Supporting Information

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SI Materials and Methods

Generation of Conditional Knockout Mice for Ikk1, Ikk2, Nemo, Ikk1/Ikk2, Ikk1/Nemo, Ikk2/Nemo, and p65. Mice carrying loxP-site-flanked [floxed (Fl)] *Ikk1* (*Ikk1*^{FL}), *Ikk2* (*Ikk2*^{FL}), and *Nemo* alleles (*Nemo*^{FL}) were generated as described (1–3). *Ikk1*$^{Fl/Fl}$, *Ikk2*$^{Fl/Fl}$, and *Nemo*$^{Fl/Y}$ mice were crossed to *Alfp-cre* transgenic mice (4) to generate liver-parenchymal-cell-specific knockouts of the respective genes (IKK1^{LPC-KO}, IKK2^{LPC-KO}, and NEMO^{LPC-KO}). Mice with double-knockout of *Ikk1* and *Ikk2* (IKK1/2^{LPC-KO}), *Ikk1*, and *Nemo* (IKK1/NEMO^{LPC-KO}) or *Ikk2* and *Nemo* (IKK2/NEMO^{LPC-KO}) in parenchymal liver cells were generated by intercrossing the respective lines. In all experiments, littermates carrying the respective loxP flanked alleles but lacking expression of *Cre* recombinase were used as WT controls. Generation of p65^{FL} mice is described in Fig. S3. p65^{LPC-KO} mice were generated by crossing p65^{FL} mice to *Alfp-cre* transgenic mice. Animals received humane care, and all experiments were performed according to European, national, and institutional regulations.

Immunoblot Analysis and Kinase Assay. Cellular protein extracts from mouse livers and primary hepatocyte cultures were prepared as described in refs. 5 and 6, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting. Membranes were probed with antibodies against IKK1 (Imgenex), NEMO (6), α-tubulin (Sigma), phospho-IκBα (Ser-32/36)(5A5), cleaved caspase 3, IKK2 (2C8), phospho-p65 (Ser-536) (93H1) (Cell Signaling), IκBα, p50, p65/RelA (Santa Cruz Biotechnology), poly(ADP-ribose)polymerase (PARP) (Biomol), and claudin-23 (Zymed). Anti-rabbit-HRP and anti-mouse-HRP (Amersham) secondary antibodies were used. For detection of IκBα kinase activity, 100 μg of protein extracts from primary hepatocytes were immunoprecipitated with 2 μl of anti-NEMO antibody (Santa Cruz Biotechnology), and a kinase reaction was performed for 20 min at 30°C in the presence of adenosine 5′-[γ-^{32}P]triphosphate (Amersham) as described in ref. 5, using a recombinant truncated GST-IκBα (1–54) protein as substrate. Before the initial immunoprecipitation reaction, 20 μg of the same protein extracts were subjected to immunoblot analysis with a tubulin antibody to ensure equal protein input.

Quantitative Real-Time PCR. Total RNA was purified from primary hepatocyte cultures by using TRIzol reagent (Invitrogen). One microgram of total RNA was used to synthesize cDNA, using the SuperScript First-Strand Synthesis System (Invitrogen), and was resuspended in 100 μl of H_2O. Five-microliter cDNA samples were used for real-time PCR, in a total volume of 20 μl, using SYBR Green reagent (Finzyme) and specific primers on a qPCR machine (Opticon 2; MJ Research). Real-time PCR reactions were performed in triplicates. Primer sequences are available upon request. All values were normalized to the level of ubiquitin mRNA.

Electro-Mobility-Shift Assay (EMSA). Gel-retardation assays were performed on nuclear extracts as described in ref. 6. DNA protein complexes were resolved on a 6% polyacrylamide gel. A ^{32}P-labeled oligonucleotide representing an NF-κB consensus site (5′-CGG GCT GGG GAT TCC CCA TCT CGG TAC-3′) was used as a probe. For supershifts, high-concentrated antibodies against p50, p65, and c-Rel (Santa Cruz Biotechnology) were used.

TUNEL Assay, Histology, Immunohistochemistry, and Electron Microscopy. The TUNEL test was performed using the *In situ* Cell Death Detection Kit, POD (Roche Diagnostics) according to the manufacturer's instructions. Nuclei were visualized by using mounting medium containing DAPI (VECTASHIELD; Vector Laboratories). H&E stainings were performed by a standard protocol for paraffin sections. Immunohistochemistry for p65 nuclear translocation was performed on primary hepatocytes that were fixed in 4% paraformaldehyde and treated with 0.2% Triton solution, using an anti-p65 primary antibody (Santa Cruz Biotechnology) followed by secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes). Nuclei were counterstained with DAPI. Visualization of hepatic bile duct cells was achieved by cytokeratin staining on paraffin slides after citrate antigen retrieval, using a rabbit anti-human cytokeratin 19 antibody (Dako) at a 1:200 dilution. The staining was visualized with the polyclonal Enhanced Vision reaction (Dako). Electron microscopy was performed by using standard procedures.

1. Gareus R, *et al.* (2007) Normal epidermal differentiation but impaired skin-barrier formation upon keratinocyte-restricted IKK1 ablation. *Nat Cell Biol* 9:461–469.
2. Pasparakis M, *et al.* (2002) TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417:861–866.
3. Schmidt-Supprian M, *et al.* (2000) NEMO/IKKγ-deficient mice model incontinentia pigmenti. *Mol Cell* 5:981–992.
4. Kellendonk C, Opherk C, Anlag K, Schutz G, Tronche F (2000) Hepatocyte-specific expression of Cre recombinase. *Genesis* 26:151–153.
5. Luedde T, *et al.* (2005) Deletion of IKK2 in hepatocytes does not sensitize these cells to TNF-induced apoptosis but protects from ischemia/reperfusion injury. *J Clin Invest* 115:849–859.
6. Luedde T, *et al.* (2007) Deletion of NEMO/IKKγ in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 11:119–132.

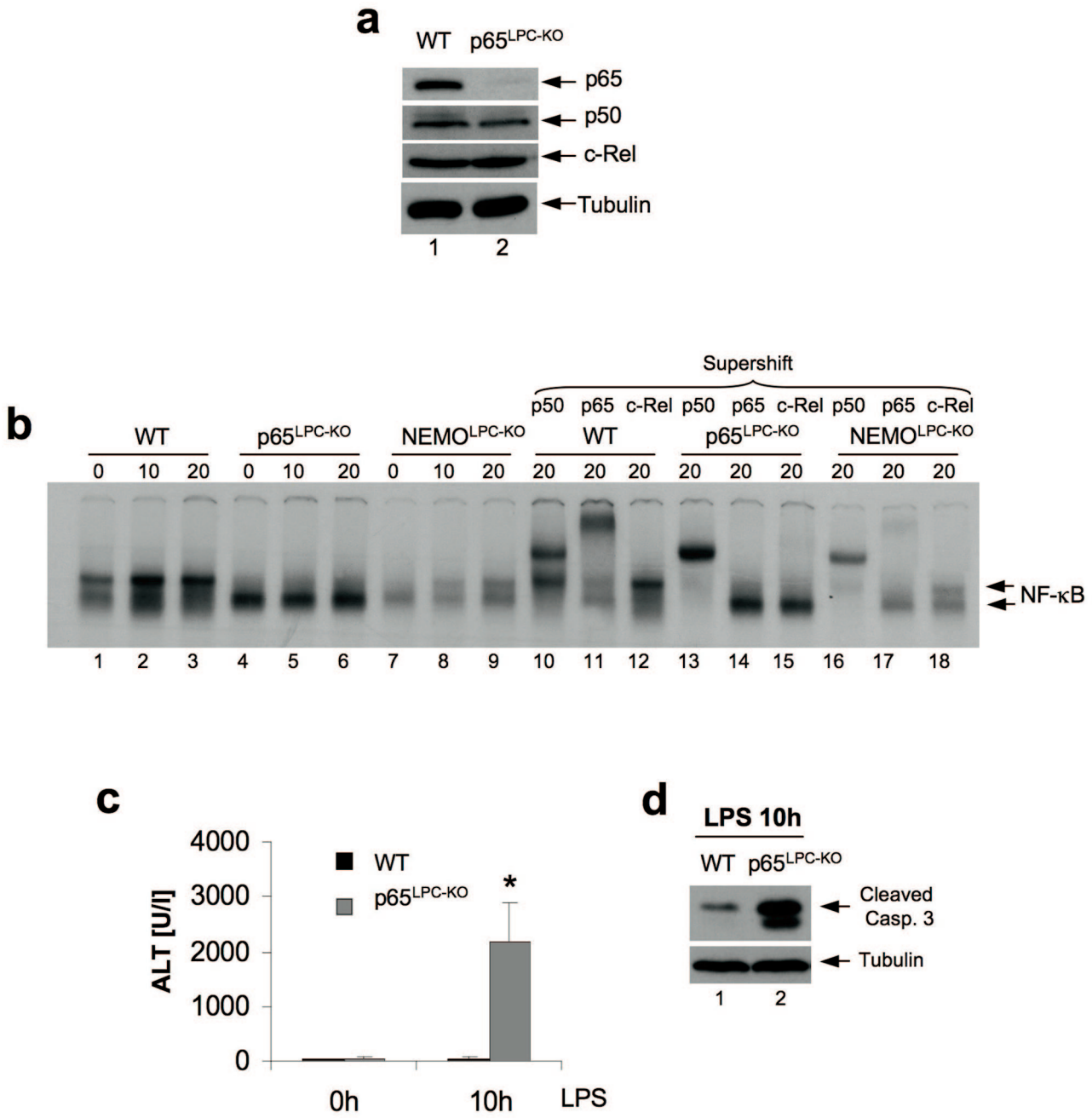

Fig. S1. p65/RelA expression in hepatocytes is crucial to protect mice from LPS-induced liver failure. (*a*) Expression of NF-κB family members in p65^{LPC-KO} mice and WT controls was assessed by immunoblot analysis on whole-liver extracts. Tubulin serves as loading control. (*b*) Primary hepatocytes were isolated from WT, p65^{LPC-KO}, and NEMO^{LPC-KO} mice and stimulated with TNF for the indicated time points. NF-κB DNA binding activity was measured by electro-mobility-shift assay (EMSA) analysis of nuclear extracts with an NF-κB consensus sequence as probe. Lanes 10–18 show supershift analysis with the indicated antibodies. (*c*) Analysis of serum alanine aminotransferase (ALT) levels in WT and p65^{LPC-KO} mice before and 10 h after LPS administration. Error bars denote SEM ($n = 3$). *, $P < 0.05$. (*d*) Immunoblot analysis for the cleaved form of caspase 3 in protein extracts from total livers dissected 10 h after LPS administration. Tubulin serves as loading control.

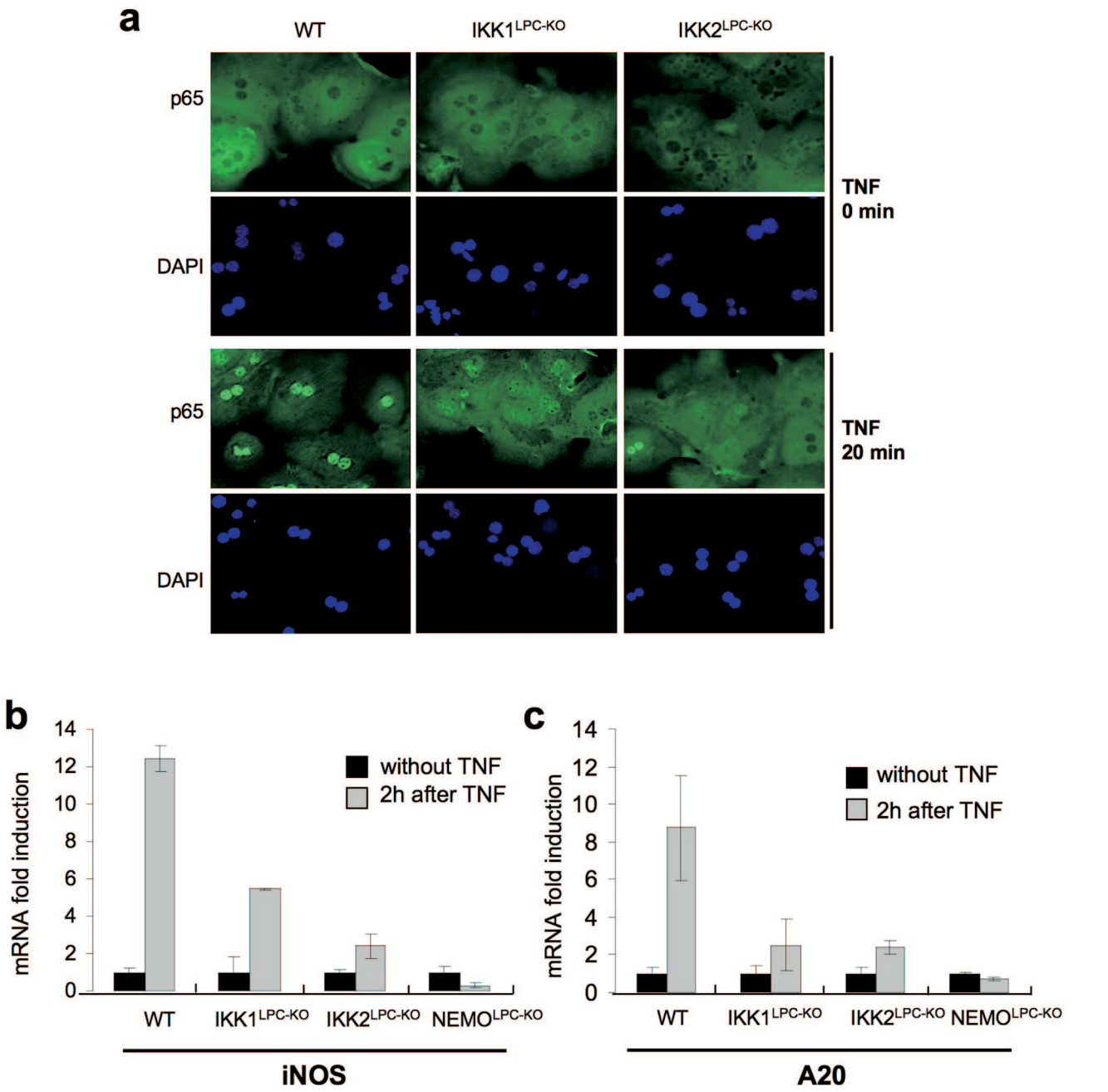

Fig. S2. Impaired p65 nuclear translocation and NF-κB-dependent gene transcription in IKK-deficient primary hepatocytes. (*a*) p65 nuclear translocation was assessed by immunofluorescent staining of primary hepatocyte cultures from WT, IKK1^{LPC-KO}, and IKK2^{LPC-KO} mice before and 20 min after TNF stimulation. Nuclei were visualized by DAPI staining. (Original magnification: ×400.) (*b* and *c*) RNA was isolated from primary hepatocytes before and 2 h after TNF stimulation (pooled from two culture dishes per time point), and relative induction of iNOS (*b*) and A20 (*c*) mRNA levels were analyzed by quantitative RT-PCR. Error bars in *b* and *c* denote SD (triplicates of PCR reactions).

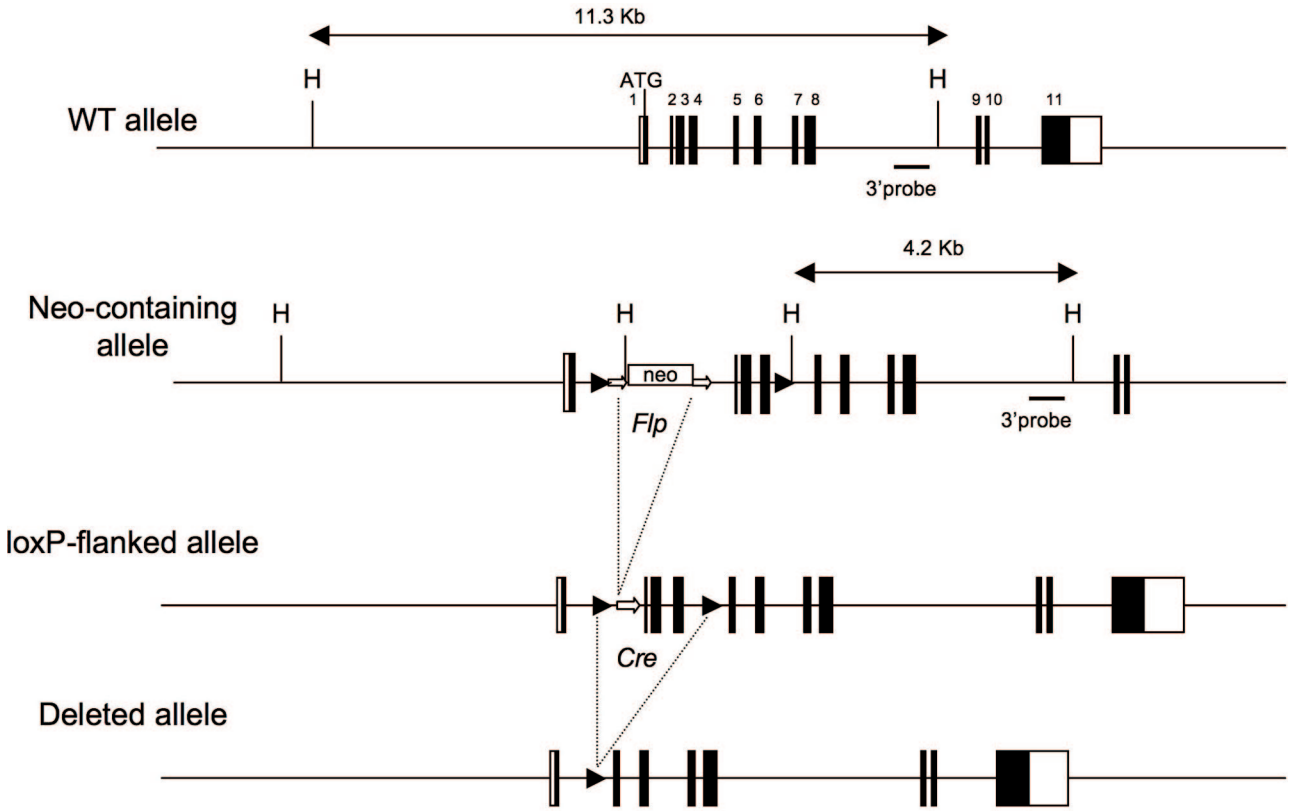

Fig. S3. Generation of mice with conditional loxP flanked p65 alleles. The targeting vector was designed to place exons 2–4 of the p65 gene between two loxP sites. An FRT-flanked neomycin cassette was introduced in the first intron for positive selection of recombinant ES clones. Germline transmitting chimeras generated using homologous recombinant ES cells were bred to FlpeDeleter mice to excise the FRT-flanked neomycin cassette generating p65FL mice. Cre-mediated recombination removes exons 2–4, resulting in out-of-frame splicing of exon 1 to exon 5 generating a null p65 allele. Black arrowheads, loxP sites; white arrows, FRT sites; filled boxes, coding exons; open boxes, nontranslated exons; H, HindIII restriction sites used for Southern blot analysis.