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LIM-Only Protein FHL2 Activates NF-κB Signaling in the Control of Liver Regeneration and Hepatocarcinogenesis

Jennifer Dahan,[a,b] Yann Nouët,[a]* Grégory Jouvion,[c] Florence Levillayer,[a,b] Minou Adib-Conquy,[d] Anne-Marie Cassard-Doulcier,[e,f] Ali Tebbi,[b] Fany Blanc,[g] Lauriane Remy,[b] Ju Chen,[h] Stefano Cairo,[i] Catherine Werts,[j] Mustapha Si-Tahar,[g]* Thierry Tordjmann,[k] Marie-Annick Buendia,[a]* Yu Wei[a,b]

Unité d'Oncogenèse et Virologie Moléculaire,[a] Laboratoire de Pathogenèse des Virus de l'Hépatite B,[b] Unité d'Histopathologie Humaine et Modèles Animaux,[c] Unité de Recherche Cytokines et Inflammation,[d] Unité Défense Innée et Inflammation,[g] and G5 Biologie et Génétique de la Paroi Bactérienne,[j] Institut Pasteur, Paris, France; Inserm UMR_S996, Clamart, France[e]; Université Paris Sud, Faculté de Médecine Paris-Sud, Kremlin-Bicêtre, France[f]; XenTech, Evry, France[i]; Inserm U757, Université Paris Sud, Orsay, France[k]; Department of Medicine, University of California, San Diego, La Jolla, California, USA[h]

Four-and-a-half LIM-only protein 2 (FHL2) is an important mediator in many signaling pathways. In this study, we analyzed the functions of FHL2 in nuclear factor κB (NF-κB) signaling in the liver. We show that FHL2 enhanced tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) activity in transcriptional activation of NF-κB targets by stabilizing the protein. TRAF6 is a binding partner of FHL2 and an important component of the Toll-like receptor–NF-κB pathway. Knockdown of FHL2 in 293-hTLR4/MD2-CD14 cells impaired lipopolysaccharide (LPS)-induced NF-κB activity, which regulates expression of inflammatory cytokines. Indeed, *FHL2*$^{-/-}$ macrophages showed significantly reduced production of TNF and interleukin 6 (IL-6) following LPS stimulation. TNF and IL-6 are the key cytokines that prime liver regeneration after hepatic injury. Following partial hepatectomy, *FHL2*$^{-/-}$ mice exhibited diminished induction of TNF and IL-6 and delayed hepatocyte regeneration. In the liver, NF-κB signaling orchestrates inflammatory cross talk between hepatocytes and hepatic immune cells that promote chemical hepatocarcinogenesis. We found that deficiency of FHL2 reduced susceptibility to diethylnitrosamine-induced hepatocarcinogenesis, correlating with the activator function of FHL2 in NF-κB signaling. Our findings demonstrate FHL2 as a positive regulator of NF-κB activity in liver regeneration and carcinogenesis and highlight the importance of FHL2 in both hepatocytes and hepatic immune cells.

Four-and-a-half LIM-only protein 2 (FHL2) plays important roles in cell proliferation, apoptosis, and transformation (1). Endowed with a unique structure exclusively composed of 4.5 LIM domains for protein-protein interaction, FHL2 has been shown to interact with more than 50 cellular partners involved in almost all major signaling pathways that govern essential cellular processes. In hormone signaling, FHL2 is the transcriptional coactivator of both androgen and estrogen receptors (2, 3). In the transforming growth factor β (TGF-β) pathway, FHL2 interacts with Smad proteins (4), the transcription repressor Ski (5), and the E3 ligase Arkadia (6) and plays the role of the positive regulator in response to TGF-β signaling. FHL2 is a serum-inducible coactivator of the transcription factor AP1 (7), the downstream effector of receptor tyrosine kinase-initiated signaling cascades. In Wnt/β-catenin signaling, FHL2 exerts both transcription corepressor and coactivator functions of β-catenin, depending on the cellular context (8–11). The main receptor of Sonic hedgehog Patched recruits a proapoptotic complex containing FHL2 and caspase 9 and triggers cell death through a caspase 9-dependent mechanism (12). In the Toll-like receptor (TLR)/tumor necrosis factor (TNF) signaling, FHL2 interacts with TNF receptor-associated factor 2 (TRAF2), TRAF4, and TRAF6 (13), which are key mediators in the signal transduction mechanisms that activate the nuclear factor κB (NF-κB) signaling pathway.

NF-κB transcription factors are master regulators of immunity, inflammation, cell survival, and proliferation (14). In steady-state conditions, NF-κB is inactive in the cytoplasm through association with the inhibitor of κB (IκB) proteins. Upon induction by signals ranging from proinflammatory cytokines to cell stress to pathogen-associated molecular patterns, IκB proteins undergo phosphorylation, ubiquitination, and degradation, releasing NF-κB for translocation to the nucleus, where NF-κB family members form homodimers or heterodimers to bind specific DNA sequences and promote transcription of target genes encoding cytokines, chemokines, and survival and growth factors (15).

The relationship between FHL2 and NF-κB is highly cell context dependent. During osteoclast formation stimulated by the receptor activator of NF-κB (RANK) ligand, FHL2 inhibits TRAF6-induced NF-κB activity (13). In muscle cells, FHL2 enhances production of interleukin 6 (IL-6) through activation of NF-κB and p38 signaling pathways (16). In HEK293 cells, FHL2 induces TRAF2-mediated activation of NF-κB by counteracting the suppressive effects of Tucan/Cardinal on NF-κB (17). The NF-κB signaling pathway is crucial for liver biology and disease (18). Knockout of the RelA/p65 component of NF-κB, IκB kinase β (IKKβ), or IKKγ in mice results in embryonic lethality due to liver apoptosis (19, 20). Deregulation of NF-κB signaling through

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Address correspondence to Yu Wei, yu.wei@pasteur.fr.
* Present address: Yann Nouët, Goodman Cancer Center and Department of Biochemistry, McGill University, Montreal, Quebec, Canada; Mustapha Si-Tahar, Inserm U1100, Faculté de Médecine, Tours, France; Marie-Annick Buendia, Inserm U785, Université Paris-Sud, Villejuif, France.
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conditional deletion of IKKβ or IKKγ or expression of a nondegradable form of IκBα in the liver leads to either enhancement or inhibition of the development of hepatocellular carcinoma (HCC) (21–23).

We have recently reported that enhanced expression of FHL2 in hepatocytes increases both cell proliferation and apoptosis and promotes liver tumorigenesis associated with activation of the Wnt/β-catenin signaling (24). Given the crucial functions of NF-κB in innate immunity and inflammation, we hypothesized that FHL2 might also have a role in liver hepatic immune cells through its implication in NF-κB signaling. Here, we used constitutive FHL2 knockout mice to investigate the relationship between FHL2 and NF-κB signaling in liver regeneration and carcinogenesis. We report that deletion of FHL2 suppresses *in vitro* and *in vivo* an array of NF-κB-mediated effects, including cytokine expression, hepatocyte proliferation, and chemical carcinogen-induced HCC. The underlying mechanisms involve regulation of TRAF6 expression by FHL2. Our findings provide evidence of the requirement of FHL2 for activation of NF-κB signaling in the liver.

MATERIALS AND METHODS

Animals. *FHL2*$^{-/-}$ mice on the Black Swiss–129-SV/J background (25) were backcrossed into C57BL/6J mice over more than eight generations. For partial hepatectomy, F1 *FHL2*$^{-/-}$ and *FHL2*$^{+/+}$ littermates generated by intercrossing *FHL2*$^{-/+}$ mice were used. For diethylnitrosamine (DEN)-induced tumorigenesis, F2 mice were used. F2 *FHL2*$^{-/-}$ mice were generated by intercrossing F1 *FHL2*$^{-/-}$ littermates. F2 wild-type (wt) mice were generated by intercrossing F1 *FHL2*$^{+/+}$ littermates. All experiments were performed with age- and sex-matched animals. Wild-type and *FHL2*$^{-/-}$ animals were housed in the same room in a pathogen-free environment at the Institut Pasteur animal facility and received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*. Experimental procedures were carried out in accordance with French government regulations.

Surgical procedures and treatments. Animal experiments were carried out according to the European Union directives for animal experimentation (decree 2001-131 published in the Journal Officiel 6 February 2001). Two-thirds hepatectomy was performed on adult male mice (8 to 12 weeks old) as described previously (26). Four to eight animals of each genotype were sacrificed at each time point posthepatectomy. To analyze hepatocyte proliferation, animals were injected intraperitoneally with bromodeoxyuridine (BrdU; 50 mg/kg of mouse body weight) 2 h before livers were harvested. After removal of the liver, part of the remnant liver lobes was fixed immediately in paraformaldehyde (PFA) and stained for BrdU. BrdU-positive cell nuclei were counted on 5 consecutive high-power fields in three animals of each genotype. Others were frozen in nitrogen and stored at −80°C. For cytokine assay on liver homogenates, livers removed from mice 1 h after partial hepatectomy (PH) were weighed and then homogenized with glass beads in 1 ml phosphate-buffered saline (PBS) using the Mixer Mill MM301 (Retsch). After centrifugation, supernatants were collected for TNF and IL-6 measurement by enzyme-linked immunosorbent assay (ELISA). Values were normalized to liver weight.

To analyze cytokine production in Kupffer cells, wt and *FHL2*$^{-/-}$ animals were fed a diet devoid of methionine-choline (MCD) (Scientific Animal Food & Engineering [SAFE]) for 2 weeks.

Immunohistochemistry and apoptosis detection. Immunohistochemistry (IHC) was described previously (24). Primary antibodies include BrdU (Dako), Ki-67 (Novocastra), and F4/80 (BM8) (Santa Cruz Biotechnology) (dilution, 1:100). Sections were probed with secondary antibodies using the Impress kit (Vector Laboratories). Revelation was performed using the 3,3′-diaminobenzidine (DAB) substrate detection kit (Vector Laboratories). Slides were counterstained with Mayer's hematoxylin solution.

Apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). TUNEL-positive cells were counted on 6 to 10 consecutive high-power fields in three animals of each genotype.

Cell culture, reagents, and reporter assay. 293T, 293-hTLR4/MD2-CD14 (InvivoGen), HEK293 cell lines expressing TLR4/MD2-CD14 and mouse embryonic fibroblasts (MEF) (27) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HepG2 cells were cultured in DMEM–F-12 with 3.5×10^{-7} M hydrocortisone and insulin (5 μg/ml). For reporter assays, 3×10^5 cells were plated in six-well plates and transfected using Lipofectamine 2000 (Invitrogen) with expression vectors coding for FHL2, TRAF6, or TRAF6 mutant C70A, Rel/p65, and luciferase reporter containing wt or mutated NF-κB-responsive elements. Luciferase activity was determined 48 h later. A renilla plasmid under the control of thymidine kinase was used as the internal control, and the total DNA amount in each transfection was kept constant by adding an empty vector. All experiments were performed in duplicate and repeated at least three times. FHL2 expression in 293-hTLR4/MD2-CD14 cells and TRAF6 expression in 293T cells were inhibited by lentiviral transduction of FHL2 or TRAF6 short hairpin RNA (shRNA) (Santa Cruz Biotechnology), respectively, followed by selection with puromycin. Lipopolysaccharide (LPS) TLR*grade* (ALX-581-012-L002; Alexis Biochemicals) (1 μg/ml) was used to stimulate FHL2-knockdown and control 293-hTLR4/MD2-CD14 cells for 18 h.

Protein analysis. Livers were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8), 200 mM NaCl, 0.1 mM EDTA, 0.5% NP-40, 10% glycerol, and protease inhibitors. For nuclear extraction, MEFs were lysed in 10 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% NP-40, and protease inhibitors. After centrifugation, the pellet was resuspended in 400 mM KCl, 50 mM Tris (pH 8), 0.5% NP-40, 1 mM DTT, and protease inhibitors and incubated for 45 min. The nuclear membrane was eliminated by centrifugation. After quantification with Bradford protein assay, equal amounts of protein extract were analyzed by immunoblotting. 293T cells transfected or not with FHL2 were lysed in Laemmli buffer, and protein extracts were resolved in 4 to 12% polyacrylamide gels (Invitrogen) and transferred to Hybond-C extra (Amersham Pharmacia Biotech). Primary antibodies include TRAF6, STAT3, phospho-STAT3, phospho-p65 (S536) (Cell Signaling), p65, PCNA, cyclin-dependent kinase 2 (CDK2), CDK4, and cyclin A (Santa Cruz Biotechnology), FHL2 (MBL), and actin and tubulin (Sigma-Aldrich). Signal intensities of nuclear p65 were scanned and quantified by using the LI-COR/Odyssey infrared image system. To determine TRAF6 turnover, 293T cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich) and harvested at the indicated times. Signal intensities were scanned and quantified by using NIH ImageJ software as described previously (28).

Cytokine assay. Bone marrow-derived macrophages (BMDMs) were prepared from wt and *FHL2*$^{-/-}$ mice as described previously (29) and stimulated for differentiation with 400 u/ml macrophage colony-stimulating factor (M-CSF). A total of 1×10^6 adherent BMDMs plated in six-well plates were cultured in RPMI with 10% heat-inactivated FBS and then induced with 1 μg/ml LPS for 24 h. TNF and IL-6 were measured in supernatants with ELISA as specified by the manufacturer (DuoSet, R&D Systems, Minneapolis, MN).

Kupffer cells were isolated from animals on MCD or normal diets as described previously (30). TNF and IL-6 were measured in supernatants with ELISA.

RNA analysis. Total RNA was extracted from Kupffer cells or homogenized liver tissues using TRIzol (Invitrogen). RNA (1 μg) was reverse transcribed using random primers (Promega) and RevertAid HMinus reverse transcriptase (Fermentas). 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

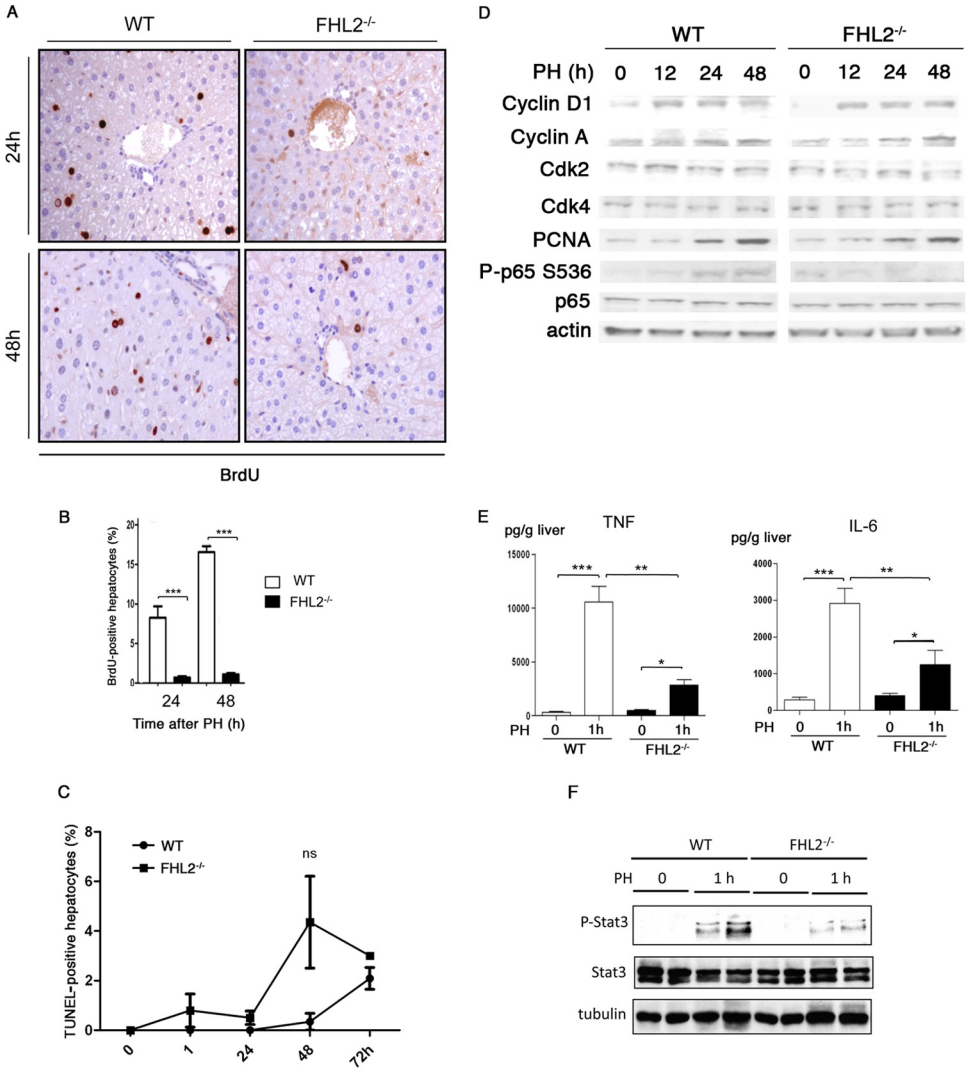


FIG 1 Delayed liver regeneration in *FHL2*$^{-/-}$ mice after partial hepatectomy (PH). (A) Immunohistochemistry on liver sections from wt and *FHL2*$^{-/-}$ mice 24 h and 48 h after PH with BrdU antibody. (B) Delayed DNA synthesis after PH in *FHL2*$^{-/-}$ liver as determined by BrdU incorporation. Asterisks indicate significant differences between *FHL2*$^{-/-}$ and wt animals. ***, $P < 0.0005$. (C) Apoptosis was detected by TUNEL. Quantification of TUNEL-positive cells in wt and *FHL2*$^{-/-}$ livers 0 h, 1 h, 24 h, 48 h, or 72 h after PH. ns, not significant. (D) Immunoblotting analysis of cell cycle regulators and phosphorylation of NF-κB p65 on S536. (E) TNF and IL-6 in wt and *FHL2*$^{-/-}$ livers 1 h after PH, as evaluated by ELISA. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. (F) Reduced phosphorylation of STAT3 in *FHL2*$^{-/-}$ livers 1 h after PH. Proteins extracted from wt and *FHL2*$^{-/-}$ livers 1 h after PH were analyzed by Western blotting.

normalized to the mean copy number of hypoxanthine-guanine phosphoribosyltransferase (HPRT). The following primers were used for real-time PCR: FHL2, 5′-GTCCTACAAGGATCGGCACT-3′, 5′-ACAGGTGAAGCAGGTCTCGT-3′; HPRT, 5′-ATGCCGAGGATTTGGAAAAA-3′, 5′-ACAATGTGATGGCCTCCCA-3′; TLR4, 5′-AACAAGAAACGGCAACTTGGA-3′, 5′-CCATAGCAGAGCCCCAGGT-3′; IL-6, 5′-TTGCCTTCTTGGGACTGATGCT-3′, 5′-GTATCTCTCTGAAGGACTCTGG-3′; TNF, 5′-GTGACCTGGACTGTGGGCCTC-3′, 5′-GGCTCTGTGAGGAAGGCTGTG-3′.

Statistical analysis. A chi-square test was used for statistical analysis of tumor incidence. Student's *t* test was used for other statistical analysis. The significant level was defined as *P* values of <0.05.

Tumor induction and analysis. Fourteen-day-old mice were injected intraperitoneally with 25 mg/kg DEN (Sigma) and sacrificed 3 h, 6 h, 15 h, 24 h, 48 h, or 8 months later. Livers were removed for RNA, protein, IHC, and TUNEL analyses.

Blood analysis. Blood samples were collected from mice, followed by centrifugation. Insulin, glucose, aspartate aminotransferase (ASAT), and triglyceride were measured in plasma by Vebiotel Laboratory (Arcueil, Val de Marne, France). A total of 30 μl of whole-blood samples was used for counting cell numbers with a vet ABC automat.

RESULTS

***FHL2*$^{-/-}$ mice exhibit diminished regeneration response after partial hepatectomy.** A previous study showed that overexpression of FHL2 in hepatocytes accelerated liver regeneration (24). To further investigate the role of FHL2 in liver regeneration, we performed a partial hepatectomy (PH) on *FHL2*$^{-/-}$ mice and sacrificed the mice at different time points to examine hepatocyte proliferation. BrdU incorporation demonstrated lower levels in DNA synthesis at 24 h and 48 h after PH in *FHL2*$^{-/-}$ mice than in wt mice but similar levels in DNA synthesis at 72 h and 96 h after PH between wt and *FHL2*$^{-/-}$ mice (Fig. 1A and B and data not shown). TUNEL analysis revealed no significant difference of apoptosis in regenerating livers from *FHL2*$^{-/-}$ and wt animals

(Fig. 1C). While cell cycle regulators cyclin D1, cyclin A, CDK2, CDK4, and PCNA were not significantly different between wt and *FHL2*$^{-/-}$ regenerating livers, the levels of NF-κB p65 phosphorylation at S536, indicative of NF-κB activation, were lower in *FHL2*$^{-/-}$ livers at 24 h and 48 h after PH (Fig. 1D). *FHL2*$^{-/-}$ mice eventually restored the same liver weight as wt mice 8 days after PH. These results showed that liver regeneration was delayed in *FHL2*$^{-/-}$ mice after PH. TNF and IL-6 are major cytokines for priming normally quiescent hepatocytes to enter the cell cycle in response to hepatic injury (31, 32). Examination of cytokine production 1 h after operation showed that TNF and IL-6 were expressed at dramatically lower levels in regenerating livers of *FHL2*$^{-/-}$ mice than those of wt mice (Fig. 1E). IL-6 stimulates compensatory hepatocyte proliferation through activation of the signal transducer and activator of transcription 3 (STAT3). Accordingly, immunoblotting analysis revealed reduced phosphorylation of STAT3 in *FHL2*$^{-/-}$ livers compared to wt counterparts at 1 h after PH (Fig. 1F). Overall, these findings suggest that loss of FHL2 impairs cytokine production and affects cytokine-regulated early proliferative response to hepatic injury.

Histological analysis of the FHL2-deficient liver revealed no notable anomaly (Fig. 2A) and no HCC after 2 years of observation. *FHL2*$^{-/-}$ mice show no spontaneous liver dysfunction, as attested by the absence of significant alterations in serum insulin, glucose, aspartate aminotransferase (ASAT), and triglyceride (Fig. 3A). Kupffer cells are the major cytokine producer in the liver. F4/80 immunostaining showed morphology and percentage of Kupffer cells similar to parenchymal cells between wt and *FHL2*$^{-/-}$ livers (Fig. 2B and C). Analysis by real-time PCR on RNA from Kupffer cells isolated from wt mice confirmed expression of FHL2 in this specific cell type (Fig. 2D). In addition, no difference was observed in cell composition in peripheral blood between wt and *FHL2*$^{-/-}$ mice (Fig. 3B). To assess the impact of FHL2 deficiency on cytokine production by Kupffer cells, we fed wt and *FHL2*$^{-/-}$ animals an MCD, which is known to induce steatosis-associated inflammation (33, 34). Kupffer cells isolated from animals on an MCD or control mice were cultured *in vitro*, and TNF and IL-6 in the supernatant were measured by ELISA. Remarkably, even though TNF and IL-6 were elevated in both wt and *FHL2*$^{-/-}$ Kupffer cells from animals on an MCD, their levels were significantly lower in *FHL2*$^{-/-}$ cells (Fig. 2E). Taken together, these results suggest that deficiency of FHL2 may affect signaling pathways that control cytokine expression.

FHL2 deficiency decreases LPS-induced cytokine production in macrophages. NF-κB signaling plays a central role in initiation of liver regeneration, as it promotes production of TNF and IL-6 (31). Lipopolysaccharide (LPS) is one of the important molecules for activation of NF-κB after PH (35), because liver injury provokes an increase of LPS levels originating from commensal intestinal bacteria. To examine the role of FHL2 in NF-κB activation induced by LPS, we knocked down FHL2 in 293-hTLR4/MD2-CD14 cells (Fig. 4A), which express the hTLR4/MD2-CD14 receptor complex for LPS, and transfected it with the NF-κB wt reporter or a reporter mutated at the NF-κB responsive element, followed by stimulation with LPS. While LPS potently activated NF-κB reporter in control cells, knockdown of FHL2 reduced the LPS-mediated NF-κB reporter activation by half (Fig. 4B). No effect of LPS was observed on the mutated NF-κB responsive elements (Fig. 4B). NF-κB regulates transcriptional programs that induce genes coding for cytokines, including TNF and IL-6. To assess the effects of FHL2 on NF-κB-mediated cytokine production, bone marrow-derived macrophages isolated from wt and *FHL2*$^{-/-}$ mice were stimulated for differentiation with M-CSF, followed by treatment with LPS. TNF and IL-6 in the supernatant were measured by ELISA. Significant reduction of TNF and IL-6 was observed in *FHL2*$^{-/-}$ macrophages after LPS stimulation (Fig. 4C), even though similar expression levels of TLR4 were detected in *FHL2*$^{-/-}$ and wt cells (Fig. 4D). To exclude the possibility that mice lacking FHL2 may harbor pathogens that elicit immune tolerance to LPS, we tested sera from wt or *FHL2*$^{-/-}$ mice in the TLR4 reporter cell line for screening LPS, which can be recognized by TLR4. None of the sera elicited any response in the line (data not shown). Taken together, these findings show that FHL2 might be required for triggering NF-κB-mediated cytokine production.

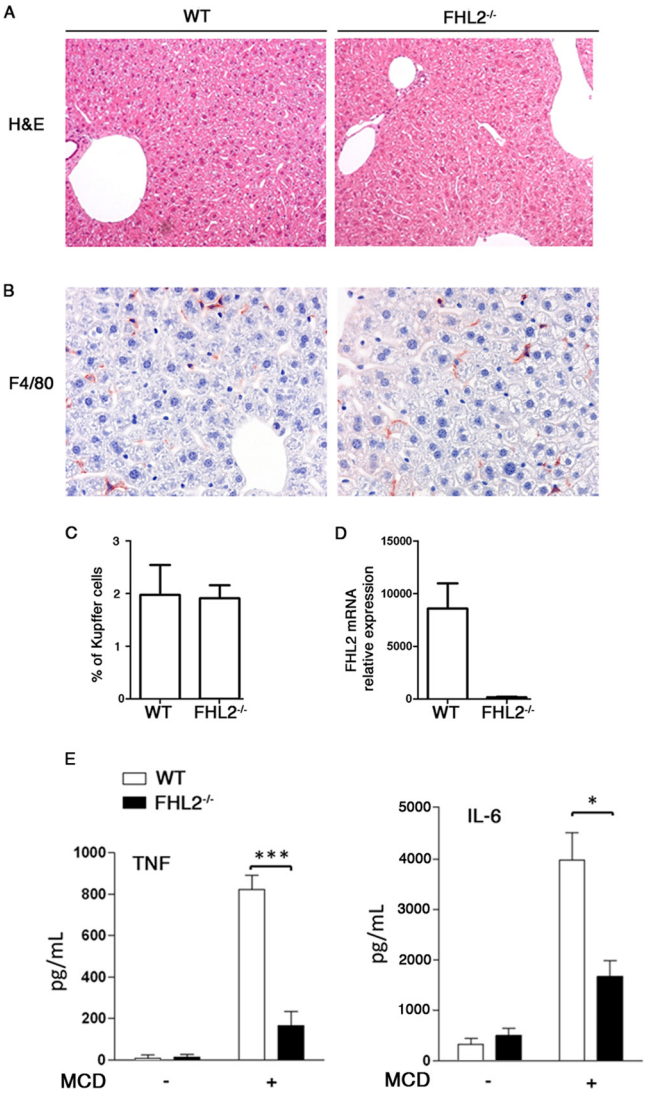


FIG 2 Loss of FHL2 has no effect on liver organization. (A) H&E shows hematoxylin and eosin staining of liver tissues. (B) Immunostaining of Kupffer cells with F4/80 antibody. (C) Quantification of Kupffer cells in wt and *FHL2*$^{-/-}$ livers. (D) Analysis of FHL2 expression by real-time reverse transcription (RT)-PCR in Kupffer cells isolated from wt and *FHL2*$^{-/-}$ mice. (E) TNF and IL-6 in wt and *FHL2*$^{-/-}$ Kupffer cells from animals on an MCD or a normal diet, as evaluated by ELISA. *, $P < 0.05$; ***, $P < 0.0005$.

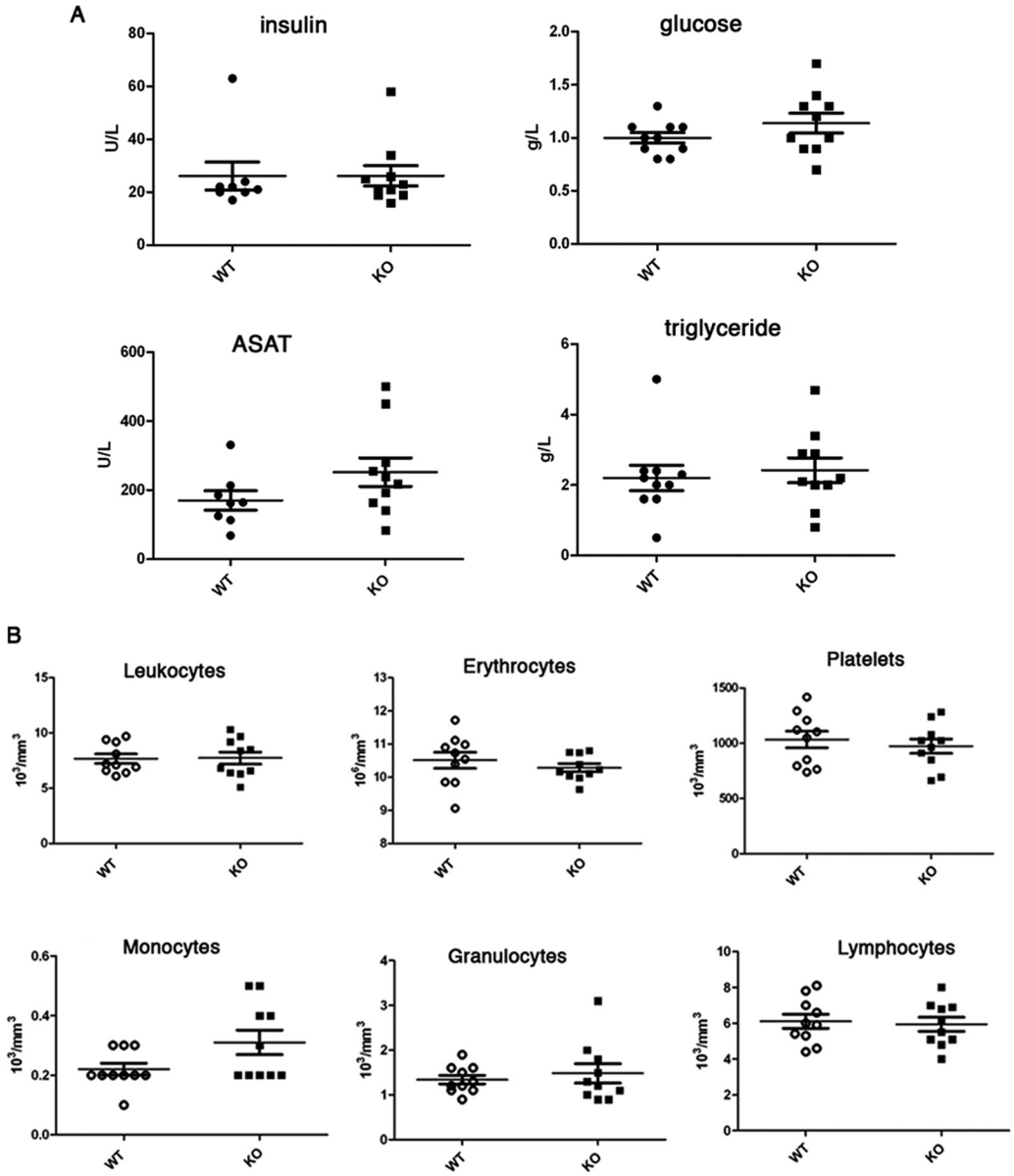


FIG 3 (A) Circulating insulin, glucose, aspartate aminotransferase (ASAT), and triglyceride levels in serum from 3-month-old wt and *FHL2*$^{-/-}$ mice. Each symbol represents one individual. (B) Full blood counts in 3-month-old wt and *FHL2*$^{-/-}$ mice. Each symbol represents one individual.

FHL2 participates in NF-κB signaling through TRAF6. FHL2 interacts with both cytoplasmic and nuclear proteins and is a cofactor of many transcription factors. We next examined whether FHL2 could synergize with NF-κB transcription factor in regulation of transcription by coexpressing FHL2 and p65 in the hepatoma cell line HepG2. FHL2 effectively potentiated p65 activity (Fig. 5A). However, we did not detect interaction between FHL2 and p65. We thus tested if FHL2 had impacts on NF-κB translocation. Wild-type or *FHL2*$^{-/-}$ mouse embryonic fibroblasts (MEF) were stimulated with LPS, and nuclear p65 expression was analyzed by immunoblotting. Deletion of FHL2 delayed nuclear translocation of NF-κB (Fig. 5B and C), suggesting that the effect of FHL2 on NF-κB is achieved mainly in the cytoplasm. It has been reported that FHL2 interacts with TRAF6 (13), an E3 ubiquitin ligase essential for the activation of NF-κB. To investigate the cooperation of FHL2 with TRAF6, we transfected the NF-κB luciferase reporter in combination with FHL2 and TRAF6 in 293T cells. Overexpression of wt TRAF6, but not the C70A mutant lacking E3 activity, readily activated NF-κB-dependent transcription (Fig. 5D, lanes 5 and 9), in agreement with previous reports (33–35). Strikingly, whereas FHL2 alone had a barely detectable effect on NF-κB reporter activity (Fig. 5D, lanes 2 to 4), it cooperated with wt TRAF6 but not the C70A mutant to enhance NF-κB signaling in a dose-dependent manner (Fig. 5D, lanes 6 to 8 and 10). Next, we knocked down TRAF6 in 293T cells and carried out NF-κB luciferase reporter assay in these cells. The activity of FHL2 on the NF-κB reporter was significantly diminished in TRAF6-knocked-down cells (Fig. 5E), indicating that TRAF6 is required for FHL2 to fully exert its function in activation of NF-κB signaling. To elucidate the mechanisms of FHL2 and TRAF6 coopera-

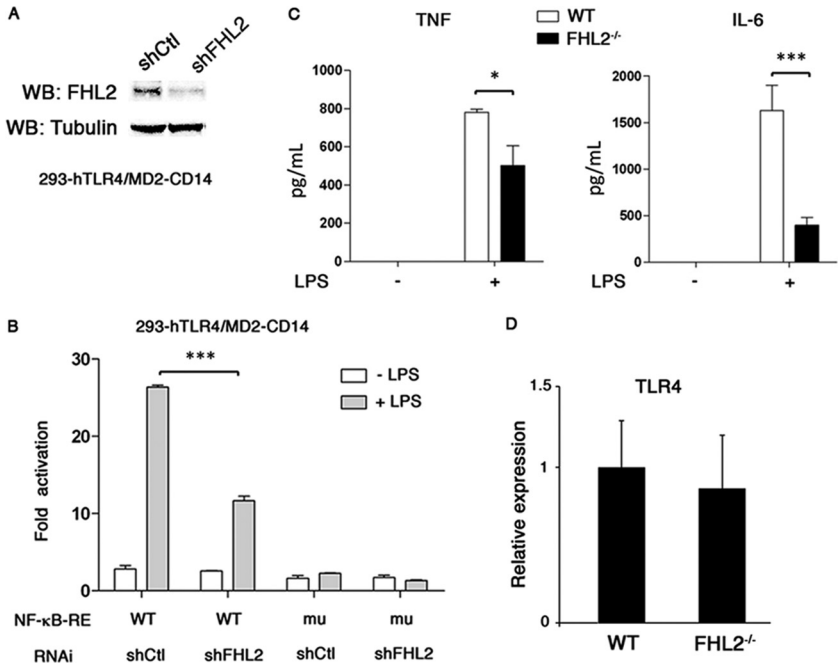


FIG 4 FHL2 deficiency results in diminished induction of cytokines TNF and IL-6 by LPS. (A) Knockdown of FHL2 in 293-hTLR4/MD2-CD14 cells. 293-hTLR4/MD2-CD14 cells were transduced with either control (shCtl) or FHL2 shRNA (shFHL2) lentiviral vectors. FHL2 expression was analyzed by Western blotting. (B) Reporter assay in 293-hTLR4/MD2-CD14 cells transduced with the control or FHL2 shRNA lentiviral vector. Cells were transfected with either NF-κB wt reporter (WT) or reporter in which the NF-κB responsive element is abolished (mu). Cells were stimulated with 1 μg/ml LPS for 18 h. The basal activity of the NF-κB wt reporter in cells transduced with shCtl without LPS stimulation was arbitrarily set at 1. Data are presented as mean induction in luciferase activity ± standard deviation (SD) from duplicate samples. The results shown are representative of those from more than three independent assays. RNAi, RNA interference. ***, $P < 0.0005$. (C) Bone marrow-derived macrophages (BMDM) isolated from wt and *FHL2*$^{-/-}$ mice were stimulated with 1 μg/ml LPS for 24 h. TNF and IL-6 were measured by ELISA. *, $P < 0.05$; ***, $P < 0.0005$. (D) Analysis of TLR4 expression in BMDM cells by real-time RT-PCR. The level of TLR4 in wt cells was arbitrarily set as 1.

tion, we assessed the impact of FHL2 on the stability of endogenous TRAF6 by transfecting FHL2 in 293T cells, followed by blocking protein synthesis with cycloheximide. Overexpression of FHL2 resulted in an increase in the stability of endogenous TRAF6 (Fig. 5F and G). These findings suggest that FHL2 participates in NF-κB signaling most likely via its ability to interact with the pathway component TRAF6.

Loss of FHL2 reduces susceptibility to DEN-induced liver carcinogenesis. NF-κB activation is intimately linked to hepatocarcinogenesis (36). A previous report demonstrated that hepatocyte-specific depletion of IKKβ increased carcinogenesis induced by the hepatic carcinogen diethylnitrosamine (DEN), whereas deletion of IKKβ in both hepatocytes and nonparenchymal cells reduced DEN-induced tumorigenesis (21). It has been argued that inactivation of NF-κB through deletion of IKKβ diminished induction of hepatomitogens in hepatic hematopoietic-derived cells, resulting in reduced compensatory proliferation of hepatocytes and thus tumorigenesis (21). As *FHL2*$^{-/-}$ mice exhibited diminished cytokine production in response to hepatic injury, we asked whether constitutive deletion of FHL2 might have similar effects as IKKβ deletion on carcinogenesis. To address it, we used the DEN model for liver carcinogenesis by injecting the chemical carcinogen into 14-day-old wt and *FHL2*$^{-/-}$ mice. As expected for wt mice, all males (8/8) developed HCCs within 8 months, versus 45.4% females with HCCs (5/11) (Table 1). Interestingly, tumor incidence was significantly lower in both *FHL2*$^{-/-}$ males ($P < 0.0005$) and females ($P < 0.005$) than in wt mice, as only 71.4% male (10/14) and 18.7% female (3/16) animals deficient for FHL2 developed tumors (Table 1). Histopathological analysis revealed HCCs of various sizes, most often multifocal and randomly distributed in the liver parenchyma in both wt and *FHL2*$^{-/-}$ mice (Fig. 6A to F). Tumors were well demarcated but not encapsulated, focally invading the remaining parenchyma (Fig. 6C and D). Tumor cells were mostly organized in nests and trabeculae, rarely in tubules, with lipids in the cytoplasm and giant nucleoli (Fig. 6E and F). Anisocytosis, anisokaryosis, and cellular atypia were frequent (data not shown). No vascular invasion was observed. Although not significant, FHL2 expression was enhanced in tumor tissues from wt mice (Fig. 6G). These data indicate that loss of FHL2 decreases susceptibility to DEN-induced hepatocarcinogenesis.

DEN-induced HCC requires NF-κB-dependent production of IL-6 by Kupffer cells and the activation of STAT3 by IL-6 in hepatocytes (37, 38). We compared early effects of DEN on IL-6 and TNF expression in wt and *FHL2*$^{-/-}$ livers and observed a trend but not a significant decrease of IL-6 in *FHL2*$^{-/-}$ mice at the 3-h time point (Fig. 7A). FHL2 expression was not enhanced by DEN administration in wt mice (Fig. 7A). Analysis of STAT3 activation by immunoblotting revealed delayed and reduced phosphorylation of STAT3 in *FHL2*$^{-/-}$ mice (Fig. 7B), suggesting that FHL2 deficiency affects the IL-6/STAT3 axis in the DEN model. Ki-67 immunostaining demonstrated a lower number of proliferating hepatocytes in *FHL2*$^{-/-}$ mice than wt mice 24 h and 48 h after DEN exposure (Fig. 7C and D). Moreover, *FHL2*$^{-/-}$ mice exhib-

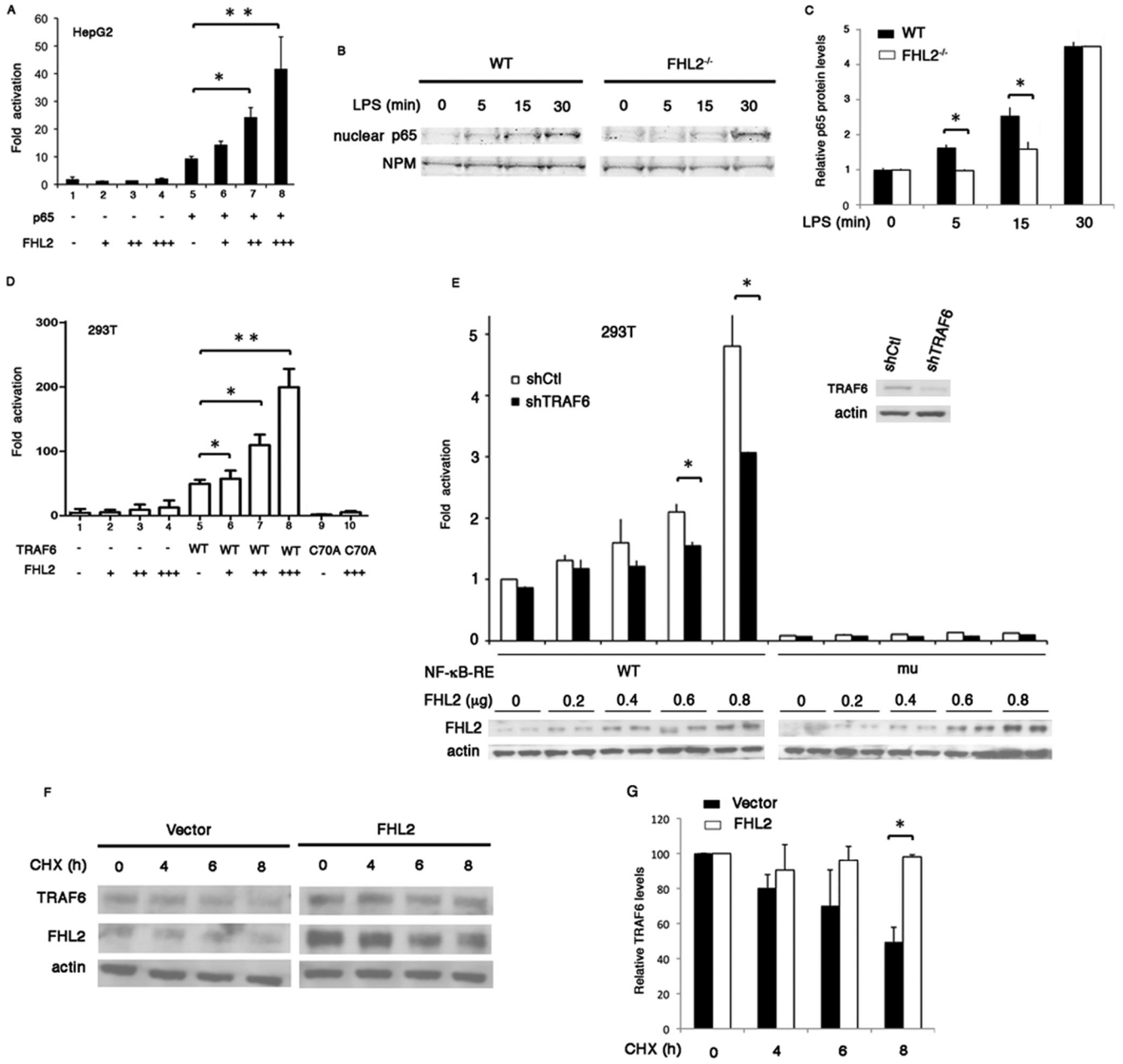


FIG 5 FHL2 cooperates with TRAF6 in activation of NF-κB. (A) HepG2 cells were transfected with NF-κB-responsive luciferase reporter together with p65 (0.05 μg) and increasing doses of FHL2 (0.1 μg, 0.3 μg, and 0.6 μg). TK-renilla was used as the internal control. The basal activity of the NF-κB reporter cotransfected with empty vector was arbitrarily set at 1. Data are presented as mean induction in luciferase activity ± SD from duplicate samples. The results shown are representative of those from more than three independent assays. *, $P < 0.05$; **, $P < 0.005$. (B and C) Loss of FHL2 delays NF-κB nuclear translocation. Wild-type and *FHL2*$^{-/-}$ mouse embryonic fibroblasts (MEF) were stimulated with 1 μg/ml LPS for 0, 5, 15, and 30 min, followed by extraction of nuclear proteins. (B) RelA/p65 expression was analyzed by Western blotting. Nucleophosmin (NPM) was used as a loading control. Signal intensities were plotted relative to 0-h values. (C) The value of p65 expression at 0 min was arbitrarily set as 1. Data are presented as the mean values ± SD from three experiments. *, $P < 0.05$. (D) 293T cells were transfected with NF-κB-responsive luciferase reporter (0.05 μg) together with TRAF6 (0.05 μg) or C70A and increasing doses of FHL2 (0.1 μg, 0.3 μg, and 0.6 μg). *, $P < 0.05$; **, $P < 0.005$. (E) NF-κB reporter assay in 293T cells transduced with control (shCtl) or TRAF6 (shTRAF6) shRNA lentiviral vectors. TRAF6 expression in 293T cells transduced with shCtl and shTRAF6 was analyzed by Western blotting (inset). Cells were transfected with either NF-κB wt or mutated reporter (mu) along with increasing doses of FHL2 (Western blots at the bottom). The basal activity of NF-κB wt reporter in cells transduced with shCtl was arbitrarily set at 1. Data are presented as mean induction in luciferase activity ± SD from duplicate samples. *, $P < 0.05$. (F and G) FHL2 overexpression increases the stability of TRAF6. 293T cells were transfected with empty vector or FHL2 and treated with cycloheximide as indicated. (F) Endogenous TRAF6 was analyzed by Western blotting. Signal intensities were plotted relative to 0-h values. (G) The value of TRAF6 expression at 0 h was arbitrarily set as 100. Data are presented as the mean values ± SD from three experiments. *, $P < 0.05$. CHX, cycloheximide.

TABLE 1 Decreased susceptibility to DEN-induced carcinogenesis in *FHL2*$^{-/-}$ mice

Mouse sex	Genotype	No. of mice studied	% (no.) of mice with tumors	*P*
Male	*FHL2*$^{-/-}$	14	71.4 (10)	<0.0005
	C57B	8	100 (8)	
Female	*FHL2*$^{-/-}$	16	18.7 (3)	<0.005
	C57B	11	45.4 (5)	
Total	*FHL2*$^{-/-}$	30	43.3 (13)	<0.005
	C57B	19	68.4 (13)	

ited lower levels of cell death at the 6-h time point (Fig. 7E and F). Taken together, loss of FHL2 diminishes STAT3 activation, hepatocyte proliferation, and apoptosis, which may account for the reduced susceptibility of *FHL2*$^{-/-}$ mice to DEN-induced hepatocarcinogenesis.

DISCUSSION

FHL2 has been shown to inhibit NF-κB activity in osteoclastogenesis (13). However, increasing evidence suggests that FHL2 has a dual role of both activating and suppressing NF-κB signaling in a cell context-dependent fashion (16, 17). Our results show that FHL2 acts as a positive regulator of NF-κB signaling in the liver at early phases of proliferative response and in chemical carcinogen-induced DNA damage *in vivo*. Loss of FHL2 produces inhibitory effects on liver regeneration by attenuating the activation of NF-κB in hematopoietic-derived cells during the initial stages of hepatic injury. Liver injury increases the levels of LPS originating from commensal intestinal bacteria in the portal circulation (35). The attenuated initial phase of inflammatory signaling in *FHL2*$^{-/-}$ livers after partial hepatectomy correlates with reduced production of inflammatory cytokines by *FHL2*$^{-/-}$ macrophages in response to LPS *in vitro*, suggesting that the hyporesponsiveness of *FHL2*$^{-/-}$ mice to hepatic injury might be mediated by suppression of TLR-induced NF-κB activity. However, this inhibitory effect is apparently insufficient to effectively restrain liver weight restoration, possibly because there exist redundant mechanisms that may functionally compensate for FHL2 deficiency. These findings underline the importance of the homeostatic functions of FHL2 in controlling NF-κB signaling for the regulation of cytokine production and hepatocyte proliferation.

Although the molecular mechanisms governing FHL2 involvement in NF-κB signaling are still unfolding, it is likely that the effects of FHL2 deficiency are operated, at least partly, by the suppression of TRAF6 activity. Activation of NF-κB signaling by TRAF6 requires polyubiquitination of TRAF6 through lysine 63 (K63) (39), which initiates phosphorylation and ubiquitination cascades in the downstream effectors, including TAK1, IκB, and IKKγ (40). The activity of these downstream effectors might be regulated by the interaction of FHL2 with TRAF6 and subsequent stabilization of TRAF6 by FHL2, which are needed for full transmission of signals that activate NF-κB, as abrogation of FHL2 delayed NF-κB nuclear translocation. FHL2 is known to interact with several RING domain E3s. Recently, we have shown that FHL2 inhibits assembly of ubiquitin chains K27 and K63 on the E3 ligase Arkadia, a regulator of TGF-β signaling (41). In the case of TRAF6, we did not observe any change of ubiquitination patterns provoked by FHL2 (data not shown). Further studies are required for elucidating the regulatory mechanisms. Functional specificity of FHL2 and TRAF6 cooperation in the balance between activation and inhibition of NF-κB signaling depending on environmental cues, such as osteoclastogenesis stimulated by RANK ligand, may explain the context-dependent and seemingly paradoxical effects of FHL2 on NF-κB signaling that have been reported (13). Interestingly, FHL2 is also a target of TRAF6 (42), which adds another layer of complexity in biological output of FHL2 relating to TRAF6.

In addition to the well-established role of FHL2 in both proliferation and apoptosis (27, 43), FHL2 is also associated with oncogenesis and cancer malignancies (24, 44). Loss of FHL2 displays inhibitory effects on liver carcinogenesis almost as effective as deletion of IKKβ in suppressing DEN-induced tumorigenesis. A previous report has demonstrated that hepatocyte proliferation in

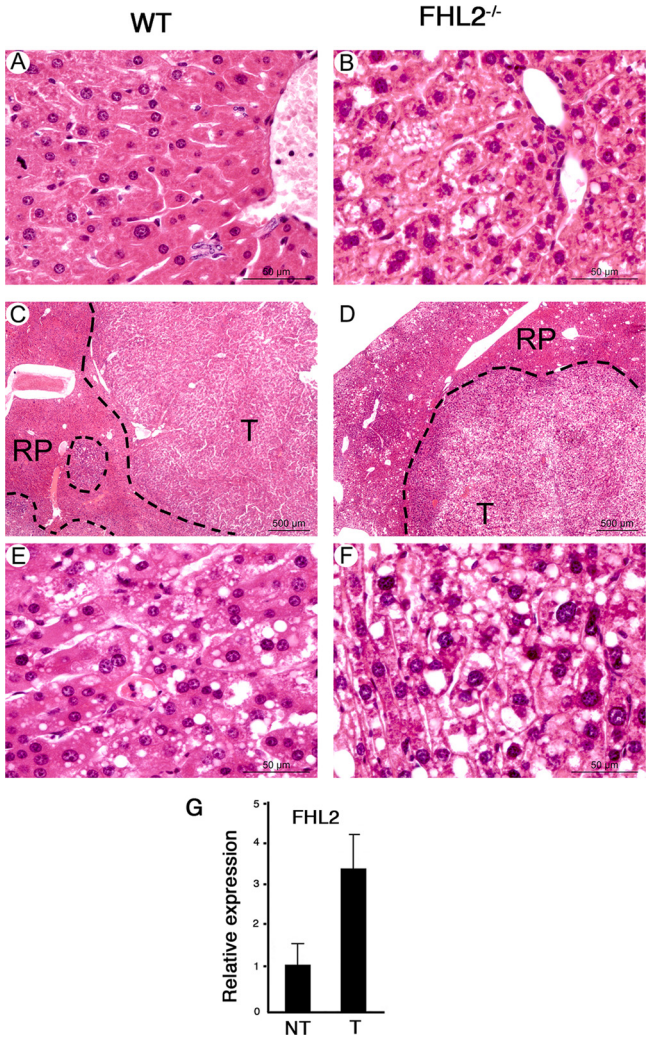


FIG 6 Hepatocarcinogenesis in wt and *FHL2*$^{-/-}$ mice 8 months after DEN exposure. H&E staining of nontumor liver parenchyma (A and B) and tumors (T) of various size (dotted lines), randomly distributed in the liver parenchyma (C and D) and tumor cells (E and F). (G) Activation of FHL2 expression in DEN-induced tumors developed in wt mice analyzed by real-time RT-PCR. The data are presented as mean values in FHL2 expression ± SD from 5 mice. NT, nontumor; T, tumor; RP, remaining parenchyma.

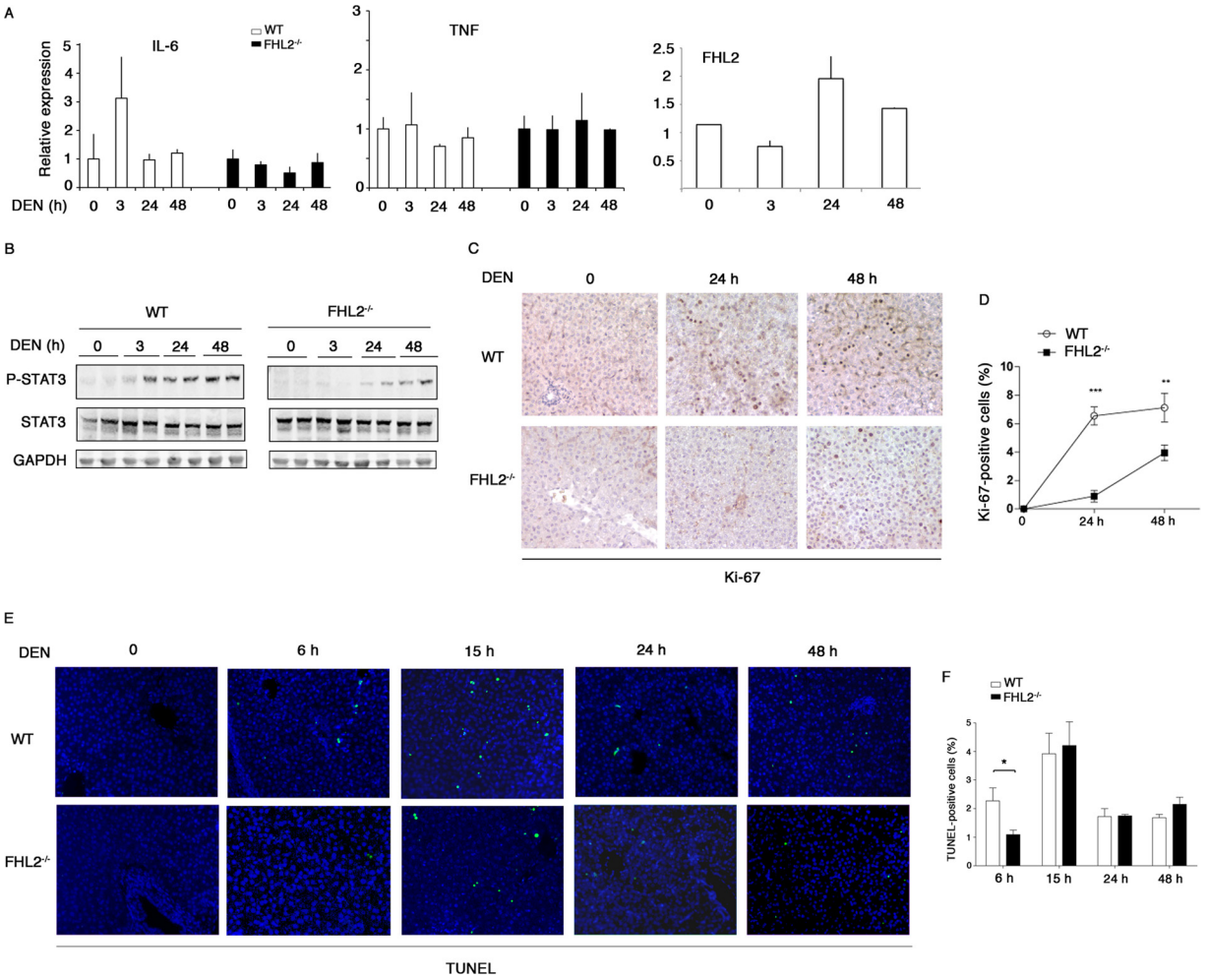


FIG 7 Loss of FHL2 reduces hepatocyte proliferation after DEN exposure. (A) Liver RNA was extracted at the indicated times after DEN injection. The levels of IL-6, TNF, and FHL2 expression were determined by real-time RT-PCR. (B) Livers from DEN-exposed mice were isolated at the indicated times and homogenized. Phosphorylation of STAT3 was analyzed by Western blotting. (C) Immunohistochemistry on liver sections with Ki-67 antibody. (D) Reduced DNA synthesis in *FHL2*$^{-/-}$ livers 24 h and 48 h after DEN exposure as determined by Ki-67 immunostaining. **, $P < 0.005$; ***, $P < 0.0005$. Apoptosis was detected by TUNEL. (E) Apoptosis was detected by TUNEL. (F) Quantification of TUNEL-positive cells in wt and *FHL2*$^{-/-}$ livers 6 h, 15 h, 24 h, or 48 h after DEN exposure. *, $P < 0.05$.

response to DEN exposure that induces DNA damage depends on cytokines, including TNF and IL-6, which are produced in an NF-κB-dependent manner. These cytokines promote surviving DEN-initiated hepatocytes, resulting in tumor development (21). Accordingly, a defect of TNF and IL-6 production in *FHL2*$^{-/-}$ mice constitutes a mechanism for reduced susceptibility to DEN-induced carcinogenesis. We have previously shown that deficiency of FHL2 decreases cell proliferation, whereas overexpression of FHL2 in hepatocytes induces cell growth and apoptosis (24, 27). It is thus also possible that loss of FHL2 constrains neoplastic transformation through the growth-suppressive properties. These results support the proposal of a role for FHL2 as a key mediator of NF-κB activation in the liver and an important component of regulatory mechanisms in liver carcinogenesis.

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