Supplemental Methods

Expressing Vectors

The pCMV6-ALK2$^{MYC-FLAG}$, the pCMV6-ALK3$^{MYC-FLAG}$ and the pCMV6-FKBP12$^{MYC-FLAG}$ expressing vectors were from OriGene (Rockwille, MD, USA). The pcDEF/FLAG-mSmad1 was kindly provided by Prof. Takenobu Katagiri (Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, Japan). The ALK2 mutants R206H, Q207E and R258S were generated by mutagenesis using the QuickChange site- directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol. The MYC-tagged ALK2 variants and ALK3 were obtained by mutagenesis through insertion of a STOP codon after the MYC sequence. The pCMV6-FKBP12 was generated from the pCMV6-FKBP12$^{MYC-FLAG}$ expressing vector by STOP codon insertion before the MYC-tag. Primers used for mutagenesis are in **Table S2**. Mutagenesis were verified by DNA sequencing.

Cell Culture conditions

Cell culture media were from Thermo Fisher Scientific (Waltham, MA, USA). HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Hep3B cells were cultured in Minimal essential medium (MEM), supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, 1 mM sodium pyruvate, and 10% heat-inactivated FBS.

Primary hepatocyte culture and treatment

Primary hepatocytes isolation was performed following the standard two-step perfusion method described in literature (Gonçalves et al., 2007; Klaunig et al., 1981; Shen et al., 2012) with minor modifications. Male mice (8-11 weeks old; sv129) were anesthetized with Avertin. For in situ perfusion, Vena Cava Inferior was cannulated and perfused (pump flux: 5ml/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 into the medium. Debris and membranes were removed by a 100 µm cell strainer. Hepatocytes (HCs) were separated from non parenchymal cells (NPCs) through low speed centrifugation (50 g for 3 minutes). HCs were resuspended in Williams-E medium (4% FBS, 1% P/S, Glutamax) (Thermo Fisher Scientific) and plated into collagen-coated 12-well ($3*10^5$ cells/well). Four hours after isolation, in a group of hepatocytes cell culture media was replaced with 4% FBS Williams-E medium (1% P/S, Glutamax) for 18 hrs. Another group of hepatocytes was serum starved for 3 hrs. Serum supplemented cells were incubated for 3 hrs with tacrolimus (1 µg/ml) in serum free media and then Activin A (10 ng/ml) was added for additional 5 hrs. Serum starved hepatocytes were incubated for 18 hours in serum free media with Torin1 (100 nM), rapamycin (100 nM), tacrolimus (1 µg/ml), Cyclosporin A (1 µg/ml) and GPI-1046 (100 µg/ml). Cells were lyzed and RNA isolated for gene expression analysis.

Quantification of Liver and Spleen Iron Content (LIC)

Liver and spleen were dried at 110°C. 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

Serum iron concentrations was assessed using SFBC (Biolabo, Maizy, FRA) kit, according to the manufacturer's protocol.

Luciferase Assay

Hepcidin promoter activation was studied by using the pGL2-HAMP-Luc in which the firefly luciferase cDNA is under the control of the 2.9 Kb human hepcidin promoter region (Pagani et al., 2008). SMAD1/5/8 activation was measured by using the pGL3-BRE-Luc plasmid, in which the firefly luciferase cDNA was under the control of BMP Responsive Element (BRE) sequences obtained from human ID1. SMAD2/3 activation was assayed by using the pGL3-CAGA-Luc vector, in which the luciferase cDNA was under the control of the CAGA sequences. The pGL3-BRE and pGL3-CAGA vectors were kindly provided by Stefano Piccolo, University of Padua, Padua, Italy[16].

Hep3B or HuH7 cells, seeded at 70%–80% of confluence in a 48-well plate, were transiently transfected using Lipotectamine, according to manufacturer's instructions (Invitrogen, Carlsbad, CA), with 250 ng HAMP-Luc, 200 ng BRE-Luc, 200 ng CAGA-Luc in combination with 15 ng of pRL-TK Renilla luciferase vector (Promega) as a control of

transfection efficiency, and with or without expressing vectors encoding wild type or mutant ALK2 (10-100 ng), or ALK3 (10 ng). Eighteen hours after transfection cells were serum-starved (MEM + 2% FBS), and, when indicated, incubated with tacrolimus, rapamycin, Torin1, GPI-1046, DMH1, LDN212854 or dorsomorphin in low-serum media.

After 24 hrs, cells were lysed and luciferase activity was determined according to manufacture's protocol (Dual Luciferase Reporter Assay, Promega). Relative luciferase activity was calculated as the ratio of Firefly (reporter) to Renilla luciferase activity and expressed as a multiple of the activity of cells transfected with the reporter alone.

Western Blot Analysis of phospho-SMAD proteins

HuH7 cells were transfected with wild type or mutants ALK2. When indicated, cells were transfected also with $Smad1^{FLAG}$ expressing vector. After 40 hrs, cells were lysed in NET/Triton buffer plus protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). Protein extracts (20-50 µg) were diluted in Laemmli sample buffer, incubated 5 minutes at 95°C, subjected to 10% or 12% SDS-PAGE and then transferred to Hybond C membrane (Amersham Bioscience Europe GmbH, Freiburg im Breisgau, Germany) by standard Western blot techniques. Blots were incubated with anti-PhosphoSMAD1/5/8 (1:1000, Cell Signaling, Danvers, MA), anti-SMAD1 (1:1000, Cell Signaling), anti-FLAG (1:1000, Sigma-Aldrich), anti-MYC (1:1000, Cell Signaling), according to standard procedures. Blots were incubated with relevant HRP-conjugated antisera and developed using a chemiluminescence detection kit (ECL; Amersham Biosciences Europe GmbH).

Immunoprecipitation

HuH7 cells transfected with $FKBP12^{FLAG}$ and $ALK2^{MYC}$ (wild type or mutants) expressing vectors were treated with tacrolimus (1 μg/ml), rapamycin (100 nM) or GPI-1046 (100 μg/ml), when indicated. Five hundred μg of cell lysates was incubated with the anti-FLAG M2 affinity gel (Sigma Aldrich) at 4°C for 2 hrs. After gel washing, samples were eluted with 20 μl of Laemmli sample buffer (without β-mercaptoethanol) and incubated at 95°C for 5 minutes. After centrifugation, β-mercaptoethanol was added to supernatants. Samples were then subjected to 12% SDS-PAGE and immunodetection was performed as described in Western Blot Analysis.

Legend to Supplemental Figures

Figure S1. The non-immunosuppressive drug GPI-1046 activates SMAD1/5/8 signaling and hepcidin expression

Hep3B cells were transfected with the SMAD1/5/8 (BRE-Luc) (**A**) or the hepcidin promoter (HAMP-Luc) (**B**) luciferase reporter vectors and treated with GPI-1046 (100 μg/ml) or vehicle for 20 hrs. Cells were lysed and luciferase activity was analyzed and normalized to an untreated mean value of 1. A representative experiment, made in triplicate, is shown. **C, D)** Total RNA was purified from primary murine hepatocytes treated with GPI-1046 (100 μg/ml) for 18 hrs. Hepcidin (*Hamp*) (**C**) and *Id1* (**D**) expression were quantified by qRT-PCR. *Hprt1* was used as housekeeping gene. Mean ΔCt values in each group were subjected to a change of origin by subtracting the mean ΔCt of vehicle-treated cells (panel C: 0.3; panel D: 1.4). RQ: relative quantification. Error bars indicate SD. ***p< .001; ****p< .0001. Estimates of the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) are shown on the graphs.

Figure S2. The ALK2 inhibitor DMH1 abrogates the FKBP12-dependent hepcidin regulation

Hep3B cells were pre-treated for 3 hrs with DMH1 (0,5 μg/ml) and then incubated for 15 hrs with rapamycin (RAPA, 100 nM), Torin1 (T1, 100 nM) (**A**), tacrolimus (TAC, 1 μg/ml) or Cyclosporin A (CA, 1 μg/ml) (**B**). Total RNA was isolated and Hepcidin (*HAMP*) expression quantified by qRT-PCR and normalized to the housekeeping gene *GAPDH*. Mean ΔCt values in each group were subjected to a change of origin by subtracting the mean ΔCt of untreated cells (panel A: 7.5; panel B: 7.1). **C**) HuH7 cells were transfected with the hepcidin promoter

luciferase reporter vector (HAMP-Luc) and with $ALK2^{wt\text{-}MYC\text{-}FLAG}$ or $ALK2^{R206H\text{-}MYC\text{-}FLAG}$ or $ALK3^{MYC\text{-}FLAG}$, or empty vector, and treated with LDN212854 (150 nM) or vehicle for 18 hrs. Cells were lysed and luciferase activity was analyzed and normalized to the luciferase mean value of cells transfected with empty vector untreated and treated. **D**) HuH7 cells, transfected with $FKBP12^{FLAG}$ and $ALK3^{MYC}$, were lyzed and total lysate immunoprecipitated with the anti-FLAG M2 affinity gel. Total extract and immunoprecipitated proteins were loaded onto a 12% SDS-PAGE and analyzed by WB. FKBP12 was revealed using the anti-FKBP12 antibody. $ALK3^{MYC}$ was detected using the anti-MYC antibody. Gel loading was normalized using the anti-beta actin antibody. Molecular weight markers are indicated on the right. **E**) HuH7 cells were transfected with the HAMP-luc vector and treated for 8 hours with LDN and then incubated for 18 hours with tacrolimus. RQ: relative quantification. Error bars indicate SD. ns: non significant; * p< .05; **p< .01; ****p< .0001. Point estimates of the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) are shown on the graphs.

Figure S3. ALK2-FKBP12 binding capacity influences hepcidin activation through BMP-SMAD pathway.

A) Hep3B cells were transfected with the hepcidin promoter luciferase reporter vector (HAMP-Luc) in the presence of $ALK2^{wt\text{-}MYC\text{-}FLAG}$ (black line), $ALK2^{R206H\text{-}MYC\text{-}FLAG}$ (red line), $ALK2^{Q207E\text{-}MYC\text{-}FLAG}$ (blue line) or $ALK2^{R258S\text{-}MYC\text{-}FLAG}$ (green line) expressing vectors. Cells, treated with increasing concentrations of tacrolimus, were lysed and analyzed for luciferase activity. Luciferase activity was normalized to an untreated-ALK2 (wt or mutants) mean value of 1. A representative experiment, made in triplicate, is shown. The ANOVA two-way analysis was applied (ALK2 wt vs ALK2 mutants)**. B)** Hep3B cells, transfected with the

HAMP-Luc reporter vector in the presence of $ALK2^{wt\text{-}MYC}$, $ALK2^{R206H\text{-}MYC}$ or $ALK2^{R258S\text{-}MYC}$, were treated for 6 hrs with DMH1 (0,5 µg/ml) and then incubated for 15 hrs with Activin A (10 ng/ml) ± DMH1. Cells were lysed and luciferase activity was quantified and normalized to an untreated mean value of 1. RQ: relative quantification. A t-test analysis was applied. Asterisks in black refer to: ActA or DMH1 vs ut; Asterisks in blue refer to: ActA+DMH1 vs ActA. Error bars indicate SD. **p< .01; ***p< .001; ****p< .0001.

Figure S4. Hepcidin expression, liver and serum iron content in mice treated with tacrolimus. A) Liver hepcidin (*Hamp*) expression was quantified by qRT-PCR and normalized to the housekeeping gene *Hprt1*. Mean ΔCt values in each group were subjected to a change of origin by subtracting the mean ΔCt (-5.6) of vehicle-treated mice. **B)** Liver iron content (LIC). **C)** Serum iron content. Error bars indicate SD. **p< .01. Estimates of the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) are shown on the graphs.

Figure S5. The drug-competitive inhibition of ALK2-FKBP12 interaction renders ALK2 responsive to Activin A

A) Primary murine hepatocytes were treated for 3 hrs with Tacrolimus (TAC, 1 µg/ml) or vehicle and then incubated for 5 hrs with increasing concentrations of Activin A (0-10 ng/ml) in the presence or absence of TAC. Total RNA was isolated and *Id1* expression was quantified by qRT-PCR. *Hprt1* was used as housekeeping gene. **B)** RNA was purified from primary murine hepatocytes treated with Torin1 (T1, 100nM), rapamycin (RAPA, 100 nM) or a combination of RAPA plus Activin A (10 ng/ml). *Id1* expression was measured by qRT-PCR as described in **A**. Mean ΔCt values in each group were subjected to a change of

origin by subtracting the mean ΔCt of untreated hepatocytes (panel A: 2.7; panel B: 3.7). **C, D)** Hep3B cells, transfected with the hepcidin promoter reporter vector (HAMP-Luc) (**C**) or the BRE luciferase reporter vector (BRE-Luc) (**D**), were treated with GPI-1046 (100 µg/ml) or vehicle for 5 hrs and then incubated or not with Activin A (10 ng/ml) ± GPI-1046 for 15 hrs. Luciferase activity was normalized to an untreated mean value of 1. Representative experiments, made in triplicate, are shown. RQ: relative quantification. Error bars indicate SD. A t-test analysis was applied. Asterisks in black refer to: -GPI-1046 vs +GPI-1046. Asterisks in green refer to: GPI-1046/-ActA vs GPI-1046/+ActA. * p< .05; ***p< .001; ****p< .0001. Estimates of the fold changes in gene expression ($2^{-\Delta\Delta Ct}$) are shown on the graphs.

Figure S6. Modulation of hepcidin by FKBP12-ALK2 interaction. A) In basal condition hepcidin levels are preferentially maintained through BMP2-ALK3-HJV signaling. ALK2 is inactive since bound to FKBP12. **B)** FKBP12 displacement (*) favors ALK2-dependent SMAD1/5/8 activation through canonical (BMP6) and non-canonical ligands (Activin A). High BMP6 level displaces FKBP12 from ALK2.

FIGURE S1

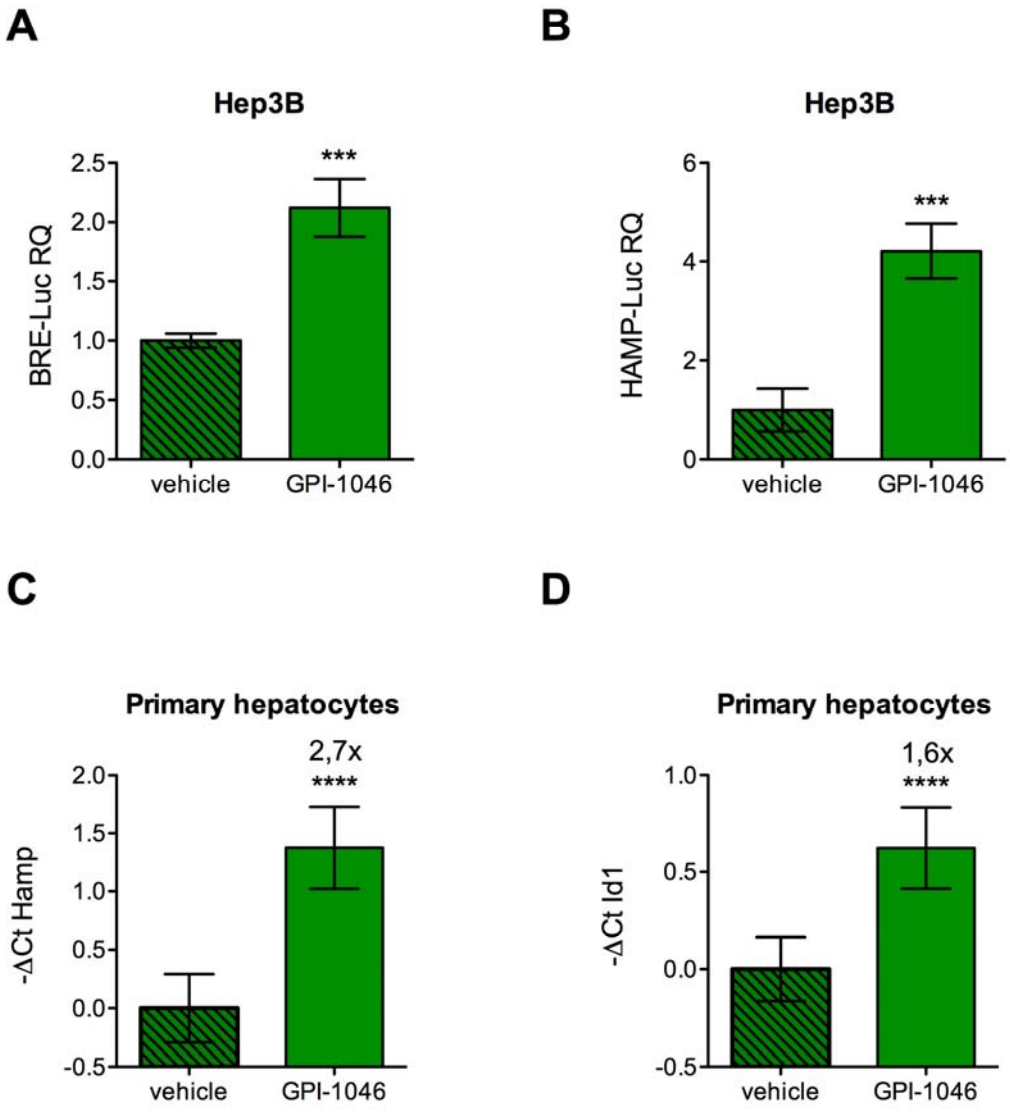

A
Hep3B
BRE-Luc RQ
2.5
2.0
1.5
1.0
0.5
0.0
***
vehicle
GPI-1046
B
Hep3B
HAMP-Luc RQ
6
4
2
0
***
vehicle
GPI-1046
C
Primary hepatocytes
-ΔCt Hamp
2,7x
****
2.0
1.5
1.0
0.5
0.0
-0.5
vehicle
GPI-1046
D
Primary hepatocytes
-ΔCt Id1
1,6x
****
1.0
0.5
0.0
-0.5
vehicle
GPI-1046

FIGURE S2

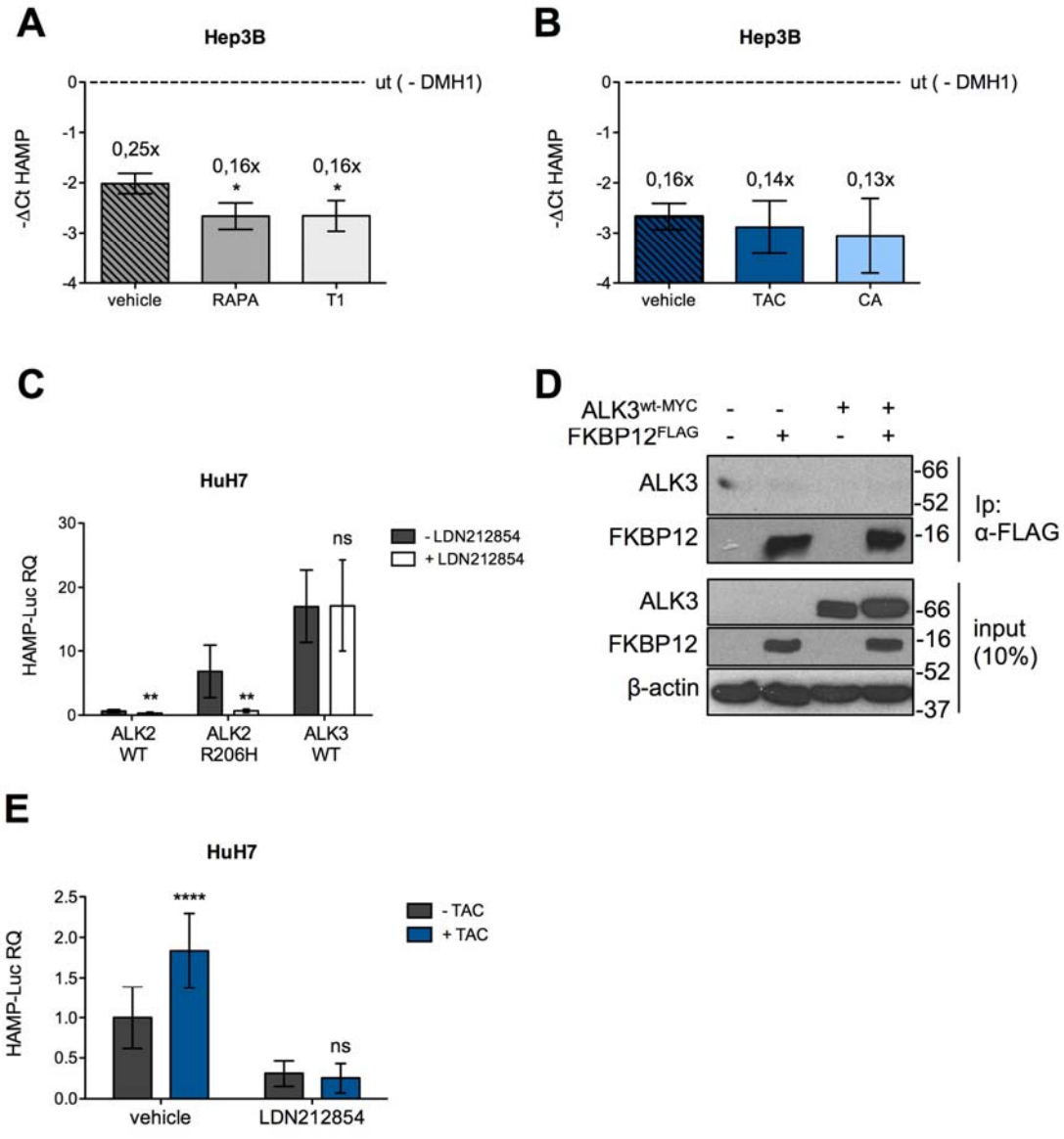

A
Hep3B
ut (- DMH1)
-ΔCt HAMP
0,25x
0,16x
0,16x
vehicle
RAPA
T1
B
Hep3B
ut (- DMH1)
-ΔCt HAMP
0,16x
0,14x
0,13x
vehicle
TAC
CA
C
HuH7
HAMP-Luc RQ
- LDN212854
+ LDN212854
ns
ALK2 WT
ALK2 R206H
ALK3 WT
D
ALK3wt-MYC
FKBP12FLAG
ALK3
FKBP12
Ip: α-FLAG
β-actin
input (10%)
E
HuH7
HAMP-Luc RQ
- TAC
+ TAC
****
ns
vehicle
LDN212854

FIGURE S3

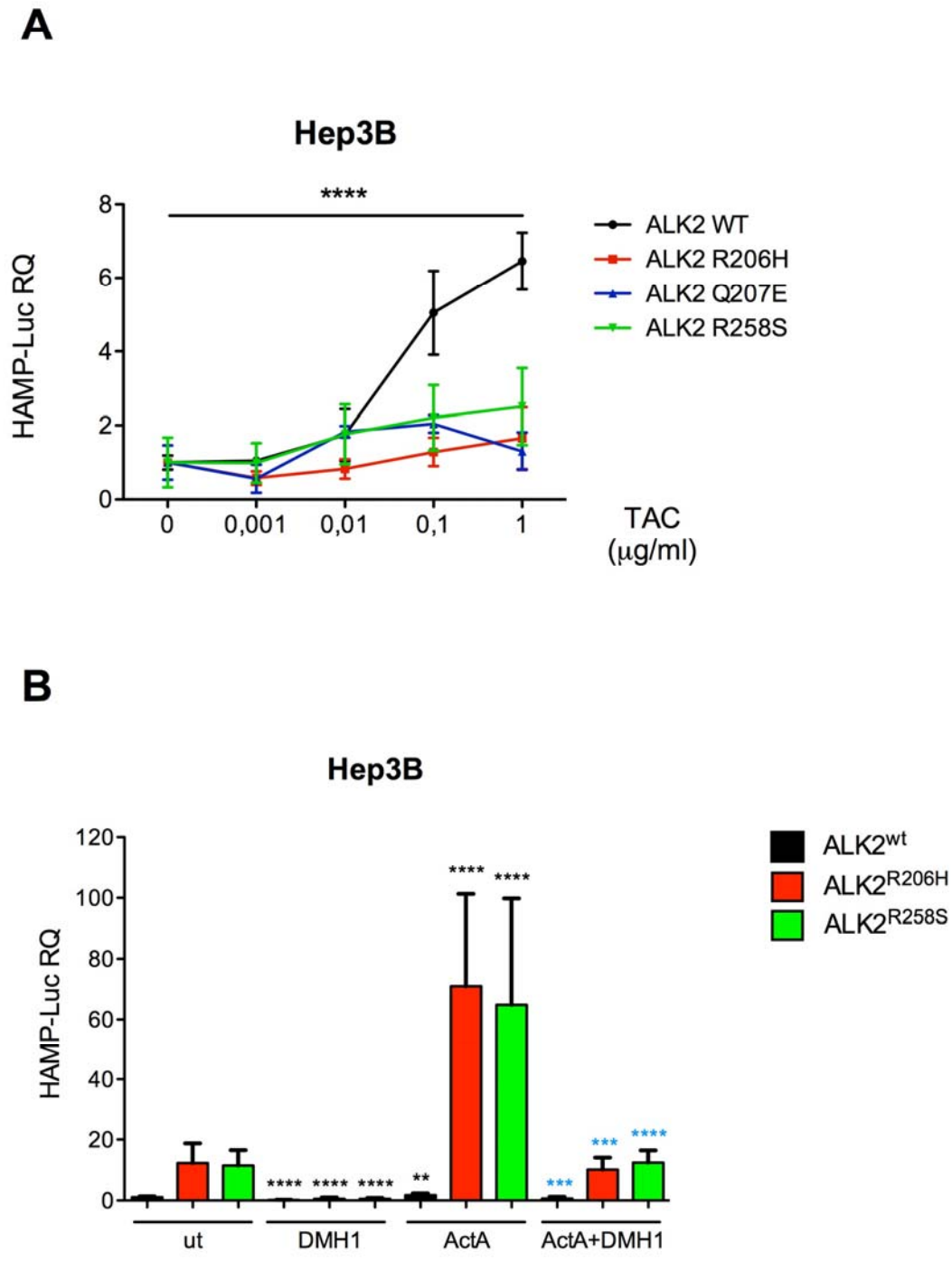
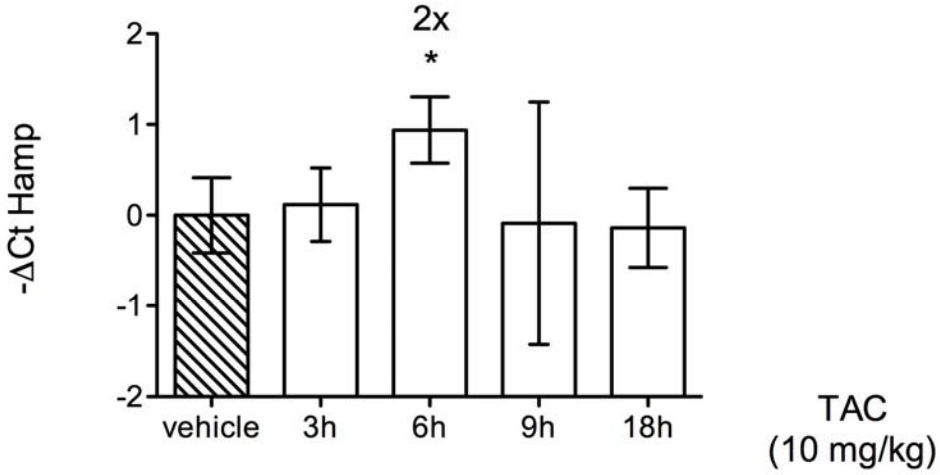
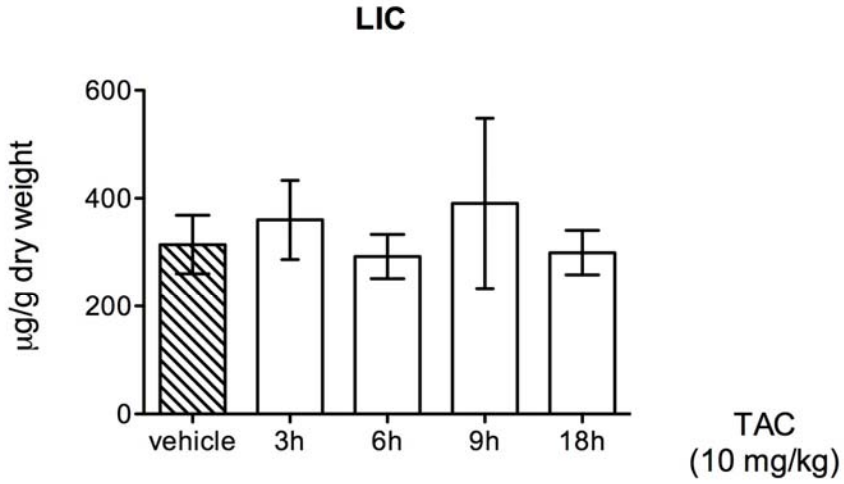

A
Hep3B
****
HAMP-Luc RQ
8
6
4
2
0
0
0,001
0,01
0,1
1
TAC
(μg/ml)
ALK2 WT
ALK2 R206H
ALK2 Q207E
ALK2 R258S
B
Hep3B
HAMP-Luc RQ
120
100
80
60
40
20
0
ut
DMH1
ActA
ActA+DMH1
ALK2wt
ALK2R206H
ALK2R258S

FIGURE S4

A



B



C

	TAC (10mg/kg)			
Serum Iron (µg/dl)	**vehicle**	**3h**	**6h**	**18h**
	241±55	331±77	178±44	161±32

FIGURE S5

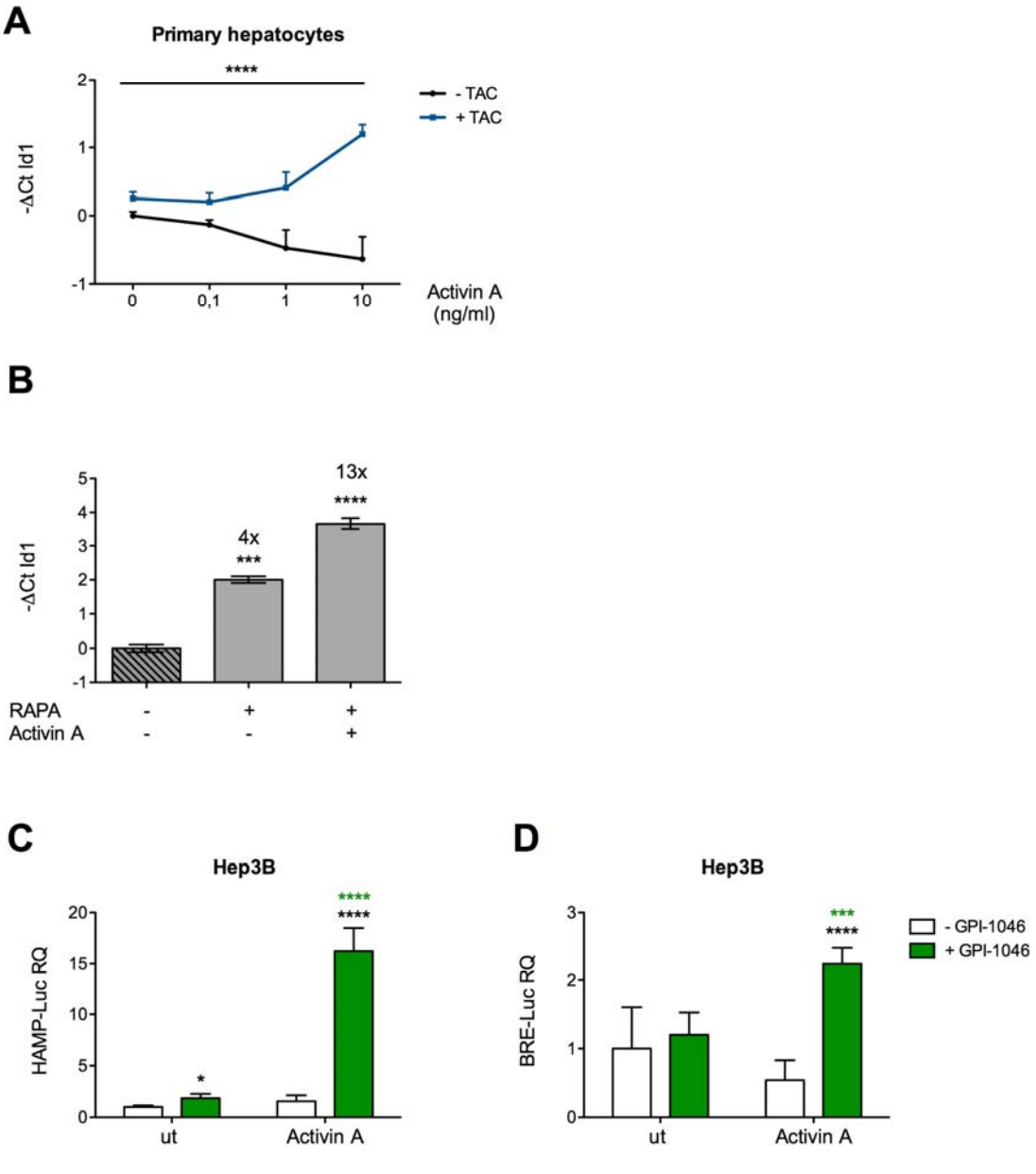
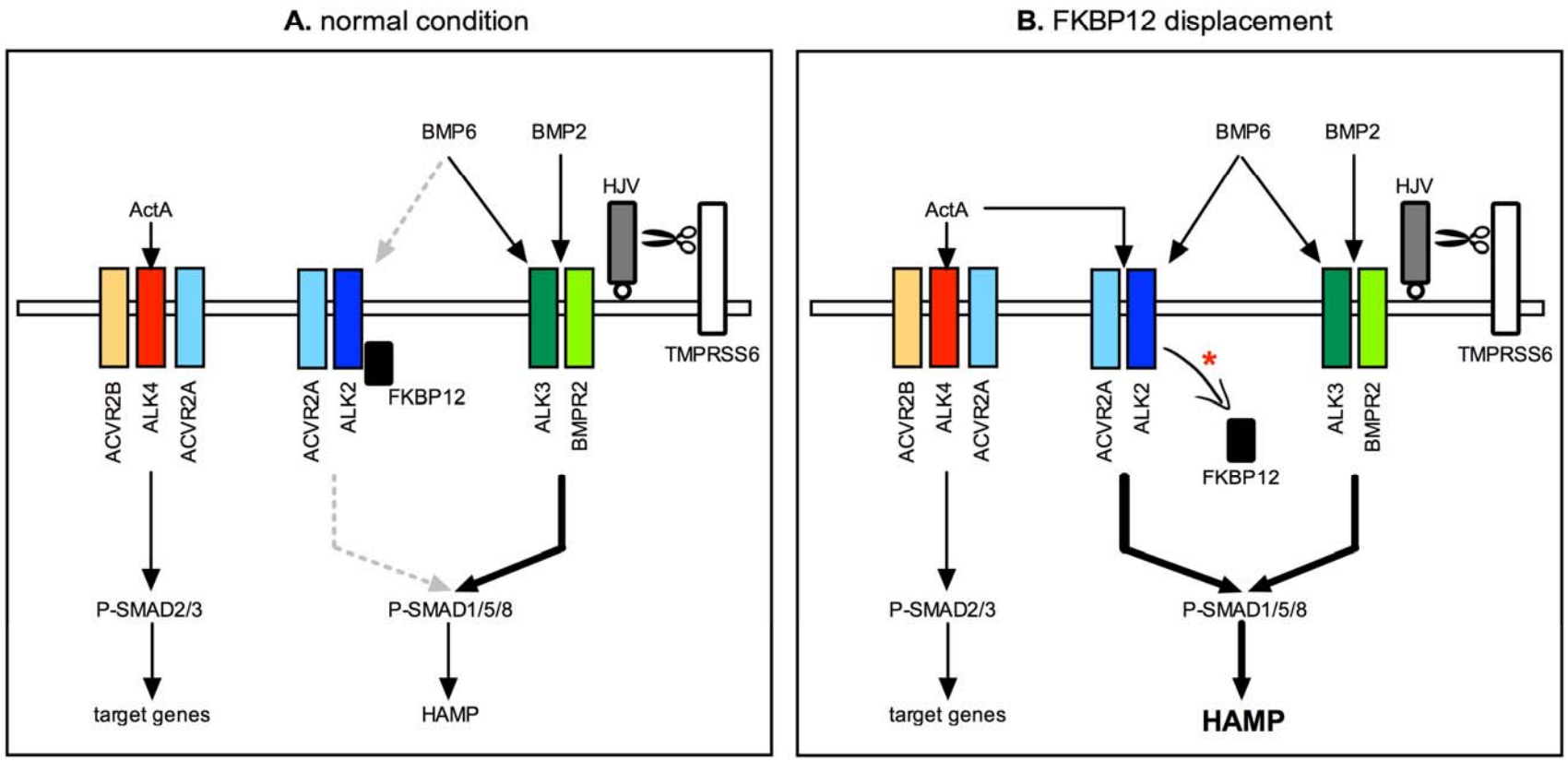

A
Primary hepatocytes
****
- TAC
+ TAC
-ΔCt Id1
2
1
0
-1
0
0,1
1
10
Activin A
(ng/ml)
B
13x
****
4x
***
-ΔCt Id1
5
4
3
2
1
0
-1
RAPA
Activin A
C
Hep3B
****
****
HAMP-Luc RQ
20
15
10
5
0
*
ut
Activin A
D
Hep3B
***
****
- GPI-1046
+ GPI-1046
BRE-Luc RQ
3
2
1
0
ut
Activin A

FIGURE S6


A. normal condition
B. FKBP12 displacement
BMP6
BMP2
HJV
ActA
TMPRSS6
ACVR2B
ALK4
ACVR2A
ACVR2A
ALK2
FKBP12
ALK3
BMPR2
P-SMAD2/3
P-SMAD1/5/8
target genes
HAMP
*

Supplemental Tables

Table S1 - Oligonucleotides for qRT-PCR

	Gene	Sequence (5' → 3') or Id
SybrGreen	*GAPDH*	FW: CCCCGGTTTCTATAAATTGAGC REV: CACCTTCCCCATGGTGTCT
	HAMP	FW: CTGTTTTCCCACAACAGACG REV: AGATGGGGAAGTGGGTGTCT
	ID1	FW: TCCAGCACGTCATCGACTAC REV: TCAGCGACACAAGATGCG
TaqMan	*Hprt1*	Mm01318743_m1
	Hamp	Mm00519025_m1
	Id1	Mm00775963_g1

Table S2 – Oligonucleotides for mutagenesis

Plasmid mutated	Sequences (5' → 3')
pCMV6-ALK2$^{R206H\text{-}MYC\text{-}FLAG}$	FW: ACAAAGAACAGTGGCTCACCAGATTACACTGTTGG Rev: CCAACAGTGTAATCTGGTGAGCCACTGTTCTTTGT
pCMV6-ALK2$^{Q207E\text{-}MYC\text{-}FLAG}$	FW: AAAGAACAGTGGCTCGCGAGATTACACTGTTGGAG Rev: CTCCAACAGTGTAATCTCGCGAGCCACTGTTCTTT
pCMV6-ALK2$^{R258S\text{-}MYC\text{-}FLAG}$	FW: GTACAACACTGTGATGCTGAGCAATGAAAATATCTTAGGTTTC Rev: GAAACCTAAGATATTTTCATGGCTCAGCATCACAGTGTTGTAC
pCMV6-ALK2 $^{R206H/Q207E/R258S\text{-}MYC}$	FW: CTCATCTCAGAAGAGGATCTGTGAGCAAATGATATCCTGGATTA Rev: TAATCCAGGATATCATTTGCTCACAGATCCTCTTCTGAGATGAG
pCMV6-FKBP12	FW: GTGGAGCTTCTAAAACTGGAATAGCGTACGCGGCC Rev: GGCCGCGTACGCTATTCCAGTTTTAGAAGCTCCAC