LIM-Only Protein FHL2 Is a Negative Regulator of Transforming Growth Factor β1 Expression

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The LIM-only protein FHL2 is a negative regulator of TGF-β1 expression

Running title: regulation of TGF-β1 by FHL2

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§ We dedicate this paper to Dr. Minou Adib-Conquy who passed away on November 3rd 2013.

Key words: FHL2, TGF-β1, transcription regulation, fibrosis

Abstract

TGF-β1 is a master cytokine in many biological processes including tissue homeostasis, epithelial to mesenchymal transition and wound repair. Here we report that the four and a half LIM-only protein 2 (FHL2) is a critical regulator of TGF-β1 expression. Devoid of DNA-binding domain, FHL2 is a transcriptional co-factor that plays the role of co-activator or co-repressor depending on the cell and promoter contexts. We detected association of FHL2 with the TGF-β1 promoter, which showed higher activity in *Fhl2*$^{-/-}$ cells than WT cells in a reporter assay. Overexpression of FHL2 abrogates the activation of the TGF-β1 promoter, whereas the upregulation of TGF-β1 transcription correlates with reduced occupancy of FHL2 on the promoter. Moreover, ablation of FHL2 facilitates recruitment of RNA polymerase II on the TGF-β1 promoter, suggesting that FHL2 may be involved in chromatin remodeling in the control of TGF-β1 transcription. Enhanced expression of TGF-β1 mRNA and cytokine was evidenced in the liver of *Fhl2*$^{-/-}$ mice. We tested the *in vivo* impact of Fhl2 loss on hepatic fibrogenesis that involves TGF-β1 activation. *Fhl2*$^{-/-}$ mice developed more severe fibrosis than WT counterparts. These results demonstrate the repressive function of FHL2 on TGF-β1 expression and contribute to the understanding of TGF-β-mediated fibrogenic response.

INTRODUCTION

Transforming growth factor β (TGF-β) family is a multifunctional regulator of cell differentiation, immunity, morphogenesis, tissue homeostasis and repair (1). TGF-β can be released from immune cells, epithelial cells, endothelial cells and fibroblasts. Binding of TGF-β to their receptors activates Smad transcription factors that mediate transcription of TGF-β target genes. Among the three mammal TGF-β isoforms that have distinct biological activities, TGF-β1 is the strongest inducer of fibrosis. In response to injury, TGF-β1 is critical for the activation of fibrogenic myofibroblasts, which express α-smooth muscle actin (α-SMA) and secrete extracellular matrix proteins, mostly fibronectin and collagen type I and III. The TGF-β1 promoter contains AP1 and Sp1 transcription factor regulatory elements and responds positively to TGF-β1 cytokine and Ras oncoprotein (2-4).

The four and a half LIM-only protein 2 (FHL2) is involved in the regulation of various signaling pathways through protein-protein interaction. Thanks to its unique structure composed exclusively of four and a half LIM domains (for lin 11, isl-1 and mec-3), FHL2 has been identified in diverse protein complexes of multiple signaling pathways. In the cytoplasm, FHL2 interacts with integrins and focal adhesion kinases and plays a role in transmission of extracellular matrix (ECM)/integrin receptors-mediated signals (5-7). FHL2 binds to tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and mediates NF-κB signalling pathway (8). In the nucleus, FHL2 interacts with a diverse group of DNA binding factors to control a broad range of transcription programs including β-catenin/TCF, AP1 and androgen receptor (9-12).

Accumulating evidence indicates that the effects of FHL2 in the cell are different, even opposite, depending on the cell type and environment. In osteoclasts, FHL2 inhibits TRAF6-induced NF-κB activation (8), whereas FHL2 potently enhances NF-κB activity in

epithelial cells (13). Similarly, interaction of FHL2 with β-catenin in myoblasts results in inhibition of β-catenin-mediated activation of a TCF/LEF-dependent reporter gene (10), whereas the same reporter was strongly activated by FHL2 in epithelial cancer cell lines (12). *In vivo* experience showed that injection of FHL2 into *Xenopus* embryos suppressed β-catenin-induced axis duplication (10), while enhanced expression of FHL2 in hepatocytes promoted liver tumorigenesis by activation of the Wnt/β-catenin signaling (14).

FHL2 is intimately linked to the TGF-β signaling pathway at different levels. FHL2 acts as a co-activator of Smad proteins including Smad2, Smad3, Smad4 and plays a role to stabilize the E3 ligase Arkadia in enhancing TGF-β signaling (15, 16). Moreover, FHL2 is a target of TGF-β, responding positively to TGF-β stimulation in a Smad4 independent manner (17, 18). In human melanoma and colon cancer, enhanced expression of FHL2 is correlated with an increase of TGF-β, which prognosticates invasion, metastasis and poor survival (18-20).

Here we report a new aspect of relationship between FHL2 and TGF-β in which deficiency of FHL2 up-regulates TGF-β1 expression. In *Fhl2*$^{-/-}$ mice, enhanced TGF-β1 expression results in severe fibrosis in experimental models for liver fibrogenesis.

MATERIALS AND METHODS

Animals and fibrosis induction

Fhl2$^{-/-}$ mice on Black Swiss–129-SV/J background (21) were backcrossed into C57BL/6J mice over more than eight generations. All animals were hosted in a pathogen-free environment at the Institut Pasteur animal facility and received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals".

Animal experiments were carried out according to the European Commission directives for animal experimentation (Decree 2001-131, 'Journal Officiel' February 06, 2001). Bile duct ligation (BDL) was performed on 6 WT and 7 *Fhl2*$^{-/-}$ mice (8-12 weeks old) by certificated researchers. Mice were sacrificed 15 days post-operation. Livers were removed and either fixed in paraformaldehyde (PFA) 4% for staining or frozen for RNA extraction. 10^7 bioluminescent *Leptospira interrogans* were used to infect five WT and five *Fhl2*$^{-/-}$ 8-week-old mice by intraperitoneal (i.p.) injection in 200 µl of PBS as described previously (22). Mice were sacrificed 21 days postinfection. These experiments were repeated twice. Kidneys from infected and naive mice were removed and fixed in PFA 4% for staining. The experimental procedures have been approved by the Institut Pasteur ethic committee (#2013-0034).

RNA analysis

Total RNA was extracted from 10 *Fhl2*$^{-/-}$ and 10 WT mice using TRIZOL (Invitrogen) from liver, lung, heart, spleen and kidney after tissue homogenization. 1 µg of RNA was reverse-transcribed using random primers (Promega) and cDNA was generated using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). The relative levels of mRNAs were determined by real-time PCR using Fast Start Universal SYBR Green (Roche). The mean copy number of each gene from a triplicate determination was normalized

to the mean copy number of hypoxanthine-guanine phosphoribosyltransferase (HPRT). The following primers were used for real-time PCR: human FHL2: 5'-GGCTGAGAACTGTGTCTTCC-3', 5'-ACAAGTCCTTGCAGTCACAG-3', murine FHL2: 5'- GTCCTACAAGGATCGGCACT-3', 5'-ACAGGTGAAGCAGGTCTCGT-3', HPRT: 5'-ATGCCGAGGATTTGGAAAAA-3', 5'-ACAATGTGATGGCCTCCCA-3', murine TGF-β1: 5'-GCGTATCAGTGGGGGTCA-3', 5'-GTCAGACATTCGGGAAGCAG-3', murine TGF-β2: 5'-GCAGATCCTGAGCAAGCTG-3', 5'-GTAGGGTCTGTAGAAAGTGG-3', murine TGF-β3: 5'-AGCGCAGACACAACCCATAG-3', 5'-ACACGACTTCACCACCATGT-3', murine Col1a1: 5'-GACCAGGAGGACCAGGAAGT-3', 5'-GAAACCCGAGGTATGCTTGA-3', murine Timp1: 5'-GTCACTCTCCAGTTTGCAAG-3', 5'-GACCACCTTATACCAGCGTT-3'.

Cytokine assay

Kupffer cells were isolated from WT and *Fhl2*$^{-/-}$ animals as described previously (23). 10^6 cells were plated in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin for 24 hours. Active form of TGF-β1 was measured in supernatants by ELISA as specified by the manufacturer (R&D Systems, Minneapolis, MN).

Cell culture, reagents and reporter assay

293T, HeLa cells, mouse embryonic fibroblasts (MEF), HepG2 and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). *Fhl2*$^{-/-}$ MEFs were reported previously (24). Human FHL2 gene was cloned in the retroviral pBabe-puromycin vector. Phoenix ecotropic virus packaging cells were transfected with either pBabe-empty or pBabe-hFHL2 and virus-containing supernatant was collected. To restore FHL2 expression in *Fhl2*$^{-/-}$ MEFs, *Fhl2*$^{-/-}$ MEFs were transduced with virus-containing

supernatant supplemented with 8 μg/ml polybrene. Cells were selected with 4 μg/ml puromycin.

The 1.6 kb fragment encompassing 800 bp upstream and 800 bp downstream of the transcription initiation site of murine TGF-β1 gene was synthesized by Eurofins MWG Operon. For reporter assays, $3X10^5$ *Fhl2*$^{-/-}$, WT MEFs, 293T cells were plated in six-well plates and transfected using Lipofectamine 2000 (Invitrogen) with luciferase reporter containing the murine TGF-β1 promoter (0.05 μg). Luciferase activity was determined 48 h later using The Dual-Glo® Luciferase Assay System (Promega). A renilla plasmid under the control of thymidine kinase was used as internal control and the total DNA amount in each transfection was kept constant by adding empty vector plasmid. All experiments were performed in duplicate and repeated at least three times.

To test the effects of FHL2 on the TGF-β1 promoter, MEFs and 293T cells were co-transfected with 0.2 μg, 0.8 μg of plasmid expressing human FHL2 or empty vector and the murine TGF-β1 promoter reporter -800/+510. 36 h later, cells were treated with 10 μM SB431542 (Sigma) for 16 h to inhibit the effects of endogenous TGF-β (25) followed by induction for 1 h with human TGF-β1 (2 ng/ml) (PeproTech) and then collected for luciferase activity assay.

Putative transcription factor binding sites were searched in sequences 800 bp upstream and 800 bp downstream of the TGF-β1 transcription start site, using PROMO (26) with dissimilarity of 10%. Transcription factor recognition sites with a length shorter than five nucleotides were not taken into account.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously (24). To investigate the association of FHL2 on the murine or human TGF- β1 promoter, MEFs, HeLa, HepG2 or Huh7 cells were used for ChIP assays with anti-FHL2 (MBL, c-76204). HeLa, HepG2 or Huh7 cells were treated with 10 μM SB431542 for 16 h followed by induction for 1 h or 5 h with human TGF-β1 (2 ng/ml) and then were subjected to ChIP-qPCR assay. Because *Tgfb1* promoter region contains highly GC-rich sequences, only two pairs of primers among ten pairs of primers tested were able to amplify the murine TGF- β1 promoter by quantitative PCR (Fig. 2A). Their sequences are: (I) 5' – ATGGAGTGGAGTGTTGAGGG – 3'; 5' – CTTGCAGTCCATGGCATAGG – 3' and (II) 5'- CTATGCCATGGACTGCAAGG -3'; 5' – GAGGCACCTTACCCATGAGA -3'. Two pairs of primers were used to amplify the human TGF-β1 promoter by quantitative PCR: 1) 5'-GGCAGTTGGCGAGAACAGT-3', 5'-CTGGGGTCAGCTCTGACAGT-3'; 2) 5'-TGGGAGGTGCTCAGTAAAGG-3', 5'-ACCCAGAACGGAAGGAGAGT-3'. To examine the effect of FHL2 on chromatin accessibility, *Fhl2*$^{-/-}$ and WT MEFs were used for ChIP assays with anti-RNA polymerase II antibodies (Santa Cruz Biotechnology, sc-9001X). Immunoprecipitated chromatin was amplified with the primers I and II by qPCR.

Immunohistochemistry and Immunoblotting

Immunohistochemistry (IHC) was performed on PFA-fixed paraffin-embedded sections as previously described (14). Briefly, tissues were dewaxed in xylene and unmasked in a citric acid solution. After blocking with normal horse serum, sections were incubated with primary antibody against α□smooth muscle actin, (Sigma, A5228). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide. The sections were then incubated with secondary antibodies (Vector Laboratories) for 30 min. The peroxidase reaction was developed using DAB Substrate Kit (SK-4100, Vector Laboratories).

A combination of hematoxylin and eosin was used for counterstaining. For Sirius red staining, after deparafination and rehydratation, tissue sections were labeled for 30 min with a solution of 0.1% (W/V) Sirius red in saturated picric acid. For fibrosis quantification, Sirius Red/Fast Green Collagen Staining Kit (Amsbio, #9046) was used.

To test the effects of FHL2 on the downstream effectors of TGF-β signaling and FHL2 expression in cells, protein lysates were prepared from liver tissues and MEFs and 293T cells as described previously (13). Protein extract was subjected to electrophoresis on NuPAGE 4-12% Bis-Tris Gel (Invitrogen, NP0323BOX) in NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific, NP000102). The following antibodies were used for immunoblotting analysis: Smad2/3 (Cell Signaling, 8685S), phosphorylated Smad2/3 (Cell Signaling, 8828S), Ski (Santa Cruz, sc-33693), SnoN (Santa Cruz, sc-9141), FHL2, actin (Sigma, MA1-744) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 2118S).

Statistic analysis

Statistic analysis was performed by Mann-Whitney U test.

RESULTS

Deficiency of FHL2 increases TGF-β□ expression

Previous studies have pointed to an important role of FHL2 in cooperation with several components of the TGF-β signaling pathway to enhance TGF-β signaling (15, 16). To investigate the effects of FHL2 on the expression of TGF-β, we examined TGF-β1 cytokine production in liver resident macrophage Kupffer cells derived from *Fhl2*$^{-/-}$ animals, because Kupffer cells are the major source of TGF-β1, which is the most important member of TGF-β family in the liver (27). We previously reported that FHL2 is expressed in Kupffer cells (13). We isolated Kupffer cells from WT and *Fhl2*$^{-/-}$ animals and cultured them *in vitro* for 24 h. The active form of TGF-β1 cytokine in the supernatant was measured by ELISA. Remarkably, *Fhl2*$^{-/-}$ Kupffer cells expressed significantly higher levels of TGF-β1 than WT cells (Fig. 1A). To assess if the upregulation of TGF-β1 expression is at the transcription level and to have a broad view of *FHL2* deficiency on the expression of three TGF-β isoforms, we extracted RNA from different tissues including liver, kidney, lung, spleen and heart derived from *Fhl2*$^{-/-}$ and WT mice and examined TGF-β1, TGF-β2 and TGF-β3 mRNA by qPCR. As shown in Fig. 1B, transcription of the TGF-β1 gene was significantly upregulated in the liver but not in the other organs from *Fhl2*$^{-/-}$ mice. Expression of TGF-β2 and TGF-β3 showed no difference in all the organs analyzed except the heart where the mRNA levels of TGF-β2 and TGF-β3 were decreased in *Fhl2*$^{-/-}$ mice. We then examined if FHL2 deficiency could have impact on the expression of TGF-β targets in the liver where FHL2 is weakly expressed in hepatocytes (14). TGF-β induces phosphorylation of Smad2/3 and degradation of the transcription repressors Ski and SnoN (28). Protein lysates prepared from WT and *Fhl2*$^{-/-}$ livers were analyzed for expression of Smad2/3, SnoN and Ski and phosphorylation of

Smad2/3. Besides individual heterogeneity, no significant difference was observed for P-Smad2/3, SnoN and Ski between WT and *Fhl2*$^{-/-}$ mice (Fig. 1C).

FHL2 is present in transcriptional complexes on the TGF-β1 promoter

We focused the study on the liver where FHL2 deletion appeared to create a condition that promotes specifically TGF-β1 expression at the transcription level. To explore how FHL2 is involved in TGF-β1 regulation, we first synthesized the genomic fragment comprising 800 bp upstream and 800 bp downstream of the transcription start site of mouse TGF-β1 gene and constructed a series of deletion fragments into the firefly luciferase reporter vector for fine mapping the region containing promoter activity (Fig. 2A). The fusion fragments were transiently transfected in 293T cells and luciferase activities were determined after normalization to the expression of a control reporter. As shown in Fig. 2B, the fragment -190 to +340 displayed the highest level in luciferase activity, which is in agreement with a previous report (2). Potential binding sites of numerous transcription factors were identified in the sequence encompassing -800 to +800 of the TGF-β1 gene using the program PROMO (26) (Supplementary file 1). Notably, sequences recognized by the AP-1 complex components c-Jun and c-Fos are localized in multiple regions of the promoter. AP1 binding sites were also identified in the human TGF-β1 promoter (29).

To determine whether the TGF-β1 gene is a direct transcriptional target of FHL2, we carried out quantitative chromatin immunoprecipitation (ChIP-qPCR) assays to examine the potential association of FHL2 with the TGF-β1 promoter. Absent of DNA-binding domain, FHL2 could be part of transcription regulatory complexes linking to the promoter through transcription factors. For ChIP assay, WT MEFs were cross-linked with formaldehyde and protein-DNA complexes were immunoprecipitated with anti-FHL2 antibody or control IgG. *Fhl2*$^{-/-}$ MEFs were used as negative control cells. Analysis by qPCR of immunoprecipitated chromatin with two pairs of primers (see Fig. 2A and Materials and Methods) revealed the

association of FHL2 with the TGF-β1 promoter (Figs. 2C and 2D). To prove that FHL2 is also linked to the human TGF-β1 promoter which shares 66% nucleotide identify with murine TGF-β1 promoter (2), we performed ChIP-qPCR in HeLa cells and two hepatic cell lines HepG2 and Huh7. Using chromatin immunoprecipitated by FHL2 antibody as template, quantitative PCR analysis with two pairs of primers specific to human TGF-β1 promoter successfully amplified the promoter region (Figs. 2E and 2F). Interestingly, when cells were treated with TGF-β1 cytokine, which is known to induce TGF-β1 transcription (4), FHL2 occupancy on the promoter of TGF-β1 was significantly reduced (Figs. 2E and 2F), suggesting that FHL2 exerts a repressive effect on the promoter of TGF-β1. These results indicate that the TGF-β1 promoter is a direct target of FHL2.

FHL2 negatively regulates the TGF-β1 promoter

The upregulated expression of TGF-β1 in *Fhl2*-deficient cells boded for the repressor effect of FHL2 on the TGF-β1 promoter. To test it, we carried out TGF-β1 luciferase reporter assay in WT or *Fhl2*$^{-/-}$ MEFs. Remarkably, the activity of all the reporters except -800/-150 was significantly increased in *Fhl2*$^{-/-}$ MEFs compared to WT MEFs (Fig. 3A). To further link the suppressive effect to FHL2, we restored FHL2 expression in *Fhl2*$^{-/-}$ MEFs with human FHL2 (*FHL2*-r) (Figs. 3B and 3C). We tested the activity of the reporter -800/+510 in *Fhl2*$^{-/-}$, FHL2-r and WT MEFs treated with TGF-β cytokine. As shown in Fig. 3D, the reporter activity was inversely correlated with the levels of FHL2 expression *Fhl2*$^{-/-}$, FHL2-r and WT cells in the presence and absence of TGF-β cytokine, indicating that the repressive effect of FHL2 on the TGF-β1 promoter is dose-dependent. But this dose-dependency of FHL2 was not linear as transfection of 0.2 μg and 0.8 μg of plasmid expressing FHL2 in FHL2-r cells (Figs. 3B and 3C) did not further repress the promoter activity (Fig. 3D). On the other hand, the promoter activity in FHL2-r cells was not significantly augmented by TGF-β cytokine

treatment compared to untreated FHL2-r cells, while it was even higher in untreated WT MEFs than treated WT MEFs (Fig. 3D). TGF-β cytokine acts on diverse regulatory elements of the TGF-β1 promoter of which FHL2 is a part. TGF-β cytokine can inhibit FHL2 association with the TGF-β1 promoter, thus relieving the repressive function of FHL2 (see Figs. 2E and 2F). The ultimate activity of the TGF-β1 promoter under the stimulation of TGF-β cytokine, however, depends on the sum of the actions of the diverse regulatory elements to which FHL2 makes contribution.

Next, we examined the effects of FHL2 on TGF-β1 promoter in 293T cell line. When 293T cells were subjected to the treatment with TGF-β cytokine, the activity of the reporter -800/+510 was modestly but significantly activated (Fig. 3E). This activation was blunted by overexpression of FHL2 (Figs. 3C and 3E). Overexpression of human FHL2 did not have effect on the promoter activity without TGF-β1 stimulation (Fig. 3E), suggesting that in the steady-state condition, endogenous FHL2 may not be the limiting factor. Taken together, these results indicate that FHL2 is a negative regulator of the TGF-β1 promoter in both murine and human cells.

To further decipher the mechanisms of the negative regulation of the TGF-β1 promoter by FHL2, we used WT and *Fhl2*$^{-/-}$ MEFs to carry out ChIP-qPCR assays with antibody against RNA polymerase II (Pol II), which transcribes all the protein-coding genes. The primers I and II were employed for qPCR to amplify the endogenous TGF-β1 promoter using chromatin immunoprecipitated with anti-Pol II antibody as template (see Fig. 2A). The recruitment of Pol II to the TGF-β1 promoter was significantly increased in *Fhl2*$^{-/-}$ MEFs in comparison to WT cells (Figs. 4A and 4B). These data suggest that loss of FHL2 may alter chromatin architecture to allow Pol II to gain access to the TGF-β1 promoter.

Fhl2$^{-/-}$ mice show increased susceptibility to fibrogenesis in the liver

TGF-β1 is considered the most powerful mediator of fibrogenesis. The high expression level of TGF-β1 in *Fhl2*$^{-/-}$ liver suggested that *Fhl2*$^{-/-}$ mice might be pro-fibrogenic. We subjected *Fhl2*$^{-/-}$ and WT mice to bile duct ligation (BDL) operation, which is widely used model for liver fibrosis through cholestatic liver injury (30). We sacrificed mice 15 days after BDL. Livers derived from BDL-treated mice lacking FHL2 developed severe fibrosis when compared to WT animals, showing wider fibrotic scars and substantially more scar branching as assessed by staining with hematoxylin-eosin and Sirius red, which directly marks the extracellular matrix deposited by activated hepatic stellate cells (HSC) (Figs. 5, A-C, E-G, I-K, M-O and Q). Moreover, FHL2 KO livers harbored a large increase in activated HSCs as determined by α-smooth muscle actin (α-SMA) staining (Figs. 5D, H, L and P). We further confirmed our findings in a second well-established model of liver fibrosis induced by thioacetamide (TAA) administration. *Fhl2*$^{-/-}$ mice showed increased fibrosis after 20 weeks of TAA treatment (data not shown). These results are in agreement with a previous report showing enhanced fibrosis in *Fhl2*$^{-/-}$ mice using carbon tetrachloride (CCl_4)-induced model of liver fibrosis (31).

To investigate the molecular characteristics of fibrosis developed in *Fhl2*$^{-/-}$ liver, we examined expression by qRT-PCR of fibrogenesis markers including alpha-1 type I collagen, tissue inhibitor of metalloproteinases-1 (TIMP1) and TGF-β1 encoded respectively by *Col1a1, Timp1* and *Tgfb1* genes, in the liver of *Fhl2*$^{-/-}$ and WT mice 15 days after BDL or control mice. This analysis revealed that expression of *Col1a1* and *Timp1* was activated after BDL in both *Fhl2*$^{-/-}$ and WT mice (Fig. 6). Moreover, the levels of *Col1a1* and *Timp1* activation following BDL were significantly higher in *Fhl2*$^{-/-}$ liver than those in WT liver, correlating with the aggravated fibrosis in *Fhl2*$^{-/-}$ mice (Fig. 6).

In accordance with the results in Fig. 1B, the mRNA level of TGF-β1 in the liver was higher in *Fhl2*$^{-/-}$ mice than WT mice at the steady-state condition (Fig. 6). Transcription of

TGF-β1 was significantly enhanced in fibrotic liver compared to control liver in both *Fhl2*$^{-/-}$ and WT mice (Fig. 6). Moreover, livers from *Fhl2*$^{-/-}$ animals displayed higher levels of TGF-β1 mRNA following BDL treatment compared to WT counterparts (Fig. 6), demonstrating that fibrogenic signaling was enhanced in the livers from *Fhl2*$^{-/-}$ animals. Of note, FHL2 expression was upregulated in fibrotic liver of WT mice (Fig. 6), which is correlated with previous observations in human liver fibrosis (14, 31). Therefore, ablation of FHL2 leads to increased level of TGF-β1 and enhanced susceptibility to fibrogenesis.

FHL2 deficiency has no effect on renal fibrosis

A previous report showed that *Fhl2*$^{-/-}$ mice developed stronger fibrotic alterations in bleomycin-induced model for lung fibrosis following an unresolved inflammation (32). To test if FHL2 deficiency had a broad effect on fibrogenesis, we chose to examine fibrogenesis in the kidney, as FHL2 plays roles in the development of kidney disease (33). We used the bacteria *Leptospira* infection model for renal fibrosis (22) and infected age- and sex-matched WT and *Fhl2*$^{-/-}$ mice with equal amounts of bacteria *Leptospira* expressing luciferase. All the mice were successfully infected in the kidney, as attested by *in vivo* imaging (data not shown). After 21 days, kidneys were examined for fibrosis using Sirius red staining and expression of TGF-β1. As shown in Figs. 7A-7P, bacteria *Leptospira* induced fibrosis in the kidney in both WT and *Fhl2*$^{-/-}$ mice. But no significant difference in the accumulation of fibrotic tissue was observed between WT and *Fhl2*$^{-/-}$ animals (Fig. 7Q). Analysis of TGF-β1 expression by qPCR showed no change in mRNA after bacteria infection between WT and *Fhl2*$^{-/-}$ mice (Fig. 7R). These results attest the tissue-dependent feature in TGF-β1 activation and fibrogenesis associated with FHL2 deficiency.

DISCUSSION

Given the importance of TGF-β1 in many biological processes, it is fundamental to understand the mechanisms controlling its expression. In this report, we show that FHL2 acts as an important negative regulator of TGF-β1 transcription. The action of FHL2 in inhibiting TGF-β1 transcription is likely to involve both direct and indirect mechanisms.

Previous studies demonstrate that the effects of FHL2 on transcription can be positive or negative depending on the gene target and the cellular context (10, 34). Associated with the TGF-β1 promoter, FHL2 plays the role of co-repressor in the transcription of the TGF-β1 gene. We show that overexpression of FHL2 inhibits the activity of TGF-β1 promoter, whereas ablation of FHL2 increases its transcription. The inhibition of TGF-β1 promoter by FHL2 is dose-dependent, suggesting that the repressive activity could be intrinsic to FHL2 or FHL2-associated factors with repressive functions. The transcription factors with which FHL2 interacts on the TGF-β1 promoter remain to be determined. Our search of transcription factor binding sites on the TGF-β1 promoter has identified multiple responsive elements recognized by Fos and Jun, which are known FHL2-associated transcription factors (11). FHL2 could act through Jun and Fos to inhibit the transcription of TGF-β1. In the face of TGF-β1 cytokine treatment, which activates the TGF-β1 promoter, transient overexpression of FHL2 can successfully abrogate the activation, correlating with the repressive effects of FHL2 on the TGF-β1 transcription. Under the physiological condition, TGF-β1-induced activation of the TGF-β1 promoter depends on a multitude of signaling pathways through the binding of signal-responsive activators and repressors, including FHL2, which also positively responds to the TGF-β1 cytokine (17, 18). The feedback loop of FHL2 and TGF-β1 may constitute one of the complex transcriptional regulation networks that determine the level and the outcome of TGF-β1 in cells. It is not clear how TGF-β1 induces hepatic fibrosis in *Fhl2*$^{-/-}$

mice. Our observation that expression of TGF-β targets Ski, SnoN and phosphorylation of Smad2/3 were not altered in the liver deficient for FHL2 suggests that the canonical TGF-β signaling may not be involved in this process. It remains to be determined if the profibrotic action of TGF-β1 in *Fhl2*$^{-/-}$ liver is operated through noncanonical TGF-β signaling.

Our finding that loss of FHL2 resulted in increased recruitment of RNA polymerase II on the TGF-β1 promoter confers a novel role to FHL2 in regulation of gene transcription. The question of how FHL2 is involved in the dynamic process of genome packaging to allow or restrict transcriptional regulators access to the regulatory sequences remains to be addressed. Composed of multiple LIM-domains for protein-protein interaction, FHL2 binds with several chromatin modifiers including histone acetyltransferase CBP/p300 (35), histone ubiquitin ligase Brca1 (36) and histone demethylase LSD1 (37) that actively participate to covalently modifying nucleosomes. It is possible that FHL2 could collaborate with these enzymes to maintain a repressed architecture by virtue of the restricted chromatin environment for transcription regulators. Ablation of FHL2 may tailor the way in which the chromatin is packaged to increase the access of RNA polymerase II to the TGF-β1 promoter.

Correlating with enhanced expression of TGF-β1, the fibrotic response in *Fhl2*$^{-/-}$ mice was dramatic in the liver. Previous report showed aggravated liver fibrosis after carbon tetrachloride injection in *Fhl2*$^{-/-}$ mice, but failed to detect increased expression of TGF-β1 mRNA at the steady-state condition as reported here (31). The reason behind this difference is unclear but may reflect different sensibility in the detection of TGF-β1 transcription. Yet, Huss et al revealed higher level of TGF-β1 in fibrotic tissues from *Fhl2*$^{-/-}$ mice than WT mice, consistent with our findings that activation of TGF-β1 gene transcription is stronger in the absence of FHL2. Previous report pointed to higher fibrosis score in the lung of *Fhl2*$^{-/-}$ mice following bleomycin administration (32). It showed that loss of FHL2 resulted in increased expression of the pro-inflammatory matrix protein tenascin C and downregulation of the

macrophage activating C-type lectin receptor DC-SIGN. This unresolved inflammation in *Fhl2*$^{-/-}$ mice was the major driver of increased pulmonary fibrosis (32). In all organ systems, the wound repair process is initiated with inflammation, followed by proliferation and remodeling. It is thus highly likely that FHL2 interferes in distinct processes in lung and liver fibrosis. Our observations that loss of FHL2 had no drastic effect on renal fibrosis further underscore the tissue specificity of FHL2 actions.

Effects of FHL2 loss on fibrosis stress the medical relevance of the repressive activity of FHL2 on the TGF-β1 promoter. However, we and others previously reported that FHL2 was upregulated in human liver fibrotic tissues and higher level of FHL2 was associated with advanced fibrosis (14, 31). This is correlated with our finding of FHL2 activation in liver fibrotic tissues of WT mice (see Fig. 6). In line with this observation, in human melanoma and colon cancer, enhanced expression of FHL2 is correlated with an increase of TGF-β in tumors with invasion, metastasis and poor survival prognostics (18-20). This multifaceted nature of the relationship between FHL2 and TGF-β1 further supports the notion that the cellular context and the expression levels of each protein dictate the process and the outcome of the complex signal transduction. Our data described here contribute to unveiling new facets of TGF-β regulation and should facilitate additional investigations aimed at further exploring TGF-β biology and dysfunctions.

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543

544

Figure Legends

Figure 1. Loss of FHL2 enhances TGF-β1 expression in the liver. (A) TGF-β1 cytokine in Kupffer cells isolated from WT and *Fhl2*$^{-/-}$ animals, as evaluated by ELISA. **, $p < 0.005$. The data are the mean ± S.D. obtained from five animals in each genotype. (B) Analysis of TGF-β1, TGF-β2 and TGF-β3 mRNA by real-time reverse transcription (RT)-PCR in indicated tissues from WT and *Fhl2*$^{-/-}$ mice. The values in the WT tissues were arbitrarily set as 1. The average and S.D. values for three independent experiments are shown. *, $p < 0.05$. (C) Analysis of expression of SnoN, Ski, Smad2/3 and phosphorylation of Smad2/3 in the liver of four WT and four *Fhl2*$^{-/-}$ animals by immunoblotting. Arrow head: FHL2, NS: nonspecific.

Figure 2. FHL2 is associated with the TGF-β1 promoter. (A) Schematic representation of murine TGF-β1 promoter constructs in luciferase reporter assay. (B) Reporter assay in 293T cells. The activity of the -800/+800 reporter was arbitrarily set at 1. Data are presented as mean induction in luciferase activity ± S.D. from duplicate samples. The results shown are representative of those from more than three independent assays. (C and D) Association of FHL2 with murine TGF-β1 promoter. WT and *Fhl2*$^{-/-}$ MEFs were analyzed by ChIP-qPCR using chromatin immunoprecipitated by anti-FHL2 antibody or IgG with the primers I (C) and II (D), which are indicated in A. (E and F) Association of FHL2 with human TGF-β1 promoter. HeLa, HepG2 and Huh7 cells were treated with SB431542 to inhibit the effects of endogenous TGF-β followed by induction for 1 h and 5 h with human TGF-β1 (2 ng/ml) before subjected to ChIP-qPCR with two independent primers (primers 1 in E and primers 2 in F) specific to human TGF-β1 promoter. *, $p < 0.05$. The data presented are the mean ± S.D. obtained from three independent experiments.

Figure 3. FHL2 is a negative regulator of the TGF-β1 promoter. (A) Luciferase reporter assay in WT and *Fhl2*$^{-/-}$ MEFs. The activity of the -800/+800 reporter in WT MEFs was arbitrarily

570 set at 1. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$, n.s.: non significant. (B) Analysis of
571 FHL2 transcription by qPCR. Primers to detect murine Fhl2 were used in WT and *Fhl2*$^{-/-}$
572 MEFs. To restore FHL2 expression in *Fhl2*$^{-/-}$ MEFs, human FHL2 was stably expressed in
573 *Fhl2*$^{-/-}$ MEFs (FHL2-r). Transient transfection of 0.2 μg, 0.8 μg of human FHL2 was carried
574 out in FHL2-r cells. Human FHL2 primers were used to analyze mRNA in FHL2-r related
575 cells. (C) Analysis of FHL2 protein in MEFs and 293T cells with anti-FHL2 antibody reactive
576 to both human and mouse FHL2 protein. (D) The TGF-β1 promoter is negatively regulated by
577 FHL2. The reporter -800/+510 was transfected in MEFs cells expressing different doses of
578 FHL2. Cells were treated with 10 μM SB431542 to inhibit the effects of endogenous TGF-β
579 followed by induction for 1 h with human TGF-β1 (2 ng/ml) before subjected to luciferase
580 assay. (E) Reporter assay in 293T cells. 293T cells were co-transfected with 0.2 μg, 0.8 μg of
581 plasmid expressing FHL2 and the murine TGF-β1 promoter reporter -800/+510. 36 h later,
582 cells were treated with 10 μM SB431542 followed by induction for 1 h with human TGF-β1
583 before subjected to luciferase assay. The data presented are the mean ± S.D. obtained from
584 three independent experiments.

585 **Figure 4.** Loss of FHL2 enhances the recruitment of RNA polymerase II (pol II). Chromatin
586 immunoprecipitated with anti-pol II antibody or IgG was amplified with either primer I (A) or
587 primer II (B), which are indicated in Fig. 2A. The value of amplification in chromatin
588 immunoprecipitated with IgG was arbitrarily set at 1. *, $p < 0.05$. The data presented are the
589 mean ± S.D. obtained from three independent experiments.

590 **Figure 5.** Enhanced hepatic fibrosis in *Fhl2*$^{-/-}$ mice. *Fhl2*$^{-/-}$ mice (n = 7) and WT mice (n = 6)
591 underwent bile duct ligation (BDL). H&E staining of liver parenchyma was performed with
592 control WT and *Fhl2*$^{-/-}$ mice (A, B, E and F) or mice subjected to BDL (I, J, M and N).
593 Hepatic fibrosis was assessed by Sirius red staining (C, G, K and O). Quantification is shown

in Q. Expression of α-SMA was determined by immunohistochemistry (D, H, L and P). The data presented are the mean ± S.D. obtained from at least five mice in each group.

Figure 6. Expression of *Col1a1*, *Tgfb1*, *TIMP1* and *Fhl2* mRNA in the liver of WT and *Fhl2*$^{-/-}$ mice after BDL. Hepatic levels of *Col1a1*, *Tgfb1*, *TIMP1* and *Fhl2* mRNA were examined by qPCR in control and operated WT and *Fhl2*$^{-/-}$ mice. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$. The data presented are the mean ± S.D. obtained from at least five mice in each group.

Figure 7. No effect of FHL2 deficiency on renal fibrosis. Kidneys were derived from naive or leptospira-infected WT and *Fhl2*$^{-/-}$ mice. Light microscopy of kidney stained with H&E staining (A, B, E, F, I, J, M, N) and collagen deposition stained with Sirius red (C, D, G, H, K, L, O, P). Original magnifications : (A, C, E, G, I, K, M, O) 40 x, (B, D, F, H, J, L, N, P) 100 x. Sirius red quantification is shown in Q. TGF-β1 mRNA was analyzed by qPCR in kidney from naive or leptospira-infected WT and *Fhl2*$^{-/-}$ mice (R). The data presented are the mean ± S.D. obtained from five mice in each group.

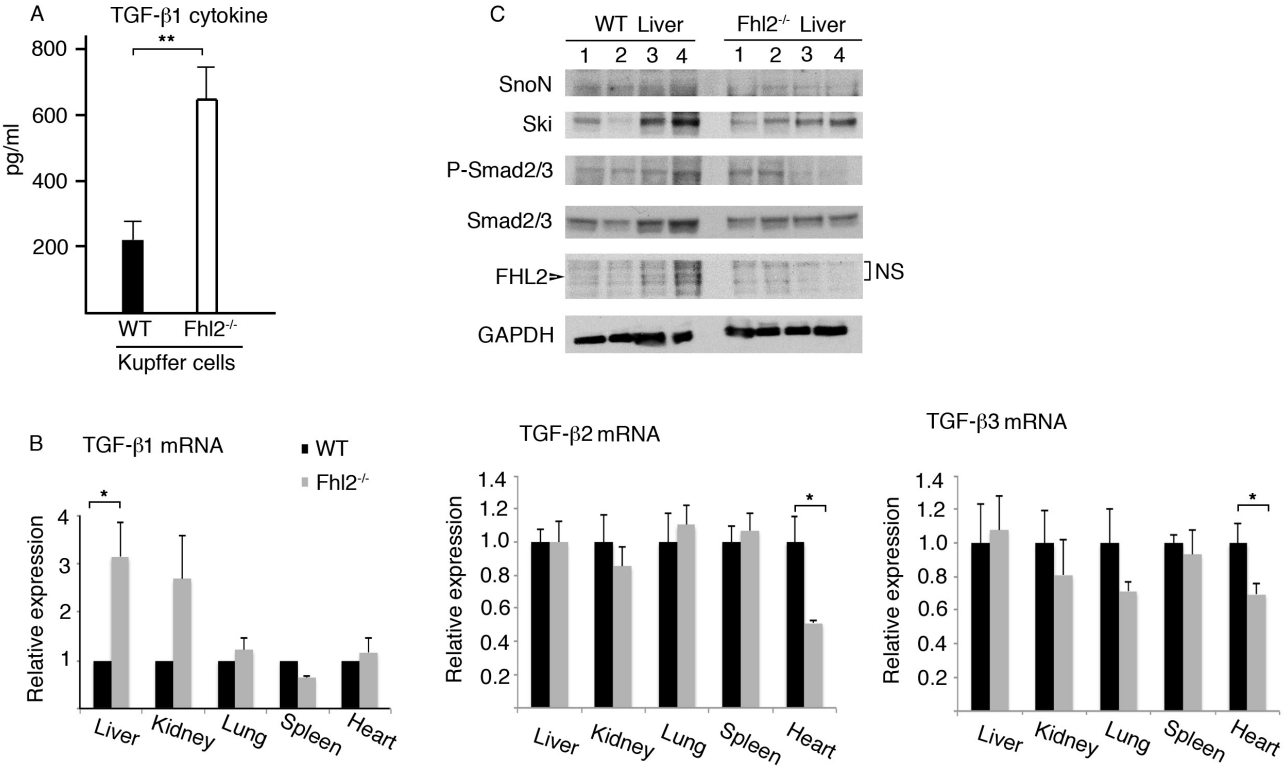
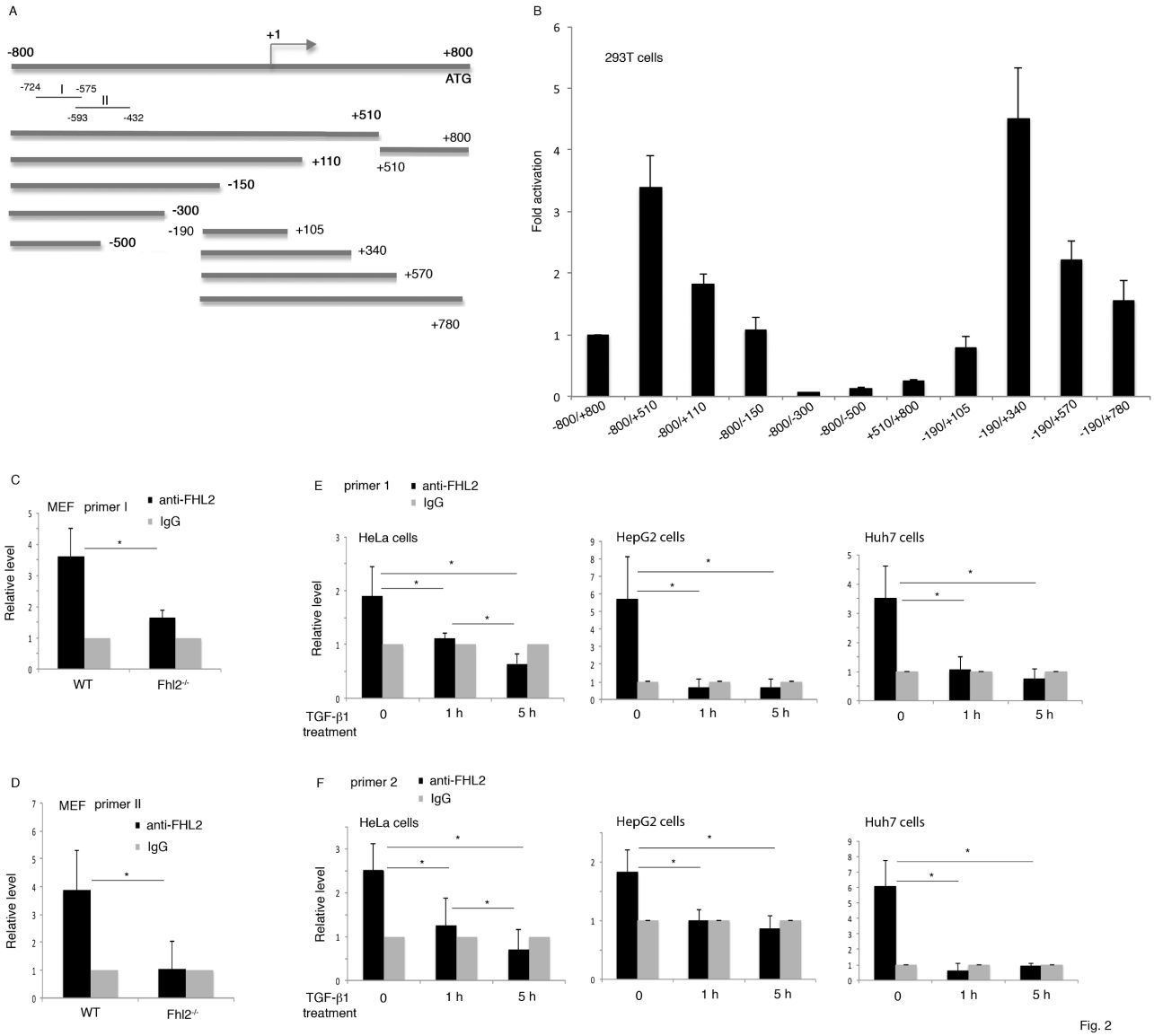

A
TGF-β1 cytokine
**
800
600
400
200
pg/ml
WT
Fhl2-/-
Kupffer cells
C
WT Liver
Fhl2-/- Liver
1 2 3 4
1 2 3 4
SnoN
Ski
P-Smad2/3
Smad2/3
FHL2
NS
GAPDH
B
TGF-β1 mRNA
WT
Fhl2-/-
*
Relative expression
4
3
2
1
Liver
Kidney
Lung
Spleen
Heart
TGF-β2 mRNA
*
Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Liver
Kidney
Lung
Spleen
Heart
TGF-β3 mRNA
*
Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Liver
Kidney
Lung
Spleen
Heart

Fig. 1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+1
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+800
ATG
-724
I
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II
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+510
+800
+110
+510
-150
-300
-190
+105
-500
+340
+570
+780
B
293T cells
Fold activation
-800/+800
-800/+510
-800/+110
-800/-150
-800/-300
-800/-500
+510/+800
-190/+105
-190/+340
-190/+570
-190/+780
C
MEF primer I
anti-FHL2
IgG
Relative level
WT
Fhl2-/-
D
MEF primer II
anti-FHL2
IgG
Relative level
WT
Fhl2-/-
E
primer 1
anti-FHL2
IgG
HeLa cells
HepG2 cells
Huh7 cells
Relative level
TGF-β1 treatment
0
1 h
5 h
F
primer 2
anti-FHL2
IgG
HeLa cells
HepG2 cells
Huh7 cells
Relative level
TGF-β1 treatment
0
1 h
5 h

Fig. 2

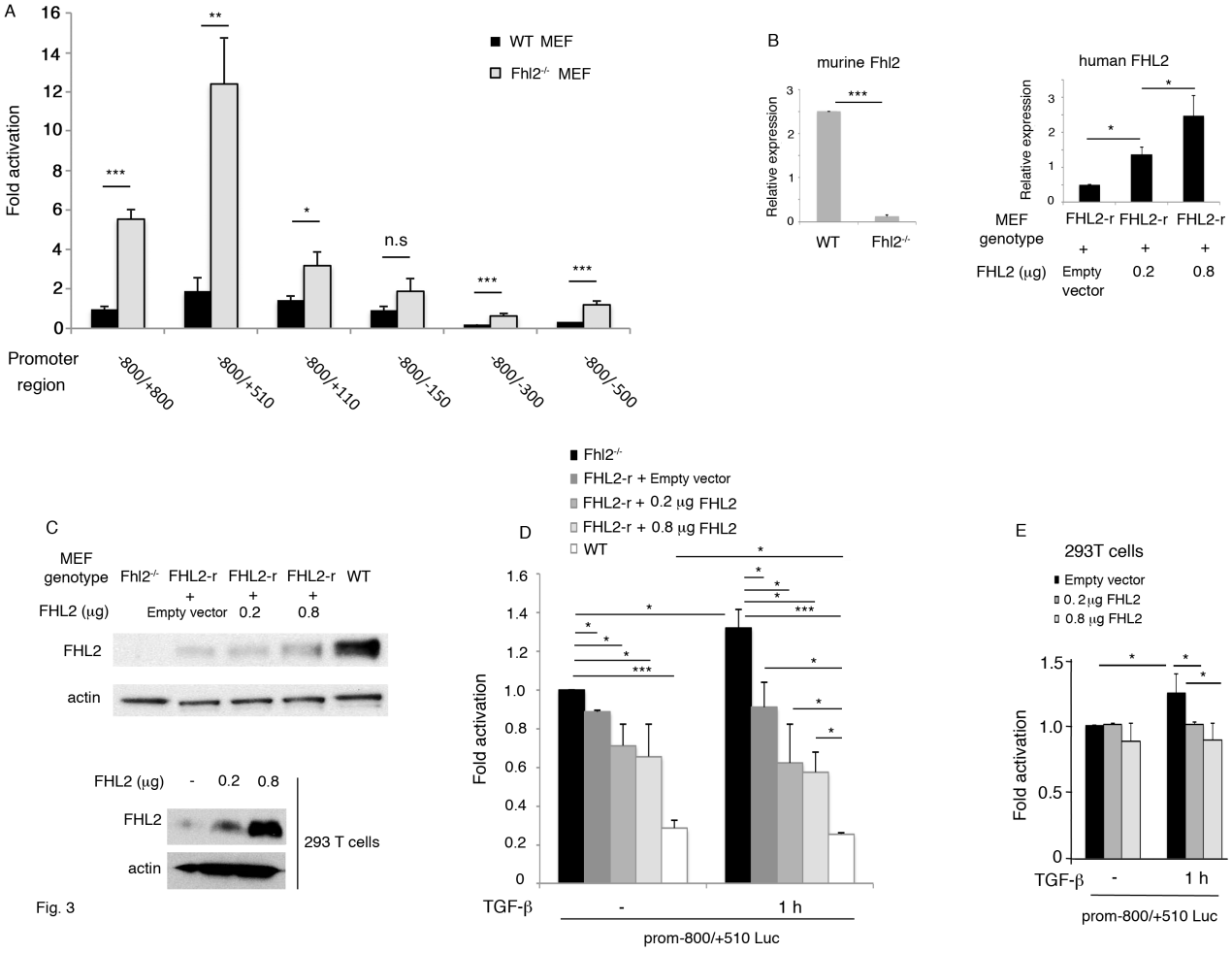

A
Fold activation
WT MEF
Fhl2-/- MEF
Promoter region
-800/+800
-800/+510
-800/+110
-800/-150
-800/-300
-800/-500
n.s
B
murine Fhl2
Relative expression
WT
Fhl2-/-
human FHL2
MEF genotype
FHL2-r
FHL2 (μg)
Empty vector
0.2
0.8
C
MEF genotype
Fhl2-/-
WT
FHL2
actin
293 T cells
D
Fhl2-/-
FHL2-r + Empty vector
FHL2-r + 0.2 μg FHL2
FHL2-r + 0.8 μg FHL2
WT
TGF-β
1 h
prom-800/+510 Luc
E
293T cells
Empty vector
0.2 μg FHL2
0.8 μg FHL2

Fig. 3

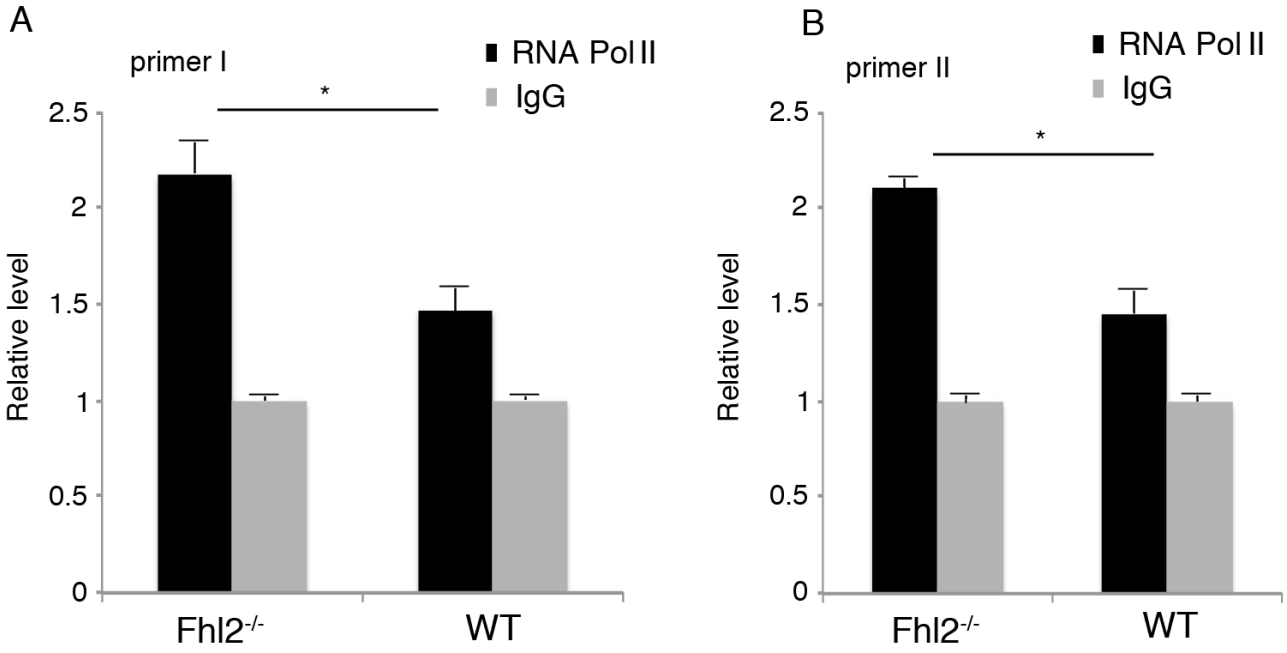
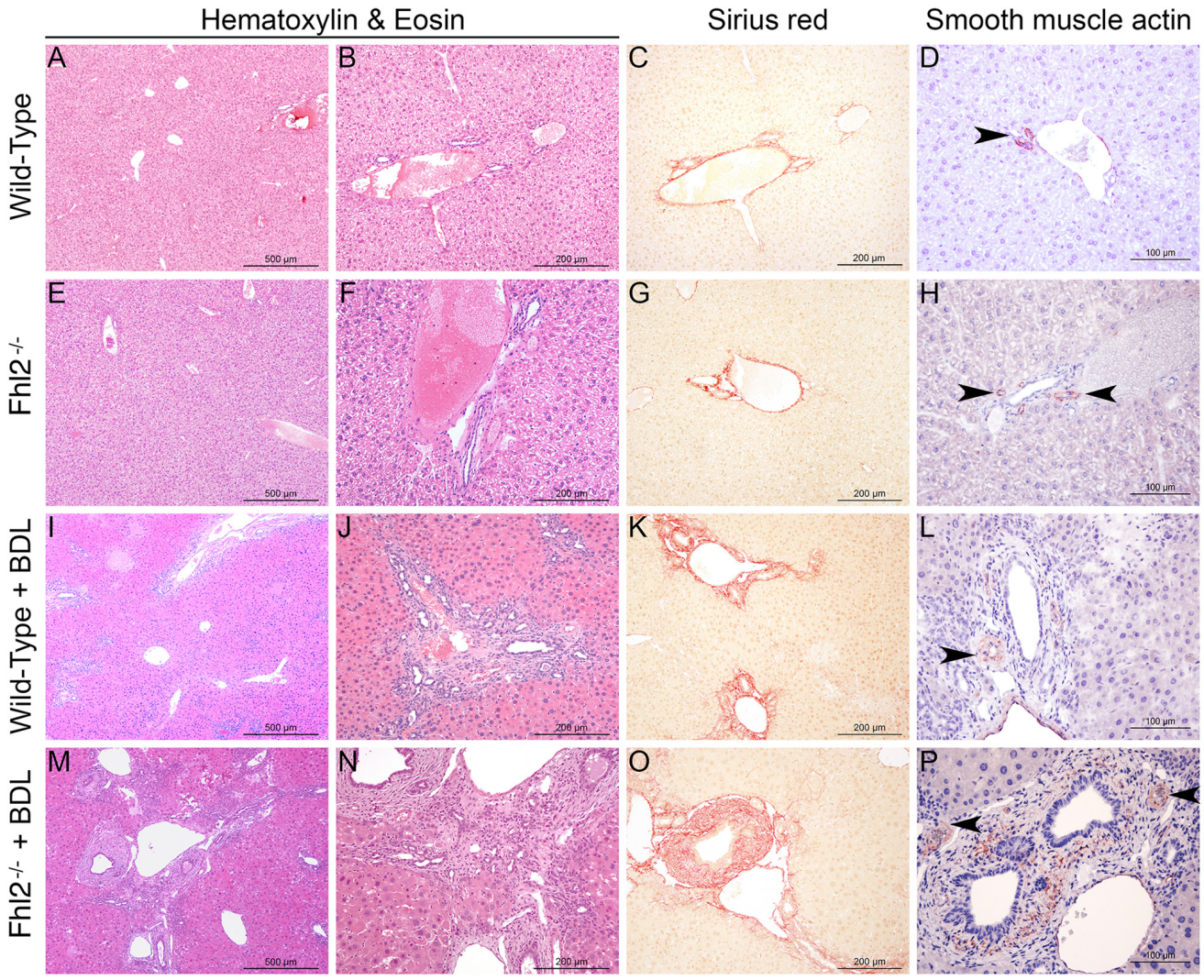

A
primer I
RNA Pol II
IgG
*
2.5
2
1.5
1
0.5
0
Relative level
Fhl2-/-
WT
B
primer II
RNA Pol II
IgG
*
2.5
2
1.5
1
0.5
0
Relative level
Fhl2-/-
WT

Fig. 4


Hematoxylin & Eosin
Sirius red
Smooth muscle actin
Wild-Type
Fhl2-/-
Wild-Type + BDL
Fhl2-/- + BDL
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
500 µm
200 µm
100 µm

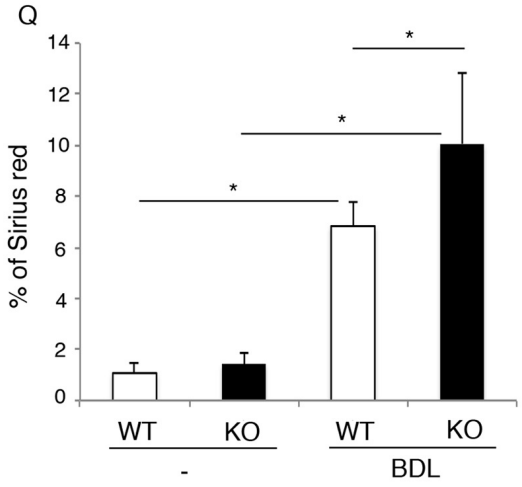

Q
% of Sirius red
0
2
4
6
8
10
12
14
*
*
*
WT
KO
WT
KO
-
BDL

Fig. 5

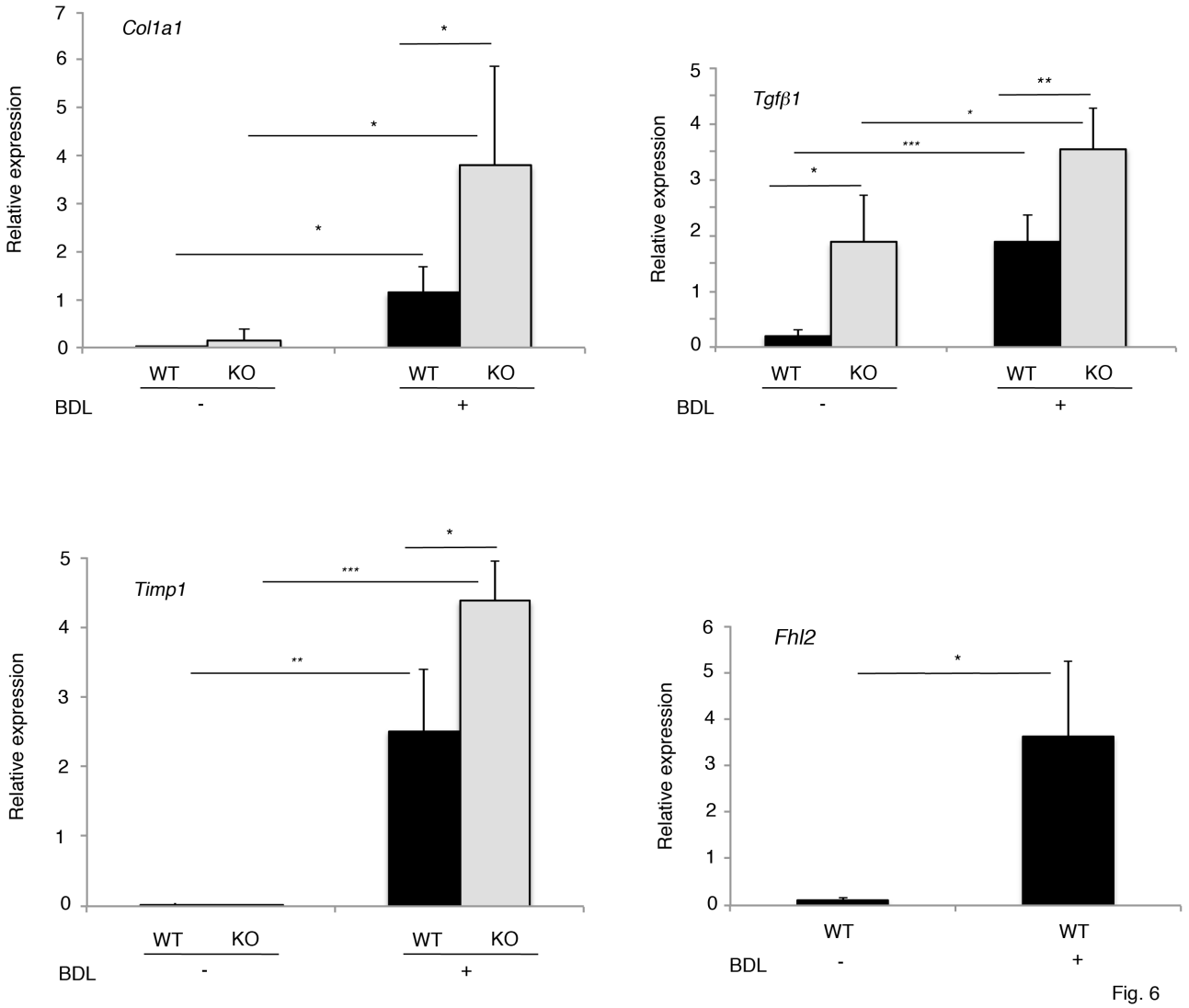

Col1a1
Relative expression
0
1
2
3
4
5
6
7
*
*
*
WT
KO
WT
KO
BDL
-
+
Tgfβ1
Relative expression
0
1
2
3
4
5
*
***
*
**
WT
KO
WT
KO
BDL
-
+
Timp1
Relative expression
0
1
2
3
4
5
*
***
**
WT
KO
WT
KO
BDL
-
+
Fhl2
Relative expression
0
1
2
3
4
5
6
*
WT
WT
BDL
-
+

Fig. 6

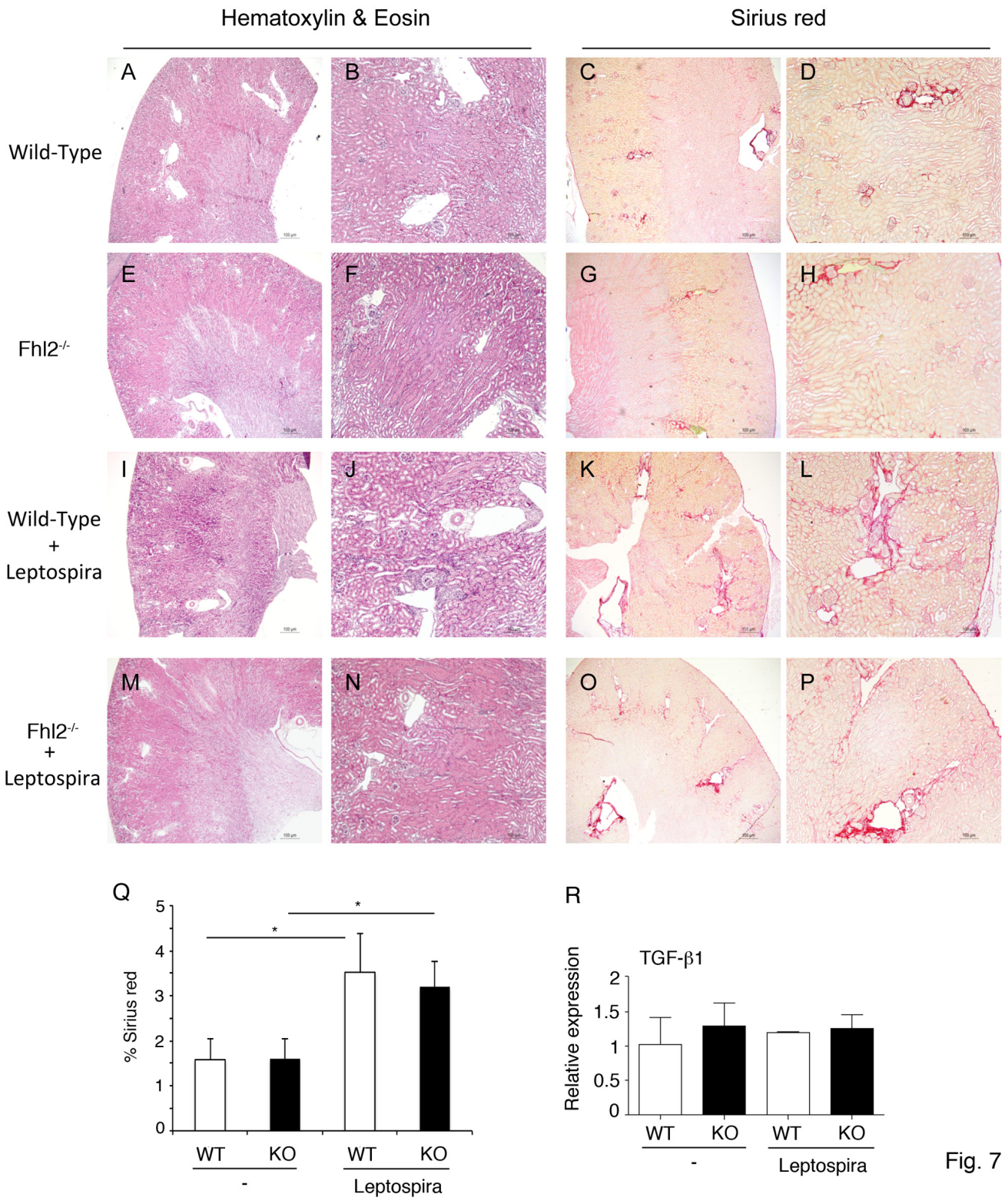

Hematoxylin & Eosin
Sirius red
Wild-Type
Fhl2-/-
Wild-Type + Leptospira
Fhl2-/- + Leptospira
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
% Sirius red
0
1
2
3
4
5
*
*
WT
KO
WT
KO
-
Leptospira
R
TGF-β1
Relative expression
0
0.5
1
1.5
2
WT
KO
WT
KO
-
Leptospira

Fig. 7