The SMAD Pathway Is Required for Hepcidin Response During Endoplasmic Reticulum Stress

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Hepcidin, the iron hormone, is regulated by a number of stimulatory and inhibitory signals. The cAMP responsive element binding protein 3-like 3 (CREB3L3) mediates hepcidin response to endoplasmic reticulum (ER) stress. In this study we asked whether hepcidin response to ER stress also requires the small mother against decapentaplegic (SMAD)-1/5/8 pathway, which has a major role in hepcidin regulation in response to iron and other stimuli. We analyzed hepcidin mRNA expression and promoter activity in response to ER stressors in HepG2 cells in the presence of the bone morphogenetic protein (BMP) type I receptor inhibitor LDN-193189, mutated hepcidin promoter or small interfering RNA against different SMAD proteins. We then used a similar approach in vivo in wild-type, *Smad1/5*, or *Creb3l3*$^{-/-}$ animals undergoing ER stress. In vitro, LDN-193189 prevented hepcidin mRNA induction by different ER stressors. Seemingly, mutation of a BMP-responsive element in the hepcidin promoter prevented ER stress-mediated up-regulation. Moreover, in vitro silencing of SMAD proteins by small interfering RNA, in particular SMAD5, blunted hepcidin response to ER stress. On the contrary, hepcidin induction by ER stress was maintained when using antibodies against canonical BMP receptor ligands. In vivo, hepcidin was induced by ER stress and prevented by LDN-193189. In addition, in *Smad1/5* knockout mice, ER stress was unable to induce hepcidin expression. Finally, in *Creb3l3* knockout mice, in response to ER stress, SMAD1/5 were correctly phosphorylated and hepcidin induction was still appreciable, although to a lesser extent as compared with the control mice. In conclusion, our study indicates that hepcidin induction by ER stress involves the central regulatory SMAD1/5 pathway. **(*Endocrinology* 157: 3935–3945, 2016)**

Hepcidin (encoded by the *HAMP* gene) is a defensin-like cysteine-rich antimicrobial peptide now recognized as the major player in iron homeostasis and a central humoral mediator of innate immunity and host defense (1–4). The peptide retains some antifungal and antimicrobial activity in vitro (2), but its main biological effect is to prevent invading pathogens from using iron sources to grow and proliferate during infection. To do so, hepcidin binds and degrades the sole iron importer in mammals (5), a multidomain transmembrane protein encoded by the *SLC40A1* gene named ferroportin (FPN1) (6–8). Hepcidin binding to FPN1 at the cell surface through its N-terminus domain results in FPN1 ubiquitination and internalization, ultimately leading to degradation in lysosomes (9, 10). This causes cessation of iron transfer through the basolateral sites of absorbing enterocytes, placental syncityotrophoblasts, hepatocytes, and tissue macrophages.

Hepcidin production by the hepatocytes is induced by a number of stimuli, in particular, iron, through the bone morphogenetic protein (BMP)-small mother against de-

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Abbreviations: ALK, activin receptor-like kinase; BFA, brefeldin A; BMP, bone morphogenetic protein; BMP-RE, BMP responsive element; CREB3L3, cAMP responsive element binding protein 3-like 3; ER, endoplasmic reticulum; FPN1, ferroportin; 4-PBA, 4-phenylbutyric acid; qRT-PCR, real-time quantitative RT-PCR; siRNA, small interfering RNA; SMAD, small mother against decapentaplegic; Tm, tunicamycin; UPR, unfolded protein response; US RNAi, unspecific RNA interference.

capentaplegic family of protein (SMAD) pathway (11, 12), and inflammatory signals, particularly IL-6 (13–17), IL-1 (18), IL-22 (19, 20), and activin B (21, 22).

The SMAD1/5/8 signaling pathway has a central role in the physiological regulation of hepcidin transcription. Iron sensing occurs through the interaction of transferrin-iron and BMPs within a multiprotein complex at the plasma membrane made by BMP receptors, a BMP coreceptor (hemojuvelin), and a number of ancillary proteins (including HFE and the second transferrin receptor). BMP ligands bind to type I (ie, activin receptor-like kinase [ALK]-1, ALK2, ALK3, and ALK6) and type II (ie, BMP receptor II, activin receptor ACVR2A, or ACVR2B) receptors and turn on a signaling cascade involving the phosphorylation of the SMAD1/5/8 complex (receptor-associated SMADs) that binds to SMAD4 (common partner SMAD) (23). Once formed, the SMAD complex translocates to the nucleus and binds specific sequence motifs in *Hamp* promoter (the proximal BMP responsive element [BMP-RE]-1 and the distal BMP-RE2) (17, 24, 25), activating the transcription of hepcidin (26). In inflammation, activin B uses a similar signaling cascade via BMP type I receptors ALK2 and ALK3 and SMAD1/5/8 to induce hepcidin (21, 22) in conjunction with the IL-6-STAT3 pathway.

In addition to extracellular stimuli, hepcidin has been recently reported to respond to intracellular stress, namely endoplasmic reticulum (ER) stress. We have found that cAMP response element binding protein 3-like 3 (CREB3L3; also known as CREBH), an ER stress-associated, liver-specific transcription factor originally involved in the induction of acute-phase response genes such as serum amyloid protein (*SAP*) and C-reactive protein (*CRP*), is constitutively engaged on hepcidin promoter and transactivates it in response to ER stress, both in vitro and in vivo (27). This finding has been confirmed and expanded to other pathophysiologic models by subsequent studies (28–30). More recently, CREBH has been also involved in hepcidin response to gluconeogenic signals during nutrient dysregulation (31) and to hypoxia (32).

Due to its central role in hepcidin transcription (23), we wondered whether the SMAD1/5/8 pathway might be also involved in the hepcidin response to ER stress. To address this question we used an in vitro and in vivo approach.

Materials and Methods

Cell cultures

HepG2 human hepatoma cells were cultured in MEM (containing 1 g/L glucose) supplemented with 1 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum, in a 5% CO_2 atmosphere at 37°C.

For ER stress experiments, HepG2 cells were incubated in the presence of tunicamycin (Tm; 10 μg/mL dissolved in dimethylsulfoxide) or brefeldin A (BFA; 20 μg/mL dissolved in dimethylsulfoxide) for the indicated time (all reagents were from Sigma-Aldrich). LDN-193189 (100 nM, provided by Professor H. Y Lin, Massachusetts General Hospital-Harvard Medical School, Boston, Massachusetts) was used as the inhibitor of the BMP-SMAD pathway. 4-Phenylbutyric acid (4-PBA; Sigma-Aldrich) was used as inhibitor of ER stress.

In specific experiments, cells were incubated with 30 μg/mL anti human activin B beta B subunit (antiactivin B), anti human BMP2/BMP4 (anti-BMP2/4), and antihuman BMP6 (anti-BMP6) antibodies, all from R&D Systems.

Real-time quantitative RT-PCR (qRT-PCR) and semiquantitative RT-PCR

Total cellular RNA was obtained by incubating cells in iScript RT-qPCR sample preparation reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Total liver RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions.

cDNA was generated by reverse transcription of 2 μL of iScript buffer (for HepG2 RNA) or 1 μg (for liver RNA) with 200 U ImProm-II reverse transcriptase (Promega) following the manufacturer's instruction. Expression of mRNAs was analyzed using SsoFast EVAGreen supermix (Bio-Rad Laboratories). Primer sequences are listed in Table 1.

Cycling conditions were 30 seconds at 98°C followed by 40 cycles of 2 seconds at 98°C and 10 seconds at 60°C. After 40 amplification cycles, threshold cycle values were automatically calculated using the default settings of the CFX Manager (Bio-Rad Laboratories; version 2.0). At the end of the PCR run, melting curves of the amplified products were obtained and used to determine the specificity of the amplification reaction. In each experiment, the change of specific mRNA expression was normalized on RPL19 housekeeping mRNAs.

XBP1 mRNA spliced forms were analyzed by semiquantitative RT-PCR using cDNA obtained as specified above from cells or mouse livers. PCR products were run on 3% agarose gels. Primers are reported in Table 1.

Western blot analysis

Liver sections were homogenized in radioimmunoprecipitation assay buffer (1× Tris buffered saline, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) to obtain the total protein extracts. After centrifugation at 14 000 × *g* at 4°C for 15 minutes, the supernatant was collected and proteins concentration assayed by the Bradford method. Total extracts were run on 12% SDS-PAGE or NuPAGE NOVEX 4%–12% bis-tris gel (Invitrogen), transferred to nitrocellulose membranes, and incubated with rabbit antiphospho-SMAD1/5 (Thermo Scientific), rabbit monoclonal antihepcidin (Abcam), or mouse anti-β-actin (Sigma-Aldrich) followed by the appropriate horseradish peroxidase-conjugate secondary antibodies. Western blot analysis was performed using Western Lightning Plus ECL (PerkinElmer) according to the manufacturer's instruction, and cross-reactivity was detected by

Table 1. Sequence of the Primers Used in Quantitative and Semiquantitative PCR Reactions

Primers	Sequences (5′–3′)
hRPL19_fw	GGGCATAGGTAAGCGGAAGG
hRPL19_rev	TCAGGTACAGGCTGTGATACA
hHAMP_fw	TGTTTTCCCACAACAGACGGG
hHAMP_rev	CGCAGCAGAAAATGCAGATGG
hXBP1_fw	CCTTGTAGTTGAGAACCAGG
hXBP1_rv	GGGGCTTGGTATATATGTGG
hBiP_fw	CGAGGAGGAGGACAAGAAGGAGGA
hBiP_rv	GAGTGAAGGCGACATAGGACGGC
hCHOP_fw	GCCAAAATCAGAGCTGGAACCT
hCHOP_rv	ACAGTGTCCCGAAGGAGAAAGG
hSMAD1_fw	AGAGACTTCTTGGGTGGAAACA
hSMAD1_rv	ATGGTGACACAGTTACTCGGT
hSMAD5_fw	TCTCCAAACAGCCCTTATCCC
hSMAD5_rv	GCAGGAGGAGGCGTATCAG
hSMAD8_fw	CTGTGCTCGTGCCAAGACA
hSMAD8_rv	TGGAAAGAGTCAGGATAGGTGG
mRpl19_fw	ATGAGTATGCTCAGGCTACAGA
mRpl19_rv	GCATTGGCGATTTCATTGGTC
mHamp_fw	GCCTGTCTCCTGCTTCTCCT
mHamp_rv	GCTCTGTAGTCTGTCTCATCTGTT
mId1_fw	GCATCTTGTGTCGCTGAG
mId1_rv	GGCTGAAAGGTGGAGAGG
mBip_fw	GCCTGTTGCTGGACTCCTAAG
mBip_rv	GCCCACATCCTCCTTCTTGTC
mActB_fw	TCAGCTTTGCAGAGACAG
mActB_rv	GAAGAAGTACAGGCGGAC
mSMAD1_fw	GCTTCGTGAAGGGTTGGGG
mSMAD1_rv	CGGATGAAATAGGATTGTGGGG
mSMAD5_fw	TTGTTCAGAGTAGGAACTGCAAC
mSMAD5_rv	GAAGCTGAGCAAACTCCTGAT
mSMAD8_fw	CTTCCGAGTCAGACAGTCCCT
mSMAD8_rv	GGTTGTTTAGTTCGTAGTAGGCA
mXbp1_fw	GAACCAGGAGTTAAGAACACG
mXbp1_rv	AGGCAACAGTGTCAGACTCC

Molecular Imager ChemiDoc XRS+with Image Lab Software (Bio-Rad Laboratories).

Plasmids, small interfering RNAs (siRNAs) transfection and luciferase assay

HepG2 cells were transfected with siRNAs against SMAD1, SMAD5, and SMAD8 (OnTARGET-Plus siGenome; Dharmacon) to prevent single SMAD expression. Negative control siRNA (unspecific RNA interference [US RNAi], scrambled sequence) was used as control.

Forty-eight hours after transfection, cells were treated for 5 hours with tunicamycin (10 μg/mL) and analyzed for specific mRNA expression normalized on *RPL19* housekeeping gene expression.

The wild-type murine hepcidin promoter region as well as luciferase analysis were described elsewhere (27).

Mutation of the BMP-RE was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and subjected to gene sequencing, as reported elsewhere (17).

Animal studies

Animal protocols were approved by the Ethics Committee for Animal Studies at the University of Modena and Reggio Emilia and the Institutional Animal Care and Use Committee at Massachusetts General Hospital. All animal experiments were carried out in accordance with the European Union Directive 2010/63/EU.

C57BL/6Crl male mice aged 8–10 weeks were allowed free access to water and fed a standard chow diet in pellets. For ER stress treatment, animals were treated with Tm (2 mg/kg, ip) or vehicle (dextrose) as reported elsewhere (27) and killed at 1, 3, or 5 hours after injection. To inhibit BMP-SMAD signaling, mice received a single dose of vehicle or Tm (2 mg/kg) and LDN-193189 (3 mg/kg, ip) or vehicle (cyclodextrin) at time 0, and a second dose of LDN-193189 or vehicle after 2 hours. All animals were killed 5 hours after the first injection for analysis.

Creb3l3 wild type and *Creb3l3*$^{-/-}$ null male and female mice (The Jackson Laboratory) at 8 weeks of age were treated with Tm or vehicle as above and killed at 5 hours.

Mice homozygous for floxed *Smad1* and *Smad5* alleles (*Smad1/5*) on a mixed C57BL/6-129-CD1 background were kindly provided by Elizabeth Robertson (University of Oxford, Oxford, United Kingdom) and An Zwijsen (VIB and KU Leuven, Leuven, Belgium) (33, 34). *Smad1/5* mice were crossed with albumin-Cre mice on a C57BL/6J background (Jackson; stock number 003574) to generate littermate *Smad1/5 Cre*$^{-}$ (control) and *Smad1/5 Cre*$^{+}$ (hepatocyte-specific double knockout) mice. Littermate *Smad1/5 Cre*$^{-}$, and *Smad1/5 Cre*$^{+}$ male mice at 8 weeks of age were treated with Tm or vehicle as described above and killed at 5 hours.

In each experimental set, the liver was collected at the time the animals were killed, and total RNA and proteins were extracted and analyzed.

Iron and transferrin saturation measurements

Serum iron and transferrin saturation measurements have been performed as reported elsewhere (22).

Statistics

All data presented in the figures were expressed as means ± SEM. All data were tested for normal distribution (Kolmogorov-Smirnov's test). Statistical significance was evaluated by a Student's *t* test when making comparisons between two groups. When making multiple statistical comparisons, a one-way ANOVA with a Tukey or Dunnett's post hoc tests was used for normally distributed data, depending on the presence or absence of homoscedasticity. For skewed data, the Kruskal-Wallis test was used. In all statistical analyses, $P < .05$ was considered significant.

All analyses were conducted using Prism 5 for Mac OS X version 5.0a software (GraphPad Software Inc).

Results

Hepcidin response to ER stress requires the SMAD1/5/8 pathway in vitro

To demonstrate the involvement of the SMAD1/5/8 pathway mediated by the BMP type I receptors ALK2 and ALK3 (21, 35–37) in hepcidin activation, we used a complementary approach by blocking either the ligand-receptor interaction or receptor activity. To the first end, we

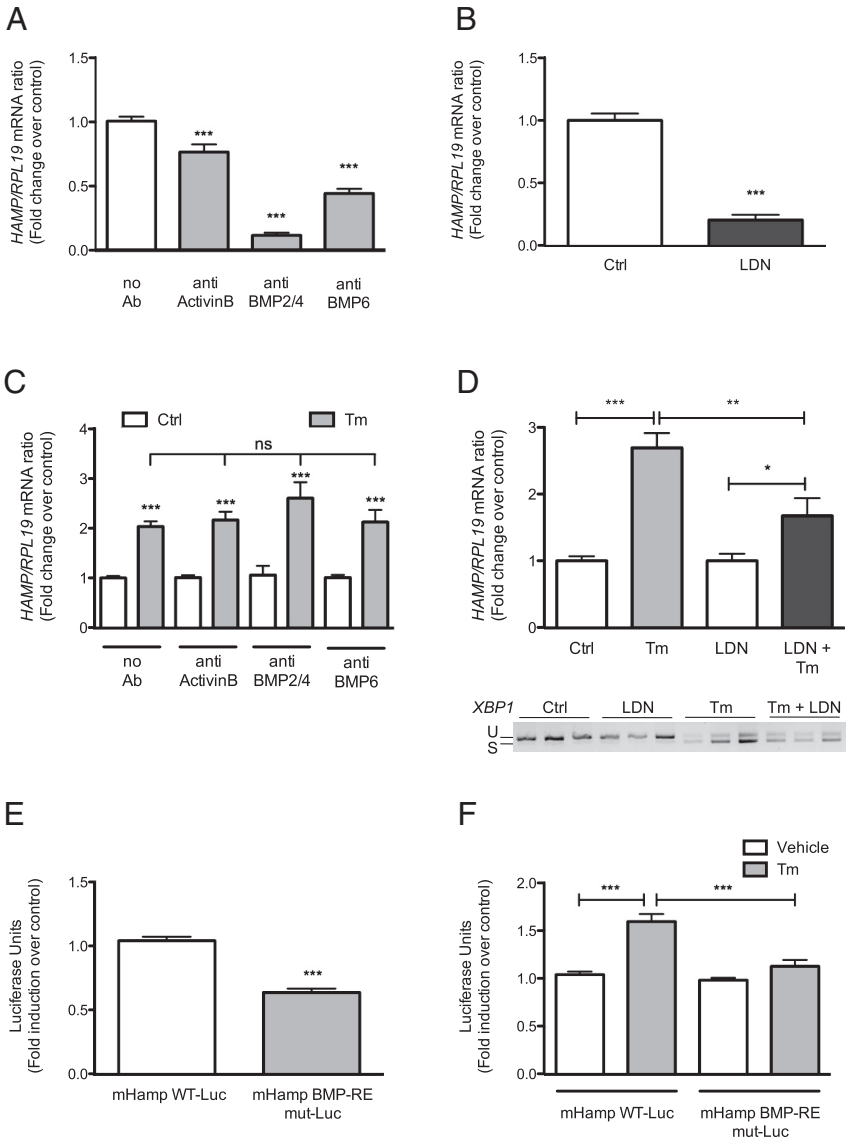

Figure 1. The SMAD pathway is required for ER stress-mediated *HAMP* induction in vitro. A and C, HepG2 cells were treated for 5 hours with 30 μg/mL antiactivin B, anti BMP2/4, anti-BMP6 either alone (A) or in the presence of Tm (C). Total RNA was extracted and analyzed for hepcidin (*HAMP*) expression normalized to *RPL19* housekeeping mRNA expression. B and D, HepG2 cells were treated for 5 hours in the presence of LDN-193189 (LDN) either alone (B) or in the presence of Tm (D). Hepcidin (*HAMP*) mRNA was analyzed by real-time PCR and normalized to *RPL19* housekeeping mRNA expression. *XBP1* mRNA splicing forms were analyzed by semiquantitative PCR and run on 3% agarose gel (D, lower panel). E, HepG2 cells were transfected with WT and BMP-RE (BMP responsive element) mutated *Hamp* promoter luciferase constructs. Luciferase units were analyzed in respect to total proteins content and expressed as fold change over wild-type control promoter activity. F, HepG2 cells were transfected as in panel E and treated with Tm for 5 hours. Luciferase units were analyzed in respect to total proteins content and expressed as fold induction over control (vehicle treated cells). Data are expressed as fold change over the control cells set to 1. Data are the mean ± SEM of three (A and C) or five (B, D, E, and F) independent experiments, each conducted in triplicate. *P* values are reported for comparisons between indicated groups. Ab, antibody; Ctrl, control. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

used specific antibodies against ALK2 and ALK3 receptor ligands, namely activin-B, BMP2/4, and BMP6 (Figure 1A). Then we used LDN-193189 (38), a small molecule that strongly inhibits the activity of the BMP type I receptors ALK2 and ALK3 (Figure 1B). In both cases, hepcidin mRNA expression was reduced, particularly when using antibody against BMP ligands or LDN-193189 (Figure 1A). A known ER stressor, Tm, which prevents the N-glycosylation of nascent proteins, led to a significant increase of hepcidin expression (Figure 1, C and D, light gray bars); however, whereas ligand-specific antibodies were unable to block the stimulatory effect of Tm (Figure 1C), cotreatment with LDN-193189 and Tm reduced ER stress-mediated activation of hepcidin (Figure 1D, dark gray bars). The appearance of the *XBP1* spliced forms, a specific event that occurs during ER stress response, indicated proper induction of ER stress (Figure 1D, lower panels). A similar effect was obtained when using BFA, an ER stress inducer that inhibits the transport of proteins from ER to Golgi, in association with LDN-193189 (Supplemental Figure 1A).

To prove that ER stress was directly involved in hepcidin up-regulation, we used a well-known ER stress inhibitor, 4-PBA (39). Exposure of HepG2 cells to increasing doses of 4-PBA led to a progressive and consistent reduction of hepcidin activation by Tm (Supplemental Figure 2A). Accordingly, induction of *BiP* (binding immunoglobulin protein) and *CHOP* (CCAAT enhancer binding protein homologous protein), two well-known ER stress markers, was similarly reduced by 4-PBA (Supplemental Figure 2, B and C).

The SMAD1/5/8 signaling requires a BMP-RE, a palindromic GGCGCC sequence, highly conserved between human, rat, and mouse hepcidin promoters (17). To prove the critical importance of this signaling pathway for hepcidin activation under ER stress, HepG2 cells were transfected with wild-type (mHamp WT-Luc) or hepcidin promoter construct mutated in the conserved palindromic sequence (mHamp BMP-REmut-Luc), fused to the luciferase gene. The activity of the hepcidin promoter was significantly reduced when the BMP-RE element was mutated (Figure 1E).

Cells were then treated with either Tm or BFA. The wild-type promoter activity was up-regulated by ER stressors, whereas the presence of the BMP-RE mutation in the promoter completely abolished the induction (Figure 1F and Supplemental Figure 1B). Altogether these results prove that a preserved SMAD1/5/8 pathway is required in vitro for proper induction of hepcidin expression during ER stress.

To clarify the role of different SMADs in hepcidin response to ER stress, we used siRNA technologies to prevent the expression of single receptor-associated SMAD molecules in HepG2 cells (Figure 2). As shown in Figure 2A (white bars), basal hepcidin mRNA expression was dramatically down-regulated by SMAD5 silencing but not by the inhibition of SMAD1 and SMAD8. After treatment with Tm, cells transfected with SMAD1 or SMAD8 RNAi, similarly to cells transfected with US RNAi, responded by activating hepcidin expression, whereas cells silenced with SMAD5 RNAi did not (Figure 2A, gray bars). Efficient silencing of single SMADs was confirmed by the reduction of SMAD1 (Figure 2B), SMAD5 (Figure 2C), and SMAD8 (Figure 2D) mRNAs. Interestingly, *SMAD5* mRNA was induced by Tm in all in vitro settings (Figure 2C).

Hepcidin response to ER stress requires induction of the SMAD1/5/8 pathway in vivo model

To confirm a role for SMAD1/5/8 signaling in regulating hepcidin gene expression during ER stress in vivo, we studied hepcidin expression in a murine model of ER stress. C57BL/6 male wild-type mice were injected with a single dose of Tm (2 mg/kg) or vehicle alone (Ctrl) ip and killed at different time points. Tm treatment triggered *Hamp* expression, peaking at 5 hours (Figure 3A). Induction of the SMAD1/5/8 pathway was confirmed by the time-dependent appearance of Smad1/5 phosphorylation and a concurrent *Id1* mRNA up-regulation in animals treated with Tm (Figure 3, B and C). Interestingly, mRNA expression of activin B (Figure 3D) was significantly up-regulated by Tm treatment (Figure 3D), whereas *Bmp6* mRNA was not induced (Figure 3E). Individual SMAD mRNA expression showed a trend toward increased levels, which reached statistical significance at 3–5 hours after Tm treatment (Supplemental Figure 3, A–C). ER stress markers were properly induced as shown by the increase of *Bip* mRNA and the appearance of the splicing forms of *Xbp1* mRNA (Supplemental Figure 3D).

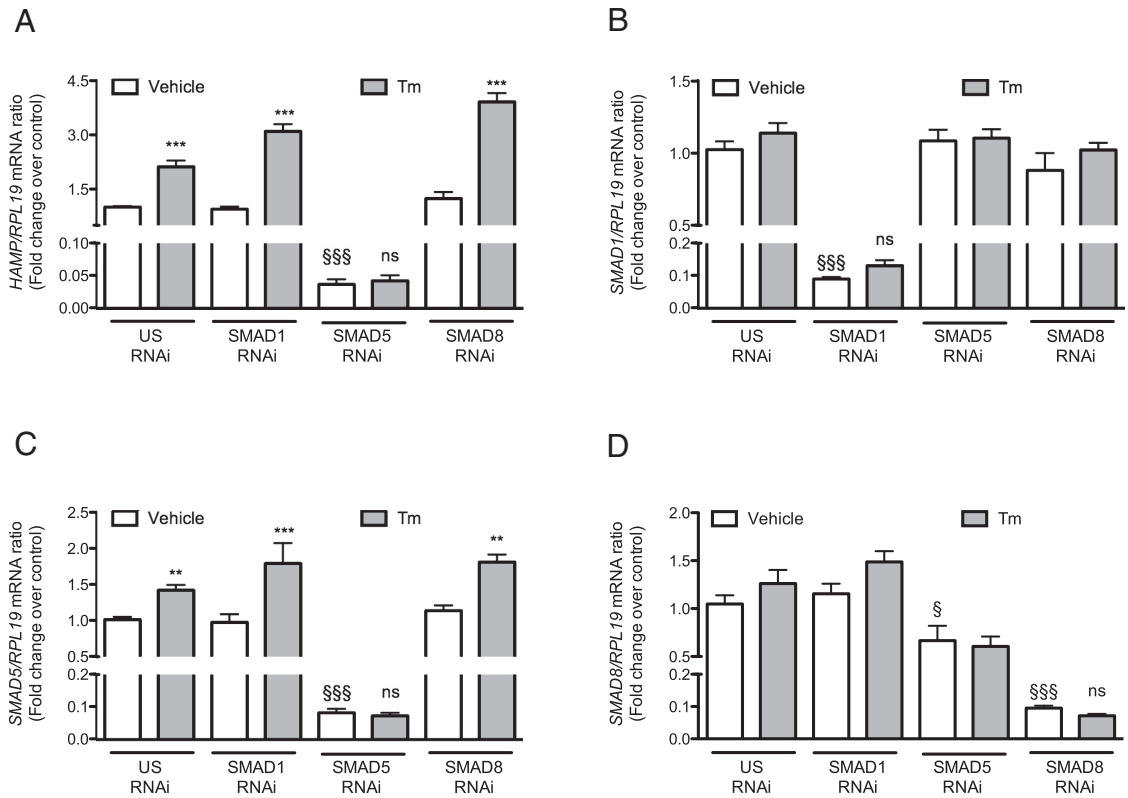

Figure 2. ER stress requires SMAD5 to induce hepcidin expression in vitro. A, HepG2 cells were transfected with indicated siRNAs to prevent the expression of specific *SMADs* (*SMAD1*, *SMAD5*, or *SMAD8*) and treated for 5 hours with Tm or vehicle. US RNAi was used as control. Hepcidin (*HAMP*) mRNA was analyzed by real-time PCR and normalized as compared with *RPL19* housekeeping mRNA expression. B–D, Specific *SMAD* mRNA expression was analyzed as in panel A. Data are expressed as fold change over control cells (vehicle treated cells) set to 1. Data are the mean ± SEM of three independent experiments, each conducted in triplicate. *P* values are reported for comparisons between control and Tm-treated cells. ns, not significant. **, $P < .01$, ***, $P < .001$. §, $P < .05$, §§§, $P < .001$, statistical significance as compared with US RNAi treated with vehicle.

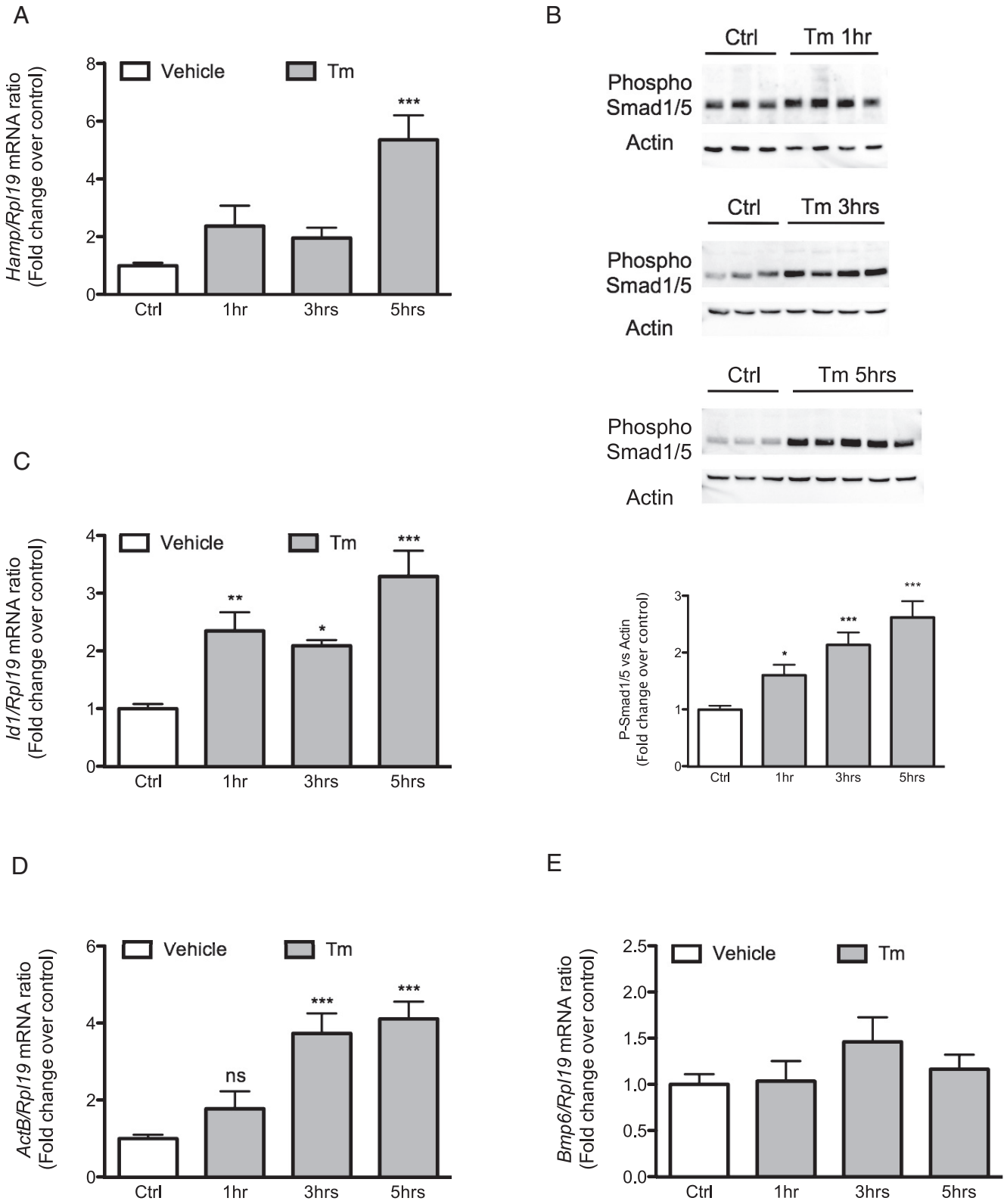

Figure 3. ER stress induces *Hamp* expression and the SMAD pathway in vivo. A, *Hamp* mRNA expression was analyzed by real-time PCR and normalized to *Rpl19* housekeeping mRNA expression using total cDNA from liver of C57BL/6 male mice treated with Tm for the indicated time points. B, Phosphorylation of SMAD1/5 was analyzed in total liver extracts obtained from C57BL/6 male mice treated with Tm for the indicated time points. Densitometric analysis of phospho-SMAD1/5 signal normalized to β-actin expression is shown in the lower panel. C–E, *Id1* (C), *ActB* (D), and *Bmp6* (E) mRNA expressions were analyzed as in panel A. Data are expressed as fold change over the control mice set to 1. Data are the mean ± SEM of six to eight mice per group. *P* values are reported for comparisons between control and each time point Tm-treated mice. Ctrl, control; ns not significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

To further clarify the role of the SMAD1/5/8 pathway, we cotreated mice with Tm and the BMP-SMAD inhibitor LDN-193189. Tm induced both *Hamp* (Figure 4A) and *Id1* (Figure 4B) mRNA expression in vivo, whereas the concomitant injection of LDN-193189 completely abolished *Hamp* and *Id1* mRNA induction (Figure 4, A and B, dark gray bars). Accordingly, hepcidin protein levels were correctly increased by Tm, whereas this effect was blunted by the concomitant administration of LDN-193189 (Figure 4C). ER stress induction is demonstrated by the up-regulation of *Bip* mRNA and the appearance of *Xbp1* spliced forms (Figure 4D).

In view of the results from both in vitro and in vivo experiments, we investigated hepcidin response in the double *Smad1/5* hepatocyte conditional knockout mice model undergoing ER stress. *Smad1/5 Cre*$^{-}$ (control) and *Smad1/5 Cre*$^{+}$ (double knockout) male mice were treated with an ip injection of Tm (2 mg/kg) and killed at 5 hours. Although hepcidin mRNA induction was correctly up-regulated in *Smad1/5 Cre*$^{-}$ mice treated with Tm, *Hamp* response was abolished in *Smad1/5 Cre*$^{+}$ mice, indicating the key role of SMAD1/5 complex for *Hamp* expression after ER stress (Figure 5A). Similar results were obtained when assessing hepcidin protein levels: in *Smad1/5 Cre*$^{-}$ mice, hepatic hepcidin synthesis was correctly induced after Tm treatment, whereas *Smad1/5 Cre*$^{+}$ mice were unable to do so (Figure 5B). As expected, the SMAD1/5/8 pathway was not induced in double-knockout mice, as

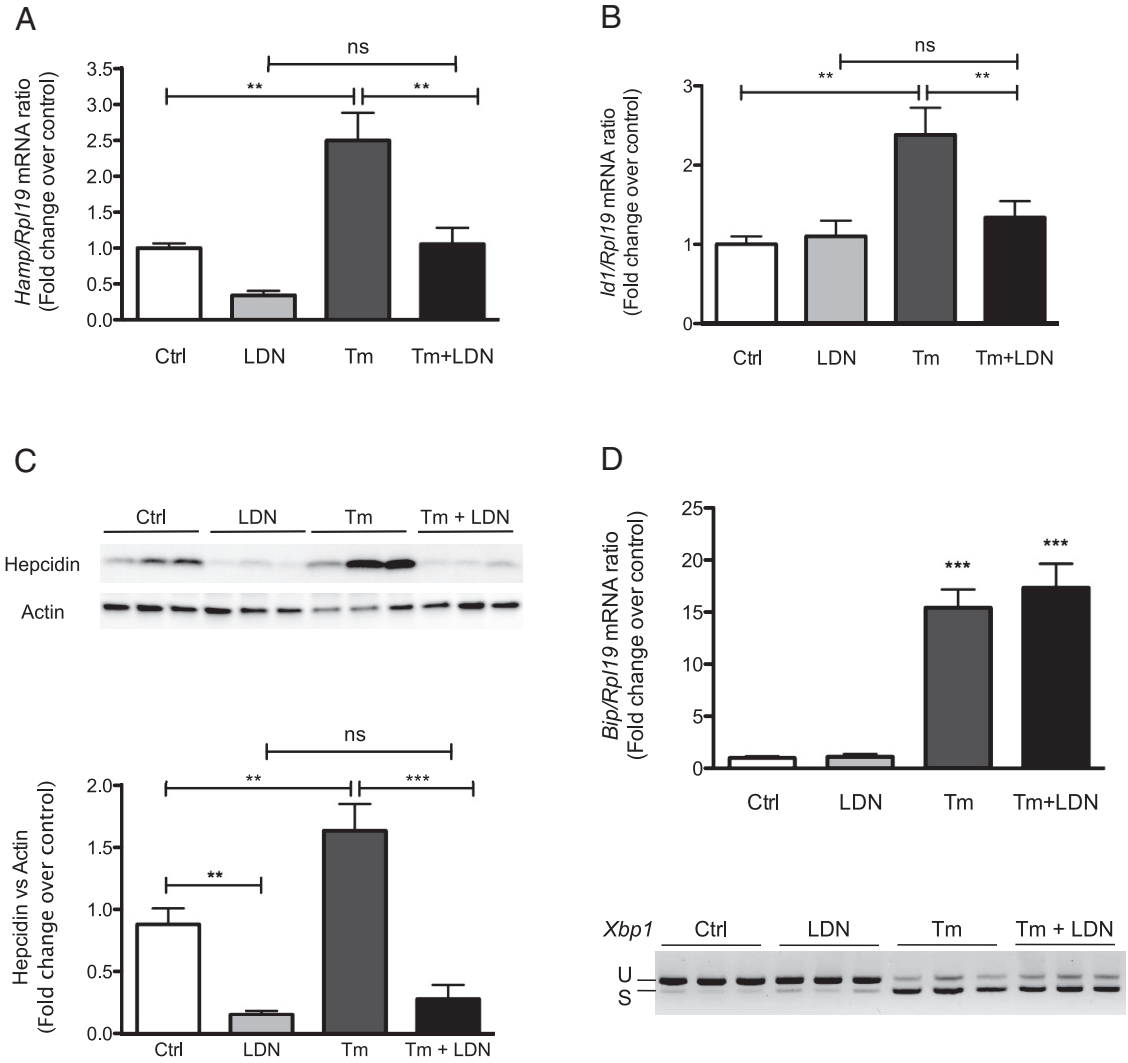

Figure 4. The SMAD pathway is required for *Hamp* induction in vivo by tm. A and B, *Hamp* (A) and *Id1* (B) mRNA expressions were analyzed by real-time PCR and normalized to *Rpl19* housekeeping mRNA expression using total cDNA from the liver of C57BL/6 male mice treated with Tm together with LDN-193189 (LDN) for 5 hours. C, Hepcidin protein expression was analyzed using total liver extracts obtained from mice treated as in panel A. Densitometric analysis of hepcidin signal normalized to β-actin expression is shown in the lower panel. A representative immunoblot is shown. D, Upper panel, *Bip* mRNA expression was analyzed by real-time PCR as in panel A. Lower panel, *Xbp1* mRNA splicing forms were analyzed by semiquantitative PCR and run on 3% agarose gel. Data are expressed as fold change over the control mice set to 1. Data are the mean of five to six mice per group ± SEM. *P* values are reported for comparisons between indicated groups. Ctrl, control; ns, not significant. **, $P < .01$; ***, $P < .001$.

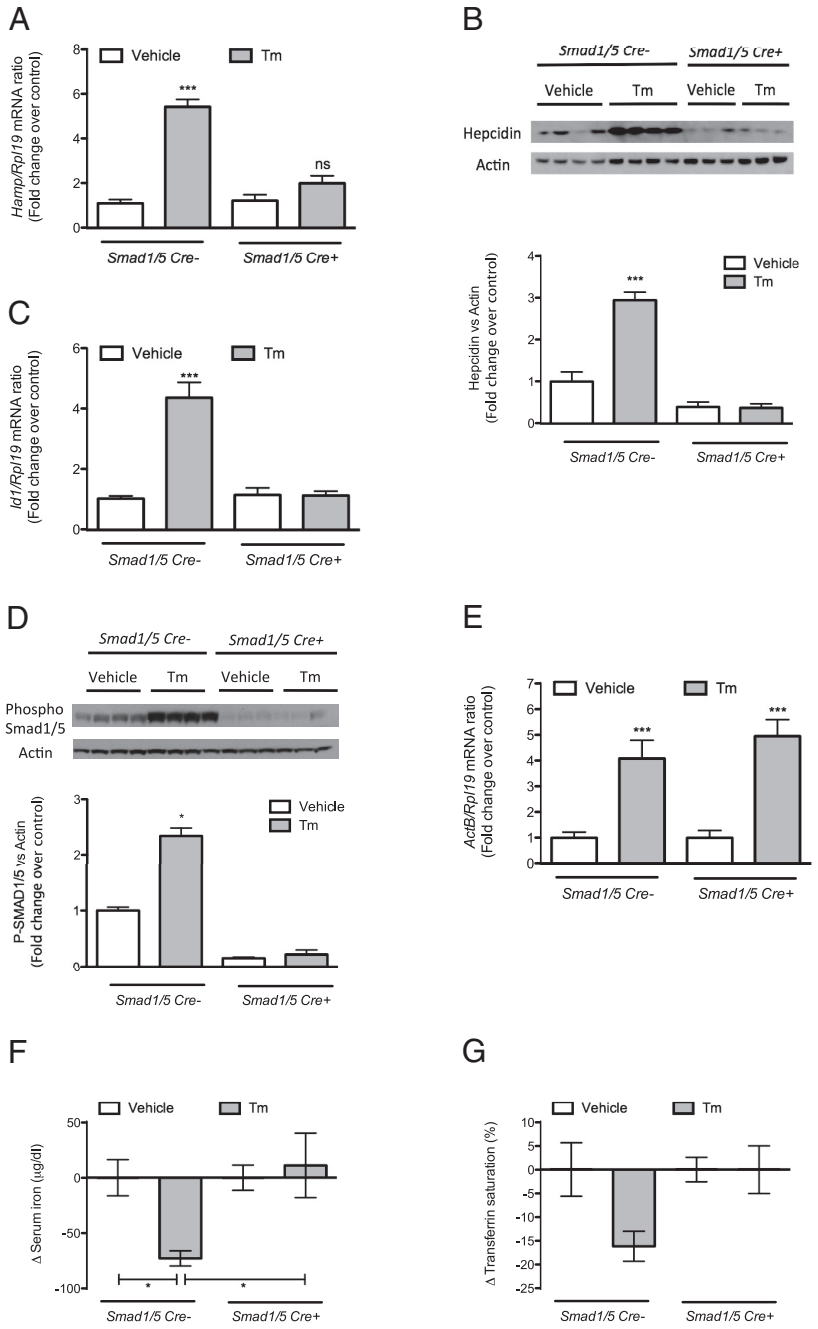

Figure 5. The SMAD1/5 complex is essential for *Hamp* induction by ER stress in vivo. A, *Hamp* mRNA expression was analyzed by real-time PCR and normalized to *Rpl19* housekeeping mRNA expression using total cDNA from liver of *Smad1/5 Cre*$^{-}$ (control) or *Smad1/5 Cre*$^{+}$ (double knockout) male mice treated with vehicle or Tm for 5 hours. B, Hepcidin protein expression was analyzed using total liver extracts obtained from mice treated as in panel A. Densitometric analysis of hepcidin signal normalized to β-actin expression is shown in the lower panel. A representative immunoblot is shown. C, *Id1* mRNA expression was analyzed as in panel A. D, Phosphorylation of SMAD1/5 complex was analyzed in total liver extracts obtained from *Smad1/5 Cre*$^{-}$ (control) or *Smad1/5 Cre*$^{+}$ (double knockout) male mice treated with Tm for 5 hours. Densitometric analysis of specific phospho-SMAD1/5 complex signal normalized to β-actin expression is shown in the lower panel. E, *ActB* mRNA expression was analyzed as in panel A. F, Change in serum iron (δ serum iron) relative to vehicle-treated mice is reported as the mean ± SEM of measured serum iron − mean serum iron of the vehicle-treated mice for each genotype. G, Change in transferrin saturation (δ Tf sat) was analyzed as in panel F. Specific mRNA expression of vehicle-treated mice was set to 1. Data are expressed as mean ± SEM of seven to eight mice per group. *P* values are reported for comparisons between control and Tm-treated mice, within each genotype. ns not significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

demonstrated by the loss of *Id1* mRNA up-regulation in *Smad1/5 Cre*$^{+}$ mice (Figure 5C) and lack of phospho-Smad1/5 complex induction (Figure 5D). Activin B mRNA was increased in both *Smad1/5 Cre*$^{-}$ and *Smad1/5 Cre*$^{+}$ mice after Tm treatment (Figure 5E). Knockout of *Smad1* and *Smad5* was confirmed by qRT-PCR (Supplemental Figure 4, A and B), whereas, in agreement with the in vitro data, Tm stimulated *Smad1* and *Smad5* mRNA expression in the *Smad1/5 Cre*$^{-}$ mice (Supplemental Figure 4, A and B, gray bars). Proper activation of ER stress was proved by *Bip* mRNA induction and *Xbp1* splicing appearance (Supplemental Figure 4C). As expected, upon Tm treatment and after hepcidin down-regulation, serum iron and transferrin saturation were significantly suppressed in *Smad1/5 Cre*$^{-}$ control mice; however, this was not the case in *Smad1/5 Cre*$^{+}$ mice after Tm treatment (Figure 5, F and G). Altogether these data confirm the requirement for the SMAD1/5/8 pathway and the involvement of specific SMADs in hepcidin activation during ER stress.

We then investigated the role of the BMP-SMAD pathway in the context of genetic loss of CREBH because we had previously shown that the CREBH transcription factor is involved in hepcidin response to ER stress (27). In 8-week-old male and female *Creb3l3* knockout mice treated with Tm for 5 hours, *Hamp* expression was still induced as compared with control but to a significantly lesser extent than in wild-type mice (Figure 6A). Activation of the SMAD1/5/8 pathway was confirmed by the *Id1* mRNA up-regulation (Figure 6B) that occurred in both wild-type and, to a lesser extent, in *Creb3l3* knockout mice. Interestingly, the phospho-Smad1/5 induction was not prevented in the *Creb3l3* knockout mice (Figure 6C),

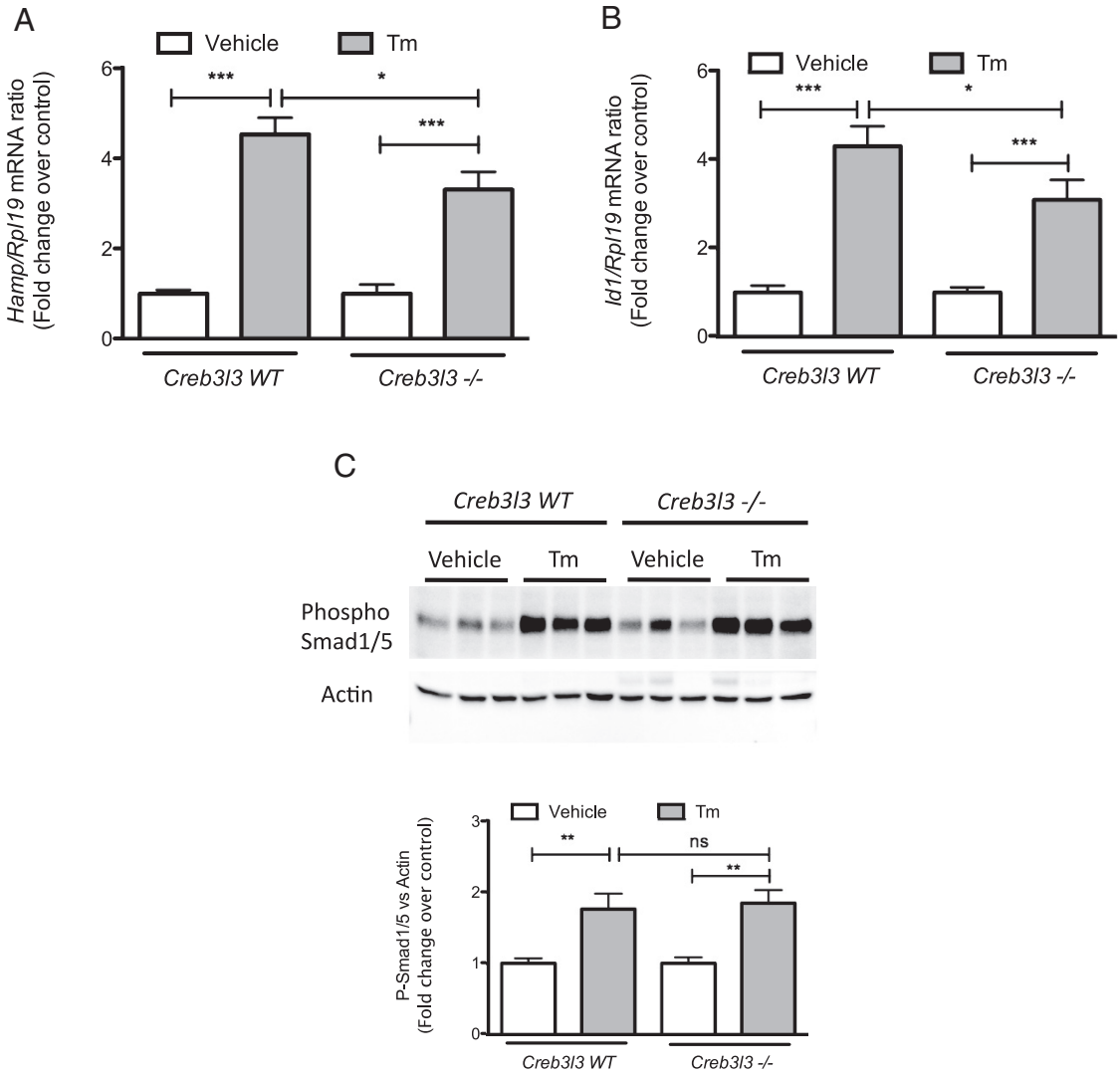

Figure 6. CREBH contributes to *Hamp* induction by ER stress in vivo. A, *Hamp* mRNA expression was analyzed by real time PCR and normalized in respect to *Rpl19* housekeeping mRNA expression using total cDNA from liver of *Creb3l3* wild-type (WT) or *Creb3l3*$^{-/-}$ (KO) mice treated with Tm for 5 hours. B, *Id1* mRNA expression was analyzed as in panel A. C, Phosphorylation of SMAD1/5 complex was analyzed using total liver extracts from *Creb3l3* WT or *Creb3l3* $^{-/-}$ (KO) mice treated with Tm for 5 hours. Densitometric analysis of specific phospho-SMAD1/5 complex signal normalized on β-actin expression is shown in the lower graph. Specific mRNA expression of vehicle-treated mice was set to 1. Data are expressed as mean ± SEM of 8–10 mice per group. *P* values are reported for comparisons between indicated groups. ns, not significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

indicating that even in the absence of CREBH, ER stress still activates the SMAD1/5/8 pathway.

Single *Smad1 and Smad5* mRNAs were similarly expressed in both WT and knockout mice (Supplemental Figure 5, A and B). Knockout of the *Creb3l3* gene was confirmed in the knockout mice by qRT-PCR (Supplemental Figure 5C).

Discussion

ER stress is associated with disruption of ER homeostasis and accumulation of unfolded or misfolded proteins in the ER (40). Homeostasis in the ER is tightly monitored through a series of adaptive programs, called the unfolded protein response (UPR). The UPR not only regulates ER capacity and the secretory pathway but also modulates fundamental physiological processes, such as differentiation of specialized cell types or cell metabolism (41). As such, ER stress has been involved in a number of pathophysiological states, including inflammatory response, nutrient disorders, and viral infection. Recently we reported that the CREB3L3 (also known as CREBH), an ER stress-associated liver-specific transcription factor that activates the transcription of acute-phase response genes in the liver (42), stably occupies hepcidin promoter and upregulates hepcidin transcription during ER stress, leading

to iron retention in vivo (27). Interestingly, CREBH also controls the expression of genes involved in hepatic lipogenesis, fatty acid oxidation and lipolysis (30), and key gluconeogenic genes (43), emerging as a key metabolic regulator in the liver.

Recent studies have shown the complexity of the regulation of hepcidin transcription, indicating that, whereas specific mediators/signals are involved in discrete regulatory pathways (eg, BMP-SMAD in the iron signaling pathway and STAT3 in the IL-6/inflammatory signaling pathway), an interplay among distinct regulatory pathways may also contribute to the regulation of hepcidin in vivo. In fact, in vitro studies using *Hamp* promoter constructs have indicated that the proximal BMP-RE is required for a maximal hepcidin response to IL-6 (24). Activin B, which is up-regulated by inflammation in vivo, may contribute to the SMAD1/5/8 pathway activation in this setting (21, 22).

In the present study, we show that hepcidin induction by different ER stressors require an intact SMAD1/5/8 pathway and hepcidin promoter BMP-RE element for optimal response in vitro. This induction is likely due to a direct effect of ER stress on the postreceptor signaling pathway because antibodies against BMP receptor ligands, including activin B, which is stimulated by ER stress (this paper) and could engage the BMP receptor complex in a paracrine fashion and enhance SMAD phosphorylation, were unable to prevent hepcidin up-regulation by ER stress. In fact, using a cell-permeable inhibitor of BMP receptor activity, LDN-193189, hepcidin response was blunted. In vivo, we confirm that the stimulatory effect by ER stressors activates the SMAD1/5/8 signaling cascade. A key role is likely played by SMAD5, which appears to be required in vitro for constitutive hepcidin expression and whose transcription is distinctly stimulated by ER stress. These results are corroborated by in vivo studies showing that ER stress stimulate SMAD1/5 expression and phosphorylation, whereas this effect was blunted in hepatocyte-specific *Smad1/5* double knockout mice. Interestingly, in the latter model, the SMAD1/5/8 pathway was not induced by ER stress, as shown by the lack of *Id1* mRNA up-regulation (Figure 5C).

CREBH is required for correct hepcidin activation following ER stress (27). Here we show that the genetic loss of CREBH impairs to some extent *HAMP* expression, as expected, but does not prevent induction of the SMAD1/5/8 pathway that still sustains residual hepcidin expression in vivo. In the same mouse model, we consistently found a reduced activation *Id1* mRNA in *Creb3l3* knockout mice (Figure 6B), a known target of the SMAD1/5/8 pathway. We speculate this might be due to a role of CREBH in regulating *Id1* promoter activity, as suggested by previous studies (44, 45).

In conclusion, our study indicates that hepcidin induction by ER stress involves the central SMAD1/5/8 pathway. This also implies a possible cooperation between the CREBH and the SMAD1/5/8 pathway. Both seem to be required for full response of hepcidin to ER stressors in vivo. Most likely, an induction of SMADs early in the pathway is critical, and a late cooperation of CREBH on hepcidin promoter is important to elicit a full response of the hepcidin gene to ER stress. The UPR is characterized by the activation of three distinct signal transduction pathways mediated by inositol-requiring protein 1α, protein kinase RNA-like ER kinase, and activating transcription factor 6, which are variably and often collectively induced (40, 41). Future studies may address whether these pathways individually or cooperatively trigger SMAD/CREBH-driven hepcidin induction during ER stress.

Acknowledgments

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