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## LIM-Only Protein FHL2 Is a Negative Regulator of Transforming Growth Factor $\beta$ 1 Expression

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1 **The LIM-only protein FHL2 is a negative regulator of TGF- $\beta$ 1 expression**

2 Running title: regulation of TGF- $\beta$ 1 by FHL2

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19 § We dedicate this paper to Dr. Minou Adib-Conquy who passed away on November 3<sup>rd</sup>  
20 2013.

21 Key words: FHL2, TGF- $\beta$ 1, transcription regulation, fibrosis

22

23 **Abstract**

24 TGF- $\beta$ 1 is a master cytokine in many biological processes including tissue  
25 homeostasis, epithelial to mesenchymal transition and wound repair. Here we report that the  
26 four and a half LIM-only protein 2 (FHL2) is a critical regulator of TGF- $\beta$ 1 expression.  
27 Devoid of DNA-binding domain, FHL2 is a transcriptional co-factor that plays the role of co-  
28 activator or co-repressor depending on the cell and promoter contexts. We detected  
29 association of FHL2 with the TGF- $\beta$ 1 promoter, which showed higher activity in *Fhl2*<sup>-/-</sup> cells  
30 than WT cells in a reporter assay. Overexpression of FHL2 abrogates the activation of the  
31 TGF- $\beta$ 1 promoter, whereas the upregulation of TGF- $\beta$ 1 transcription correlates with reduced  
32 occupancy of FHL2 on the promoter. Moreover, ablation of FHL2 facilitates recruitment of  
33 RNA polymerase II on the TGF- $\beta$ 1 promoter, suggesting that FHL2 may be involved in  
34 chromatin remodeling in the control of TGF- $\beta$ 1 transcription. Enhanced expression of TGF-  
35  $\beta$ 1 mRNA and cytokine was evidenced in the liver of *Fhl2*<sup>-/-</sup> mice. We tested the *in vivo*  
36 impact of Fhl2 loss on hepatic fibrogenesis that involves TGF- $\beta$ 1 activation. *Fhl2*<sup>-/-</sup> mice  
37 developed more severe fibrosis than WT counterparts. These results demonstrate the  
38 repressive function of FHL2 on TGF- $\beta$ 1 expression and contribute to the understanding of  
39 TGF- $\beta$ -mediated fibrogenic response.

40

41

## 42 INTRODUCTION

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

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

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

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

72 FHL2 is intimately linked to the TGF- $\beta$  signaling pathway at different levels. FHL2  
73 acts as a co-activator of Smad proteins including Smad2, Smad3, Smad4 and plays a role to  
74 stabilize the E3 ligase Arkadia in enhancing TGF- $\beta$  signaling (15, 16). Moreover, FHL2 is a  
75 target of TGF- $\beta$ , responding positively to TGF- $\beta$  stimulation in a Smad4 independent manner  
76 (17, 18). In human melanoma and colon cancer, enhanced expression of FHL2 is correlated  
77 with an increase of TGF- $\beta$ , which prognosticates invasion, metastasis and poor survival (18-  
78 20).

79 Here we report a new aspect of relationship between FHL2 and TGF- $\beta$  in which  
80 deficiency of FHL2 up-regulates TGF- $\beta$ 1 expression. In *Fhl2*<sup>-/-</sup> mice, enhanced TGF- $\beta$ 1  
81 expression results in severe fibrosis in experimental models for liver fibrogenesis.

## 82 MATERIALS AND METHODS

### 83 Animals and fibrosis induction

84 *Fhl2*<sup>-/-</sup> mice on Black Swiss-129-SV/J background (21) were backcrossed into  
85 C57BL/6J mice over more than eight generations. All animals were hosted in a pathogen-free  
86 environment at the Institut Pasteur animal facility and received human care according to the  
87 criteria outlined in the “Guide for the Care and Use of Laboratory Animals”.

88 Animal experiments were carried out according to the European Commission  
89 directives for animal experimentation (Decree 2001-131, ‘Journal Officiel’ February 06,  
90 2001). Bile duct ligation (BDL) was performed on 6 WT and 7 *Fhl2*<sup>-/-</sup> mice (8-12 weeks old)  
91 by certificated researchers. Mice were sacrificed 15 days post-operation. Livers were removed  
92 and either fixed in paraformaldehyde (PFA) 4% for staining or frozen for RNA extraction. 10<sup>7</sup>  
93 bioluminescent *Leptospira interrogans* were used to infect five WT and five *Fhl2*<sup>-/-</sup> 8-week-  
94 old mice by intraperitoneal (i.p.) injection in 200 µl of PBS as described previously (22).  
95 Mice were sacrificed 21 days postinfection. These experiments were repeated twice. Kidneys  
96 from infected and naive mice were removed and fixed in PFA 4% for staining. The  
97 experimental procedures have been approved by the Institut Pasteur ethic committee (#2013-  
98 0034).

### 99 RNA analysis

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

106 to the mean copy number of hypoxanthine-guanine phosphoribosyltransferase (HPRT). The  
107 following primers were used for real-time PCR: human FHL2: 5'-  
108 GGCTGAGAACTGTGTCTTCC-3', 5'-ACAAGTCCTTGCAGTCACAG-3', murine FHL2:  
109 5'-GTCCTACAAGGATCGGCACT-3', 5'-ACAGGTGAAGCAGGTCTCGT-3', HPRT: 5'-  
110 ATGCCGAGGATTTGGAAAAA-3', 5'-ACAATGTGATGGCCTCCCA-3', murine TGF-  
111  $\beta$ 1: 5'-GCGTATCAGTGGGGGTCA-3', 5'-GTCAGACATTCGGGAAGCAG-3', murine  
112 TGF- $\beta$ 2: 5'-GCAGATCCTGAGCAAGCTG-3', 5'-GTAGGGTCTGTAGAAAGTGG-3',  
113 murine TGF- $\beta$ 3: 5'-AGCGCAGACACAACCCATAG-3', 5'-  
114 ACACGACTTCACCACCATGT-3', murine Colla1: 5'-GACCAGGAGGACCAGGAAGT-  
115 3', 5'-GAAACCCGAGGTATGCTTGA-3', murine Timp1: 5'-  
116 GTCACTCTCCAGTTTGCAAG-3', 5'-GACCACCTTATACCAGCGTT-3'.

#### 117 **Cytokine assay**

118 Kupffer cells were isolated from WT and *Fhl2*<sup>-/-</sup> animals as described previously (23).  
119 10<sup>6</sup> cells were plated in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin  
120 for 24 hours. Active form of TGF- $\beta$ 1 was measured in supernatants by ELISA as specified by  
121 the manufacturer (R&D Systems, Minneapolis, MN).

#### 122 **Cell culture, reagents and reporter assay**

123 293T, HeLa cells, mouse embryonic fibroblasts (MEF), HepG2 and Huh7 cells were  
124 cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum  
125 (FBS). *Fhl2*<sup>-/-</sup> MEFs were reported previously (24). Human FHL2 gene was cloned in the  
126 retroviral pBabe-puromycin vector. Phoenix ecotropic virus packaging cells were transfected  
127 with either pBabe-empty or pBabe-hFHL2 and virus-containing supernatant was collected. To  
128 restore FHL2 expression in *Fhl2*<sup>-/-</sup> MEFs, *Fhl2*<sup>-/-</sup> MEFs were transduced with virus-containing

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

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

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

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

#### 150 **Chromatin immunoprecipitation (ChIP) assay**



151           ChIP was performed as described previously (24). To investigate the association of  
152 FHL2 on the murine or human TGF-  $\beta$ 1 promoter, MEFs, HeLa, HepG2 or Huh7 cells were  
153 used for ChIP assays with anti-FHL2 (MBL, c-76204). HeLa, HepG2 or Huh7 cells were  
154 treated with 10  $\mu$ M SB431542 for 16 h followed by induction for 1 h or 5 h with human TGF-  
155  $\beta$ 1 (2 ng/ml) and then were subjected to ChIP-qPCR assay. Because *Tgfb1* promoter region  
156 contains highly GC-rich sequences, only two pairs of primers among ten pairs of primers  
157 tested were able to amplify the murine TGF-  $\beta$ 1 promoter by quantitative PCR (Fig. 2A).  
158 Their sequences are: (I) 5' - ATGGAGTGGAGTGTGAGGG - 3'; 5' -  
159 CTTGCAGTCCATGGCATAGG - 3' and (II) 5'- CTATGCCATGGACTGCAAGG -3'; 5' -  
160 GAGGCACCTTACCCATGAGA -3'. Two pairs of primers were used to amplify the human  
161 TGF- $\beta$ 1 promoter by quantitative PCR: 1) 5'-GGCAGTTGGCGAGAACAGT-3', 5'-  
162 CTGGGGTCAGCTCTGACAGT-3'; 2) 5'-TGGGAGGTGCTCAGTAAAGG-3', 5'-  
163 ACCCAGAACGGAAGGAGAGT-3'. To examine the effect of FHL2 on chromatin  
164 accessibility, *Fhl2*<sup>-/-</sup> and WT MEFs were used for ChIP assays with anti-RNA polymerase II  
165 antibodies (Santa Cruz Biotechnology, sc-9001X). Immunoprecipitated chromatin was  
166 amplified with the primers I and II by qPCR.

#### 167 **Immunohistochemistry and Immunoblotting**

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

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

179 To test the effects of FHL2 on the downstream effectors of TGF- $\beta$  signaling and FHL2  
180 expression in cells, protein lysates were prepared from liver tissues and MEFs and 293T cells  
181 as described previously (13). Protein extract was subjected to electrophoresis on NuPAGE 4-  
182 12% Bis-Tris Gel (Invitrogen, NP0323BOX) in NuPAGE MOPS SDS Running Buffer  
183 (Thermo Fisher Scientific, NP000102). The following antibodies were used for  
184 immunoblotting analysis: Smad2/3 (Cell Signaling, 8685S), phosphorylated Smad2/3 (Cell  
185 Signaling, 8828S), Ski (Santa Cruz, sc-33693), SnoN (Santa Cruz, sc-9141), FHL2, actin  
186 (Sigma, MA1-744) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell  
187 Signaling, 2118S).

#### 188 **Statistic analysis**

189 Statistic analysis was performed by Mann-Whitney U test.

190

191 **RESULTS**

192 **Deficiency of FHL2 increases TGF- $\beta$  expression**

193 Previous studies have pointed to an important role of FHL2 in cooperation with  
194 several components of the TGF- $\beta$  signaling pathway to enhance TGF- $\beta$  signaling (15, 16). To  
195 investigate the effects of FHL2 on the expression of TGF- $\beta$ , we examined TGF- $\beta$ 1 cytokine  
196 production in liver resident macrophage Kupffer cells derived from *Fhl2*<sup>-/-</sup> animals, because  
197 Kupffer cells are the major source of TGF- $\beta$ 1, which is the most important member of TGF- $\beta$   
198 family in the liver (27). We previously reported that FHL2 is expressed in Kupffer cells (13).  
199 We isolated Kupffer cells from WT and *Fhl2*<sup>-/-</sup> animals and cultured them *in vitro* for 24 h.  
200 The active form of TGF- $\beta$ 1 cytokine in the supernatant was measured by ELISA.  
201 Remarkably, *Fhl2*<sup>-/-</sup> Kupffer cells expressed significantly higher levels of TGF- $\beta$ 1 than WT  
202 cells (Fig. 1A). To assess if the upregulation of TGF- $\beta$ 1 expression is at the transcription level  
203 and to have a broad view of *FHL2* deficiency on the expression of three TGF- $\beta$  isoforms, we  
204 extracted RNA from different tissues including liver, kidney, lung, spleen and heart derived  
205 from *Fhl2*<sup>-/-</sup> and WT mice and examined TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 mRNA by qPCR. As  
206 shown in Fig. 1B, transcription of the TGF- $\beta$ 1 gene was significantly upregulated in the liver  
207 but not in the other organs from *Fhl2*<sup>-/-</sup> mice. Expression of TGF- $\beta$ 2 and TGF- $\beta$ 3 showed no  
208 difference in all the organs analyzed except the heart where the mRNA levels of TGF- $\beta$ 2 and  
209 TGF- $\beta$ 3 were decreased in *Fhl2*<sup>-/-</sup> mice. We then examined if FHL2 deficiency could have  
210 impact on the expression of TGF- $\beta$  targets in the liver where FHL2 is weakly expressed in  
211 hepatocytes (14). TGF- $\beta$  induces phosphorylation of Smad2/3 and degradation of the  
212 transcription repressors Ski and SnoN (28). Protein lysates prepared from WT and *Fhl2*<sup>-/-</sup>  
213 livers were analyzed for expression of Smad2/3, SnoN and Ski and phosphorylation of

214 Smad2/3. Besides individual heterogeneity, no significant difference was observed for P-  
215 Smad2/3, SnoN and Ski between WT and *Fhl2*<sup>-/-</sup> mice (Fig. 1C).

### 216 **FHL2 is present in transcriptional complexes on the TGF- $\beta$ 1 promoter**

217 We focused the study on the liver where FHL2 deletion appeared to create a condition  
218 that promotes specifically TGF- $\beta$ 1 expression at the transcription level. To explore how  
219 FHL2 is involved in TGF- $\beta$ 1 regulation, we first synthesized the genomic fragment  
220 comprising 800 bp upstream and 800 bp downstream of the transcription start site of mouse  
221 TGF- $\beta$ 1 gene and constructed a series of deletion fragments into the firefly luciferase reporter  
222 vector for fine mapping the region containing promoter activity (Fig. 2A). The fusion  
223 fragments were transiently transfected in 293T cells and luciferase activities were determined  
224 after normalization to the expression of a control reporter. As shown in Fig. 2B, the fragment  
225 -190 to +340 displayed the highest level in luciferase activity, which is in agreement with a  
226 previous report (2). Potential binding sites of numerous transcription factors were identified in  
227 the sequence encompassing -800 to +800 of the TGF- $\beta$ 1 gene using the program PROMO  
228 (26) (Supplementary file 1). Notably, sequences recognized by the AP-1 complex components  
229 c-Jun and c-Fos are localized in multiple regions of the promoter. AP1 binding sites were also  
230 identified in the human TGF- $\beta$ 1 promoter (29).

231 To determine whether the TGF- $\beta$ 1 gene is a direct transcriptional target of FHL2, we  
232 carried out quantitative chromatin immunoprecipitation (ChIP-qPCR) assays to examine the  
233 potential association of FHL2 with the TGF- $\beta$ 1 promoter. Absent of DNA-binding domain,  
234 FHL2 could be part of transcription regulatory complexes linking to the promoter through  
235 transcription factors. For ChIP assay, WT MEFs were cross-linked with formaldehyde and  
236 protein-DNA complexes were immunoprecipitated with anti-FHL2 antibody or control IgG.  
237 *Fhl2*<sup>-/-</sup> MEFs were used as negative control cells. Analysis by qPCR of immunoprecipitated  
238 chromatin with two pairs of primers (see Fig. 2A and Materials and Methods) revealed the

239 association of FHL2 with the TGF- $\beta$ 1 promoter (Figs. 2C and 2D). To prove that FHL2 is  
240 also linked to the human TGF- $\beta$ 1 promoter which shares 66% nucleotide identify with murine  
241 TGF- $\beta$ 1 promoter (2), we performed ChIP-qPCR in HeLa cells and two hepatic cell lines  
242 HepG2 and Huh7. Using chromatin immunoprecipitated by FHL2 antibody as template,  
243 quantitative PCR analysis with two pairs of primers specific to human TGF- $\beta$ 1 promoter  
244 successfully amplified the promoter region (Figs. 2E and 2F). Interestingly, when cells were  
245 treated with TGF- $\beta$ 1 cytokine, which is known to induce TGF- $\beta$ 1 transcription (4), FHL2  
246 occupancy on the promoter of TGF- $\beta$ 1 was significantly reduced (Figs. 2E and 2F),  
247 suggesting that FHL2 exerts a repressive effect on the promoter of TGF- $\beta$ 1. These results  
248 indicate that the TGF- $\beta$ 1 promoter is a direct target of FHL2.

#### 249 **FHL2 negatively regulates the TGF- $\beta$ 1 promoter**

250 The upregulated expression of TGF- $\beta$ 1 in *Fhl2*-deficient cells boded for the repressor  
251 effect of FHL2 on the TGF- $\beta$ 1 promoter. To test it, we carried out TGF- $\beta$ 1 luciferase reporter  
252 assay in WT or *Fhl2*<sup>-/-</sup> MEFs. Remarkably, the activity of all the reporters except -800/-150  
253 was significantly increased in *Fhl2*<sup>-/-</sup> MEFs compared to WT MEFs (Fig. 3A). To further link  
254 the suppressive effect to FHL2, we restored FHL2 expression in *Fhl2*<sup>-/-</sup> MEFs with human  
255 FHL2 (*FHL2*-r) (Figs. 3B and 3C). We tested the activity of the reporter -800/+510 in *Fhl2*<sup>-/-</sup>,  
256 *FHL2*-r and WT MEFs treated with TGF- $\beta$  cytokine. As shown in Fig. 3D, the reporter  
257 activity was inversely correlated with the levels of FHL2 expression *Fhl2*<sup>-/-</sup>, *FHL2*-r and WT  
258 cells in the presence and absence of TGF- $\beta$  cytokine, indicating that the repressive effect of  
259 FHL2 on the TGF- $\beta$ 1 promoter is dose-dependent. But this dose-dependency of FHL2 was  
260 not linear as transfection of 0.2  $\mu$ g and 0.8  $\mu$ g of plasmid expressing FHL2 in *FHL2*-r cells  
261 (Figs. 3B and 3C) did not further repress the promoter activity (Fig. 3D). On the other hand,  
262 the promoter activity in *FHL2*-r cells was not significantly augmented by TGF- $\beta$  cytokine

263 treatment compared to untreated FHL2-r cells, while it was even higher in untreated WT  
264 MEFs than treated WT MEFs (Fig. 3D). TGF- $\beta$  cytokine acts on diverse regulatory elements  
265 of the TGF- $\beta$ 1 promoter of which FHL2 is a part. TGF- $\beta$  cytokine can inhibit FHL2  
266 association with the TGF- $\beta$ 1 promoter, thus relieving the repressive function of FHL2 (see  
267 Figs. 2E and 2F). The ultimate activity of the TGF- $\beta$ 1 promoter under the stimulation of  
268 TGF- $\beta$  cytokine, however, depends on the sum of the actions of the diverse regulatory  
269 elements to which FHL2 makes contribution.

270         Next, we examined the effects of FHL2 on TGF- $\beta$ 1 promoter in 293T cell line. When  
271 293T cells were subjected to the treatment with TGF- $\beta$  cytokine, the activity of the reporter -  
272 800/+510 was modestly but significantly activated (Fig. 3E). This activation was blunted by  
273 overexpression of FHL2 (Figs. 3C and 3E). Overexpression of human FHL2 did not have  
274 effect on the promoter activity without TGF- $\beta$ 1 stimulation (Fig. 3E), suggesting that in the  
275 steady-state condition, endogenous FHL2 may not be the limiting factor. Taken together,  
276 these results indicate that FHL2 is a negative regulator of the TGF- $\beta$ 1 promoter in both  
277 murine and human cells.

278         To further decipher the mechanisms of the negative regulation of the TGF- $\beta$ 1  
279 promoter by FHL2, we used WT and *Fhl2*<sup>-/-</sup> MEFs to carry out ChIP-qPCR assays with  
280 antibody against RNA polymerase II (Pol II), which transcribes all the protein-coding genes.  
281 The primers I and II were employed for qPCR to amplify the endogenous TGF- $\beta$ 1 promoter  
282 using chromatin immunoprecipitated with anti-Pol II antibody as template (see Fig. 2A). The  
283 recruitment of Pol II to the TGF- $\beta$ 1 promoter was significantly increased in *Fhl2*<sup>-/-</sup> MEFs in  
284 comparison to WT cells (Figs. 4A and 4B). These data suggest that loss of FHL2 may alter  
285 chromatin architecture to allow Pol II to gain access to the TGF- $\beta$ 1 promoter.

286 ***Fhl2*<sup>-/-</sup> mice show increased susceptibility to fibrogenesis in the liver**

287 TGF- $\beta$ 1 is considered the most powerful mediator of fibrogenesis. The high  
288 expression level of TGF- $\beta$ 1 in *Fhl2*<sup>-/-</sup> liver suggested that *Fhl2*<sup>-/-</sup> mice might be pro-  
289 fibrogenic. We subjected *Fhl2*<sup>-/-</sup> and WT mice to bile duct ligation (BDL) operation, which is  
290 widely used model for liver fibrosis through cholestatic liver injury (30). We sacrificed mice  
291 15 days after BDL. Livers derived from BDL-treated mice lacking FHL2 developed severe  
292 fibrosis when compared to WT animals, showing wider fibrotic scars and substantially more  
293 scar branching as assessed by staining with hematoxylin-eosin and Sirius red, which directly  
294 marks the extracellular matrix deposited by activated hepatic stellate cells (HSC) (Figs. 5, A-  
295 C, E-G, I-K, M-O and Q). Moreover, FHL2 KO livers harbored a large increase in activated  
296 HSCs as determined by  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) staining (Figs. 5D, H, L and P). We  
297 further confirmed our findings in a second well-established model of liver fibrosis induced by  
298 thioacetamide (TAA) administration. *Fhl2*<sup>-/-</sup> mice showed increased fibrosis after 20 weeks of  
299 TAA treatment (data not shown). These results are in agreement with a previous report  
300 showing enhanced fibrosis in *Fhl2*<sup>-/-</sup> mice using carbon tetrachloride (CCl<sub>4</sub>)-induced model of  
301 liver fibrosis (31).

302 To investigate the molecular characteristics of fibrosis developed in *Fhl2*<sup>-/-</sup> liver, we  
303 examined expression by qRT-PCR of fibrogenesis markers including alpha-1 type I collagen,  
304 tissue inhibitor of metalloproteinases-1 (TIMP1) and TGF- $\beta$ 1 encoded respectively by  
305 *Colla1*, *Timp1* and *Tgfb1* genes, in the liver of *Fhl2*<sup>-/-</sup> and WT mice 15 days after BDL or  
306 control mice. This analysis revealed that expression of *Colla1* and *Timp1* was activated after  
307 BDL in both *Fhl2*<sup>-/-</sup> and WT mice (Fig. 6). Moreover, the levels of *Colla1* and *Timp1*  
308 activation following BDL were significantly higher in *Fhl2*<sup>-/-</sup> liver than those in WT liver,  
309 correlating with the aggravated fibrosis in *Fhl2*<sup>-/-</sup> mice (Fig. 6).

310 In accordance with the results in Fig. 1B, the mRNA level of TGF- $\beta$ 1 in the liver was  
311 higher in *Fhl2*<sup>-/-</sup> mice than WT mice at the steady-state condition (Fig. 6). Transcription of

312 TGF- $\beta$ 1 was significantly enhanced in fibrotic liver compared to control liver in both *Fhl2*<sup>-/-</sup>  
313 and WT mice (Fig. 6). Moreover, livers from *Fhl2*<sup>-/-</sup> animals displayed higher levels of TGF-  
314  $\beta$ 1 mRNA following BDL treatment compared to WT counterparts (Fig. 6), demonstrating  
315 that fibrogenic signaling was enhanced in the livers from *Fhl2*<sup>-/-</sup> animals. Of note, FHL2  
316 expression was upregulated in fibrotic liver of WT mice (Fig. 6), which is correlated with  
317 previous observations in human liver fibrosis (14, 31). Therefore, ablation of FHL2 leads to  
318 increased level of TGF- $\beta$ 1 and enhanced susceptibility to fibrogenesis.

### 319 **FHL2 deficiency has no effect on renal fibrosis**

320 A previous report showed that *Fhl2*<sup>-/-</sup> mice developed stronger fibrotic alterations in  
321 bleomycin-induced model for lung fibrosis following an unresolved inflammation (32). To  
322 test if FHL2 deficiency had a broad effect on fibrogenesis, we chose to examine fibrogenesis  
323 in the kidney, as FHL2 plays roles in the development of kidney disease (33). We used the  
324 bacteria *Leptospira* infection model for renal fibrosis (22) and infected age- and sex-matched  
325 WT and *Fhl2*<sup>-/-</sup> mice with equal amounts of bacteria *Leptospira* expressing luciferase. All the  
326 mice were successfully infected in the kidney, as attested by *in vivo* imaging (data not  
327 shown). After 21 days, kidneys were examined for fibrosis using Sirius red staining and  
328 expression of TGF- $\beta$ 1. As shown in Figs. 7A-7P, bacteria *Leptospira* induced fibrosis in the  
329 kidney in both WT and *Fhl2*<sup>-/-</sup> mice. But no significant difference in the accumulation of  
330 fibrotic tissue was observed between WT and *Fhl2*<sup>-/-</sup> animals (Fig. 7Q). Analysis of TGF- $\beta$ 1  
331 expression by qPCR showed no change in mRNA after bacteria infection between WT and  
332 *Fhl2*<sup>-/-</sup> mice (Fig. 7R). These results attest the tissue-dependent feature in TGF- $\beta$ 1 activation  
333 and fibrogenesis associated with FHL2 deficiency.



334 **DISCUSSION**

335           Given the importance of TGF- $\beta$ 1 in many biological processes, it is fundamental to  
336 understand the mechanisms controlling its expression. In this report, we show that FHL2 acts  
337 as an important negative regulator of TGF- $\beta$ 1 transcription. The action of FHL2 in inhibiting  
338 TGF- $\beta$ 1 transcription is likely to involve both direct and indirect mechanisms.

339           Previous studies demonstrate that the effects of FHL2 on transcription can be positive  
340 or negative depending on the gene target and the cellular context (10, 34). Associated with the  
341 TGF- $\beta$ 1 promoter, FHL2 plays the role of co-repressor in the transcription of the TGF- $\beta$ 1  
342 gene. We show that overexpression of FHL2 inhibits the activity of TGF- $\beta$ 1 promoter,  
343 whereas ablation of FHL2 increases its transcription. The inhibition of TGF- $\beta$ 1 promoter by  
344 FHL2 is dose-dependent, suggesting that the repressive activity could be intrinsic to FHL2 or  
345 FHL2-associated factors with repressive functions. The transcription factors with which  
346 FHL2 interacts on the TGF- $\beta$ 1 promoter remain to be determined. Our search of transcription  
347 factor binding sites on the TGF- $\beta$ 1 promoter has identified multiple responsive elements  
348 recognized by Fos and Jun, which are known FHL2-associated transcription factors (11).  
349 FHL2 could act through Jun and Fos to inhibit the transcription of TGF- $\beta$ 1. In the face of  
350 TGF- $\beta$ 1 cytokine treatment, which activates the TGF- $\beta$ 1 promoter, transient overexpression  
351 of FHL2 can successfully abrogate the activation, correlating with the repressive effects of  
352 FHL2 on the TGF- $\beta$ 1 transcription. Under the physiological condition, TGF- $\beta$ 1-induced  
353 activation of the TGF- $\beta$ 1 promoter depends on a multitude of signaling pathways through the  
354 binding of signal-responsive activators and repressors, including FHL2, which also positively  
355 responds to the TGF- $\beta$ 1 cytokine (17, 18). The feedback loop of FHL2 and TGF- $\beta$ 1 may  
356 constitute one of the complex transcriptional regulation networks that determine the level and  
357 the outcome of TGF- $\beta$ 1 in cells. It is not clear how TGF- $\beta$ 1 induces hepatic fibrosis in *Fhl2*<sup>-/-</sup>

358 mice. Our observation that expression of TGF- $\beta$  targets Ski, SnoN and phosphorylation of  
359 Smad2/3 were not altered in the liver deficient for FHL2 suggests that the canonical TGF- $\beta$   
360 signaling may not be involved in this process. It remains to be determined if the profibrotic  
361 action of TGF- $\beta$ 1 in *Fhl2*<sup>-/-</sup> liver is operated through noncanonical TGF- $\beta$  signaling.

362 Our finding that loss of FHL2 resulted in increased recruitment of RNA polymerase II  
363 on the TGF- $\beta$ 1 promoter confers a novel role to FHL2 in regulation of gene transcription. The  
364 question of how FHL2 is involved in the dynamic process of genome packaging to allow or  
365 restrict transcriptional regulators access to the regulatory sequences remains to be addressed.  
366 Composed of multiple LIM-domains for protein-protein interaction, FHL2 binds with several  
367 chromatin modifiers including histone acetyltransferase CBP/p300 (35), histone ubiquitin  
368 ligase Brca1 (36) and histone demethylase LSD1 (37) that actively participate to covalently  
369 modifying nucleosomes. It is possible that FHL2 could collaborate with these enzymes to  
370 maintain a repressed architecture by virtue of the restricted chromatin environment for  
371 transcription regulators. Ablation of FHL2 may tailor the way in which the chromatin is  
372 packaged to increase the access of RNA polymerase II to the TGF- $\beta$ 1 promoter.

373 Correlating with enhanced expression of TGF- $\beta$ 1, the fibrotic response in *Fhl2*<sup>-/-</sup> mice  
374 was dramatic in the liver. Previous report showed aggravated liver fibrosis after carbon  
375 tetrachloride injection in *Fhl2*<sup>-/-</sup> mice, but failed to detect increased expression of TGF- $\beta$ 1  
376 mRNA at the steady-state condition as reported here (31). The reason behind this difference is  
377 unclear but may reflect different sensibility in the detection of TGF- $\beta$ 1 transcription. Yet,  
378 Huss et al revealed higher level of TGF- $\beta$ 1 in fibrotic tissues from *Fhl2*<sup>-/-</sup> mice than WT mice,  
379 consistent with our findings that activation of TGF- $\beta$ 1 gene transcription is stronger in the  
380 absence of FHL2. Previous report pointed to higher fibrosis score in the lung of *Fhl2*<sup>-/-</sup> mice  
381 following bleomycin administration (32). It showed that loss of FHL2 resulted in increased  
382 expression of the pro-inflammatory matrix protein tenascin C and downregulation of the

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

389         Effects of FHL2 loss on fibrosis stress the medical relevance of the repressive activity  
390 of FHL2 on the TGF- $\beta$ 1 promoter. However, we and others previously reported that FHL2  
391 was upregulated in human liver fibrotic tissues and higher level of FHL2 was associated with  
392 advanced fibrosis (14, 31). This is correlated with our finding of FHL2 activation in liver  
393 fibrotic tissues of WT mice (see Fig. 6). In line with this observation, in human melanoma  
394 and colon cancer, enhanced expression of FHL2 is correlated with an increase of TGF- $\beta$  in  
395 tumors with invasion, metastasis and poor survival prognostics (18-20). This multifaceted  
396 nature of the relationship between FHL2 and TGF- $\beta$ 1 further supports the notion that the  
397 cellular context and the expression levels of each protein dictate the process and the outcome  
398 of the complex signal transduction. Our data described here contribute to unveiling new facets  
399 of TGF- $\beta$  regulation and should facilitate additional investigations aimed at further exploring  
400 TGF- $\beta$  biology and dysfunctions.

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403

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545 **Figure Legends**

546 **Figure 1.** Loss of FHL2 enhances TGF- $\beta$ 1 expression in the liver. (A) TGF- $\beta$ 1 cytokine in  
547 Kupffer cells isolated from WT and *Fhl2*<sup>-/-</sup> animals, as evaluated by ELISA. \*\*,  $p < 0.005$ .  
548 The data are the mean  $\pm$  S.D. obtained from five animals in each genotype. (B) Analysis of  
549 TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 mRNA by real-time reverse transcription (RT)-PCR in  
550 indicated tissues from WT and *Fhl2*<sup>-/-</sup> mice. The values in the WT tissues were arbitrarily set  
551 as 1. The average and S.D. values for three independent experiments are shown. \*,  $p < 0.05$ .  
552 (C) Analysis of expression of SnoN, Ski, Smad2/3 and phosphorylation of Smad2/3 in the  
553 liver of four WT and four *Fhl2*<sup>-/-</sup> animals by immunoblotting. Arrow head: FHL2, NS:  
554 nonspecific.

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

568 **Figure 3.** FHL2 is a negative regulator of the TGF- $\beta$ 1 promoter. (A) Luciferase reporter assay  
569 in WT and *Fhl2*<sup>-/-</sup> 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*<sup>-/-</sup>  
572 MEFs. To restore FHL2 expression in *Fhl2*<sup>-/-</sup> MEFs, human FHL2 was stably expressed in  
573 *Fhl2*<sup>-/-</sup> 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*<sup>-/-</sup> mice. *Fhl2*<sup>-/-</sup> 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*<sup>-/-</sup> 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

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

596 **Figure 6.** Expression of *Coll1a1*, *Tgfb1*, *TIMP1* and *Fhl2* mRNA in the liver of WT and *Fhl2*<sup>-/-</sup>  
597 mice after BDL. Hepatic levels of *Coll1a1*, *Tgfb1*, *TIMP1* and *Fhl2* mRNA were examined  
598 by qPCR in control and operated WT and *Fhl2*<sup>-/-</sup> mice. \*,  $p < 0.05$ , \*\*,  $p < 0.005$ , \*\*\*,  $p <$   
599 0.0005. The data presented are the mean  $\pm$  S.D. obtained from at least five mice in each  
600 group.

601 **Figure 7.** No effect of FHL2 deficiency on renal fibrosis. Kidneys were derived from naive or  
602 leptospira-infected WT and *Fhl2*<sup>-/-</sup> mice. Light microscopy of kidney stained with H&E  
603 staining (A, B, E, F, I, J, M, N) and collagen deposition stained with Sirius red (C, D, G, H,  
604 K, L, O, P). Original magnifications : (A, C, E, G, I, K, M, O) 40 x, (B, D, F, H, J, L, N, P)  
605 100 x. Sirius red quantification is shown in Q. TGF- $\beta$ 1 mRNA was analyzed by qPCR in  
606 kidney from naive or leptospira-infected WT and *Fhl2*<sup>-/-</sup> mice (R). The data presented are the  
607 mean  $\pm$  S.D. obtained from five mice in each group.

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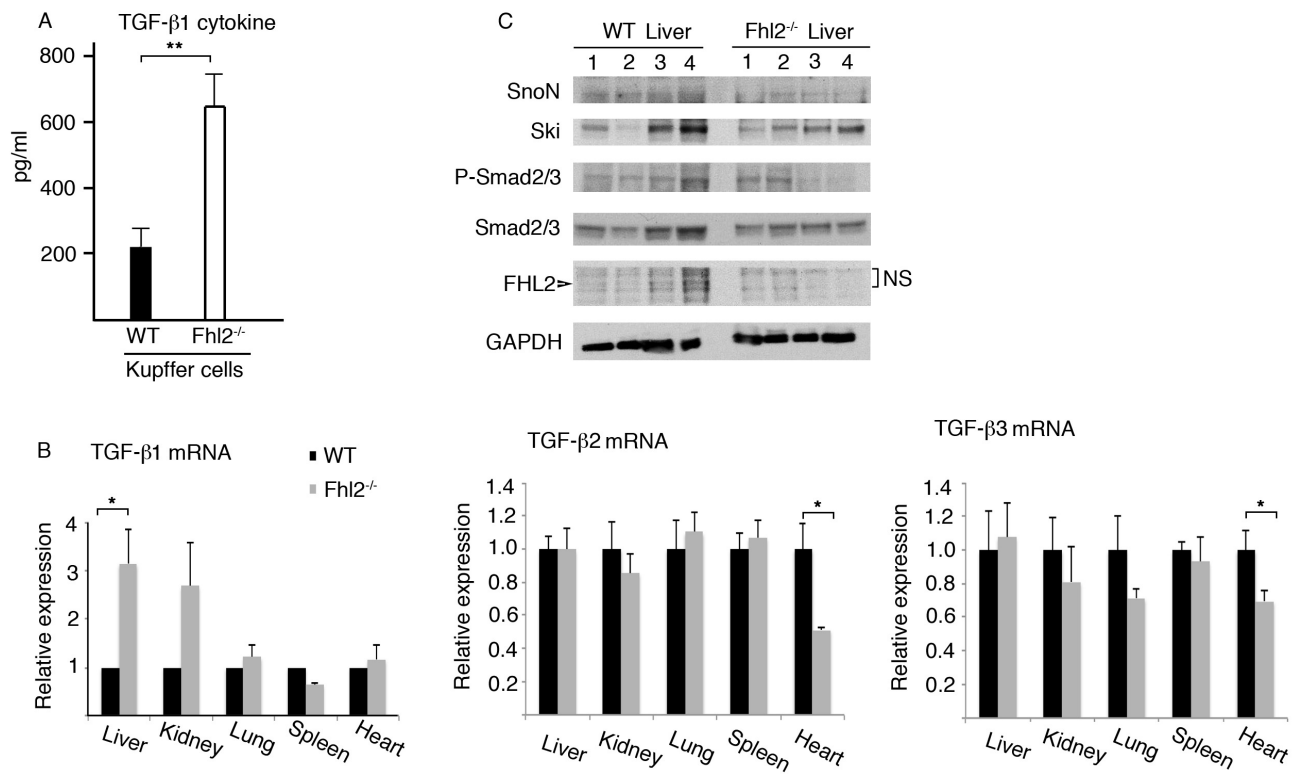


Fig. 1

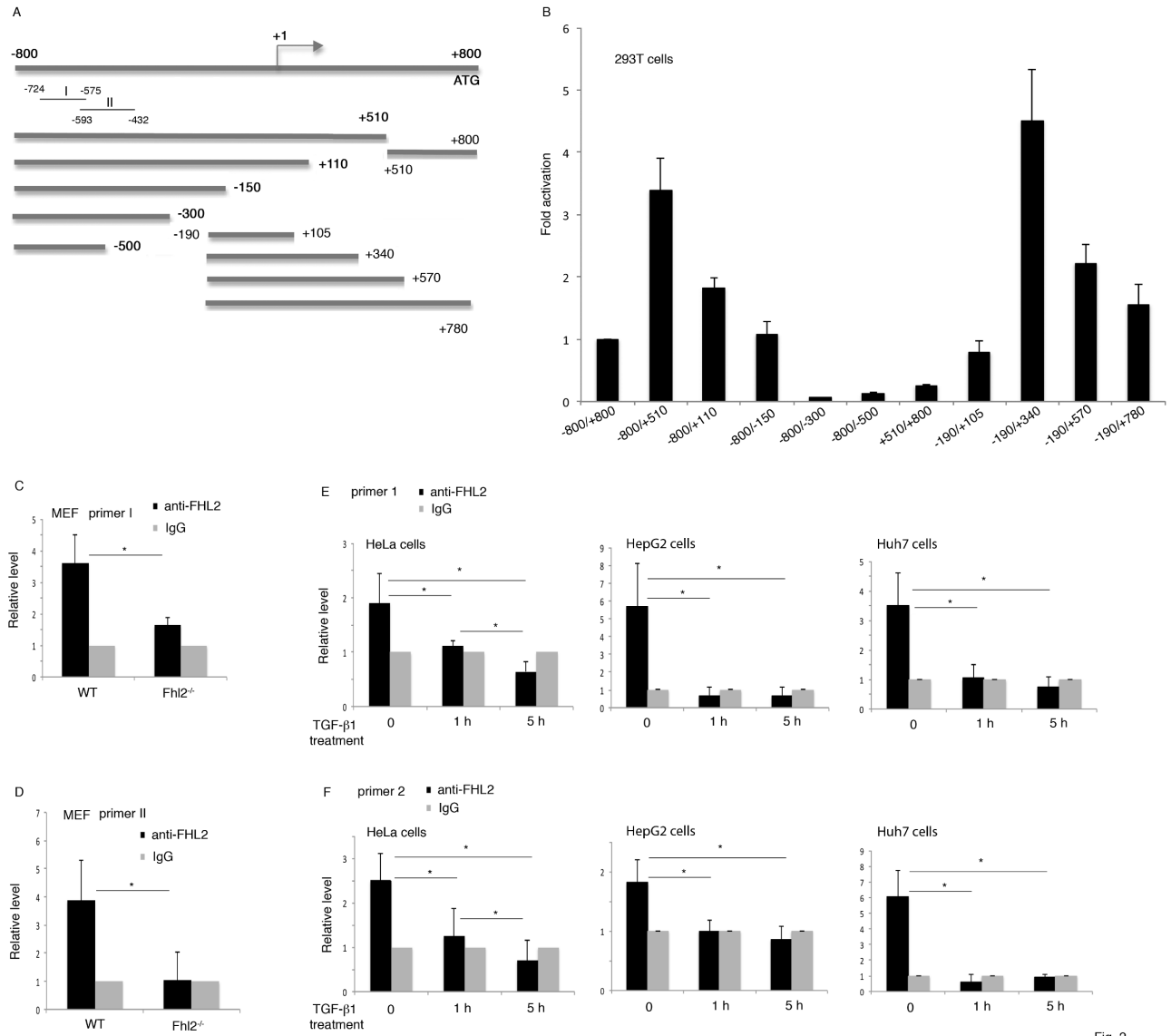


Fig. 2

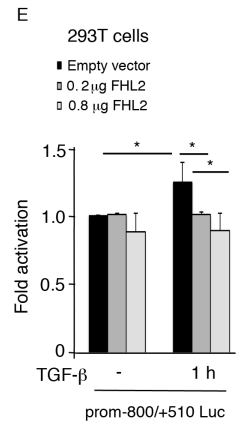
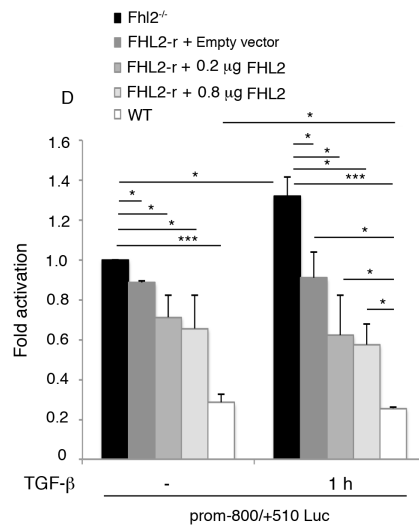
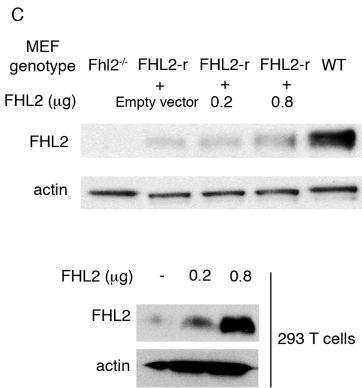
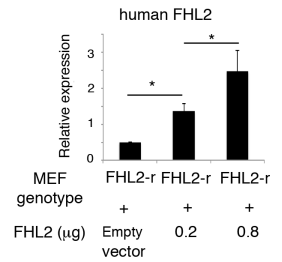
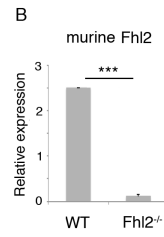
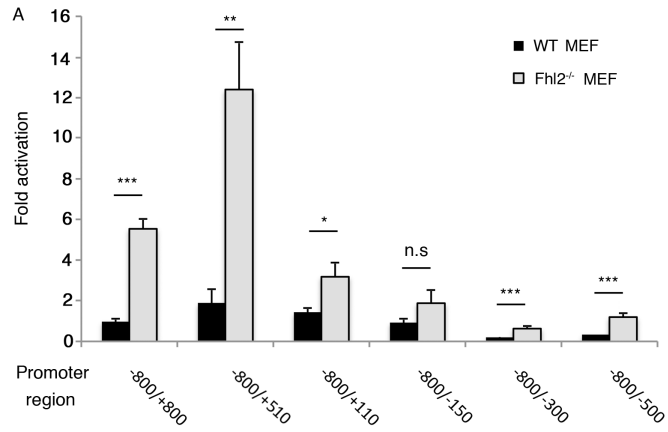


Fig. 3

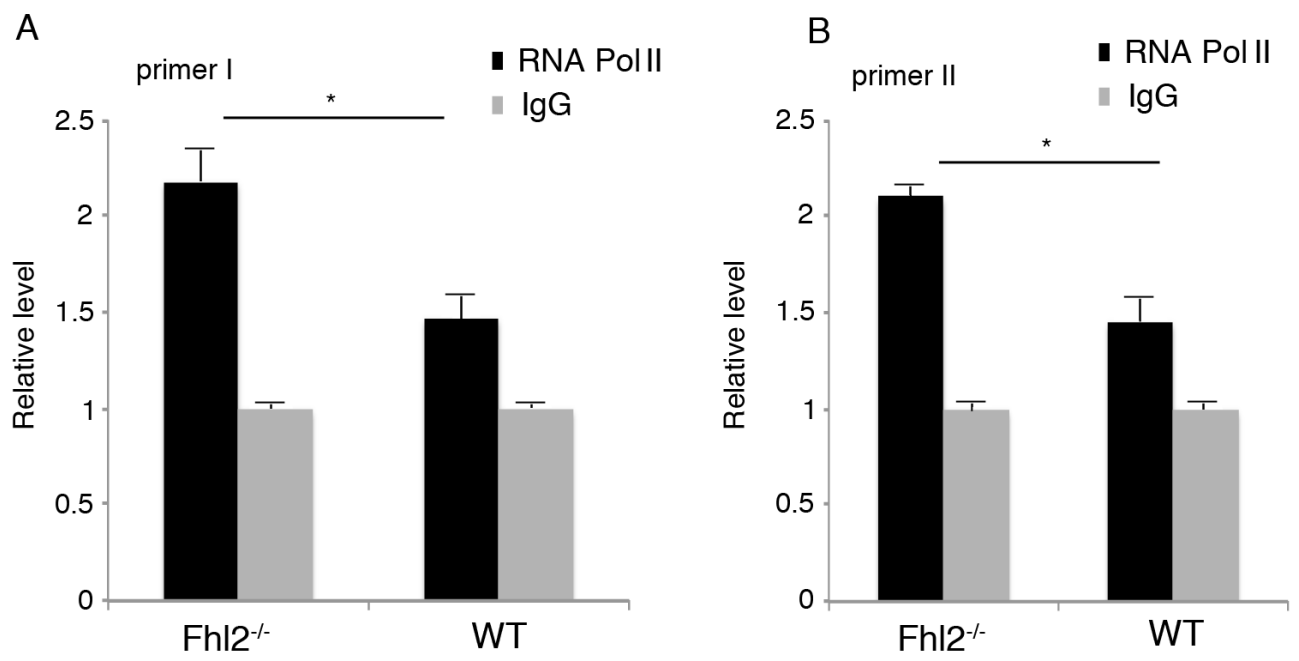


Fig. 4

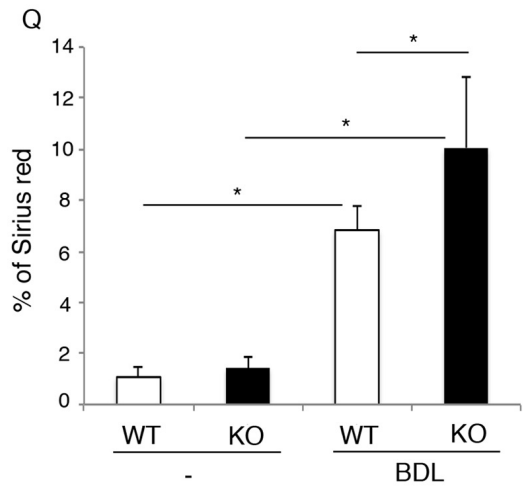
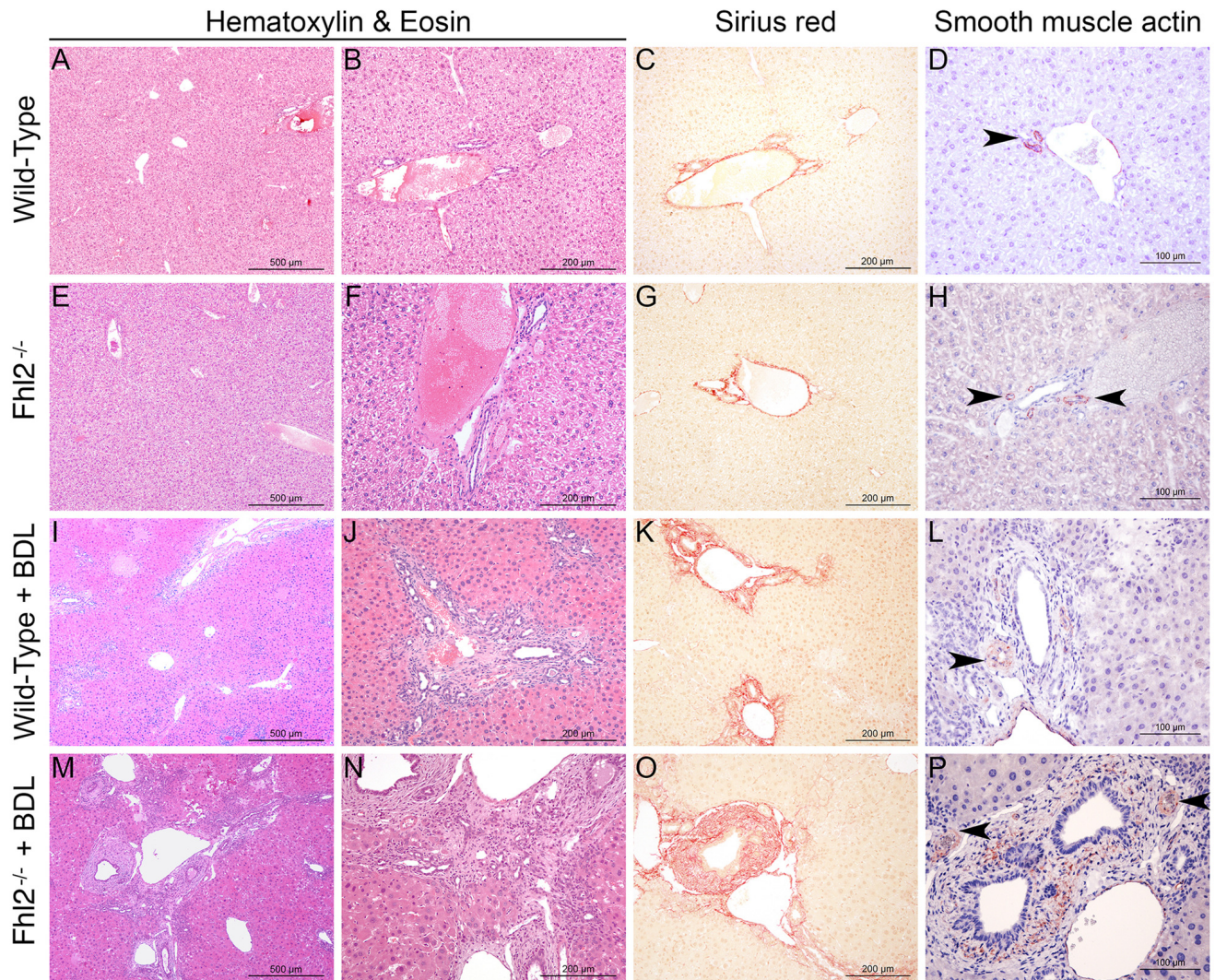


Fig. 5

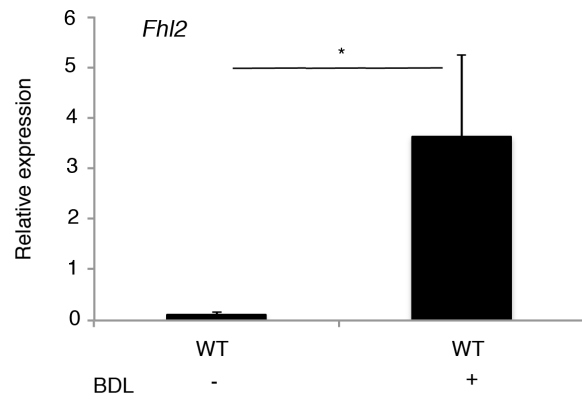
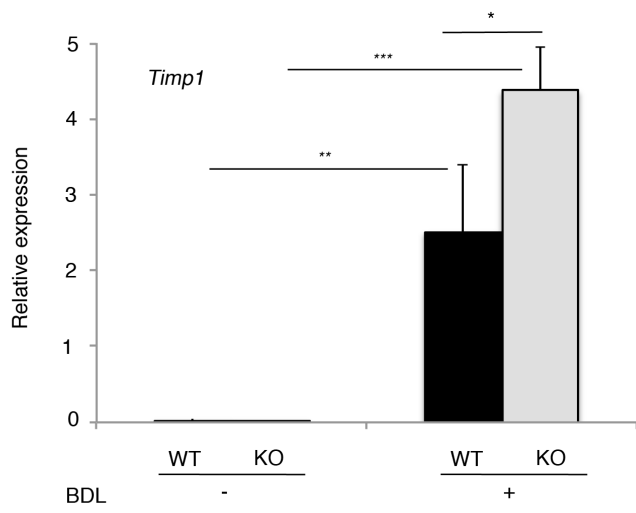
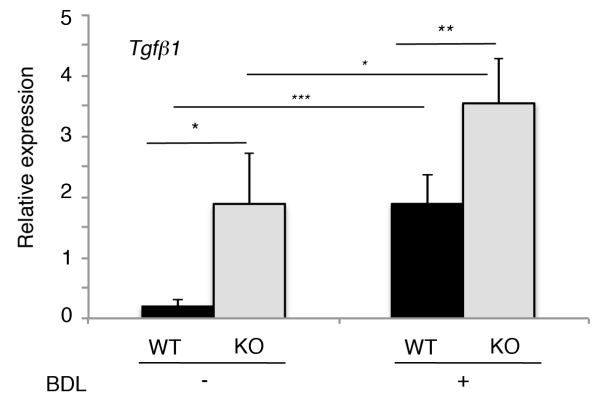
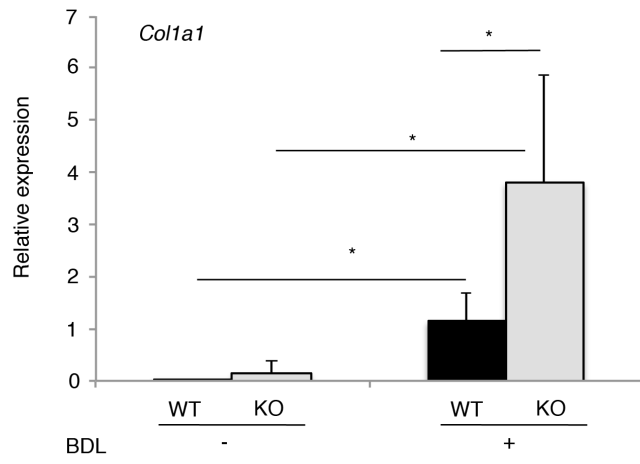


Fig. 6



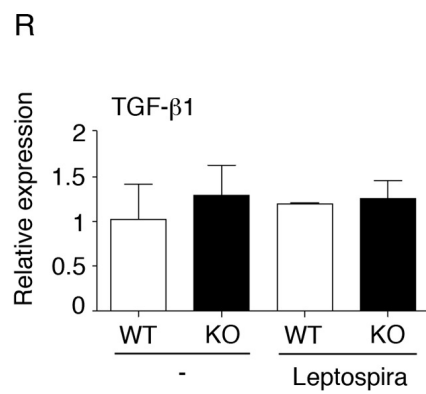
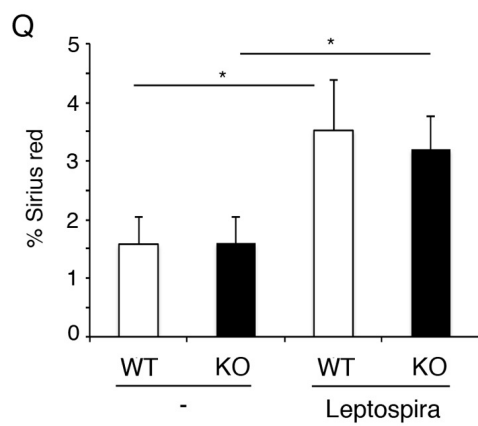
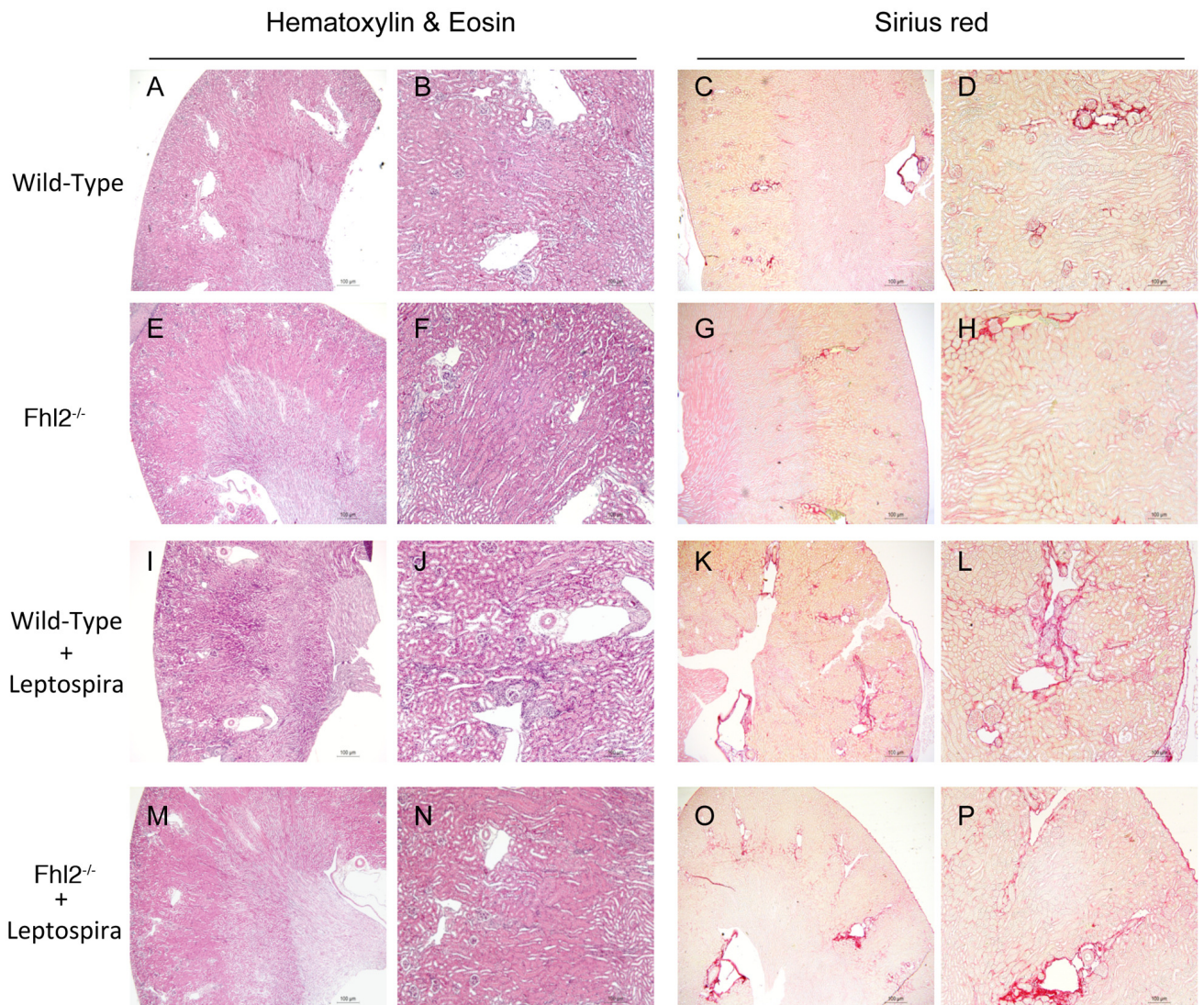


Fig. 7