SUPPLEMENTARY APPENDIX

Increased hepcidin in transferrin-treated thalassemic mice correlates with increased liver BMP2 expression and decreased hepatocyte ERK activation

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Supplemental Table I: Mouse specific primers used for qRT-PCR

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
BMP6	CAGCTTGCAAGAAGCATGAG	GGAACACTCTCCATCACAGTAG
HJV	TATGGGCCAGTCCCCTAGTC	ATCTTGCACTGGGAGTGAGC
Tfr2	CCTGATCACCCTGCTAATCTTC	TCTTCATCGACCACCAACAC
HFE	CACCGCGTTCACATTCTCTA	AAAGAGCTGGTCATCCACATAG
TWSG1	CACTCTGTGCCAGCGATGTGA	CACAGCATGCACTCCTTACAG
BMP2	GACTGCGGTCTCCTAAAGGTCG	CTGGGGAAGCAGCAACACTA
BMP4	GAGGAGTTTCCATCACGAAGAA	ATGCTGCTGAGGTTGAAGAG

Supplemental Table II: RBC parameters in transferrin-treated mice

	RBCs (x10^6 cells/L)	Hb (g/dL)	MCV (fL)	Retics (x 10^9 cells / L)	CHr (pg)	RDW (%)
WT - PBS (n=7)	10.5±0.2	14.5±0.3	48±0.3	345±34	15±0.2	13±0.4
thal - PBS (n=6)	8.5±0.3*	7.1±0.3*	36±1.1*	2514±186*	12±0.2*	34±0.7*
WT - apo (n=8)	10.5±0.4	10.6±0.6**	41±0.3**	374±21	12±0.1**	15±0.4**
thal - apo (n=6)	13.3±0.5***	10±0.4***	29±0.7***	907±141***	10±0.1***	25±0.8***

Data represented as mean ± s.e.m. *P<0.0002 thal-PBS versus WT-PBS; **P<0.003 WT-apo versus thal-apo; ***P<0.0002 thal-apo versus thal-PBS; WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$); apo = apo-transferrin; RBCs = red blood cells; Hb = hemoglobin; MCV = mean corpuscular volume; Retics = reticulocyte count; CHr = corpuscular hemoglobin of reticulocytes; RDW = red cell distribution width.

SUPPLEMENTAL METHODS

***In vivo* experiments** All mice were treated with 10 mg (400 mg/kg/day) of human apoTf (Kamada, Israel) or same volume of PBS via intraperitoneal injections daily for 20 or 60 days as described previously.[1] Blood and tissues were processed for analyses. Erythroid precursors were collected from bone marrow as reported.[2] Serum BMP4 was measured using ELISA kits (US Biological, MA).

***Ex vivo* experiments** Primary hepatocytes were isolated using two-step liver perfusion and cultured as previously described.[3] After 12 hours of starvation, cells were treated for 24 hours with 5% mouse serum or combined with 20 μg/mL monoclonal anti-human BMP2/4 antibody (R&D systems), or incubated with 5% FBS and 0, 5, 10, 20 ng/ml BMP2 (Sigma) for 24 hours. After treatments, cells were harvested for RNA and protein analyses.

Quantitative real-time RT-PCR Total RNA were extracted using PureLink RNA Mini Kit (Ambion, Life Technology) and analyzed with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Life Technology). Primers of HFE, HJV, TfR1, TfR2 TWSG1, BMP2, and BMP4 were shown in **Supplemental Table I**. Smad7 and GDF11 mRNA levels were detected using primers as previously reported.[4,5] mRNA concentrations were normalized to GAPDH.[6]

Western Blot Proteins were separated on 10% SDS-polyacrylamide gels by electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were processed and incubated with primary antibodies (Smad1, pSmad1/5/8, Ferritin H, ERK1/2, and pERK1/2 (Cell Signaling); GAPDH (Thermo Scientific)) as well as HRP-conjugated secondary antibodies (Thermo Scientific).

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Comparison of 20 and 60 days of apo-transferrin injections in thalassemic mice. (A) Spleen size (measured as a ratio of spleen to body weight) and **(B)** transferrin saturation (measured in the serum as a ratio of serum iron to total transferrin binding capacity). n=5-18 mice per group (Tf = transferrin; apo = apo-transferrin; WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$))

Supplemental Figure 2: Effect of apo-transferrin injection on hepcidin, Id1, and Smad7 expression, other genes related to hepcidin regulation in the liver and Smad phosphorylation in hepatocytes. Liver hepcidin, Id1, and Smad7 **(A)**; Tfr2, HFE and HJV **(B)**; as well as TfR1 **(C)** mRNA expression relative to GAPDH and normalized to PBS-injected WT mice (n = 8-12 per group). **(D)** Hepcidin mRNA normalized to non-heme iron (n = 8-12 per group). **(E)** Statistical analysis of phosphorylated Smad1/5/8 relative to Smad1 normalized to Ferritin H in primary hepatocytes performed using ImageJ, presented as mean ± SEM (n = 6-7 mice per group). (apo = apo-transferrin; WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$))

Supplemental Figure 3: BMP2 mRNA expression in the liver and hepatocytes. Values shown are the means ± SEM of −ΔCt values (Ct GAPDH − Ct hepcidin). (n = 4-6 mice per group; apo = apo-transferrin; WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$))

Supplemental Figure 4: Effect of apo-transferrin treatment on BMP4 serum concentration and mRNA expression in the liver and hepatocytes. (A) Serum BMP4 concentration measured by ELISA (n = 6-9 mice per group). Liver **(B)** and hepatocyte **(C)** BMP4 mRNA expression relative to GAPDH normalized to PBS-injected WT mice (n = 8-12 mice per group). (WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$); apo = apo-transferrin)

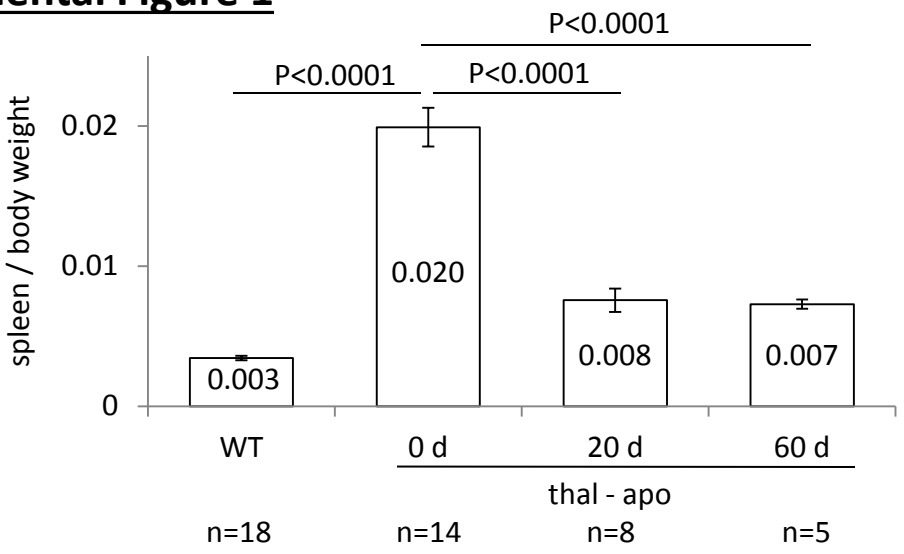
Supplemental Figure 5: Id1 mRNA expression in primary hepatocytes *in vitro*. Primary WT hepatocytes treated with serum from WT or thalassemic mice after PBS or apo-transferrin injection, normalized to untreated hepatocytes in culture. Concurrent treatment with serum and neutralizing anti-BMP2/4 antibodies compared with primary hepatocytes treated with serum or anti-BMP2/4 antibody alone. These *in vitro* results represent 4-6 independent experiments. (* P=0.04 serum alone vs. serum + Ab) (UT = untreated; apo = apo-transferrin; WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$); Ab = anti-BMP2/4 neutralizing antibody)

Supplemental Figure 6: Effects of BMP2 on hepcidin expression as well as Smad1/5/8 and ERK1/2 signaling *in vitro*. Hepcidin mRNA expression **(A)** as well as ERK1/2 and Smad1/5/8 signaling **(B)** in WT mouse primary hepatocytes treated with 0, 5, 10, and 20 ng/ml BMP2. These results represent 3 independent experiments.

Supplemental Figure 7: Effect of apo-transferrin treatment on bone marrow GDF11 expression. TWSG1 **(A)** and GDF11 **(B)** in sorted bone marrow orthochromatophilic erythroblasts (n = 4 sorted samples per group, each sorted sample from 2-3 mice). (WT = wild type; thal = thalassemic (*Hbb*$^{th1/th1}$); apo = apo-transferrin; TWSG1 = twisted gastrulation factor 1; GDF11 = growth differentiation factor 11)

Supplemental Figure 1

A



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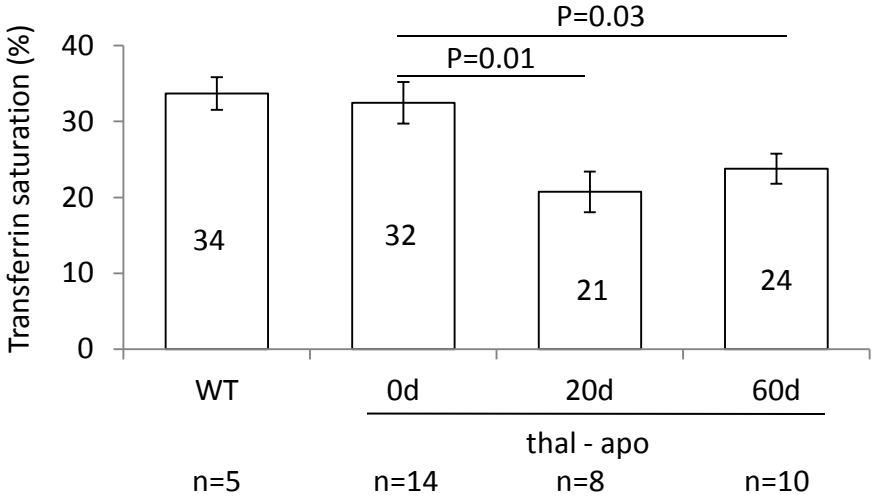
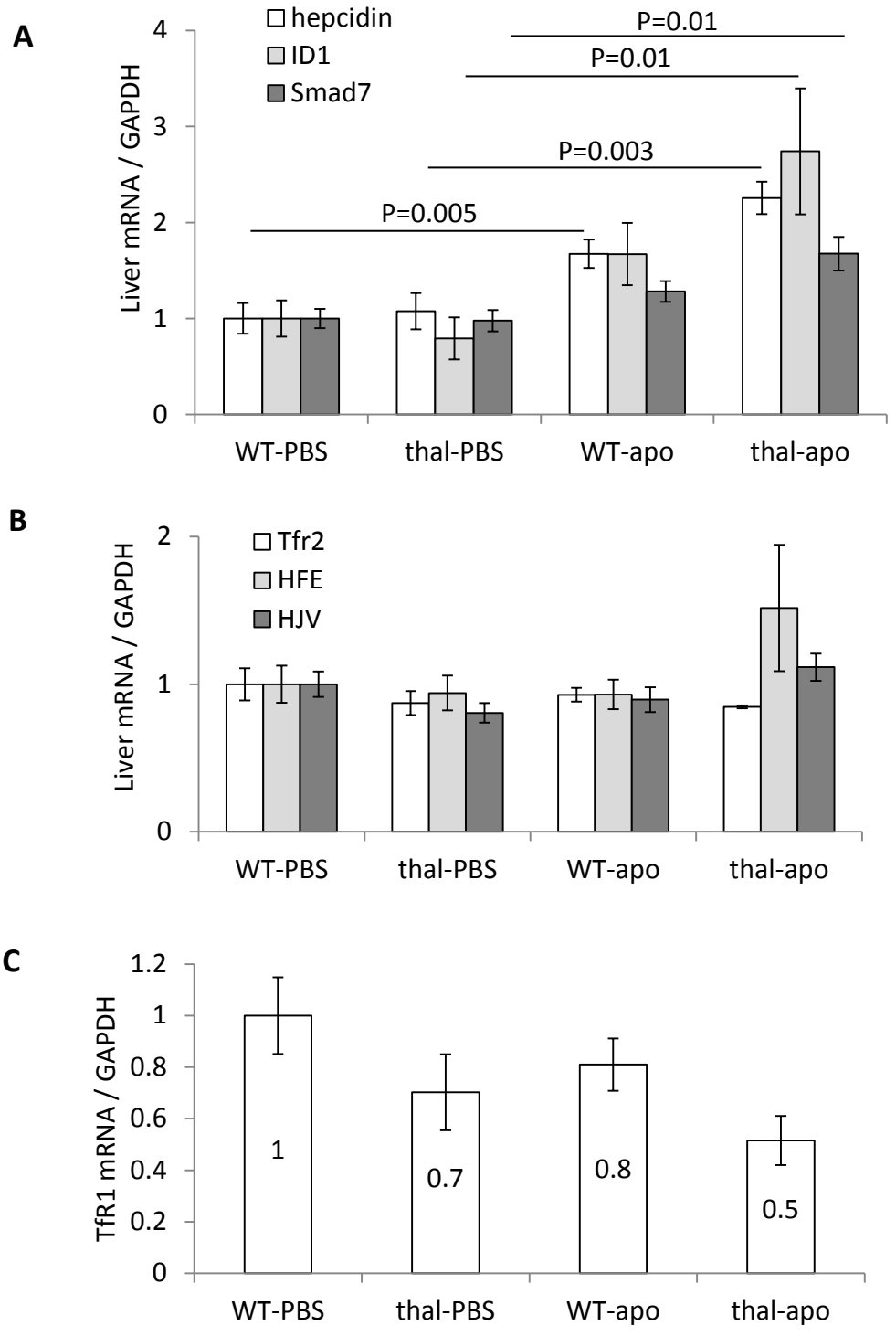
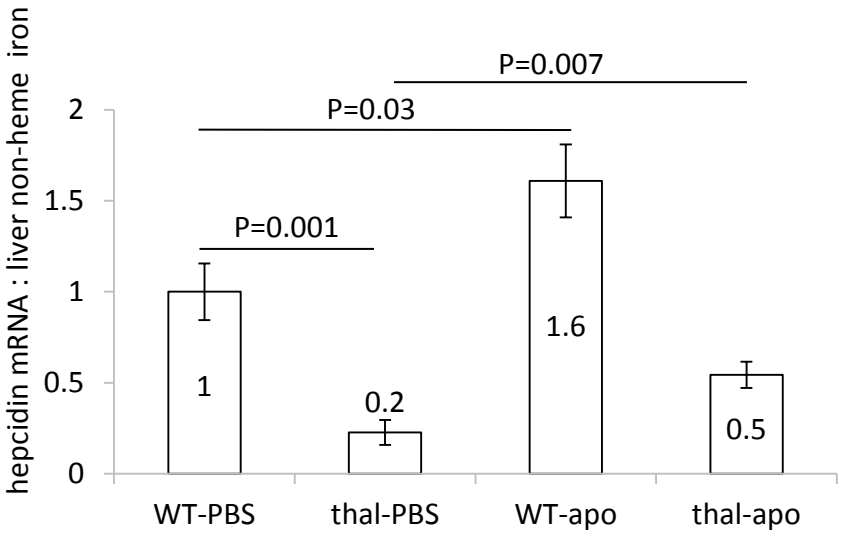


Supplemental Figure 2



Supplemental Figure 2

D



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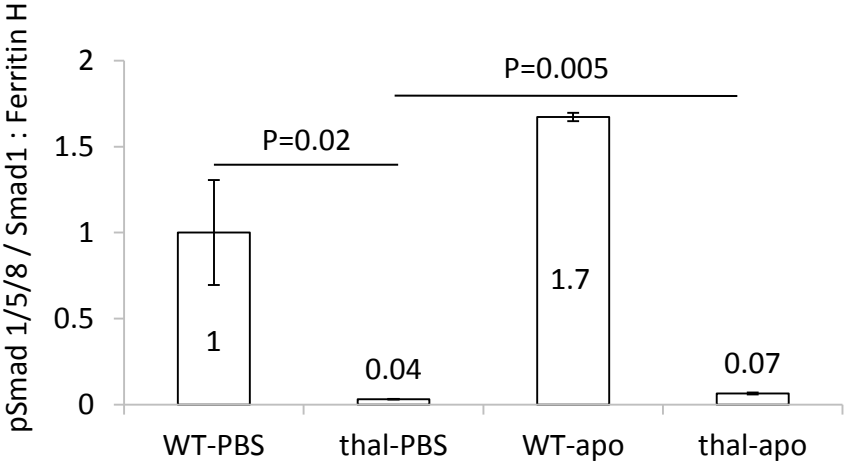

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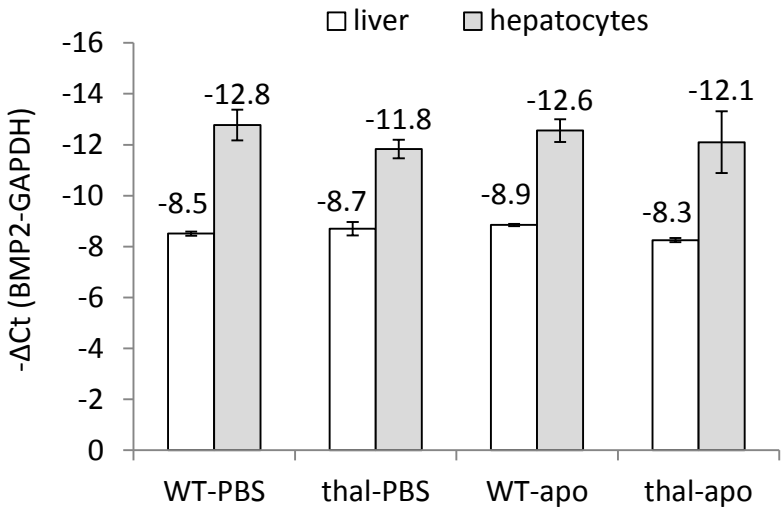

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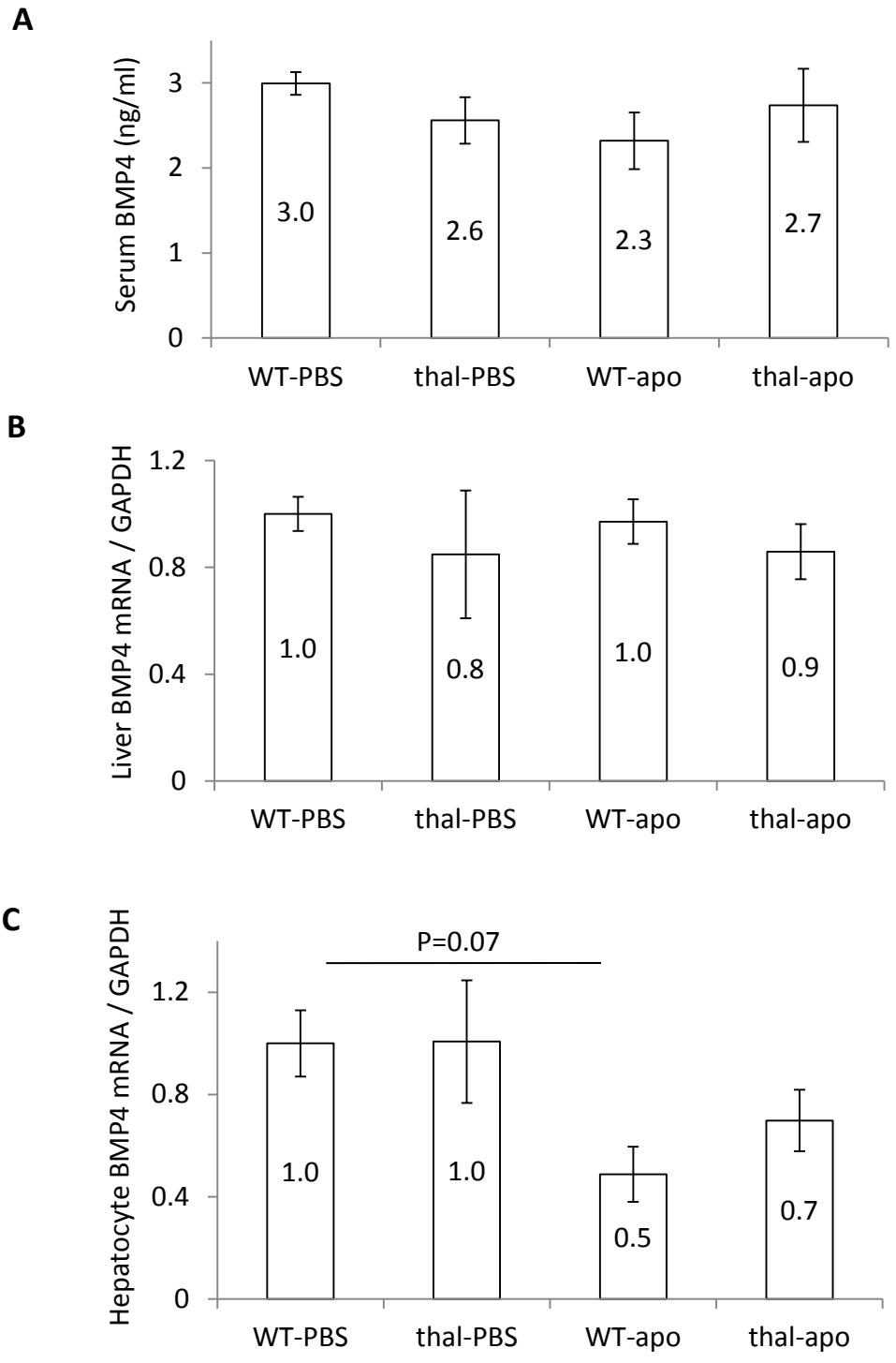
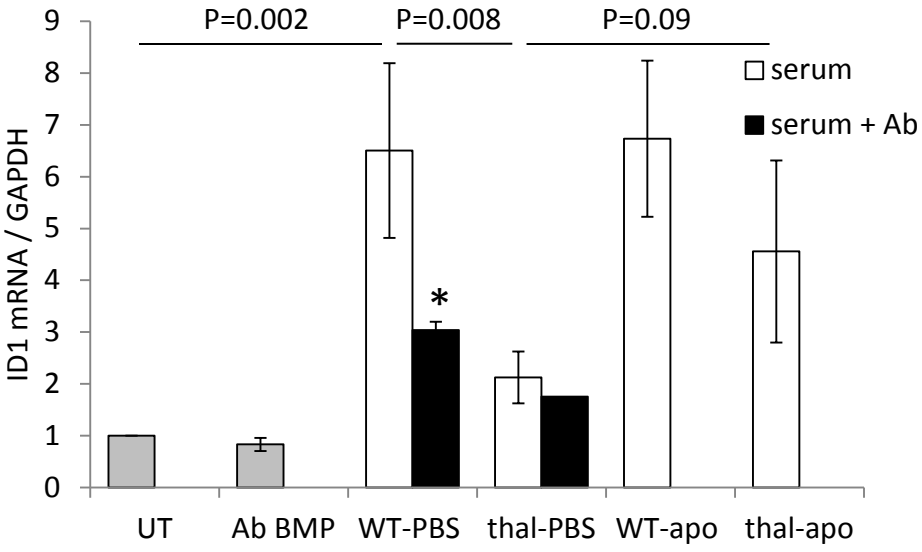
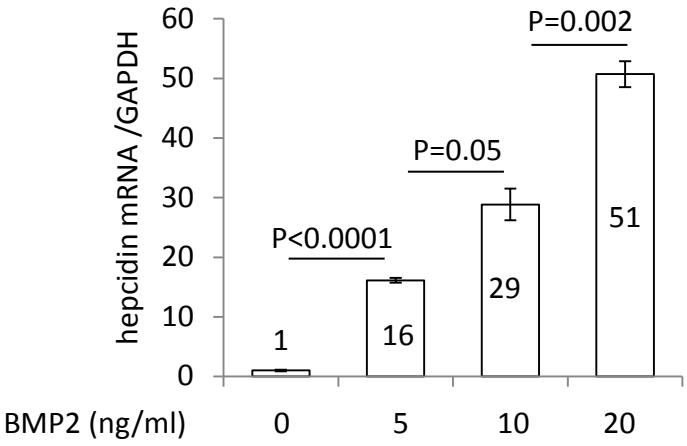


Supplemental Figure 5



Supplemental Figure 6

A



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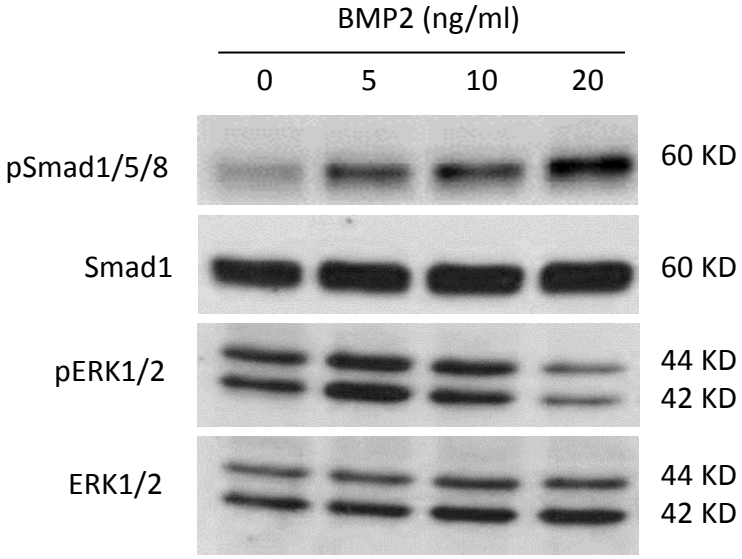

Supplemental Figure 7