Hemochromatosis proteins are dispensable for the acute hepcidin response to BMP2

The liver iron hormone hepcidin regulates body iron homeostasis by blocking iron export *via* ferroportin. Hepcidin transcription is controlled by the BMP-SMAD pathway, whose activation requires BMP2, BMP6, type I (ALK2 and ALK3) and type II (ACVR2A and BMPR2) BMP receptors.[1] BMP2 and BMP6 are mainly expressed by liver sinusoidal endothelial cells and their deletion in mice causes iron overload due to decreased hepcidin mRNA expression.[2,3] BMP type II receptors show a redundant function in hepcidin regulation *in vivo*, while ALK2 and ALK3 have a non-redundant role. The current model suggests that ALK2 is mainly involved in BMP6-dependent hepcidin upregulation in conditions of iron overload[4] and is inhibited by FKBP12,[5] whereas ALK3 maintains basal hepcidin expression[4] and signals preferentially in response to BMP2.[6,7] Hereditary hemochromatosis (HH), characterized by iron overload due to inappropriately low hepcidin production, is caused by mutations in

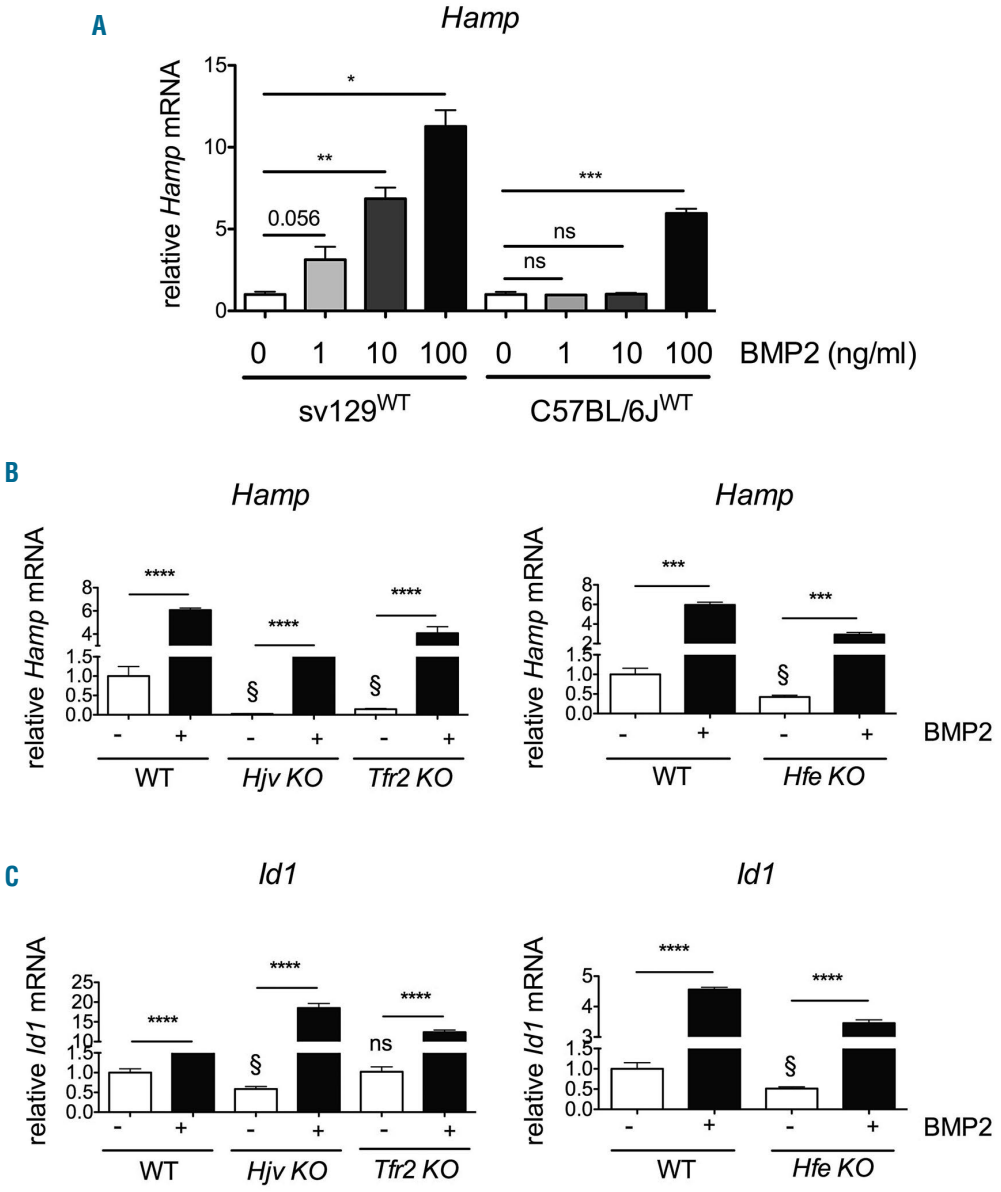

Figure 1. BMP2 up-regulates *hepcidin* and *Id1* expression in murine primary hepatocytes lacking *Hjv*, *Tfr2* or *Hfe*. (A) Primary murine hepatocytes from 3 or 4 wild-type (WT) male mice maintained on a Sv129 or C57BL/6J background, respectively, were isolated as described[5] and treated for 4 hours (h) with increasing concentrations of BMP2 (1-100 ng/mL as indicated). Total RNA was isolated and retrotranscribed, and quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyze *hepcidin (Hamp)* expression. *Gapdh* was used as housekeeping gene. mRNA expression ratio was normalized to an untreated mean value of 1. A representative experiment is shown. (B and C) Primary murine hepatocytes were isolated from 3 WT, 3 *Hjv*-knockout (KO), and 3 *Tfr2*-KO mice (on a Sv129 background) and treated with 10 ng/mL BMP2 for 4 h, or from 4 WT and 4 *Hfe*-KO mice (on a C57BL/6J background) and treated with 100 ng/mL BMP2 for 4 h. Total RNA was isolated and retrotranscribed, and qRT-PCR was performed to analyze hepcidin (*Hamp*) (B) and *Id1* (C) expression. *Gapdh* was used as housekeeping genes. mRNA expression ratio was normalized to an untreated mean value of 1. A representative experiment is shown. *Untreated versus BMP2-treated. §Untreated WT *versus* untreated hereditary hemochromatosis. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; §P<0.05; ns: not significant. Error bar indicates standard error.

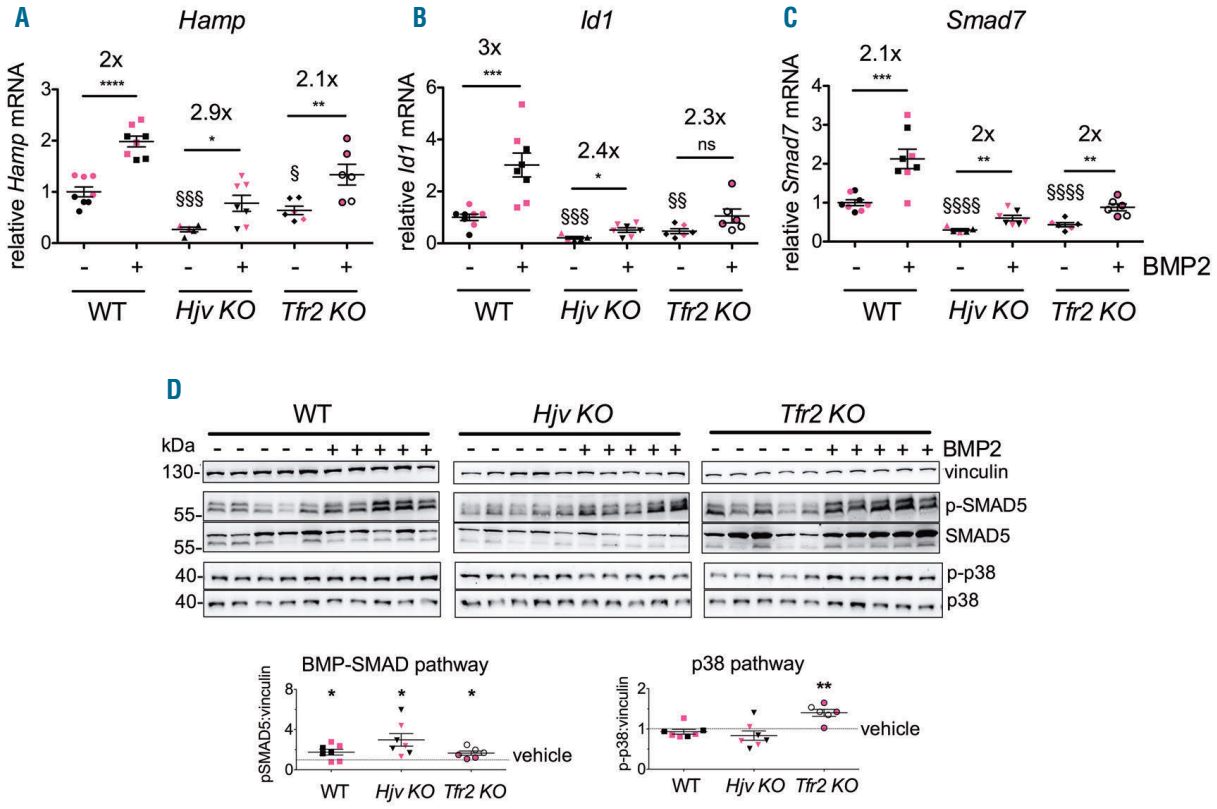

Figure 2. Acute BMP2 treatment in wild-type (WT), *Hjv*- and *Tfr2*-knockout (KO) mice up-regulates *hepcidin* through BMP-SMAD pathway activation. WT (n=16), *Hjv*-KO (n=13), and *Tfr2*-KO (n=12) mice (10-12 weeks old) were treated with vehicle or 24 μg/mouse BMP2 and sacrificed 4 hours later. Total liver RNA was isolated and retrotranscribed, and quantitative real-time polymerase chain reaction was performed. Liver *hepcidin* (*Hamp*, A), *Id1* (B) and *Smad7* (C) mRNA levels were normalized to the housekeeping gene *Hprt1*. (D) Liver extracts were separated on a 12% SDS-PAGE for western blotting and probed with antibodies specific for phospho- or unmodified SMAD5 and p38. The anti-phospho-SMAD5 and anti-SMAD5 antibodies recognize two bands: according to Wang *et al.*,[16] SMAD5 corresponds to the lower band. Vinculin was used to normalize gel loading. Molecular weight markers are indicated on the left. (Right) Densitometric analyses of p-SMAD5 and p-p38 are indicated below. Males: black symbol; females: pink symbol. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

HFE, the second transferrin receptor (*TFR2*), hemojuvelin (*HJV*) and hepcidin (*HAMP*). HJV is a BMP co-receptor that activates the BMP-SMAD pathway and hepcidin expression. It interacts with HFE and TFR2 and is functionally independent of BMP6 and likely of ALK2.[1] Recent studies imply a function of HH proteins in BMP2-ALK3 signaling: both HJV[8] and TFR2[9] interact with BMP2, and TFR2 and HFE bind ALK3[9,10] (*data not shown*). However, the functional role of the HH proteins in BMP2-mediated hepcidin regulation is still elusive.

To analyze whether HFE, TFR2 and HJV play a role in hepcidin activation by BMP2, we first studied primary murine hepatocytes (mHC) isolated from wild-type (WT) and HH mouse models. Since *Hfe*-KO mice were on the C57BL/6J and *Tfr2* and *Hjv*-KO mice on the Sv129 genetic background, responses of *Hjv, Hfe* and *Tfr2*-KO primary mHC to BMP2 were compared to those of controls on the appropriate background. Unexpectedly, mHC isolated from C57BL/6J background mice showed a more than 10-fold diminished hepcidin response to BMP2 compared to Sv129 (Figure 1A). Thus the concentration of BMP2 used was 10 ng/mL for Sv129-derived cells and 100 ng/mL for C57BL/6J-derived hepatocytes. As expected, basal *hepcidin* expression was reduced in *Hfe*-, *Tfr2*- and *Hjv*-KO mHC. However, BMP2 treatment increased hepcidin expression independently of the genotype (Figure 1B and *Online Supplementary Figure S1A*), likely in a BMP-SMAD pathway-dependent manner as suggested by the upregulation of the BMP-SMAD target genes *Id1* (Figure 1C and *Online Supplementary Figure S1B*). In the absence of HH proteins, the fold-increase of hepcidin and *Id1* was more pronounced than in WT likely because of the low basal activation of the pathway or the high iron content of hepatocytes derived from HH models. These results indicate that HH proteins are dispensable for BMP2-dependent hepcidin upregulation *ex vivo* in hepatocytes.

To explore the *in vivo* role of HH proteins in BMP2-dependent hepcidin activation, we focused on *Hjv*- and *Tfr2*-KO mice whose genetic background conferred increased BMP2 sensitivity. Adult HH mice and WT littermates were treated with a single BMP2 or saline injection and sacrificed 4 hours later. We chose the BMP2 dose (24 mg/mouse) that up-regulated both *Hamp* and *Id1* in WT mice (*Online Supplementary Figure S2*). *Hjv*-KO mice were severely iron loaded, with high liver iron (LIC) and serum iron levels (*Online Supplementary Figures S3A and C*), and low spleen iron concentration (SIC) (*Online Supplementary Figure S3B*), whereas *Tfr2*-KO mice showed milder iron overload than *Hjv*-KO mice (*Online Supplementary Figure S3A-C*). Iron parameters were not modified by the short BMP2 treatment in both models (*Online Supplementary Figure S3A-C*). The BMP-SMAD target genes *hepcidin* (Figure 2A and *Online Supplementary Figure S3E*), *Id1* (Figure 2B and *Online Supplementary Figure S3F*), and *Smad7* (Figure 2C and *Online*

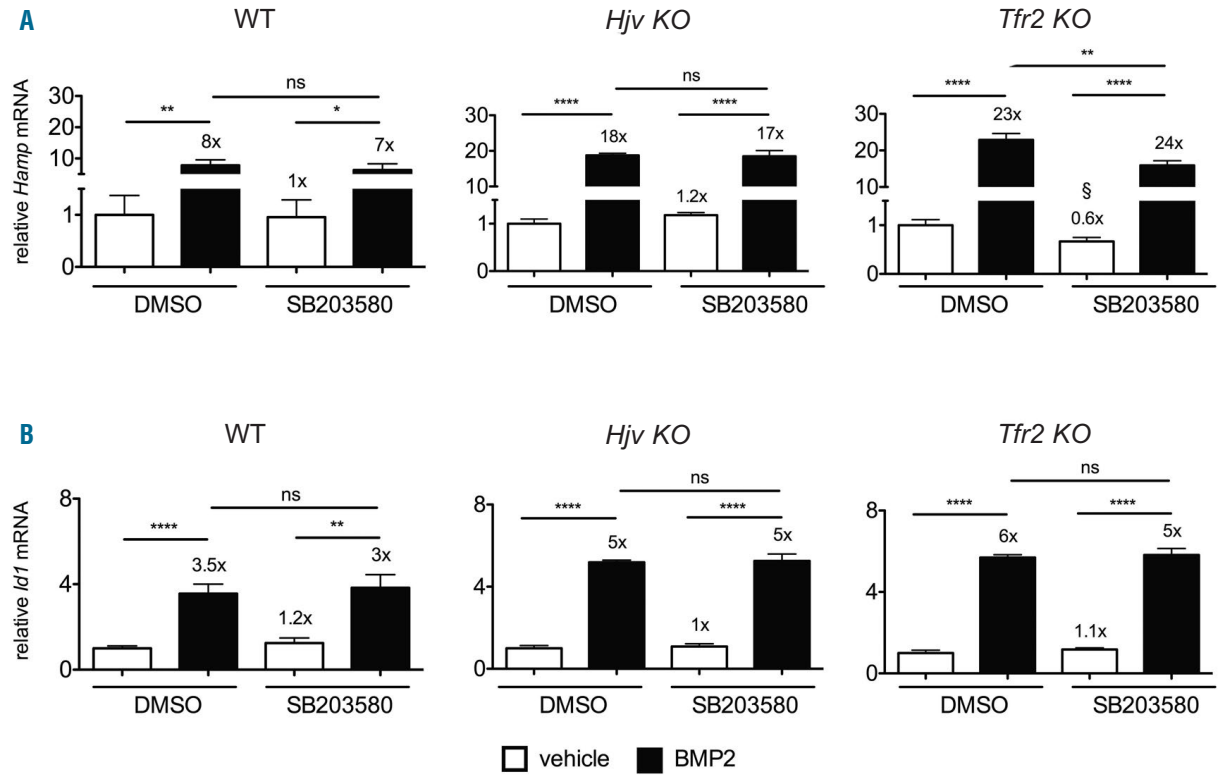

Figure 3. p38 activation contributes to BMP2-dependent *hepcidin* upregulation in *Tfr2*-knockout (KO) primary hepatocytes. (A and B) Primary hepatocytes were isolated from 3 wild-type (WT), 2 *Hjv*-KO and 2 *Tfr2*-KO (8-10 weeks old, females) and treated with BMP2 (10 ng/mL) in the presence or absence of the p38 inhibitor SB203580 (10 μM). Expression of *hepcidin* (A) and *Id1* (B) were assessed by quantitative real-time polymerase chain reaction. *Gapdh* was used as housekeeping gene. mRNA expression ratio was normalized to an untreated mean value of 1. **P*<0.05; ***P*<0.01; *****P*<0.0001; §*P*<0.05 SB203580 *versus* DMSO; ns: not significant. Error bar indicates standard error. Numbers over the white bars indicate fold changes of SB203580-treated cells *versus* DMSO-treated ones. Numbers over the black bars indicate fold changes of BMP2-treated cells *versus* vehicle-treated ones.

Supplementary Figure S3G) were significantly up-regulated in *Hjv*- and *Tfr2*-KO mice after BMP2 treatment. For our data analysis, we pooled male and female mice, since in our hands they showed comparable BMP-SMAD pathway activation and hepcidin expression. Accordingly, the analysis of male mice only showed that *hepcidin* (*Online Supplementary Figure S4A*) and *Smad7* mRNA expression (*Online Supplementary Figure S4B*) were significantly increased by BMP2 both in WT and HH mice. Serum hepcidin mirrored *Hamp* mRNA in that it was increased in WT and *Hjv*-KO mice and showed a trend towards increase in *Tfr2*-KO mice (*Online Supplementary Figure S3D*). We exclude the possibility that the absence of HJV and TFR2 decreases BMP2 sensitivity; indeed the fold-change increase of *hepcidin* (*Online Supplementary Figure S5A*) and Id1 (*Online Supplementary Figure S5B*) was augmented in HH-derived HC.

Overall these results indicate that both HJV and TFR2 are dispensable for the transcriptional activation of hepcidin by BMP2 *in vivo*, in agreement with results obtained in hepatocytes.

Bmp6 expression is strongly regulated by iron concentration.[11] Consistently, *Bmp6* mRNA levels were high in untreated *Hjv*- and *Tfr2*-KO mice compared to WT (*Online Supplementary Figure S4C*). By contrast, *Bmp2* mRNA expression, which is only mildly responsive to iron,[12] was comparable to WT mice and unaffected by iron overload in *Hjv*- and *Tfr2*-KO mice (*Online Supplementary Figure S4D*). Expression of both *Bmp2* and *Bmp6* was unchanged by BMP2 treatment.

BMP2 likely induces the activation of canonical (SMAD1/5/8) and of non-canonical signaling pathways, the latter culminating in AKT, ERK and p38 phosphorylation.[13] We next investigated whether HJV and TFR2 influence the activation of these pathways in response to BMP2. Under basal conditions phospho-SMAD5 levels were strongly reduced in *Hjv*-KO mice and similar to WT in *Tfr2*-KO mice, but still inappropriately low considering the increased LIC and *Bmp6* (*Online Supplementary Figure S6A and B*). AKT, ERK and p38 pathways were unaffected by the absence of HJV or TFR2 (*Online Supplementary Figure S6A, C-F*).

Importantly, BMP2 injection increased SMAD5 phosphorylation both in WT and in HH mice (Figure 2D), further supporting the dispensable role of HH proteins for BMP-SMAD pathway activation in response to acute BMP2 treatment. Moreover, BMP2 fails to activate the non-canonical AKT and ERK pathways in all the genotypes (*Online Supplementary Figure S7A, C-E*), likely because of their fast kinetics.[9] Surprisingly, BMP2 induces p38 phosphorylation only in *Tfr2*-KO mice (Figure 2D), a process that in turn might increase SMAD1/5/8 phosphorylation, as shown in other cell types.[14] We speculate that *in vivo Tfr2* deficiency enhances BMP2-mediated *hepcidin* expression through p38 phosphorylation. To investigate whether p38 phosphorylation contributes to hepcidin upregulation, WT, *Hjv*-KO and *Tfr2*-KO primary HC were treated with BMP2 in the presence or absence of the p38 inhibitor SB203580. SB203580 treatment in primary HC up-regulated *Egr1* expression (*Online*

Supplementary Figure S8), as described,[15] confirming p38 inhibition. It did not change basal and BMP2-mediated hepcidin upregulation in WT and *Hjv*-KO HC. Interestingly, SB203580 significantly reduced *hepcidin* expression both in untreated and in BMP2-treated *Tfr2*-KO HC (Figure 3A) in a SMAD1/5/8-independent way, as shown by comparable *Id1* expression (Figure 3B). However, the change increase by BMP2 is comparable in the untreated and SB203580-treated cells. Overall these results suggest a functional role of TFR2 in p38 regulation. Whether this signaling pathway influences hepcidin regulation still remains to be clarified.

In summary, our results demonstrate that HJV and TFR2 are necessary for the canonical BMP-SMAD pathway but not for the non-canonical pathways in steady state, and that HH proteins are not required for the activation of BMP-SMAD signaling and *hepcidin* expression by BMP2.

Our study highlights the dispensable role of the HH proteins in response to an acute BMP2 increase, likely occurring in response to increased iron levels,[12] and uncovers an unprecedented role of TFR2 in the regulation of p38 signaling.

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