Endoplasmic reticulum stress controls iron metabolism through *TMPRSS6* repression and hepcidin mRNA stabilization by RNA-binding protein HuR

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Supplementary Methods

Animal studies

All the animal protocols were approved by the Midi-Pyrénées Animal Ethics Committee. Only males were used in this study. Mice were given free access to tap water and standard laboratory mouse chow diet (250 mg iron/kg; SAFE, Augy, France).

Intraperitoneal injections of tunicamycin (Sigma-Aldrich, Allentown, PA) (2mg/kg) or vehicle (2.5% DMSO) were carried out on 7-10 week-old CD1 or C57BL/6J wild type (WT) mice, C57BL/6J *Tmprss6*$^{-/-}$ mice, CD1- C57BL/6J mixed genetic background *Bmp6*$^{-/-}$*Tmprss6*$^{-/-}$ mice, CD1 *Bmp6*$^{-/-}$ mice, C57BL/6J *Inhbb*$^{-/-}$ mice and C57BL/6J mice with hepatocyte-specific *Bmpr1a* deficiency (*Bmpr1a*$^{fl/fl}$, Alb-Cre+) or control littermates (*Bmpr1a*$^{fl/fl}$, Alb-Cre-)[1] (provided by Dr. A. Steinbicker laboratory). Except for the study of tunicamycin response over time, mice were sacrificed 6 hours after injection.

Cell culture and treatments

Human hepatoma cells (HepG2; ATCC) were cultured in high-glucose DMEM supplemented with 10% FCS and antibiotics. Cells were transferred to 24-well plates for mRNA extraction or 6-well plates for protein extraction. 48 hours after seeding, cells were treated for 6 hrs with tunicamycin (10µg/ml, Sigma-Aldrich) or mock (0.275% DMSO), in combination with actinomycin D (6.275 µg/ml, Sigma-Aldrich) for mRNA stabilization experiments.

SiRNA experiments

HepG2 cells were seeded onto 24-well plates. During seeding, cells were reverse-transfected with INTERFERin (Polyplus transfection, Illkirch-Graffenstaden, France) and either 50nM of the negative control pool of siRNAs (D-001810-10-05, Dharmacon, Chicago, IL) or a pool of siRNAs designed to silence human HuR (M-003773-04-0005, Dharmacon). 48 hours later, cells were trypsinized and reverse-transfected again in similar conditions. After 48 hours, cells were treated with mock or tunicamycin for 6 hours as described above.

RNA extraction, quantitative real-time PCR and semiquantitative RT-PCR

Total RNA was isolated using UPzol lysis reagent (biotechrabbit, Hennigsdorf, Germany). First-strand cDNA synthesis was performed using MMLV-RT (Promega, Madison, WI).

Sequences of the primers used in this study are listed in Online Supplementary Table S1. Real-time qPCR reactions were prepared with LightCycler® 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics, Meylan, France) and run on a LightCycler® 480 System (Roche Diagnostics). Box plots were used to show the distribution of −ΔCt values, with lines representing the first, second (median) and third quartiles, and the ends of the whiskers the lowest and highest –ΔCt. The higher the −ΔCt, the greater is the amount of target amplicon. Estimates of the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) are shown on the graphs.

Since HuR proteins are very stable, cells have been transfected with SiRNA for a total of 96 hours to reduce their protein level. Because this long silencing leads to variations in basal hepcidin expression between repeated experiments, results for this experiment are normalized on hepcidin expression in the SiRNA Ctl mock treated condition.

Xbp1 mRNA spliced forms were analyzed by semiquantitative RT-PCR using cDNA obtained as specified above. PCR products were run on 3% agarose gels.

Western blot analysis

For all analysis except matriptase-2 western blot, livers were homogenized in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5mM EDTA, pH 8, 0.1% NP-40) including inhibitors of proteases (complete protease inhibitor cocktail, Roche Applied Science) and of phosphatases (phosphatase inhibitor cocktail 2, Sigma-Aldrich) with a FastPrep®-24 instrument for 15 s at 4 m/s. For HepG2 cells, proteins were extracted with RIPA buffer (Sigma-Aldrich) plus inhibitors of proteases and phosphatases. Proteins were then quantified using the Bio-Rad Protein Assay.

For matriptase-2 analysis, a plasma membrane-enriched fraction of liver homogenate was used as previously reported[2]. Liver samples were homogenized in 10 mM HEPES, pH7.4, 2 mM EDTA, and protease inhibitors. Unbroken cells were discarded and supernatant was centrifuged at 3,000g for 15 min. The 3,000g pellet was then homogenized in 10 mM HEPES containing 2 M NaCl, 2mM EDTA, recentrifuged for 15 min at 3,000g, and homogenized in 0.1M sodium carbonate. After agitation at 4°C for 1h, the homogenate was centrifuged at 16,000g, and the pellet was homogenized in 10 mM HEPES containing 4 M urea, 2mM EDTA, 100mM NaCl. After 30 min on ice, the homogenate was centrifuged at 16,000g, and the pellet resuspended in 2% SDS in 25 mM of ammonium bicarbonate.

Equal amounts of proteins were subjected to SDS-PAGE before their liquid transfer to nitrocellulose membranes (Amersham, Velizy-Villacoublay, France). Blots were blocked with 5% of dry milk in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.15% Tween 20) 1 hour at RT, incubated overnight at 4°C with either a rabbit anti-phospho-Smad5 antibody (1:5000) (Epitomics, Burlingame, CA) or rabbit anti-matriptase-2 (1:2000) (provided by A-S. Zhang). After washing with TBS-T, blots were incubated for 1 hour at RT with HRP-conjugated secondary anti-rabbit antibody (Cell Signaling) and enzymatic activity was visualized using a chemiluminescence detection kit (ECL, GE Healthcare). Membranes were then stripped and reprobed with a rabbit anti-Smad5 antibody (1:5000) (Epitomics) or a rabbit anti-Na^+K^+ ATPase antibody (1:20000) (Abcam) overnight at 4°C before incubation with the HRP-conjugated secondary anti-rabbit antibody. Membranes were also reprobed with mouse anti-vinculin (1:50000) (Sigma) for normalization. Blot imaging and analysis were performed on a Chemidoc MP Imaging System (Bio-Rad, Hercules, CA) with the Image Lab software. Means of the ratio pSmad5/Smad5 or Matriptase-2/Na^+K^+ATPase are shown on the images

RNA immunoprecipitation (CLIP)

HepG2 cells were seeded onto 10 cm dishes for 72 hours and treated for 6 hours with tunicamycin (10µg/ml, Sigma-Aldrich) or mock (0.275% DMSO). RNA and proteins were UV-crosslinked once with 300 mJ/cm^2 at 254 nm using a UV BIO-LINK BLX 254 (Thermo Fisher Scientific). Cytoplasmic lysates were prepared with RIPA (Sigma-Aldrich) including inhibitors of proteases. After centrifugation, proteins were quantified using the Bio-Rad Protein Assay and treated with TURBO DNase (Invitrogen). Dynabead Protein G (Invitrogen) (30µl) were incubated with 1µg rabbit IgG antibody (PP64, Sigma-Aldrich) or mouse anti-HuR (sc-5261, Santa Cruz Biotechnology) for 3h at 4°C, then incubated with supernatants adjusted to 1mg of protein overnight at 4°C. After extensive washing and treatment with proteinase K, RNA was extracted with ReliaPrep™ RNA Miniprep Systems (Promega) and used for quantitative real-time PCR of HAMP 3'UTR.

Statistical analyses

Results were analyzed with GraphPad Prism 7.02 software. Means of quantitative PCR ΔCt values were compared with Student's t-tests or with ANOVA followed by Sidak's multiple comparison tests for planned contrasts between pairs of means. SiRNA experiments were run

in four independent experiments (run) and each run consisted of all treatments. In this case, comparisons between treatments were made by repeated measures one-way ANOVA followed by Sidak's multiple comparison test to take into account that each run is a block.

Legends of supplementary Figures

Supplementary Figure S1. Hepcidin induction by ER stress coincides with an activation of the Bmp-Smad pathway. WT CD1 mice (3-9/group) injected with tunicamycin (red boxes) were sacrificed at time points ranging from 3 to 24 hours after injection and compared to controls (blue boxes). Mice were analyzed for liver **(A)** *Atf3* mRNA expression; **(B)** *Ddit3* mRNA expression; **(C)** *Hamp* mRNA expression; **(D)** *Id1* mRNA expression; **(E)** *Tmprss6* mRNA expression and **(F)** *Elavl1* mRNA expression. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Supplementary Figure S2. Tunicamycin injection promotes ER stress in WT mice. Wild-type CD1 mice (6-9/group) were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed 6h later for liver. Representative mice are shown for **(A)** *Hspa5* mRNA expression and (B) *Xbp1* mRNA splicing. ****, p<0.0001.

Supplementary Figure 3. Bmp-Smad signaling is required for hepcidin up-regulation by ER-stress. Mice (5-8/group) with hepatocyte-specific *Bmpr1a* deficiency (*Bmpr1a*$^{fl/fl}$, Alb-Cre+) and control littermates (*Bmpr1a*$^{fl/fl}$, Alb-Cre-) were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed 6h later for liver **(A)** *Atf3* mRNA expression; **(B)** *Ddit3* mRNA expression; **(C)** *Hspa5* mRNA expression; **(D)** *Xbp1* mRNA splicing; **(E)** *Id1* mRNA expression; **(F)** pSmad-5 relative to total Smad5 protein expression; and **(G)** *Hamp* mRNA expression; **(H)** *Elavl1* mRNA. *, p<0.05; **, p<0.01; ****, p<0.0001.

Supplementary Figure S4. Activation of the Bmp-Smad pathway by ER stress is independent of Bmp6, Bmp2, Bmp4, activin B and Bmp receptors regulation. WT CD1 mice were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed 6 hours later for liver **(A)** *Bmp6* mRNA expression; **(B)** *Bmp2* mRNA expression; **(C)** *Bmp4* mRNA expression; **(D)** *Acvr1* mRNA expression; **(E)** *Bmpr1A* mRNA expression; **(F)** *Bmpr2* mRNA expression; **(G)** *Acvr2a* mRNA expression; **(H)** *Acvr2b* mRNA expression. WT and *Inhbb*$^{-/-}$ mice (5-7/group), males and females, were injected with mock (blue boxes) or tunicamycin (red boxes) and were

analyzed 6 hours later for liver **(I)** *Id1* and **(J)** *Hamp* mRNA expression. ***, p<0.001; ****, p<0.0001.

Supplementary Figure S5. ER stress suppresses matriptase-2 at protein level in WT mice. Wild-type CD1 mice (6-9/group) were injected with mock or tunicamycin and were analyzed 6h later for matriptase-2 protein level in the liver. Representative mice are shown. Proteins extracted from *Tmprss6*$^{-/-}$ mice were used as a negative control for the anti-matriptase-2 antibody.

Supplementary Figure S6. Iron treatment is able to further activate Bmp-Smad signaling in *Tmprss6*$^{-/-}$ mice.

C57Bl/6 WT mice and *Tmprss6*$^{-/-}$ mice (3-4/group) were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed 6h later for liver (**A**) *Atf3* mRNA expression; **(B)** *Ddit3* mRNA expression **(C)** *Hspa5* mRNA expression; **(D)** *Xbp1* mRNA splicing and **(E)** *Elavl1* mRNA expression. (**F-G)** *Tmprss6*$^{-/-}$ mice received one subcutaneous injection of iron-dextran (Sigma-Aldrich, Allentown, PA) weekly for 2 weeks (5mg/injection) (green box) and were analyzed for liver pSmad-5 relative to total Smad5 protein expression and *Id1* mRNA expression. Comparisons were made with data obtained in *Tmprss6*$^{-/-}$ mice injected with tunicamycin (red box) and iron (green box). **, p<0.01; ****, p<0.0001.

Supplementary Figure S7. ER stress does not activate the Bmp-Smad pathway in *Bmp6*$^{-/-}$-*Tmprss6*$^{-/-}$ mice.

Bmp6$^{-/-}$-*Tmprss6*$^{-/-}$ mice (3-5/group) were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed for liver (**A**) *Id1* mRNA expression; **(B)** pSmad-5 relative to total Smad5 protein expression and **(C)** *Hamp* mRNA expression. **, p<0.01.

Supplementary Figure S8. Lack of Bmp6 does not prevent the stimulation of Bmp signaling and hepcidin expression in *Bmp6*$^{-/-}$ mice.

Bmp6$^{-/-}$ mice (5-10/group) were injected with mock (blue boxes) or tunicamycin (red boxes) and were analyzed for liver (**A**) pSmad-5 relative to total Smad5 protein; **(B)** expression *Id1* mRNA expression and **(C)** *Hamp* mRNA expression. ***, p<0.001; ****, p<0.0001.

Supplementary Figure S9. ER stress induces hepcidin expression independently of an of a Bmp signaling activation in HepG2 cells. HepG2 cells were treated with mock (blue boxes) or tunicamycin (red boxes) were analyzed at time points ranging from 3 to 10 hours after treatment and compared to controls (blue boxes) for **(A)** *HAMP* mRNA expression and **(B)** *ID1* mRNA expression. Values shown are the result of 3 independent experiments performed in duplicate.**, p<0.01; ***, p<0.001.

Supplementary Figure S10. ER stress promotes HuR binding to HAMP 3'UTR. Total lysates from HepG2 cells treated with mock or tunicamycin were subjected to CLIP with either HuR or control normal IgG antibodies. RNA was collected from the immunoprecipitates and analyzed for **(A-B)** HAMP 3'UTR sequence and **(C-D)** a non-relevant sequence (BMPR1A) enrichment by qRT-PCR. Two representative experiments over 3 independent experiments are presented.

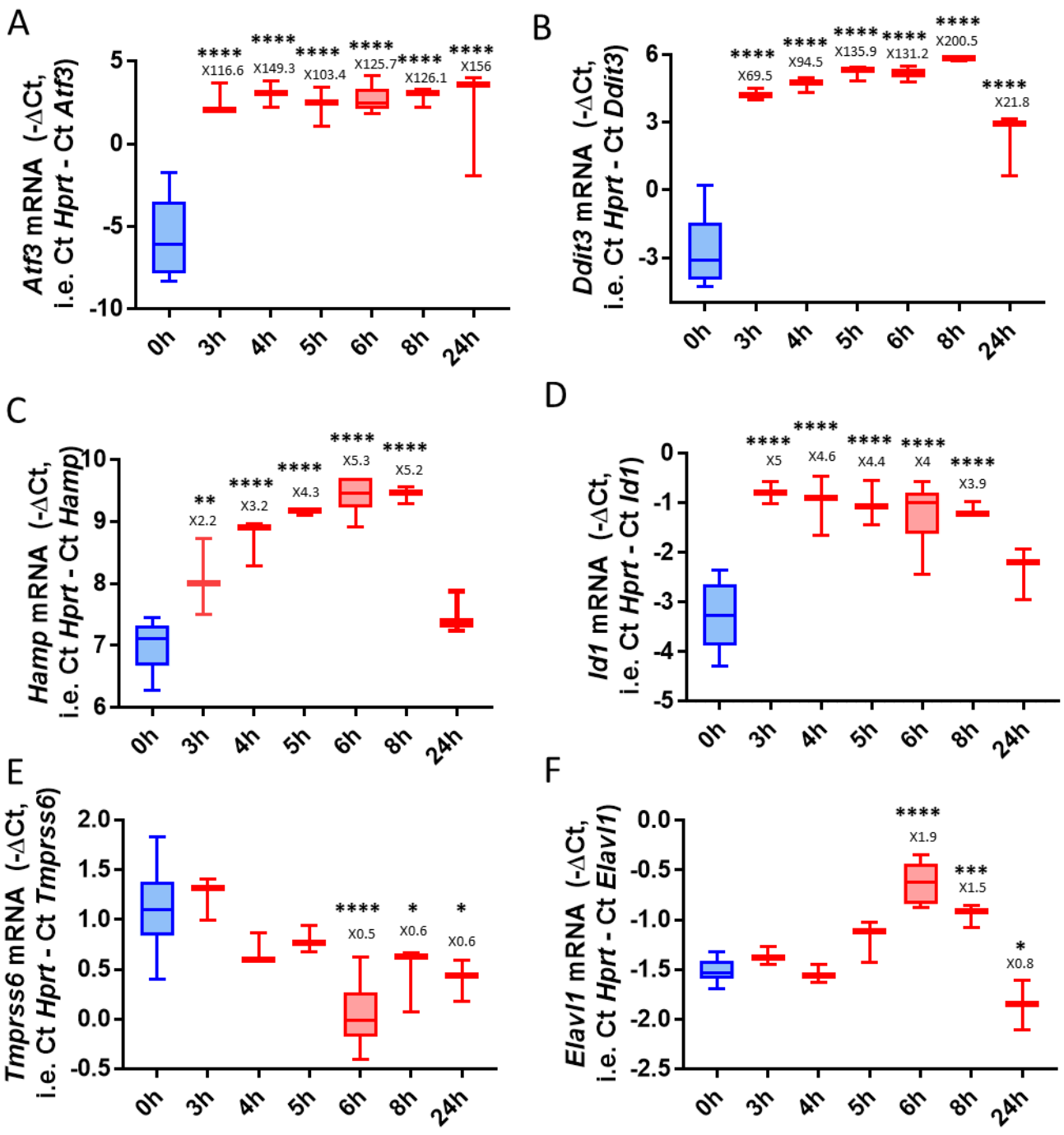
Supplementary Figure S11. Western-blot quantification. Western-blot analysis were quantified using Image Lab software. Quantification of the western-blot presented in **(A-B)** Figure 1; **(C)** Figure 2; **(D)** Supplementary figure S3; **(E)** Supplementary figure S5; **(F)** Supplementary figure S6; **(G)** Supplementary figure S7; **(H)** Supplementary figure S8. Results are expressed as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

Supplementary Table 1: **Primer sequences**

1 Steinbicker, A. U. *et al.* Perturbation of hepcidin expression by BMP type I receptor deletion induces iron overload in mice. *Blood* **118**, 4224-4230, doi:10.1182/blood-2011-03-339952 (2011).

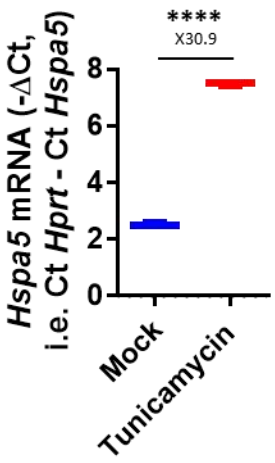
2 Frydlova, J. *et al.* Effect of Erythropoietin, Iron Deficiency and Iron Overload on Liver Matriptase-2 (TMPRSS6) Protein Content in Mice and Rats. *PLoS One* **11**, e0148540, doi:10.1371/journal.pone.0148540 (2016).

Supplementary figure 1



Supplementary figure 2

A



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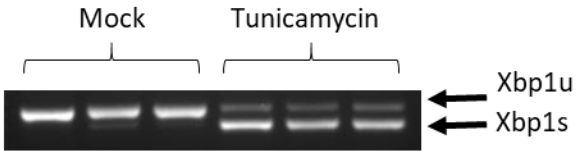

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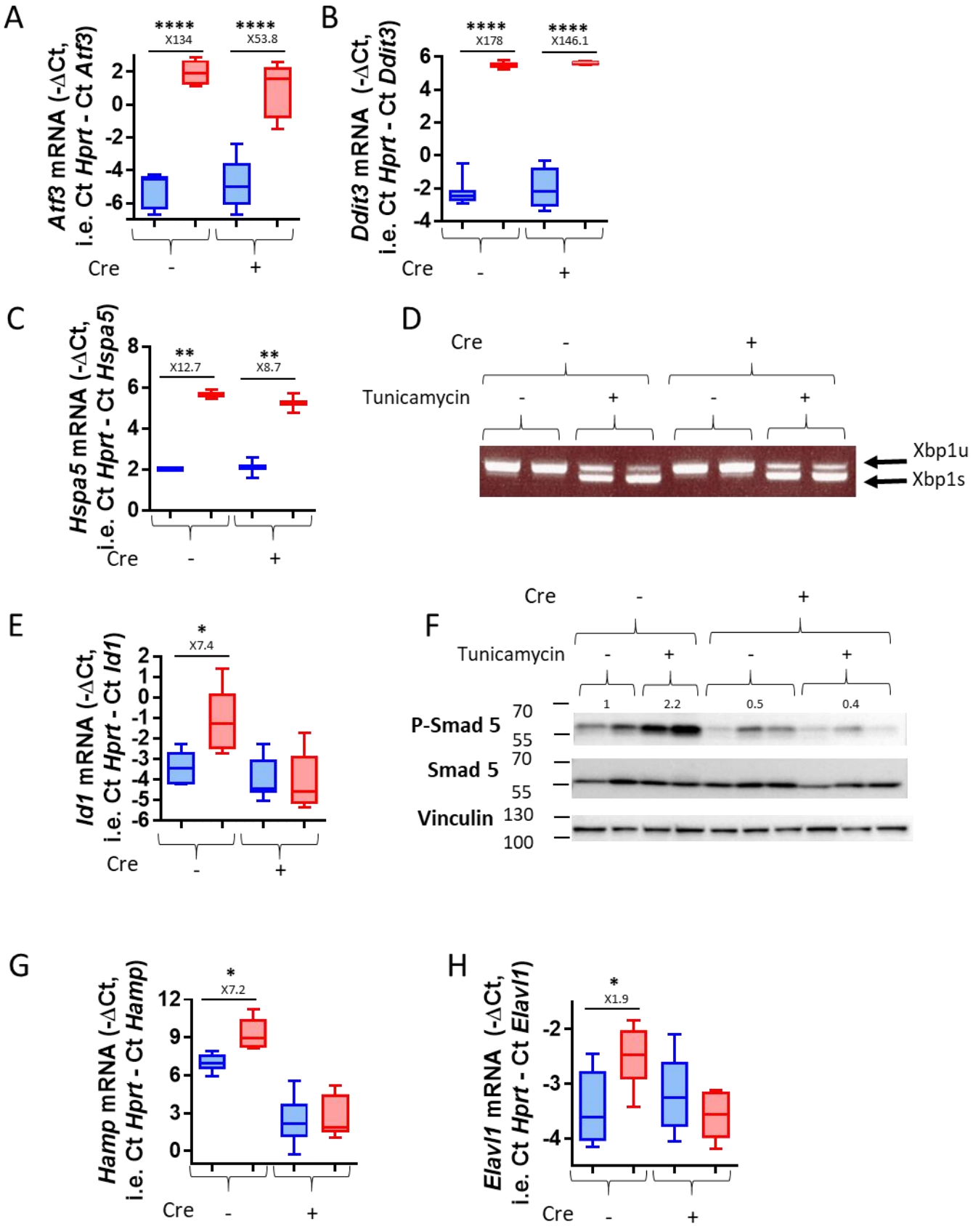

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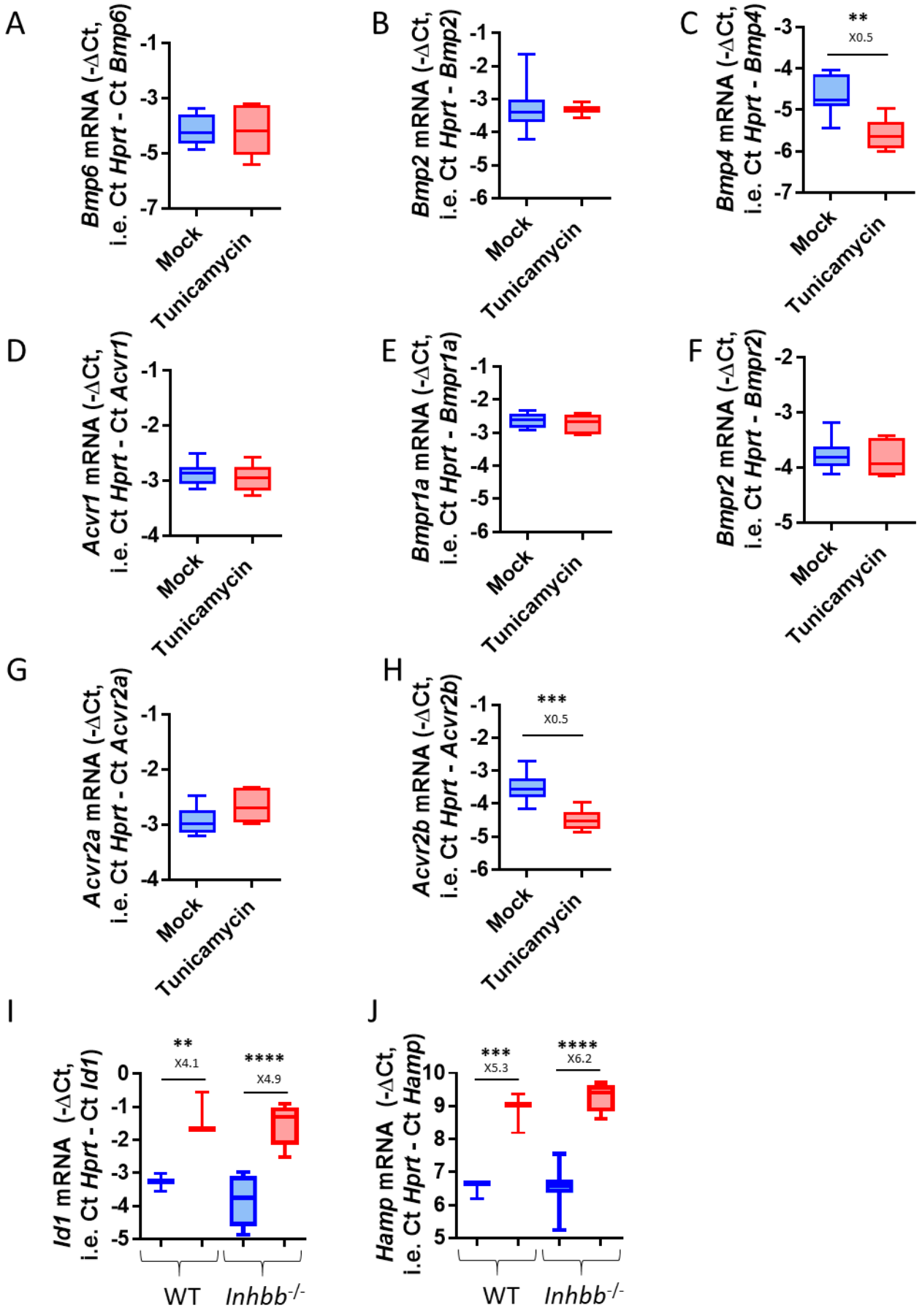

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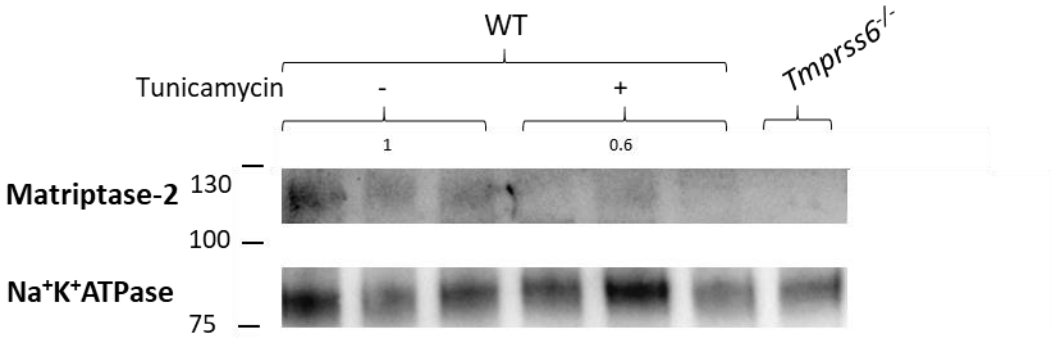
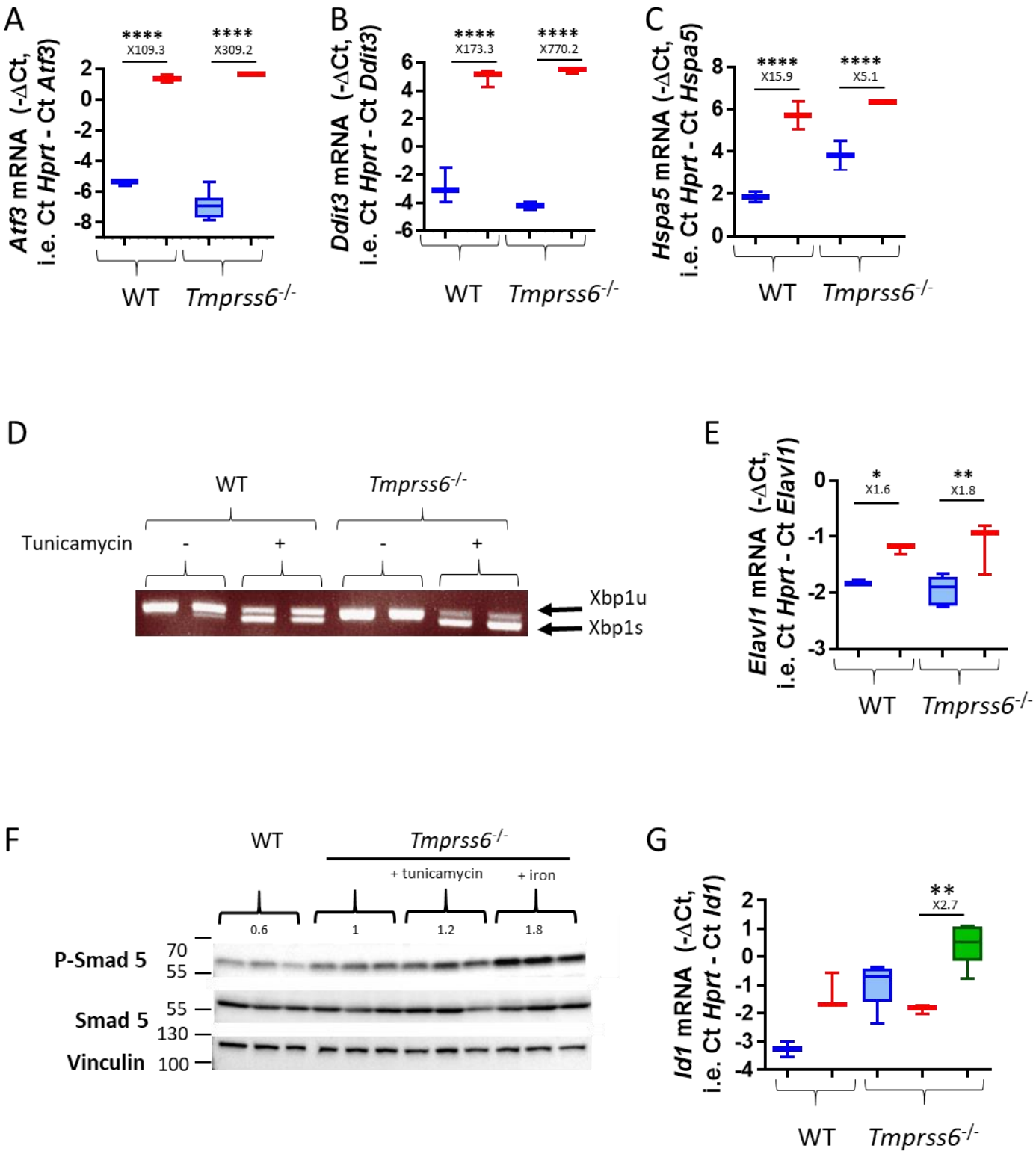


Supplementary figure 6



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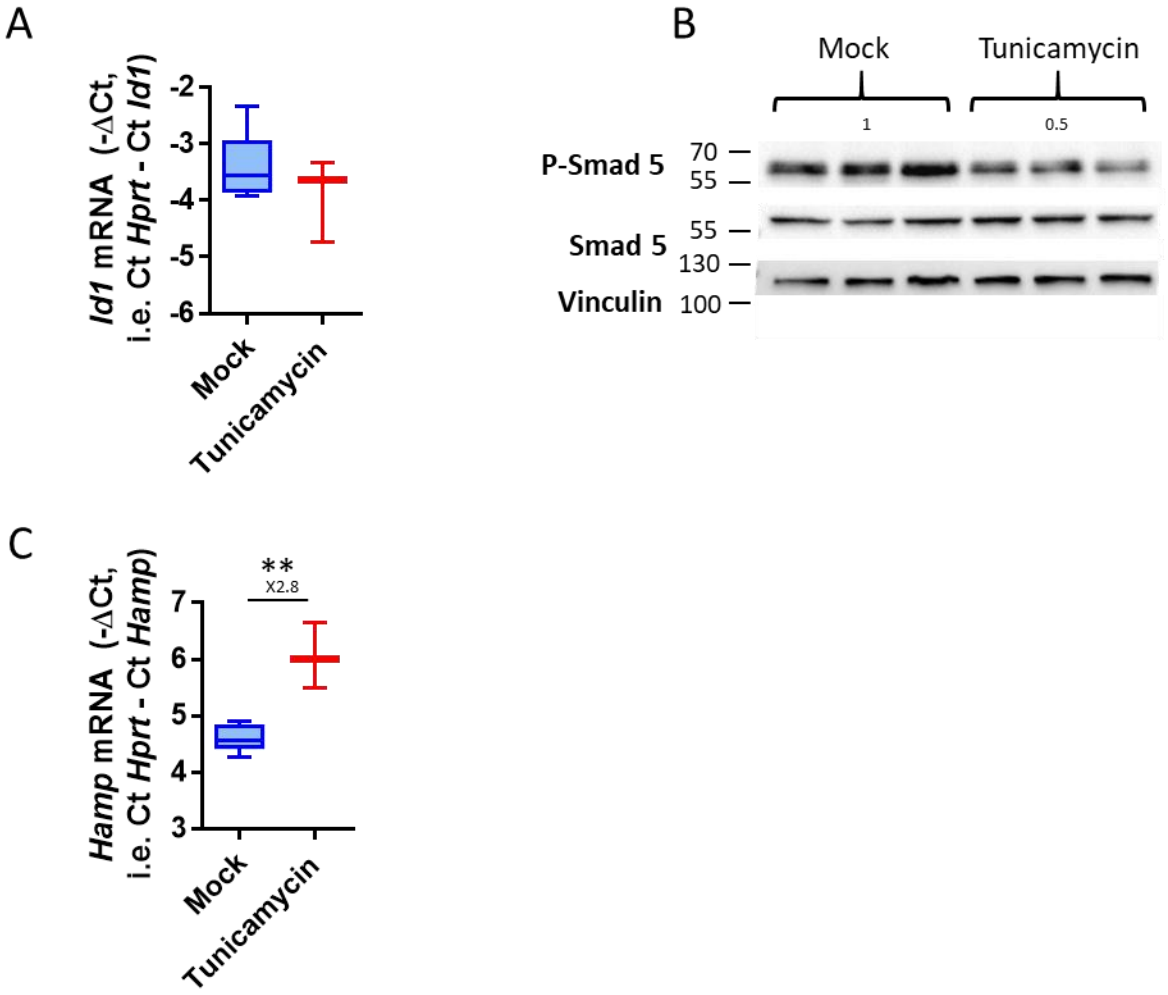

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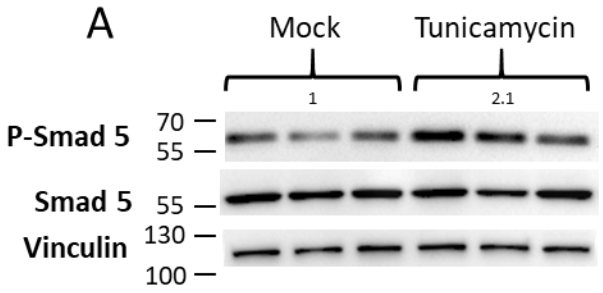
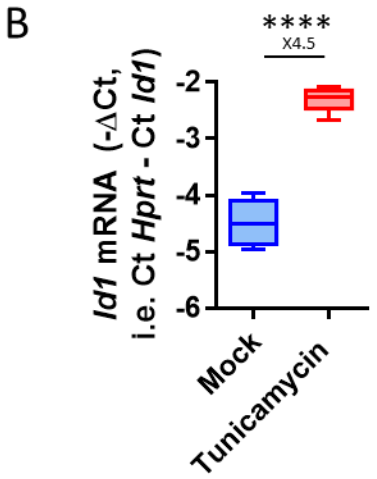
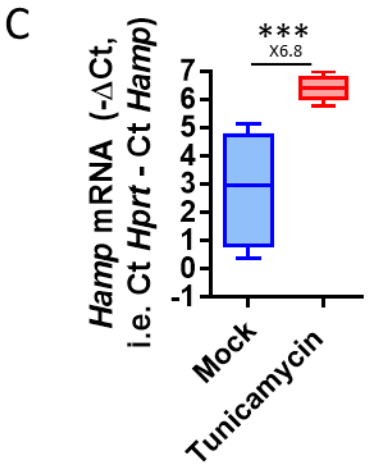
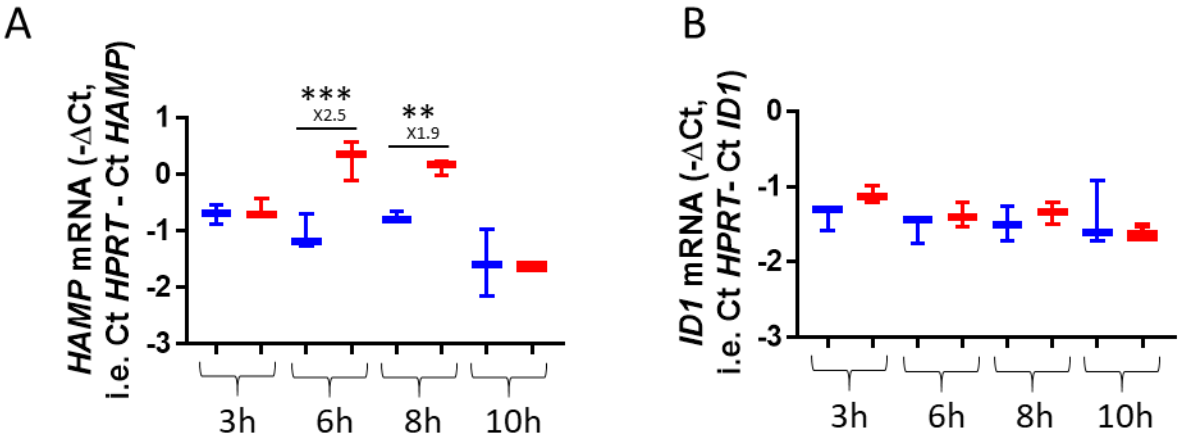






Supplementary figure 9



Supplementary figure 10

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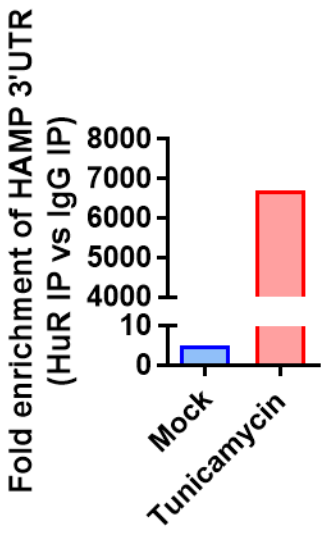

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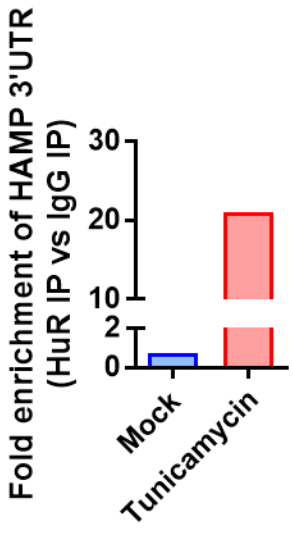

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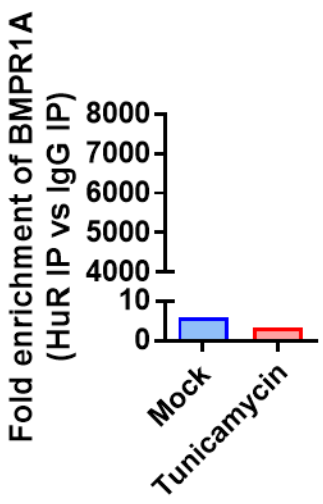

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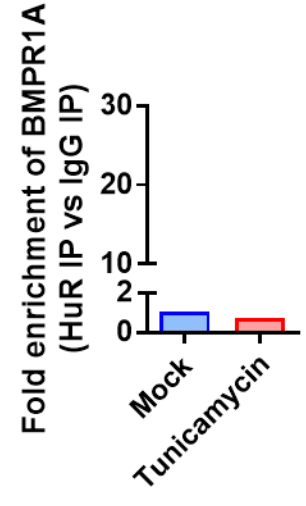
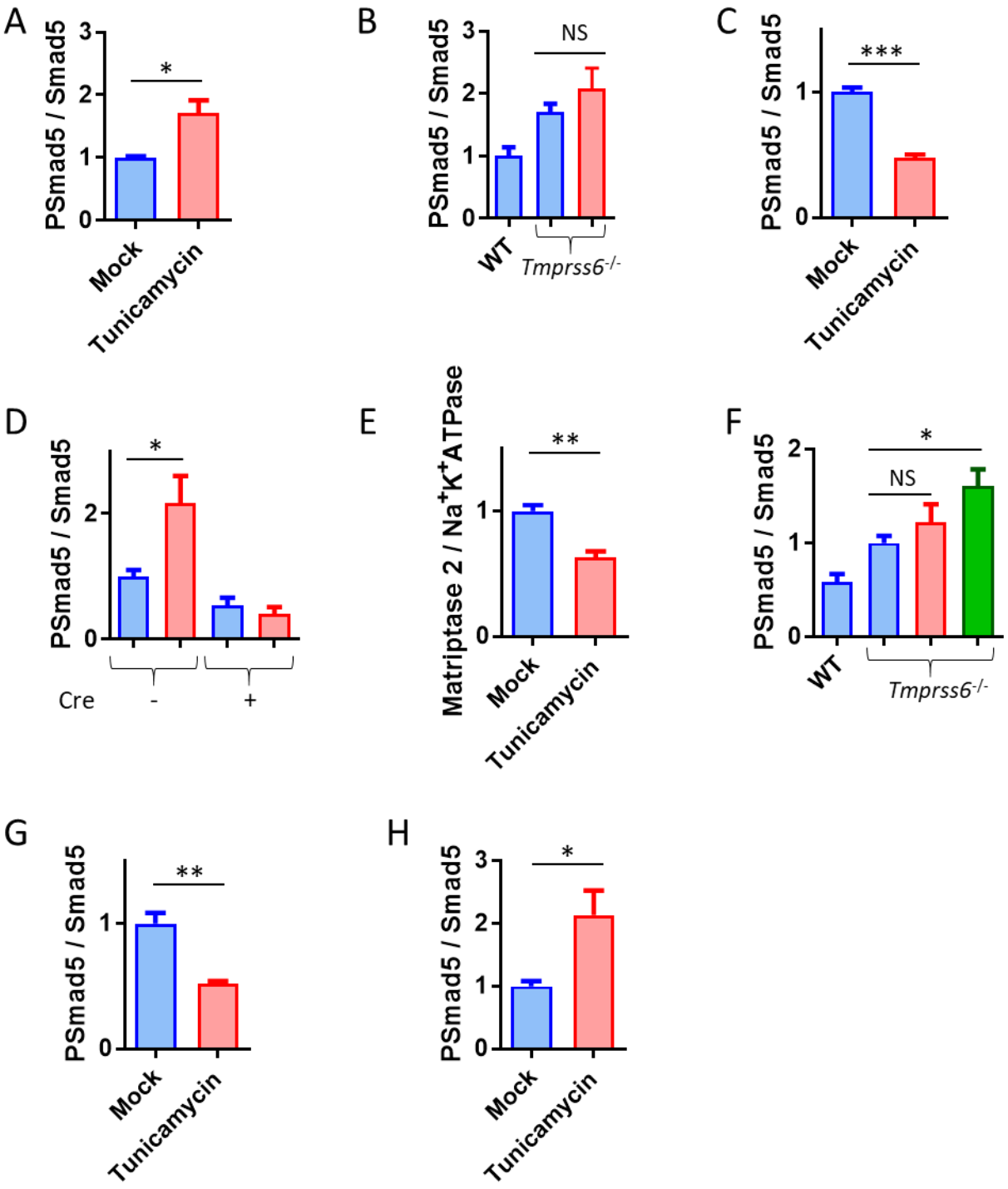


Supplementary figure 11



Supplementary table 1

	Forward	Reverse
Mouse		
Hprt	CTG-GTT-AAG-CAG-TAC-AGC-CCC-AA	CAG-GAG-GTC-CTT-TTC-ACC-AGC
Hamp	AAG-CAG-GGC-AGA-CAT-TGC-GAT	CAG-GAT-GTG-GCT-CTA-GGC-TAT-GT
Id1	ACC-CTG-AAC-GGC-GAG-ATC-A	TCG-TCG-GCT-GGA-ACA-CAT-G
Atf3	GCT-GCC-AAG-TGT-CGA-AAC-AA	TTC-TCT-GAC-TCT-TTC-TGC-AGG-C
Ddit3	AGC-CAG-AAT-AAC-AGC-CGG-AAC	TTC-TGC-TTT-CAG-GTG-TGG-TGG
Tmprss6	TCT-GTG-CTG-GCT-ACC-GCA-AG	CAG-AGT-CAC-CCT-GGC-AGG-C
Bmp6	ATG-GCA-GGA-CTG-GAT-CAT-TGC	CCA-TCA-CAG-TAG-TTG-GCA-GCG-T
Bmp2	GCG-CAG-CTT-CCA-TCA-CGA	CCC-ACT-CAT-CTC-TGG-AAG-TTC-CT
Elavl1	TTC-TCG-GTT-TGG-GCG-AAT-CA	CCT-CTG-GAC-AAC-CCT-GTG-GT
Xbp1	GAA-CCA-GGA-GTT-AAG-AAC-ACG	AAG-CAA-CAG-TGT-CAG-ACTC-C
Hspa5	GCC-TGT-TGC-TGG-ACT-CCT-AAG	GCC-CAC-ATC-CTC-CTT-CTT-GTC
Bmp4	TGA-GTA-CCC-GGA-GCG-TCC	CTC-CAG-ATG-TTC-TTC-GTG-ATG-G
Acvr1	GCC-ATT-GCC-CAT-CGA-GAT-C	ATG-CAT-GAC-TGC-CAG-GCC
Bmpr1a	GGC-TGC-AAA-TAC-TGG-TTG-CAC	TGC-AAG-GAT-TCA-CCG-AAA-GC
Bmpr2	TGT-GAG-CCC-AAC-GGT-CAA	GAC-AGG-TTG-CGT-TCA-TTC-TGC
Acvr2a	AGA-GAT-GGA-TGC-TGG-CCA-AT	TGC-AGA-TGG-ACC-CGT-AGA-TG
Acvr2b	CGC-CTT-CCT-GCG-TAT-CGA-C	TGT-ACT-CAT-CGA-CAG-GCC-CG
Human		
HPRT	TGC-TTT-CCT-TGG-TCA-GGC-AG	AAG-CTT-GCG-ACC-TTG-ACC-AT
HAMP	CCA-GCT-GGA-TGC-CCA-TGT-T	GCC-GCA-GCA-GAA-AAT-GCA
ID1	CCC-TCA-ACG-GCG-AGA-TCA-G	GTT-TCC-CCG-TCG-GTA-TAA-GGA
ATF3	GTC-CAT-CAC-AAA-AGC-CGA-GG	TGT-TTC-GGC-ACT-TTG-CAG-C
ELAVL1	AGG-CGC-AGA-GAT-TCA-GGT-TC	ATG-AAA-ATG-CAC-CAG-CCG-GA
HAMP 3'UTR	CCG-TCC-CCT-CCC-TTC-CTT-AT	GGA-AAA-CAA-AAG-AAC-CAG-CCA
BMPR1A	TGA-CAT-CTA-CAG-CTT-CGG-CCT	CTG-TGA-TAC-AAC-GAC-GAG-CCA