Supplementary data

IL-1β transcriptionally activates hepcidin by inducing C/EBPδ expression in hepatocytes

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Table S1. Nucleotide sequences used for dsRNAi

Gene	Nucleotide sequence	GenBank accession number
GFP (Control)	5′-GUUCAGCGUGUCCGGCGAG$_d$T$_d$T-3	AB296083
C/EBPδ	5′-ACAGCCUGGACUUACCACCACUAAA-3′	NM_005195
BMP2	5′-GAUGCAAGAUGCUUUAGGA$_d$T$_d$T-3′	NM_ 001200

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Table S2. Nucleotide sequence of primers used in RT-qPCR and RT-PCR analyses

Gene	Forward primer	Reverse primer	GenBank accession number
Human			
BMP2	5'- cggactgcggtctcctaaag -3'	5'- ggaagcagcaacgctagaag -3'	NM_001200
C/EBPα	5'- gaggaggggagaattcttgg -3'	5'- catttccaaggcacaaggtt -3'	NM_001285829
C/EBPβ	5'- ctctctgcttctccctctgc -3'	5'- gattgcatcaacttcgaaacc -3'	NM_001285878
C/EBPδ	5'- ggacataggagcgcaaagaa -3'	5'- gcttctctcgcagtttagtgg -3'	NM_005195
C/EBPζ	5'- cagagctggaacctgaggag -3'	5'- tgtttatggctgctttggtg -3'	NM_001195053
Hepcidin	5'- ctgttttcccacaacagacg -3'	5'- ttcgcctctggaacatgg -3'	NM_021175
IL-6	5'- agtgaggaacaagccagagc -3'	5'- atttgtggttgggtcagggg -3'	NM_001318095
TBP	5'- cgaaccacggcactgatttt -3'	5'- ctgccagtctggactgttct -3'	NM_003194
Mouse			
Albumin	5'- ggaaaagtgctgcgctgaag -3'	5'- cacacacggttcaggattgc -3'	NM_009654
BMP2	5'- cggactgcggtctcctaa -3'	5'- ggggaagcagcaacactaga -3'	NM_007553
C/EBPα	5'- caagaacagcaacgagtaccg -3'	5'- gtcactggtcaactccagcac -3'	NM_001287514
C/EBPβ	5'- acgacttcctctccgacctct -3'	5'- cgaggctcacgtaaccgtagt -3'	NM_001287738
C/EBPδ	5'- cgacttcagcgcctacattga -3'	5'- ctagcgacagaccccacac -3'	NM_007679
C/EBPζ	5'- gccagaataacagccggaac -3'	5'- gaccaggttctgctttcacgt -3'	NM_001290183
Hepcidin	5'- aagcagggcagacattgcgat -3'	5'- caggatgtggctctaggctatgt -3'	NM_032541
IL-1β	5'- tgtaatgaaagacggcacacc -3'	5'- tcttctttgggtattgcttgg -3'	NM_008361
IL-6	5'- ctctgcaagagacttccatccagt -3'	5'- cgtggttgtcaccagcatca -3'	NM_001314054
Inhibin βB	5'- cgagatcatcagctttgcag -3'	5'- ggttgccttcattagagacga -3'	NM_008381
iNOS	5'- ctctgcaagagacttccatccagt -3'	5'- cgtggttgtcaccagcatca -3'	NM_010927
Nramp-1	5'- ctcatgattagtgacaagagccc -3'	5'- ggcaccttggggtagtagaga -3'	NM_013612
Stabilin-1	5'- ttcctcatgcctgcctcttg -3'	5'- tgggaaattccgagaccagc -3'	NM_138672
TBP	5'- ccaatgactcctatgaccccta -3'	5'- cagccaagattcacggtagat -3'	NM_013684
Rat			
BMP2	5'- cggactgcggtctcctaa -3'	5'- ggggaagcagcaacactaga-3'	NM_017178
C/EBPδ	5'- gacttcagcgcctacattga -3'	5'- ctcgtcgtggcacaactcta -3'	NM_013154
Hepcidn	5'- gatggcactcagcactgga -3'	5'- gctgcagctctgtagtctgtct -3'	NM_053469
iNOS	5'- tgcagaaagagctggccgac -3'	5'- gatgcgcacatcgccacaaac -3'	NM_012611
TBP	5'- ccctatcactcctgccaca -3'	5'- ggtcaagtttacagccaagattc -3'	NM_001004198

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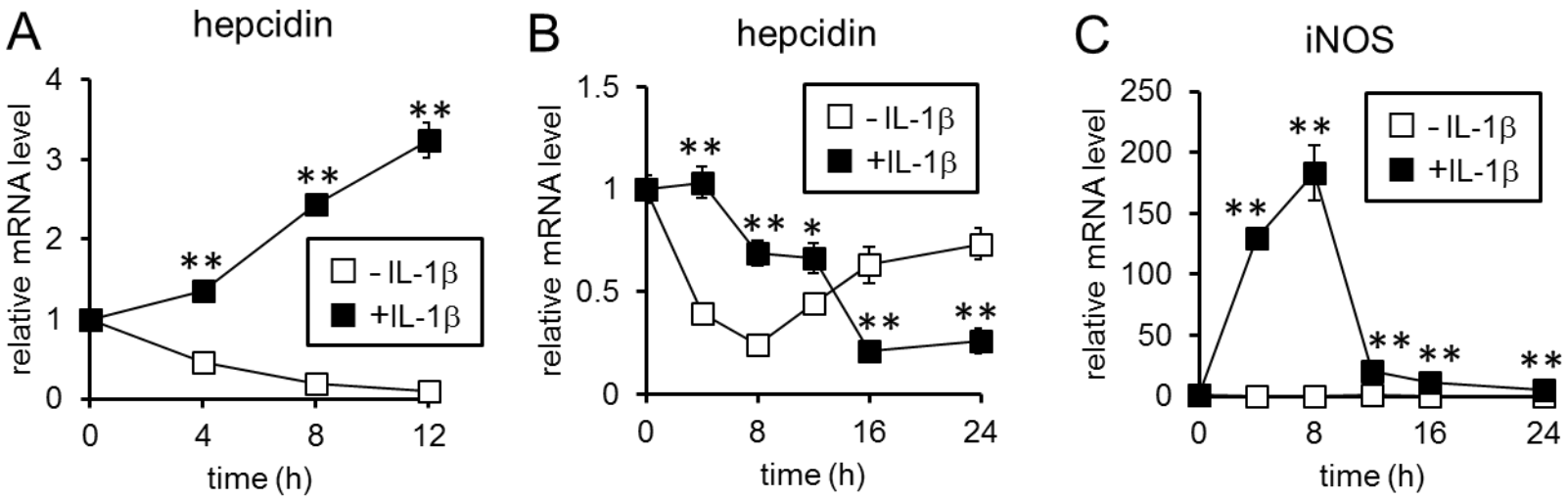

Figure S1. Efficient induction of hepcidin gene expression by IL-1β in primary mouse hepatocytes but not in primary rat hepatocytes
Mouse (A) and rat (B and C) primary hepatocytes were treated with IL-1β (25 ng/mL) for the indicated times. The expression of hepcidin (A and B) and iNOS (C) was examined by RT-qPCR analyses, and the expression level in the cells prior to IL-1β treatment was defined as 1. The data are presented as the mean ± SE (n=4). *$P < 0.05$ and **$P < 0.01$ vs. cells without IL-1β treatment at the respective time points.

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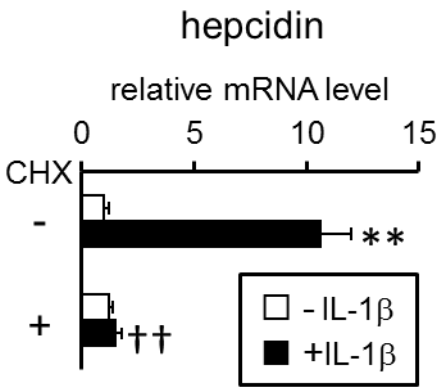

Figure S2. Blockage of IL-1β-induced hepcidin expression by cycloheximide in primary mouse hepatocytes
Primary mouse hepatocytes cells were pretreated with or without cycloheximide (CHX, 1 μg/mL) followed by treatment with IL-1β (25 ng/mL) for 24 h. The expression level in the control cells treated without CHX and IL-1β was defined as 1. The data are presented as the mean ± SE (n=4). ***P* < 0.01 vs. cells treated with the respective inhibitor (CHX) in the absence of IL-1β. ††*P* < 0.01 vs. cells with corresponding IL-1β treatments in the absence of cycloheximide.

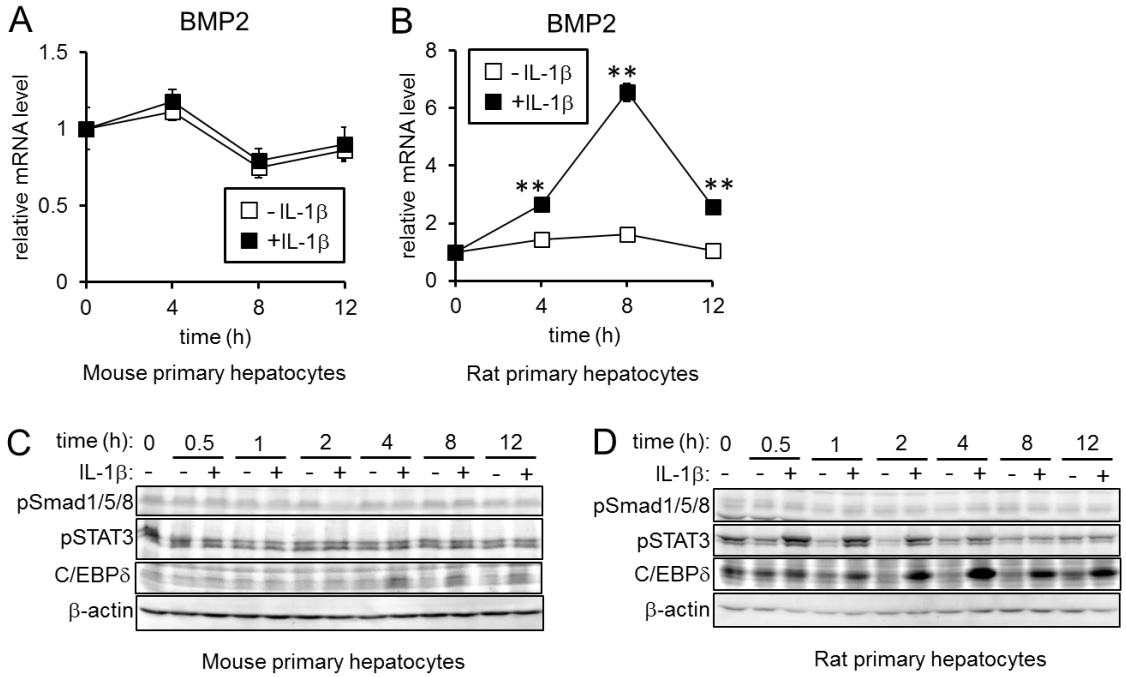

Figure S3. Failure of BMP pathway activation by IL-1β in primary mouse hepatocytes

Mouse (A and C) and rat (B and D) primary hepatocytes were treated with or without IL-1β (25 ng/mL) for the indicated times. (A and B) BMP2 expression was examined by RT-qPCR analysis with the level in the control cells prior to IL-1β treatment defined as 1. The data are presented as the mean ± SE (n=4). $^{**}P < 0.01$ vs. cells in the absence of IL-1β at the respective time point. (C and D) Levels of phosphorylated Smad1/5/8 and STAT3 as well as C/EBPδ expression was examined by Western blot analysis with β-actin as the loading control.

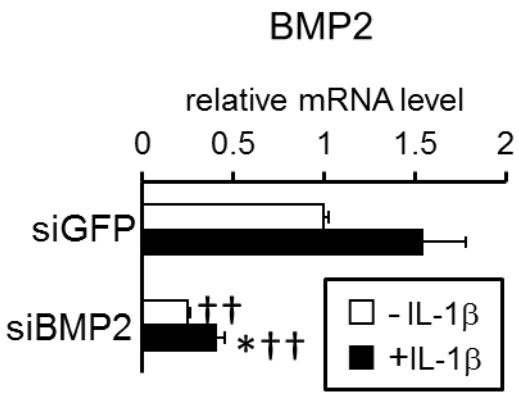

Figure S4. Down-regulation of BMP2 expression by siRNA knockdown targeting BMP2
HepG2 cells were transfected with siRNAs targeting the indicated gene. At 48 h after transfection, cells were treated with IL-1β (25 ng/mL) for 4 h. Expression of BMP2 was examined by RT-qPCR analysis with the level in cells transfected with siGFP in the absence of IL-1β defined as 1. The data are presented as the mean ± SE (n=3). *$P < 0.05$ vs. cells transfected with the respective siRNA in the absence of IL-1β. ††$P < 0.01$ vs. cells with corresponding IL-1β treatments and transfected with siGFP.

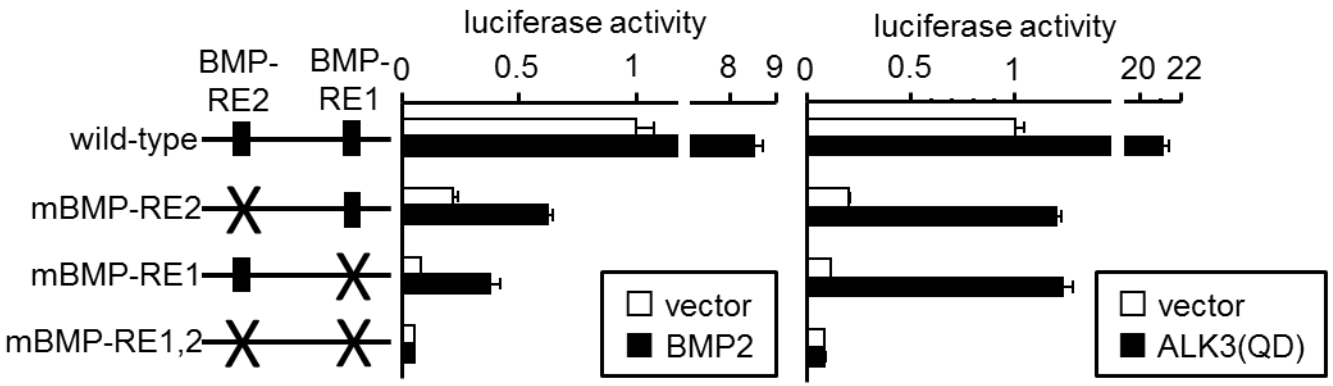

Figure S5. Hepcidin transcription via BMP-REs mediated by the BMP pathway

HepG2 cells were transfected with CMV-βGal and the indicated reporters and expression vectors. At 28 h post-transfection, the cells were harvested. Firefly luciferase activity normalized to β-galactosidase activity was calculated, and the relative luciferase activity in cells transfected with hepcidin(-2018)-luc and empty vector was defined as 1. The data are presented as the mean ± SE (n=3).

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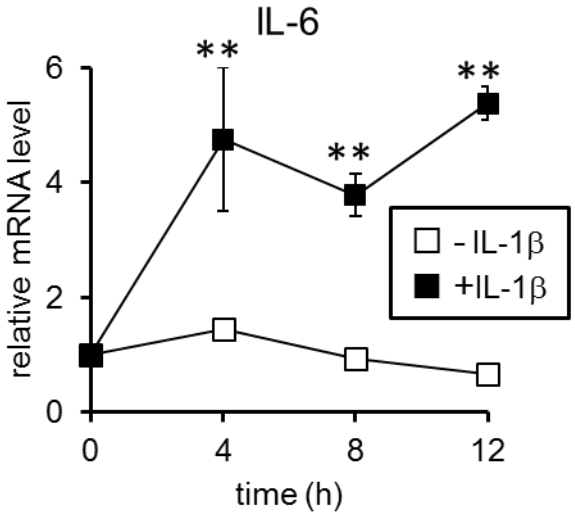

Figure S6. IL-1β-induced IL-6 expression in primary mouse hepatocytes

Primary mouse hepatocytes were treated with IL-1β (25 ng/mL) for the indicated times. Expression of IL-6 was examined by RT-qPCR analysis with the level in the control cells prior to IL-1β treatment defined as 1. The data are presented as the mean ± SE (n=4). $^{**}P < 0.01$ vs. cells in the absence of IL-1β at the respective time points.

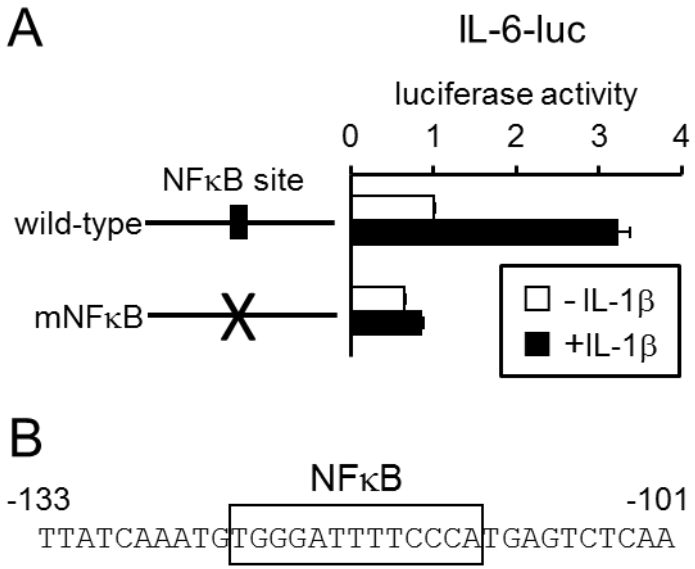

Figure S7. IL-1β-induced transcription of IL-6 via an NF-κB site

(A) HepG2 cells were transfected with tk-Renilla-luc and the indicated reporters. At 4 h post-transfection, cells were treated with or without IL-1β (10 ng/mL) for 12 h. Firefly luciferase activity normalized to *Renilla* luciferase activity was calculated, and the relative luciferase activity in cells transfected with wild-type IL-6-luc in the absence of IL-1β was defined as 1. The data are presented as the mean ± SE (n=3). (B) Nucleotide sequence surrounding the putative NF-κB site (boxed) of the IL-6 gene.

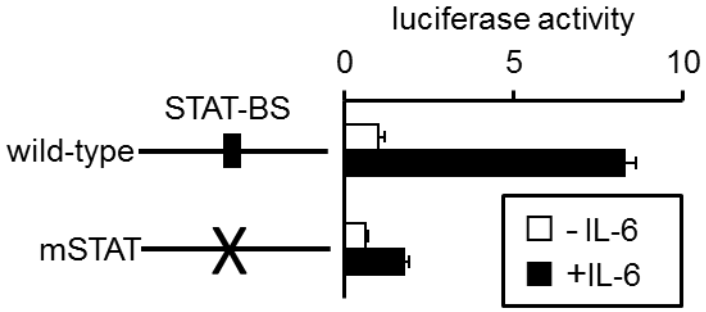

Figure S8. Dependence of the STAT-BS on IL-6-induced hepcidin transcription

HepG2 cells were transfected with thymidine kinase (tk)-Renilla-luc and the indicated reporters. At 4 h post-transfection, cells were treated with or without IL-6 (10 ng/mL) for 24 h. Firefly luciferase activity normalized to *Renilla* luciferase activity was calculated, and the relative luciferase activity in cells transfected with hepcidin(-2018)-luc in the absence of IL-6 was defined as 1. The data are presented as the mean ± SE (n=3).

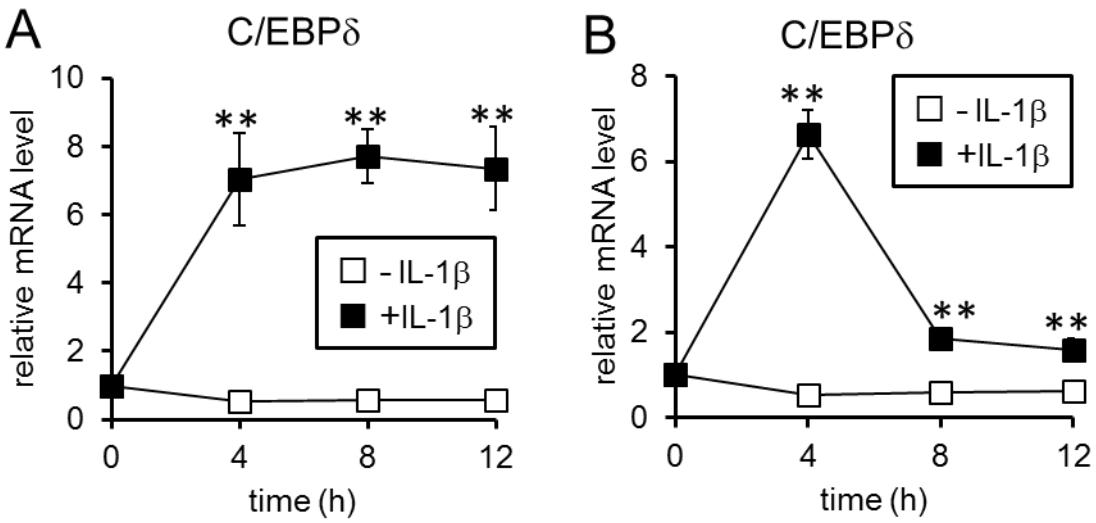

Figure S9. IL-1β-induced C/EBPδ expression in primary hepatocytes

Mouse (A) and rat (B) primary hepatocytes were treated with or without IL-1β (25 ng/mL) for the indicated times. Expression of the C/EBPδ was examined by RT-qPCR analysis. The expression levels in control cells prior to IL-1β treatment were defined as 1. The data are presented as the mean ± SE (n=4). **P* < 0.05 and ***P* < 0.01 vs. cells in the absence of IL-1β at the respective time points.

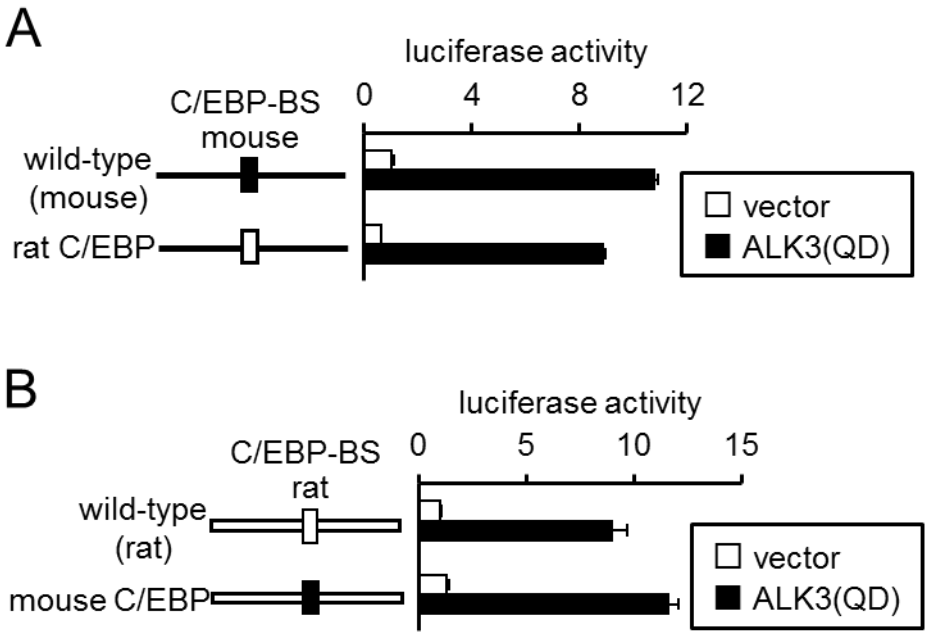

Figure S10. Swapping the mouse and rat C/EBP-BSs does not affect hepcidin transcription induced by the BMP pathway
HepG2 cells were transfected with or without ALK3(QD) in addition to tk-Renilla-luc and the indicated reporters. At 28 h post-transfection, cells were harvested. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the relative luciferase activity in cells transfected with empty vector and either mouse hepcidin(-2018)-luc (A) or rat hepcidin (-1861)-luc (B) was defined as 1. The data are presented as the mean ± SE (n=3).

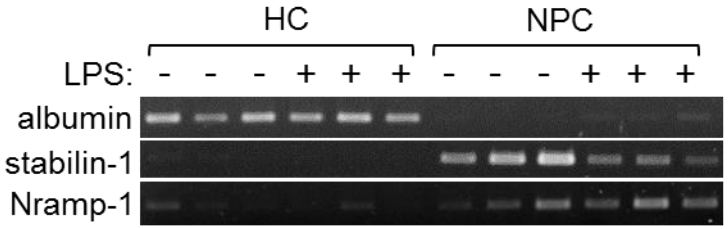

Figure S11. Separation of hepatocytes and non-parenchymal cells from the livers of LPS or PBS-injected mice
ICR mice were intraperitoneally injected with PBS or LPS (5 mg/kg). At 6 h post-injection, liver cells were separated by a centrifugation-based method. Expression of albumin (hepatocyte marker), stabilin-1 (endothelial cell marker), and Nramp-1 (Kupffer cell marker) was examined by RT-PCR.

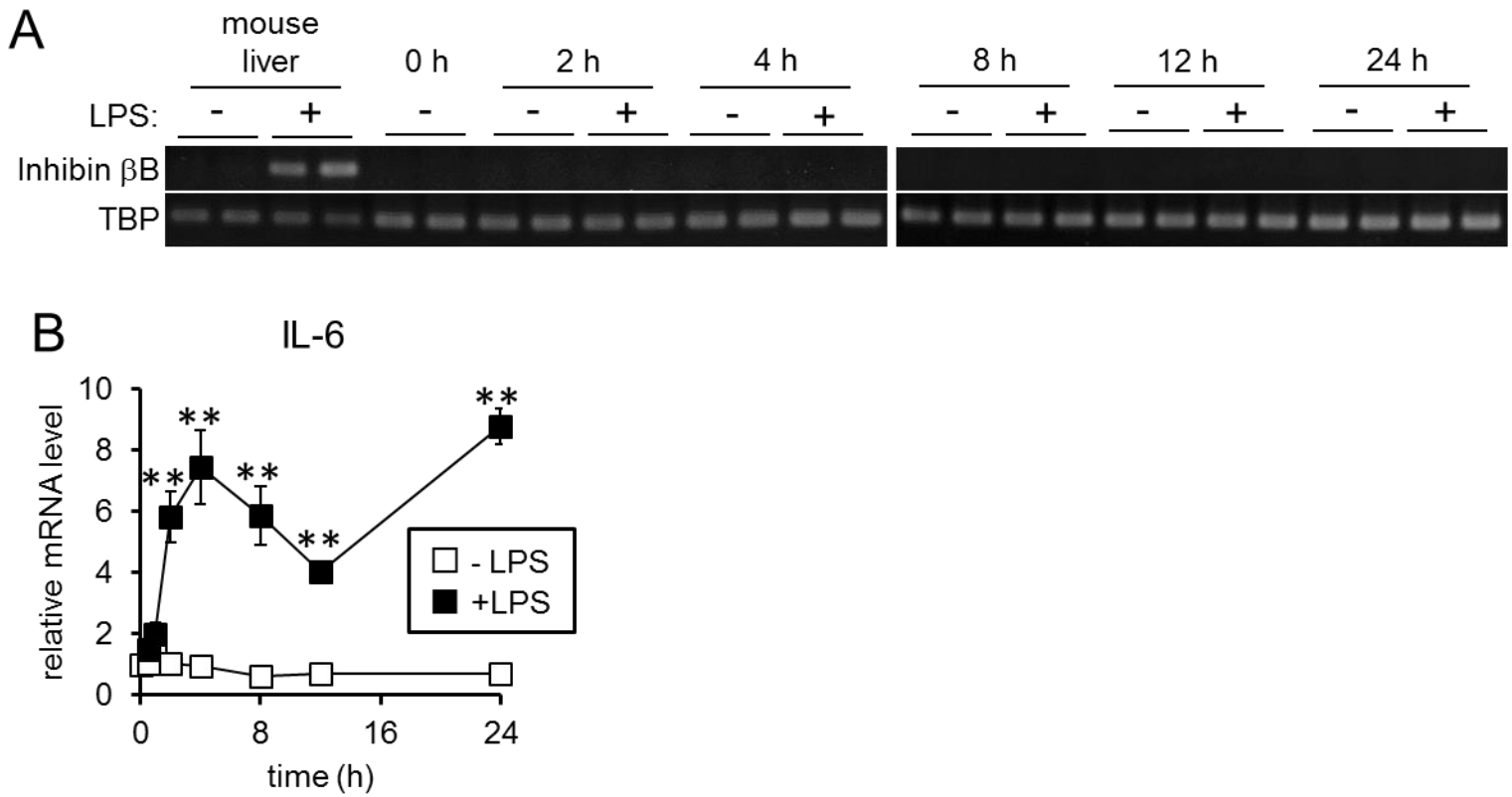

Figure S12. Expression of inhibin βB and IL-6 in RAW264.7 cells

RAW264.7 cells were treated with or without LPS (100 ng/mL) for the indicated time. The expression of inhibin βB (A) and IL-6 (B) were examined by RT-PCR and RT-qPCR analysis, respectively. cDNA from the livers of LPS-injected mice was used as a positive control. The levels in the control cells prior to IL-1β treatment were defined as 1. The data are presented as the mean ± SE (n=3). ***P* < 0.05 vs. cells treated without LPS at the respective time points.

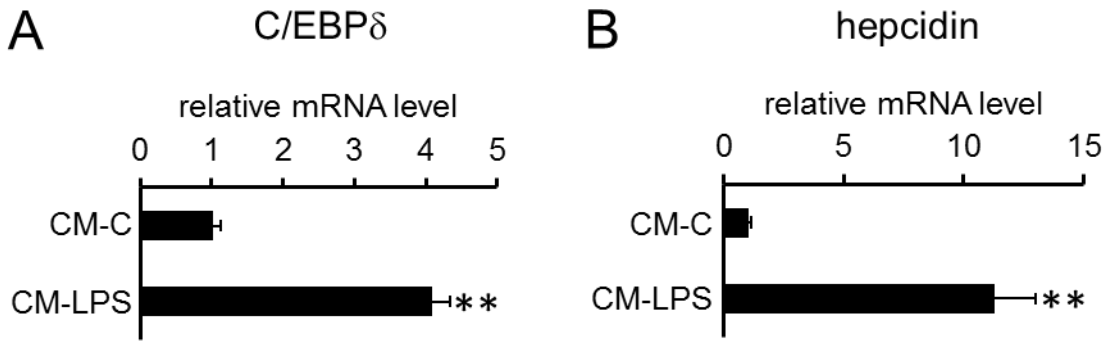

Figure S13. C/EBPδ and hepcidin induction in primary mouse hepatocytes by molecules from activated macrophages
Conditioned medium of RAW264.7 cells treated with (CM-LPS) or without (CM-C) LPS (100 ng/mL) for 30 h was prepared as described in EXPERIMENTAL PROCEDURES. Primary mouse hepatocytes were treated with either CM-C or CM-LPS for 24 h. Expression of C/EBPδ (A) and hepcidin (B) was examined by RT-qPCR analysis with the level in cells treated with control conditioned medium for 24 h defined as 1. The data are presented as the mean ± SE (n=4). $^{**}P < 0.01$ vs. cells treated with control supernatants.