# Hemochromatosis proteins are dispensable for the acute hepcidin response to BMP2

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# Supplemental Materials

## Supplemental Material and Methods

### Mice treatment

*Wild-type* (WT), *Hjv*-KO and *Tfr2*-KO mice, on a sv129 background, were housed under standard 12-hours light/dark cycle with water and chow ad libitum in pathogen-free animal facility at San Raffaele Scientific Institute, Milan, Italy. The study was approved by the Institutional Animal Care and Use Committee. *Hfe*-KO mice and WT littermates, on C57BL/6 background, were housed in a pathogen-free animal facility at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. Mice were kept under standard 12-hour light/dark cycle with water and chow ad libitum. Eight weeks old mice were fed the same iron balance diet (Teklad 2018S, containing 200 ppm iron) for 4 weeks. Mice were treated with 24 µg/mouse of BMP2 (Peprotech, Hamburg, Germany; #120-02C) or vehicle for 4 hours and then anesthetized and euthanized by cervical dislocation. Blood, liver and spleen were collected for serum, liver and spleen iron quantification. Liver was also processed for RNA and proteins analyses.

### Quantification of Liver (LIC) and Spleen Iron Content (SIC)

Liver and spleen were dried at 65°C for 1 week. 18-22 mg of dried livers were digested in 1 mL of acid solution (3M HCl, 0.6 M trichloroacetic acid) for 20 hours at 65°C. Twenty µl of acid extract was added to 1ml of working chromogen reagent (1 volume of 0.1% bathophenanthroline sulfate and 1% thioglycolic acid solution, 5 volumes of water, and 5 volumes of saturated sodium acetate), incubated at room temperature and the absorbance measured at 535 nm. The standard curve was prepared by adding increasing amount of iron diluted from a stock solution of Titrisol iron standard (Merck, Darmstadt, Germany) in the acid solution.

### Quantification of Serum Iron Content

Blood was collected at sacrifice and processed for serum separation. Serum iron concentration was assessed by using the SFBC kit (Biolabo, Maizy, FRA), according to the manufacturer's protocol.

**Quantification of serum hepcidin**

Serum hepcidin was quantified in duplicate in 12 µl of sample using the "Hepcidin Murine-Compete ELISA Kit" (Intrinsic Lifescience, United States) according to manufacturer's instructions. Hepcidin concentration was extrapolated against a standard curve by using a 4-parameter logistic model using the Graphpad Prism V7.1 software.

**Primary hepatocytes isolation and treatment**

Primary murine hepatocytes from WT, *Tfr2*-KO, *Hjv*-KO (on sv129 background) and from WT and *Hfe*-KO (on C57BL/6J background) were isolated following the standard *in situ* two-step perfusion method as described in[1]. Briefly mice were anesthetized and sacrificed. A U cut in the lower abdomen was performed to expose vena cava and vena porta. Vena cava inferior was cannulated and perfused (pump flux: 5 ml/min) with Liver Perfusion Medium (Thermo Fisher Scientific) and Liver Digest Medium (Thermo Fisher Scientific). After digestion, the liver capsule was mechanically disrupted to release cells. Debris and membranes were removed through a 100 µm cell strainer. Hepatocytes (HCs) were separated from non-parenchymal cells through low-speed centrifugation (50 g for 3 minutes), resuspended in Williams-E medium (4% FBS, 1% P/S, Glutamax) (Thermo Fisher Scientific) and plated into collagen-coated 12-well ($2.5\text{-}3*10^5$ cells/well). Four hours after isolation, HCs were attached and cell culture medium was replaced with fresh Williams-E medium (4% FBS, 1% P/S, Glutamax). Eighteen hrs later HCs were serum starved for 3-4 hrs (Williams-E medium 0% FBS, 1% P/S, Glutamax) and then treated for 4 hrs with BMP2 (10 ng/mL or 100ng/mL; Peprotech) in serum-starved media. For p38 inhibition experiments, primary hepatocytes were treated with the p38 inhibitor SB203580 (10 µM; Invivogen) for 2 hrs in serum-starved media, and then incubated for 4 hrs with 10 ng/ml BMP2 (Peprotech) with or without SB203580. Cells were lyzed and RNA isolated for gene expression analysis.

**qRT-PCR**

Total RNA was extracted from hepatocytes and liver with the peqGOLD Total RNA Kit (vwr), TRIFAST (Euroclone) or RNEasy kit (Qiagen) according to manufacturer's instruction. cDNA was synthetized with the RevertAid H Minus Reverse Transcriptase kit (Thermo SCIENTIFIC) or with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Framingham, MA). Gene expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the SybrGreen or the TaqMan Gene Expression Master Mix (Applied Biosystems). *Hprt1* or *Gapdh* were used as housekeeping genes. Primers for qRT-PCR are in Supplemental Table 1 and 2. Values shown are means of $2^{-\Delta Ct}$ (ie, Ct *Hprt1*- Ct target) or -$\Delta$Ct. Results are expressed as mean ± standard error (SE).

**Western Blot Analyses of liver tissues**

Snap-frozen liver samples were lysed in RIPA buffer (10mM Tris-HCl pH=8.0, 150mM NaCl, 1mM EDTA, 1% NP40, SDS) plus protease inhibitor cocktail (Roche Diagnostics) and phosphoSTOP (Roche Diagnostics). Protein concentration was determined by Pierce BCA protein assay (Life Technology). Fifty µg of protein samples were diluted in Laemmli sample buffer and incubated 5 minutes at 95°C. Samples were then loaded onto 12% SDS-PAGE and transferred to nitrocellulose blotting membrane (neoLab, Germany) by standard techniques. Blots were incubated with the antibodies listed below and then with HRP-conjugated antisera, and developed using a chemiluminescence detection kit. Western blot images were acquired in a quantitative way with the Vilber-Lourmat-Fusion-FX-Chemiluminescence-system. Densitometric analysis was performed by using ImageJ.

**Statistical analyses**

Unpaired 2-tailed Student's t-test (for normal distributions) or Mann-Whitney test (for non Gaussian distributions) were performed using GraphPad Prism 5.0 or 7.0. Two-way ANOVA was used to calculate significance in the dose-response experiments. Data were presented as mean ± Standard Error (SE). $P<0.05$ was considered statistically significant.

## Supplemental Table 1. Oligos for qRT-PCR (SybrGreen)

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *Gapdh* | TCCACTCACGGCAAATTCAA | TTTGATGTTAGTGGGGTCTCG |
| *Hamp* | ATACCAATGCAGAAGAGAAGG | AACAGATACCACACTGGGAA |
| *Id1* | ACCCTGAACGGCGAGATCA | TCGTCGGCTGGAACACATG |
| *Smad7* | GCAGGCTGTCCAGATGCTGT | GATCCCCAGGCTCCAGAAGA |
| *Bmp6* | AGCACAGAGACTCTGACCTATTTTTG | CCACAGATTGCTAGTTGCTGTGA |
| *Bmp2* | CGGACTGCGGTCTCCTAA | GGGGAAGCAGCAACACTAGA |

## Supplemental Table 2. Oligos for qRT-PCR (TaqMan)

| Gene | Transcription assay ID |
|---|---|
| *Hprt1* | Mm01318743_m1 |
| *Hamp* | Mm00519025_m1 |
| *Id1* | Mm00775963_m1 |
| *Egr1* | Mm00656724_m1 |

## Supplemental Table 3. Antibodies for Western Blot

| Antigen | Host | Diluition | Reference/supplier |
|---|---|---|---|
| SMAD5 (phospho S463 + S465) | Rabbit | 1:1000 | ab92698/abcam |
| SMAD5 | Rabbit | 1:1000 | ab40771/abcam |
| Phospho-p38 MAPK (Thr180/Tyr182) | Rabbit | 1:1000 | 4511S/Cell signaling |
| p38 MAPK | Rabbit | 1:1000 | 8690S/Cell Signaling |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | Rabbit | 1:1000 | 4370/Cell Signaling |
| p44/42 MAPK (Erk1/2) | Rabbit | 1:1000 | 4695/Cell Signaling |
| Phospho-Akt (Ser473) | Rabbit | 1:1000 | 4060S/Cell Signaling |
| Akt | Rabbit | 1:1000 | 9272S/Cell signaling |
| Vinculin | Mouse | 1:1000 | V4505/Sigma Aldrich |

## Supplemental References

1. Colucci S, Pagani A, Pettinato M, et al. The immunophilin FKBP12 inhibits hepcidin expression by binding the BMP type I receptor ALK2 in hepatocytes. Blood. 2017;130(19):2111-2120.
2. Wang CY, Core AB, Canali S, et al. Smad1/5 is required for erythropoietin-mediated suppression of hepcidin in mice. Blood. 2017;130(1):73-83.

**Legend to Supplemental Figures**

**Figure S1. BMP2 increases *hepcidin* and *Id1* mRNA expression in primary hepatocytes from HH mice.**

**A-B**) Primary murine hepatocytes isolated from 3 *Hjv*-KO, 3 *Tfr2*-KO (on a sv129 background) and 4 Hfe KO (on a C57BL/6J background) male mice, and the corresponding WT controls, were treated with 10 ng/ml (sv129) or 100 ng/ml (C57BL/6J) BMP2 for 4 hrs. *Hepcidin* (*Hamp*, **A**) and *Id1* (**B**) mRNA expression was analyzed as described in **Figure 1**. mRNA levels were expressed as – ΔCt values. * refers to untreated vs BMP2-treated; § refers to untreated WT vs untreated HH. **: P<.01; ***: P<.001; ****: P<.0001. §: P<.05; §§: P<.01; §§§: P<.001; ns: non significant. Error bar indicates Standard Error (SE).

**Figure S2. BMP2 dose response in sv129 wild-type mice.**

Sv129 WT mice (n=3 for each group) were treated with 6-12-24 μg/mouse of BMP2 or saline and sacrificed 4 hours later. Total RNA was isolated from the liver and qRT-PCR was performed to measure *hepcidin* (*Hamp*) and *Id1 mRNA* expression. *Hprt1* was used as housekeeping gene. mRNA expression ratio was normalized to an untreated mean value of 1. *: P<.05; **: P<.01; ***: P<.001; ns: non significant. Error bar indicates Standard Error (SE).

**Figure S3. Iron-related parameters in BMP2-treated mice.**

Liver (**A**), spleen (**B**), serum iron (**C**) and serum hepcidin levels (**D**) were analyzed in WT, *Hjv*- and *Tfr2*-KO mice treated for 4 hrs with vehicle or BMP2 (24 μg/mouse). *Hepcidin* (*Hamp*, **E**), *Id1* (**F**) and *Smad7* (**G**) mRNA expression was analyzed in the liver of BMP2-treated mice as describe in **Figure 2**. mRNA levels were expressed as – ΔCt values. * refers to untreated vs BMP2-treated; § refers to untreated WT vs untreated HH. *: P<.05; **: P<.01; ***: P<.001; ****: P<.0001 §: P<.05; §§§ **: P<.01; §§§§: P<.0001; ns: non significant. Error bar indicates Standard Error (SE).

**Figure S4. Expression of hepcidin regulatory genes in BMP2-treated mice.** Liver *hepcidin* (*Hamp*, **A**) and *Smad7* (B) were analyzed by qRT-PCR in vehicle and BMP2-treated WT and HH male mice. *Bmp6* (**C**) and *Bmp2* (**D**) mRNA expression was measured in BMP2-treated mice. *Gapdh* was used as housekeeping gene. mRNA expression ratio was normalized to an untreated mean value of 1. * refers to untreated vs BMP2-treated; § refers to untreated WT vs untreated HH. Ns: non significant. *: P<.05; **: P<.01; ***: P<.001. §§: P<.01; §§§: P<.001; §§§§: P<.0001. Error bar indicates Standard Error (SE).

**Figure S5. Dose-response curves of BMP2-treated primary hepatocytes from Hereditary Hemochromatosis mice.**

Primary hepatocytes were isolated from WT-sv129 (n=3), *Hjv*-KO (n=2) and *Tfr2*-KO (n=2) mice (8-10 wks, female) and treated with increasing concentrations of BMP2 (1-10-100 ng/ml) for 4 hrs. mRNA expression of *hepcidin* (*Hamp*, **A**) and *Id1* (**B**) was assessed by qRT-PCR, using *Gapdh* as housekeeping gene. A 2-way ANOVA analysis was performed comparing the BMP2 response of HH-derived cells vs WT HCs. ***: P<.001; ****: P<.0001. Error bar indicates Standard Error (SE).

**Figure S6. The absence of HJV or TFR2 in mice impairs the canonical but not non-canonical pathway under basal conditions.**

WT, *Hjv*- and *Tfr2*-KO mice, injected with vehicle, were sacrificed 4 hrs later. Liver extracts were separated on a 12% SDS-PAGE and analyzed by Western Blot (**A**). Phospho and unmodified proteins were detected using specific antibodies. The anti-SMAD5 antibody recognizes two bands: according to[2], SMAD5 corresponds to the lower band. Vinculin was used to normalize gel loading. Molecular weight markers are indicated on the left. Densitometric analyses of SMAD5 (**B**), AKT (**C**), ERK-p42 (**D**), ERK-p44 (**E**) and p38 (**F**) are shown. *: P<.05 (vs WT mice); ns: non significant. Pink symbol indicates female mice. Black symbol indicates male mice. Error bar indicates Standard Error (SE).

**Figure S7. BMP2 treatment activates the canonical but leaves unchanged the non-canonical BMP-SMAD pathway both in WT and in Hereditary Hemochromatosis mouse models.**

**A**) WT, *Hjv*- and *Tfr2*-KO mice were ip injected with vehicle or BMP2 (24 μg/mouse) and sacrificed 4 hrs later. Liver extracts were loaded onto a 12% SDS-PAGE and analyzed by WB using specific antibodies recognizing phospho- and unmodified AKT, p42 and p44 (ERK pathway) proteins. Vinculin was used to normalize gel loading (see also Figure 2C). Molecular weight markers are indicated on the left. Signaling pathways activation was quantified by densitometric analyses: AKT (**B**), ERK-p42 (**C**), ERK-p44 (**D**).

**Figure S8. p38 inhibition by SB203580 upregulates *Egr1* expression similarly in WT, *Hjv* KO and *Tfr2* KO primary hepatocytes.**

Primary hepatocytes were isolated from 3 WT, 2 *Hjv*-KO and 2 *Tfr2*-KO (8-10 wks old, females) and treated with the p38 inhibitor SB203580 (10 μM) in the presence (right panel) or absence (left panel) of BMP2 (10 ng/ml). Expression of *Egr1* was assessed by qRT-PCR. *Hprt1* was used as housekeeping gene. mRNA expression ratio was normalized to an untreated mean value of 1. *: P<.05; ***: P<.001; ns: non significant. Error bar indicates Standard Error (SE).

**FIGURE S1**

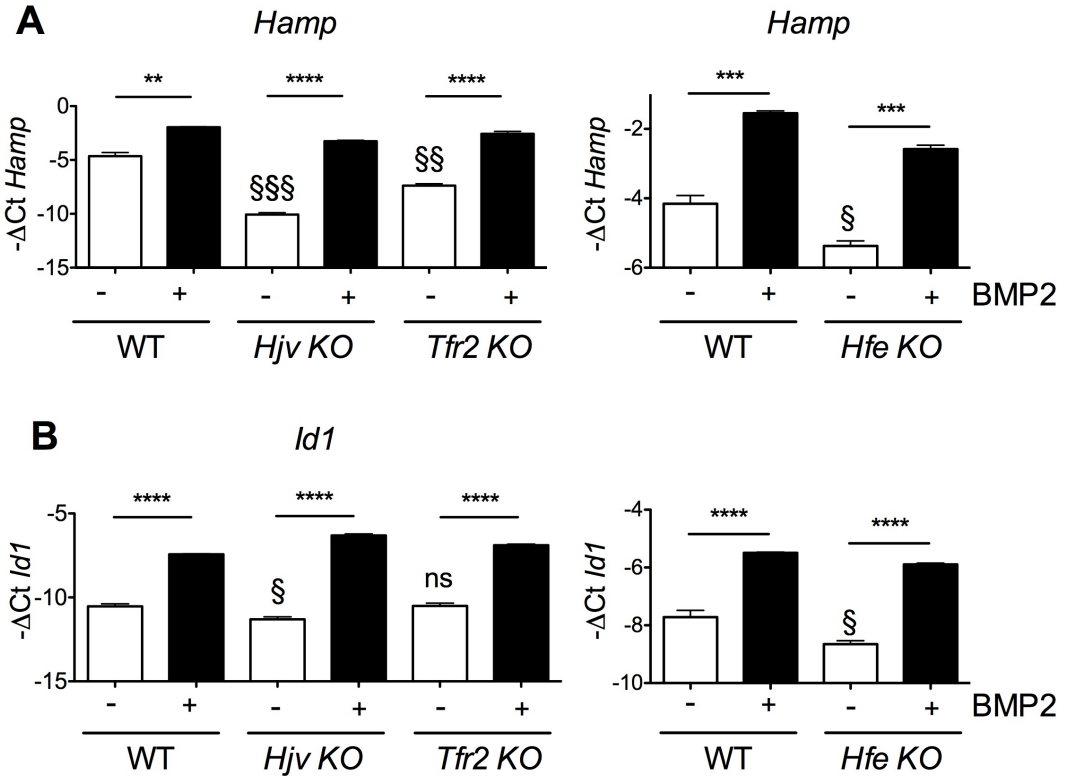
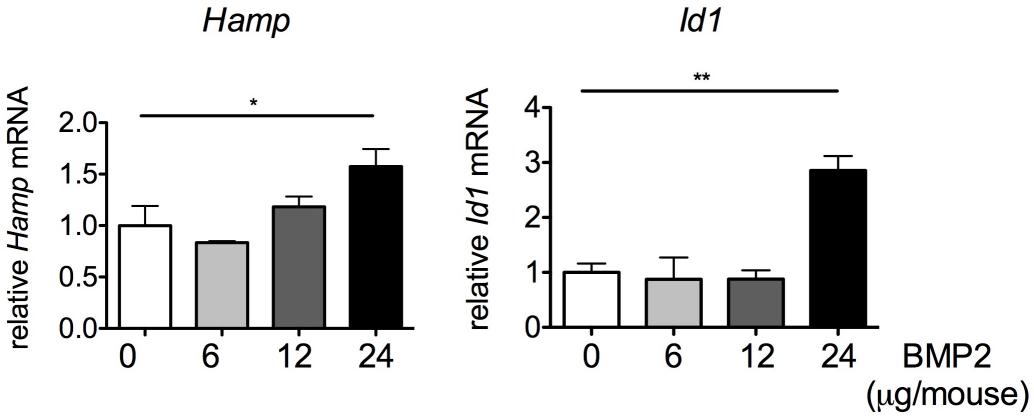

A
Hamp
Hamp
-ΔCt Hamp
**
****
****
§§§
§§
***
***
§
BMP2
WT
Hjv KO
Tfr2 KO
Hfe KO
B
Id1
-ΔCt Id1
****
****
****
§
ns
§
BMP2
WT
Hjv KO
Tfr2 KO
Hfe KO

**FIGURE S2**


Hamp
Id1
relative Hamp mRNA
relative Id1 mRNA
*
**
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
0
6
12
24
BMP2
(μg/mouse)

# FIGURE S3

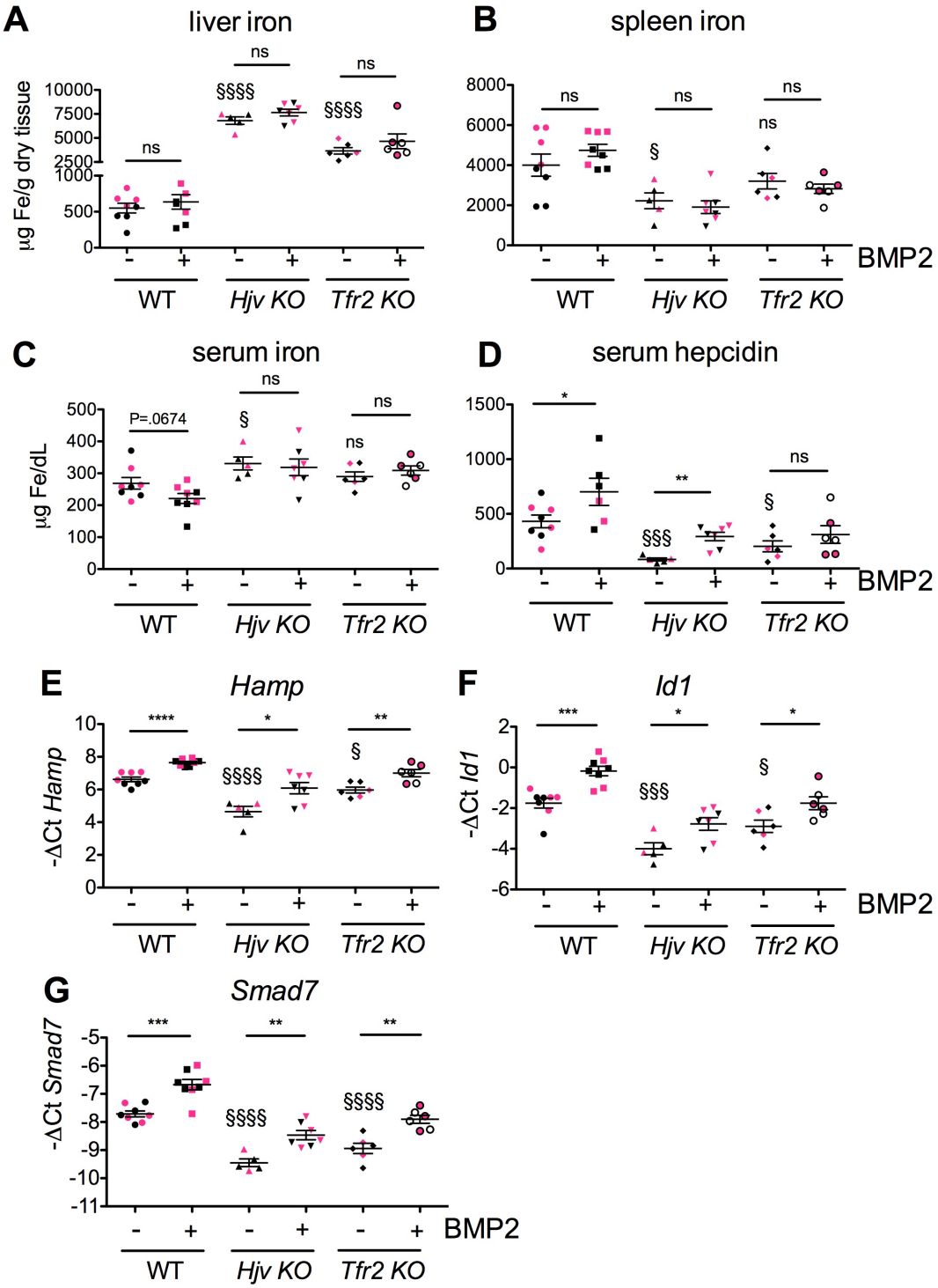
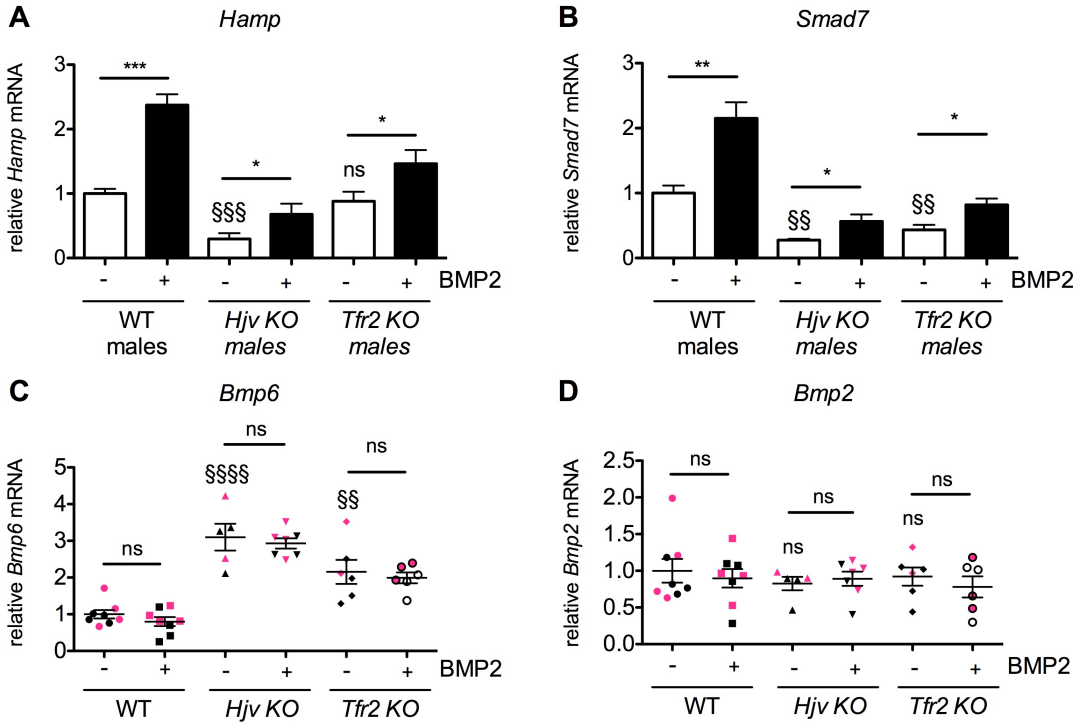
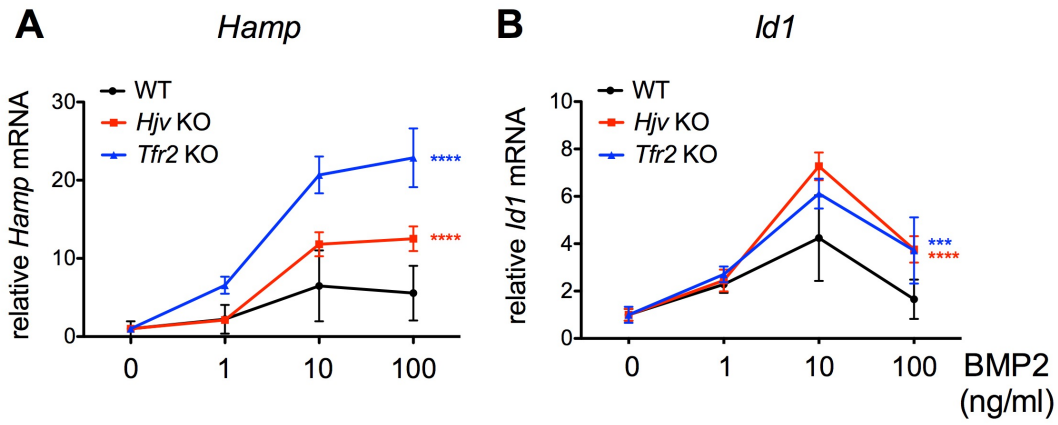

A
liver iron
µg Fe/g dry tissue
10000
7500
5000
2500
1000
500
0
ns
§§§§
ns
§§§§
ns
B
spleen iron
8000
6000
4000
2000
0
ns
§
ns
ns
ns
BMP2
WT
Hjv KO
Tfr2 KO
C
serum iron
µg Fe/dL
500
400
300
200
100
0
P=.0674
ns
§
ns
ns
D
serum hepcidin
1500
1000
500
0
*
**
§§§
§
ns
BMP2
E
Hamp
-ΔCt Hamp
10
8
6
4
2
0
****
*
§§§§
**
§
F
Id1
-ΔCt Id1
2
0
-2
-4
-6
***
*
§§§
*
§
G
Smad7
-ΔCt Smad7
-5
-6
-7
-8
-9
-10
-11
***
**
§§§§
**
§§§§
BMP2

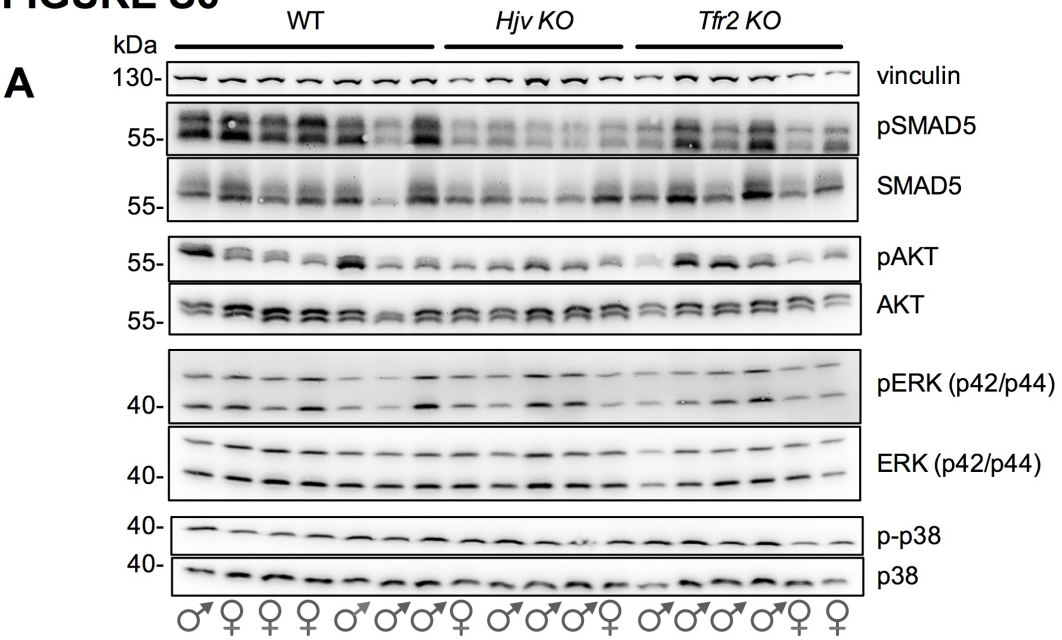
**FIGURE S4**


A
Hamp
relative Hamp mRNA
***
*
ns
*
§§§
BMP2
WT males
Hjv KO males
Tfr2 KO males
B
Smad7
relative Smad7 mRNA
**
*
*
§§
§§
BMP2
WT males
Hjv KO males
Tfr2 KO males
C
Bmp6
relative Bmp6 mRNA
ns
ns
ns
§§§§
§§
BMP2
WT
Hjv KO
Tfr2 KO
D
Bmp2
relative Bmp2 mRNA
ns
ns
ns
ns
BMP2
WT
Hjv KO
Tfr2 KO

# FIGURE S5


A
Hamp
relative Hamp mRNA
WT
Hjv KO
Tfr2 KO
****
****
0
10
20
30
0
1
10
100
B
Id1
relative Id1 mRNA
WT
Hjv KO
Tfr2 KO
***
****
0
2
4
6
8
10
0
1
10
100
BMP2
(ng/ml)

# FIGURE S6


A
WT
Hjv KO
Tfr2 KO
kDa
130-
55-
55-
55-
55-
40-
40-
40-
40-
vinculin
pSMAD5
SMAD5
pAKT
AKT
pERK (p42/p44)
ERK (p42/p44)
p-p38
p38

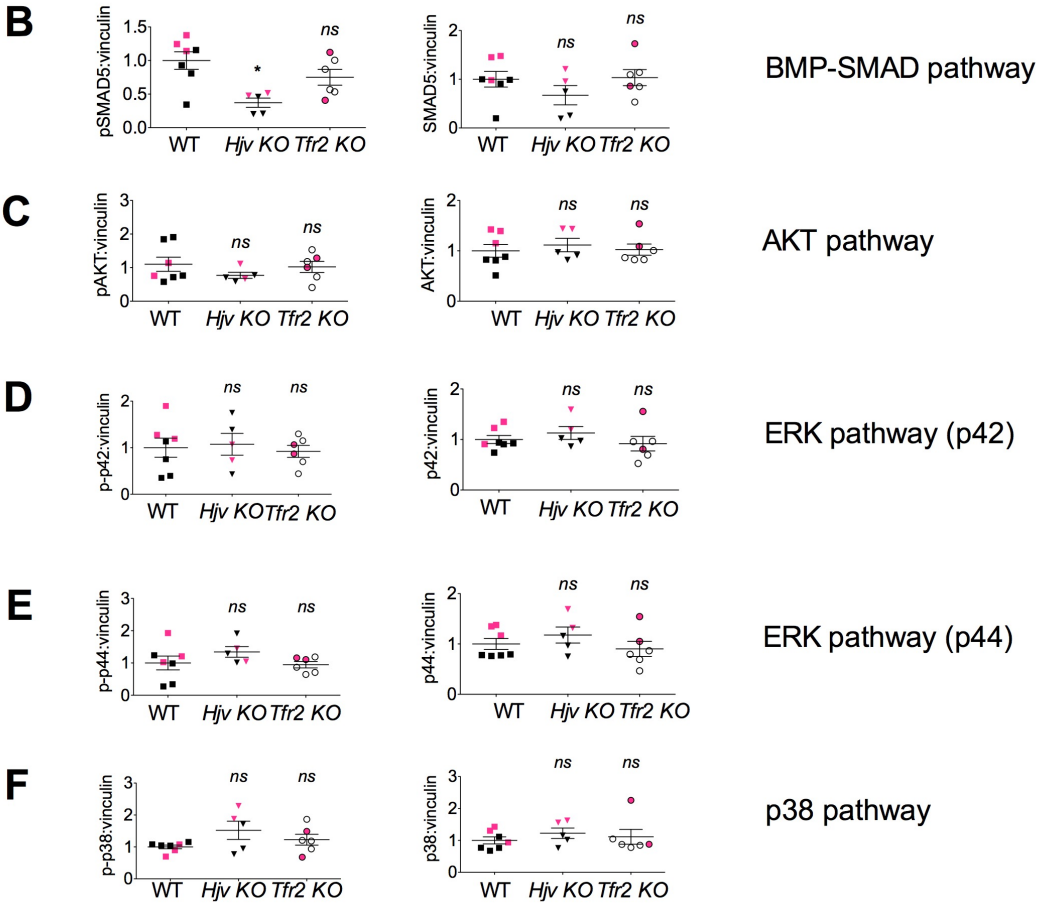

B
pSMAD5:vinculin
SMAD5:vinculin
BMP-SMAD pathway
C
pAKT:vinculin
AKT:vinculin
AKT pathway
D
p-p42:vinculin
p42:vinculin
ERK pathway (p42)
E
p-p44:vinculin
p44:vinculin
ERK pathway (p44)
F
p-p38:vinculin
p38:vinculin
p38 pathway
WT
Hjv KO
Tfr2 KO
ns
*

**FIGURE S7**

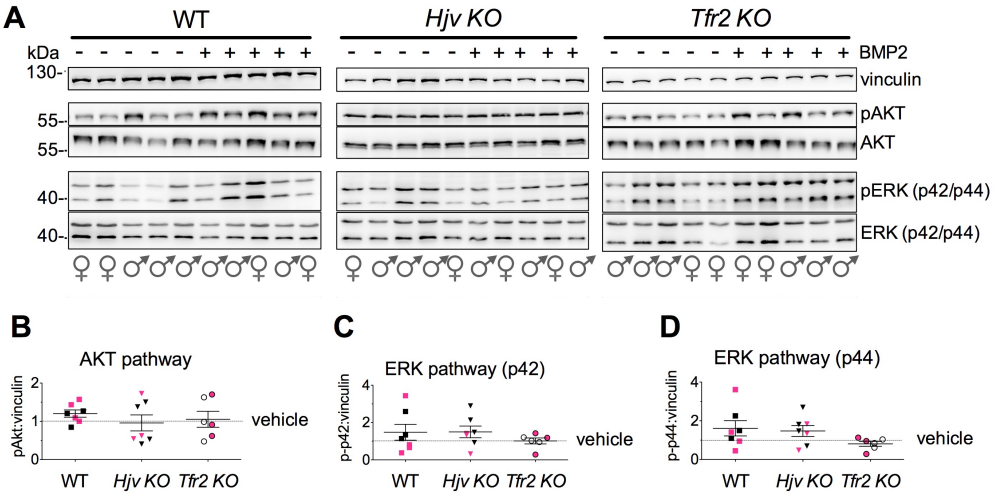
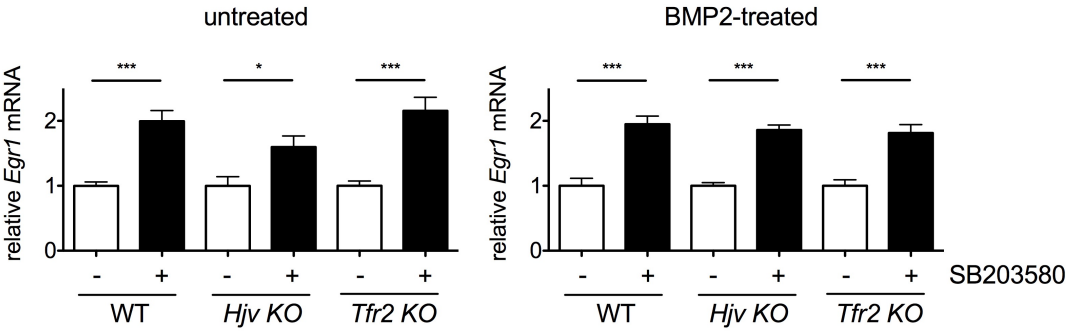

A
WT
Hjv KO
Tfr2 KO
kDa
BMP2
130-
vinculin
55-
pAKT
55-
AKT
40-
pERK (p42/p44)
40-
ERK (p42/p44)
B
AKT pathway
pAkt:vinculin
vehicle
WT
Hjv KO
Tfr2 KO
C
ERK pathway (p42)
p-p42:vinculin
vehicle
WT
Hjv KO
Tfr2 KO
D
ERK pathway (p44)
p-p44:vinculin
vehicle
WT
Hjv KO
Tfr2 KO

**FIGURE S8**


untreated
BMP2-treated
relative Egr1 mRNA
***
*
***
***
***
***
2
1
0
- + - + - +
SB203580
WT
Hjv KO
Tfr2 KO