), and CCL5(1), were up-regulated in the livers of Gab1CKO mice. Among these genes, the functions of Gbp2, Fam26f, Igtp, and Ly6a in liver fibrosis have remained unclear to date (6-8, 11, 13, 17, 19, 41). Our validation study revealed no statistically significant difference in the expression of these genes between control and Gab1CKO mouse livers 10 days after BDL (data not shown). In contrast, the gene expression of CCL5 was statistically significantly up-regulated only in the livers of Gab1CKO mice after BDL (Fig. 6A, B). CCL5 (also known as RANTES) belongs to the CC chemokine family, which has powerful chemoattractant effects on a variety of cell types (1). Importantly, in experimental mouse models, CCL5 and its receptors have been shown to promote liver fibrosis by increasing the migration, proliferation and ECM production of hepatic stellate cells (4, 34). In this study, we demonstrated that Ccl5 gene expression was increased in the hepatocytes of Gab1CKO mice but not in NPCs after BDL (Fig 6C, D).
Our immunohistochemical analysis and *in vitro* studies using primary hepatocytes isolated from control and Gab1CKO mice also showed that loss of Gab1 in hepatocytes upregulated CCL5 synthesis after BDL (Fig. 7A-D). These data suggest that hepatocytes are the main source of CCL5 in our Gab1CKO mice. In contrast, a previous report showed that the main source of CCL5 in the liver was considered to be NPCs (4).

Indeed, we detected CCL5-positive NPCs in control mice and Gab1CKO mice, but the CCL5-positive cells in the liver of Gab1CKO mice were primarily hepatocytes (Fig. 7A-C). Consistent with this finding, recent publications have also demonstrated that CCL5 is produced from hepatocytes in mice and humans (23, 33). In addition, our results are in accordance with another study using hepatocyte-specific c-Met KO mice (23) in which Marquardt et al. performed cDNA microarray analysis of hepatocytes isolated from WT and c-Met-KO livers during the early fibrotic response and identified *Ccl5* as an up-regulated gene in the hepatocytes of c-Met-KO mice (23). Together, these findings support that an increase in hepatocyte CCL5 synthesis is involved in the enhanced liver fibrosis observed in our Gab1CKO mice.

CCL5 expression is known to be regulated by transcription factors, including NF-κB (26). In addition, a previous study provided an important link between hepatocyte NF-κB activation and liver fibrosis development (38). Furthermore, a very
recent report demonstrated an important role of hepatocyte Toll-like receptor 4 (TLR4) in obesity-induced inflammation in mice (16). Based on these findings, we performed in vitro experiments using primary hepatocytes isolated from control and Gab1CKO mice to investigate the possibility of a molecular link between the deletion of Gab1 and abnormal CCL5 synthesis in hepatocytes. To activate the TLR4-NF-κB pathway in hepatocytes, we stimulated primary hepatocytes from control and Gab1CKO mice in vitro with LPS, a ligand for TLR4, and analyzed the gene and protein expression levels of CCL5. Consistent with the in vivo data, the gene and protein expression levels of CCL5 in response to LPS were significantly increased in Gab1CKO hepatocytes (Fig. 8A, B). As expected, siRNA-mediated depletion of Gab1 resulted in enhanced LPS-induced NF-κB activation in mouse hepatocyte cell lines (Fig. 8C, D). In addition, we confirmed that gene expression of other potential NF-κB targets, including TNF-α and IL-6, was increased in hepatocytes of Gab1CKO mice stimulated with LPS when compared with control mice (data not shown). Considering these findings, our in vitro data indicate that loss of hepatocytes Gab1 enhances NF-κB activation and increases subsequent CCL5 production in hepatocytes during liver fibrosis, leading to the exacerbation of liver fibrosis. Several factors might damage the hepatocytes during liver fibrosis in vivo. For example, serum bile acid levels are reported to be elevated in
patients with chronic hepatitis C and positively correlated with the degree of liver fibrosis (36), so factors including bile acid other than LPS might damage hepatocytes and then stimulate expression of CCL5 from Gab1-deficient hepatocytes \textit{in vivo}. Further study is needed to shed light on this issue.

Finally, to elucidate the functional role of abnormal CCL5 over-expression, we administered a pharmacological CCL5 antagonist during fibrosis progression in Gab1CKO mice after BDL. As expected, administration of the CCL5 antagonist significantly improved liver fibrosis in Gab1CKO mice but not in control mice after BDL (Fig. 9A-C). This result might be caused by the difference in the induction of CCL5 expression between Gab1CKO mice and control mice after BDL (Fig. 6B). In contrast, the CCL5 antagonist did not affect liver injury as assessed by areas of oncostic necrosis, apoptotic hepatocytes, and serum ALT levels in Gab1CKO mice (data not shown). These data indicate that over-expression of hepatocyte CCL5 in our Gab1CKO mice functioned to exacerbate liver fibrosis. In addition, our data showed that antagonism of CCL5 did not completely abolish the exacerbation of liver fibrosis observed in Gab1CKO mice, suggesting that there are other possible mechanisms to explain the enhanced liver fibrosis (Fig 9A-C). Because Gab1CKO mice showed exacerbated liver injury, inflammation, and decreased hepatocyte proliferation after
BDL (Fig. 5A-G), the genes related with the aforementioned pathways, in addition to CCL5, might contribute to exacerbation of liver fibrosis. Collectively, as CCL5 has been reported to be increased in patients with various chronic liver diseases (4, 18), the novel regulation of CCL5 through hepatocyte Gab1 is clinically relevant.

In conclusion, we provide evidence indicating that hepatocyte Gab1 regulates liver fibrosis in mice. Our findings also suggest that hepatocyte CCL5 could be an important contributor to enhanced liver fibrosis in our hepatocyte-specific Gab1CKO mice. Therefore, our present study provides a novel function of hepatocyte Gab1 in liver fibrosis; that is, in addition to hepatoprotective potentials (2), Gab1 has a protective role against liver fibrosis by regulating hepatic CCL5 production. As Gab1 amplifies signals downstream of a broad range of growth factors/RTKs, the results observed in our hepatocyte-specific Gab1CKO mice also highlight the important role of the signaling of growth factors/RTKs in hepatocytes during liver fibrosis. Taken together, our current data indicate that Gab1 may be a potential target for the treatment of liver fibrosis.

Acknowledgement: The authors thank Dr. Toshio Hirano (Osaka University) for helpful discussions and critical review of this paper.
Figure Legends

Figure 1. Generation of hepatocyte-specific Gab1CKO mice.

(A) Schematic representation of mouse *Gab1 WT, Gab1 flox, Gab1-deleted* alleles and targeting vectors. LoxP sequences are indicated as black triangles. (B) Western blot analysis of Gab1 protein in the liver, spleen, and heart of control and Gab1CKO mice. (C) Western blot analysis of Gab1 protein in hepatocytes and non-parenchymal cells of control and Gab1CKO mice. (D) Tyrosine-phosphorylation of c-Met, Gab1 and ERK1/2 induced by HGF in hepatocytes of control and Gab1CKO mice.

Figure 2. Involvement of Gab1 during liver fibrosis after BDL.

Tyrosine-phosphorylation of Gab1 in the livers of control and Gab1CKO mice 5 and 10 days after BDL.

Figure 3. Enhanced progression of liver fibrosis in hepatocyte-specific Gab1CKO mice after BDL.

(A) Representative views of Sirius red staining in liver sections 10 days after BDL (n=9 control, n=10 Gab1CKO) or Sham (n=5 control, n=5 Gab1CKO). Top panel, Sham; middle panel, BDL; bottom panel, BDL (enlarged view of boxed region in middle panel). Scale bars: 400 μm. (B) Quantification of Sirius red-positive areas in liver
sections. (C) Determination of hydroxyproline content in livers. (D) Gene expression of Col1a1, Col1a2, TGF-β1, and Acta2 in livers. (E) Representative views of α-SMA staining in liver sections. Top panel, Sham; middle panel, BDL; bottom panel, BDL (enlarged view of boxed region in middle panel). Scale bars: 200 μm. Data are the mean±SEM, *p< 0.05, **p< 0.01.

**Figure 4. Enhanced progression of liver fibrosis in hepatocyte-specific Gab1CKO mice after the chronic administration of carbon tetrachloride.**

(A) Representative views of Sirius red staining in liver sections of control and Gab1CKO mice after the chronic administration of carbon tetrachloride (CCl4) and oil. Control and Gab1CKO mice were subjected to intraperitoneal injections of CCl4 (0.5 ml/kg bw, n=10 control, n=11 Gab1CKO) or oil (n=5 for control, n=5 for Gab1CKO) twice per week for 6 weeks and were sacrificed 24 hours after the final injection. Top panel, oil; middle panel, CCl4; bottom panel, CCl4 (enlarged view of boxed region in middle panel). (B) Quantification of Sirius red-positive areas in liver sections. (C) Determination of hydroxyproline content in the liver. (D) Gene expression of Col1a1, Col1a2, TGF-β1, and Acta2 in livers. (E) Representative views of α-SMA staining in liver sections. Top panel, oil; middle panel, CCl4; bottom panel, CCl4 (enlarged view of boxed region in middle panel). Scale bars: 200 μm (A and E). Data are the mean±SEM,
*p< 0.05, **p< 0.01.

**Figure 5. Exacerbation of liver injury, impaired hepatocyte proliferation and enhancement of liver inflammation in hepatocyte-specific Gab1CKO mice after BDL.**

(A) Representative H&E staining of liver sections 5 days after BDL (n=9 control, n=11 Gab1CKO) or Sham (n=5 control, n=5 Gab1CKO). Scale bars: 400 μm. (B) Quantification of oncotic necrotic areas in liver sections. (C) Quantification of the number of TUNEL-positive hepatocytes in liver sections. (D) Serum levels of ALT, total bilirubin and ALP. (E) Quantification of the number of Ki67-positive hepatocytes in liver sections. (F) Representative CD68 staining of liver sections. Scale bars: 100 μm. (G) Gene expression of CD68, IL-6, and Ccl2 in livers. Data are the mean±SEM, *p< 0.05, **p< 0.01, n.s.: not significant.

**Figure 6. Microarray analysis reveals up-regulation of CCL5 gene in the livers of hepatocyte-specific Gab1CKO mice after BDL.**

(A) Heat map analysis revealed up-regulation of Ccl5 mRNA in the livers of Gab1CKO mice 10 days after BDL. (B) Gene expression of Ccl5 in the livers after BDL (n=9 control, n=10 Gab1CKO) or Sham (n=5 control, n=5 Gab1CKO). (C) Gene expression of CCL5 in isolated hepatocytes. (D) Gene expression of CCL5 in isolated
non-parenchymal cells. Data are the mean±SEM, *p< 0.05, n.s.: not significant.

**Figure 7. Loss of Gab1 up-regulates protein synthesis of CCL5 in hepatocytes during liver fibrosis after BDL.**

(A) Representative CCL5 staining of liver sections 10 days after BDL (n=9 control, n=10 Gab1CKO) or Sham (n=5 control, n=5 Gab1CKO). Top panel, Sham; middle panel, BDL; bottom panel, BDL (enlarged view of boxed region in middle panel). Scale bars: 50 μm. (B) Quantification of CCL5-positive hepatocytes. (C) Quantification of CCL5-positive non-parenchymal cells. (D) Quantification of CCL5 protein levels in whole liver extracts. Data are the mean±SEM, *p< 0.05, **p< 0.01, n.s.: not significant.

**Figure 8. Loss of Gab1 in hepatocytes enhances NF-κB activation and CCL5 synthesis in hepatocytes in vitro.**

(A) Gene expression of Ccl5 in cultured primary hepatocytes from control and Gab1CKO mice in response to LPS (n=8 per group). (B) Secretion of CCL5 protein in the supernatant of cultured primary hepatocytes. (C) Activation of NF-kB activation in Gab1-depleted BNL.CL2 cells in response to LPS (n=4 per group). (D) Western blot confirms the depletion of Gab1 in siRNA-treated BNL.CL2 cells. Data are the mean±SEM, **p< 0.01.
Figure 9. CCL5 antagonist reduces liver fibrosis after BDL in hepatocyte-specific Gab1CKO mice.

(A) Representative views of Sirius red staining in liver sections of control and Gab1CKO mice 10 days after BDL and treatment with PBS or a CCL5 antagonist. Control and Gab1CKO mice were subjected to BDL and were administered PBS or a CCL5 antagonist daily (n=8 for PBS-treated control, n=8 for CCL5 antagonist-treated control, n=8 for PBS-treated Gab1CKO, n=8 for CCL5 antagonist-treated Gab1CKO) until sacrifice. Liver fibrosis was examined 10 days after BDL. Scale bars: 400 μm. (B) Quantification of Sirius red-positive areas in liver sections. (C) Determination of hydroxyproline content in livers. Data are the mean±SEM, *p< 0.05, **p< 0.01, n.s.: not significant.
References


22. Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR.
Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 73: 249-261, 1993.


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Fig. 1.

A

\[ \text{Gab}1\text{WT} \]

\[ \text{Gab}1\text{ flox} \]

\[ \text{Gab}1\text{deleted} \]

B

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C

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Fig. 2. Time after BDL:

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- p-Gab1
- Gab1
- GAPDH
Fig. 3.

A) Control Gab1 CKO

Sham

BDL

B) Sham BDL

Cotrol Gab1 CKO

Sirius red positive area (%)

Hydroxyproline (μg/g liver tissue)

D) Col1α1 mRNA (fold changes)

Col1α2 mRNA (fold changes)

TGF-β1 mRNA (fold changes)

ACTA2 mRNA (fold changes)

E) Sham BDL

Cotrol Gab1 CKO
Fig. 4.

A

[Images of liver tissue sections for Control and Gab1 CKO treated with Oil and CCl₄, showing Sirius red positive areas and hydroxyproline levels.

B

Bar graph showing Sirius red positive area (%) for Control and Gab1 CKO treated with Oil and CCl₄.

C

Bar graph showing hydroxyproline levels (μg/g liver tissue) for Control and Gab1 CKO treated with Oil and CCl₄.

D

Graphs showing fold changes in mRNA expression for Col1α1, Col1α2, TGFβ1, and ACTA2 for Control and Gab1 CKO treated with Oil and CCl₄.

E

[Images of liver tissue sections for Control and Gab1 CKO treated with Oil and CCl₄, showing tissue changes.]
Fig. 5.

**A**

![Histological images of sham and BDL groups](image)

- **Control**
- **Gab1 CKO**

**B**

- **Necrotic areas (field)**
  - Sham: 0
  - BDL: 4

- **TUNEL positive cell count (field)**
  - Sham: 5
  - BDL: 10

**C**

- **Ki67 positive cell count (field)**
  - Sham: 1
  - BDL: 3

**D**

- **ALT (IU/L)**
  - Sham: 0, BDL: 100

- **Total bilirubin (mg/dL)**
  - Sham: 0, BDL: 20

- **ALP (IU/L)**
  - Sham: 0, BDL: 1000

- **Ki67 positive cell count (field)**
  - Sham: 1, BDL: 3

**E**

- **CD68 mRNA (fold changes)**
  - Sham: 3, BDL: 9

- **IL6 mRNA (fold changes)**
  - Sham: 5, BDL: 10

- **Ccl2 mRNA (fold changes)**
  - Sham: 100, BDL: 300
Fig. 6.

A

Sham BDL

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B

Ccl5 mRNA (fold changes)

Control Gab1 CKO

Sham BDL

C

Ccl5 mRNA (fold changes)

Hepatocytes

D

Ccl5 mRNA (fold changes)

Non-parenchymal cells

n.s.
Fig. 7.

A

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B

CCL5 positive cell count (field)

- ** Sham BDL
- N.S. Sham BDL

C

Hepatocytes

D

Non-parenchymal cells

CCL5 protein (ng/g liver tissue)

- * Sham BDL
- + Sham BDL
Fig. 8.

A. Ccl5 mRNA (fold changes) with (-) and LPS treatment.

B. CCL5 protein (pg/ml) with (-) and LPS treatment.

C. NF-κB activation (fold) with (-) and LPS treatment.

D. Western blotting showing Gab1 and GAPDH expression with Control and Gab1 siRNA treatments.
Fig. 9.

**A**

- Control
- Gab1 CKO

- PBS
- CCL5-antagonist

**B**

Sirius red positive area (%)

- Control
- Gab1 CKO

**C**

Hydroxyproline (μg/g liver tissue)

- Control
- Gab1 CKO

Statistical significance:
- *: p < 0.05
- **: p < 0.01
- n.s.: not significant