Regulation of cell surface transferrin receptor-2 by iron-dependent cleavage and release of a soluble form

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SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

Constructs

The whole *furin* and Erythropoietin receptor (EPOR) open reading frame were amplified from human cDNA and cloned in pcDNA3.1 (+) (Invitrogen, Carlsbad, CA), as described(1, 2); *PCSK7* construct was purchased from Origene (Origene Technologies, Rockville, MD). Rabbit PrlR construct is as described(3).

Cell culture conditions

HeLa, HEK293 and the hepatoma cell lines HuH7 (kindly provided by Martina Muckenthaler, University of Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM). Hep3B cells were cultured in Earle's minimum essential medium (EMEM). The erythroleukemic cell line UT7 was cultured in minimal essential medium (MEM) supplemented with 2U/ml Epo(4). CHO-K1 (from ATCC) and CHO-Trvb-0 (kindly provided by Caroline Enns, Oregon Health and Science University, Portland, Oregon, USA) were cultured in F12 medium. All media were supplemented with 2mM L-glutamine, 200U/mL penicillin, 200mg/mL streptomycin, 1mM sodium pyruvate and 10% heat-inactivated fetal bovine serum (FBS). Standard culture conditions were maintained for all cell lines used (37°C in 95% humidifier air and 5% CO_2). Cell culture media and reagents were from Gibco (Gibco Cell Culture, Portland, OR), Invitrogen and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). $CD34^+$ cells were obtained from human donors who gave informed consent in accordance with the Declaration of Helsinki Principles and approval obtained by the La Pitié-Salpétrière Hospital Institutional ethic committee. $CD34^+$ cells were purified from the peripheral blood after cytapheresis. $CD34^+$ cells were isolated by positive selection using an immunomagnetic procedure (MACS CD34 isolation Kit; Miltenyi Biotech, Bergish Badgach, Germany) and cultured in 5 % CO2 at 37°C in Iscove DMEM (IMDM) containing 15 % BIT 9500 (StemCell Technologies, Vancouver, Canada), 100 ng/ml SCF, 10 ng/ml IL6 and 10 ng/ml IL3. After 7 days of culture, $CD36^+$ cells corresponding to a highly purified population of human erythroid progenitors were obtained by positive selection on CD36

immunomagnetic beads. $CD36^+$ cells were then cultured in the presence of 2U/ml Epo, 100 ng/ml SCF and 10 ng/ml IL3 up to 12 days for erythroid differentiation.
When indicated cells were treated with the proconvertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, Alexis Biochemicals, San Diego, CA) at a concentration of 50 μM.

Pull-down of membrane and soluble TFR2

Transiently transfected cells, were treated with EZ-Link-Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Thermo Scientific, Rockford, IL) for 30 minutes at 4°C, 18 hours (for the analysis of soluble proteins) or 36 hours (for the analysis of membrane associated proteins) after transfection. Cells were then washed with 100 mM glycine to quench excess of biotin, and re-incubated for 24 hours in serum-free media to follow biotinylated proteins released in the cell cultured media or collected and lysed in NET/Triton buffer. Total lysates were quantitated using the Bio-Rad protein Assay. One mg of lysates or 100 μl of media concentrated using 3 kDa molecular weight cut-off ultrafiltration tubes Amicon Ultra (EMD Millipore Corporation, Billerica, MA) were incubated overnight with streptavidin agarose resin (Pierce Biotechnology) at 4°C. Samples were eluted with 20 μl of Laemmli sample buffer, boiled 5 minutes, subjected to SDS-PAGE on 10% acrylamide gels and then analyzed by western blot.
The media of HeLa cells transiently transfected with $TFR2^{WT\text{-}C\text{-}FLAG}$ construct and incubated in serum-free media added of H-holo-TF or B-holo-TF were collected and concentrated using Amicon Ultra 3 kDa tubes (EMD Millipore). The concentrated media were incubated overnight at 4 °C with 40 μL of anti-FLAG M2 affinity gel (Sigma-Aldrich) then the resin was resuspended in 20 μL of Laemmli sample buffer, boiled for 5 minutes and added of 4% v/v β-mercaptoethanol. Samples were subjected to SDS-PAGE on 10% acrylamide gels and then analyzed by western blot.
For erythroid cells, the cell surface proteins were biotinylated with EZ-Link-Sulfo-NHS-SS-Biotin according to the manufacturer's instructions (Pierce Biotechnology). After 24 hours of incubation in 10ml of 10% FCS supplemented medium, cells were lysed in buffer A (1% NP40, 10mM Tris/HCl pH 7.4, 150mM sodium chloride, 5mM EDTA, 2.5% glycerol, 25mM glycerophosphate, 1mM sodium pyrophosphate, 20mM sodium fluoride, 1mM sodium orthovanadate) and the supernatants were reduced to 500μl on an Amicon Ultra-15 Centrifugal Filter Units 10kDa (EMD Millipore).

Samples were incubated for 1h at 4°C with neutravidin agarose beads (Pierce Biotechnology). The beads, washed in buffer A and twice in buffer B (buffer A with 0.1% NP40 instead of 1%), were boiled 5 minutes in Laemmli buffer and analyzed by western blot.

Western Blot Analysis

Cells were seeded in 100-mm–diameter dishes until 70-80% confluence was reached and then were transfected with 12 μg plasmid DNA and 36 μL of the liposomal transfection reagent Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 18 hours media were replaced by serum free media and 24 hours later cells were collected and lysed in NET/Triton buffer (150 mM NaCl, 5 mM EDTA, and 10 mM Tris [pH 7.4] with 1% Triton X-100) and media were collected and concentrated using 3 kDa molecular weight cut-off ultrafiltration tubes Amicon Ultra (EMD Millipore Corporation, Billerica, MA). Proteins were quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich); samples (50 μg) were boiled 5 minutes in Laemmli buffer, subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond C membrane (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) following standard Western Blot procedures.

Blots were blocked with 2% ECL Advance Blocking Agent (Amersham Biosciences, GE Healthcare) in TBS (0.5 M Tris-HGI pH 7.4 and 0.15 M NaCl) containing 0.1% Tween-20 (TBST) and incubated with the primary antibodies: anti-TFR2 (1/1000; 3F8C11 and H-140, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-TFR2 (1/500; Abcam, Cambridge, UK), anti-TFR2^{hm} (1/1000, rabbit polyclonal antibody that we generated against a recombinant protein containing the human TFR2 ectodomain fused to thioredoxin), anti-HJV (1:1000,(5)), anti-FLAG (1/1000; Sigma-Aldrich), anti-TFR1 (1:1000, Zymed Laboratories, CA, USA), anti-MYC (1:1000, Novus Biologicals, Littleton, CO) anti- Phospho-ERK (T202/Y204, 1/2000, Cell Signaling, Danvers, MA), anti-Phospho-AKT (S473, 1/1000, Cell Signaling), anti-Phospho-STAT5 (Y694, 1/2000, Cell Signaling), anti-Furin (1/1000, Santa Cruz Biotechnologies), anti-βactin (1/10000, Sigma-Aldrich) anti-ERK (1/2000, Santa Cruz Biotechnologies), anti-AKT(1/1000, Cell Signaling), anti-STAT5 (1/2000, Santa Cruz Biotechnologies), anti-pan-cadherin (1/2000, Abcam), anti-EPOR

(1/1000)(6). Blots were then incubated with relevant HRP-conjugated secondary antisera and developed using a chemiluminescence detection kit (ECL, Amersham Biosciences, GE Healthcare).

In case of erythroid cells, cultured for 24h in 10 ml of serum free media, the supernatant was concentrated to 200µl on Amicon Ultra-15 Centrifugal Filter Units 10kDa (Millipore). The proteins were precipitated adding 20% of trichloroacetic acid for 1 hour at 4°C, centrifuged for 10 minutes at 12000g, washed twice with acetone and boiled 5 minutes in Laemmli buffer.

Luciferase reporter assay

HuH7 and Hep3B cells, seeded in 48-wells plates, were transiently transfected with 0.25 µg hepcidin promoter luciferase reporter construct (Hep-Luc), in combination with pRL-TK Renilla luciferase vector (Promega, Madison, WI) to control transfection efficiency, with 0.1 µg of the empty vector or *HJV* expressing vector, in the presence or not of increasing concentrations (0.01, 0.03 and 0.1 µg) of *sTFR2** expressing vector. Eighteen hours after transfection media were replace and, when indicated, cells were treated with 10 ng/mL BMP6 (R&D System, Minneapolis, MN) or incubated with conditioned media from HuH7 cells transfected with $TFR2^{WT}$ or empty vectors. Twenty-four hours later, cells were lysed and luciferase activity was determined according to manufacture's instructions (Dual Luciferase Reporter Assay, Promega).

Relative luciferase activity was calculated as the ratio of Firefly to Renilla luciferase activity and expressed as a multiple of the activity of cells transfected with the reporter alone.

SUPPLEMENTARY RESULTS

Diferric transferrin and not iron modulates TFR2 shedding

To evaluate whether modulation of cellular iron status might regulate TFR2 shedding HeLa cells were transiently transfected with wild type *TFR2* coding vector ($TFR2^{WT}$), and treated with the iron chelator deferoxamine (DFO, 100μM) or the iron donor ferric ammonium citrate (FAC, 100 μM plus Ascorbic Acid, 100 μM) or human diferric transferrin (H-holo-TF, 0.5-5 μM). The shedding was inhibited only in cells treated with holo-TF (Figure S1A) and the inhibition was dose dependent (Figure S1B).

Searching for the protease responsible for TFR2 shedding

In order to identify the protease responsible for TFR2 shedding we tested different protease inhibitors. We observed a strong decrease of TFR2 shedding only after the pro-convertases inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, Alexis Biochemicals) treatment.

Among proconvertases involved in shedding soluble forms of proteins with relevant roles in iron metabolism PCSK7, a member of the family of furin-like pro-convertases has been recently shown to shed TFR1(7) and furin, activated in iron deficiency(1), is responsible for sHJV release(1),(8).

We excluded as candidates for TFR2 shedding both PCSK7 and furin since their overexpression did not modify sTFR2 release (Fig. S3A). Moreover, we observed that the consensus cleavage site for PCSK7 is not conserved in TFR2 (Fig. S3B).

SUPPLEMENTARY LEGEND TO FIGURES

Figure S1. Holo-TF but not iron modulates sTFR2 shedding

A) HeLa cells were transiently transfected with wild type *TFR2* coding vector ($TFR2^{WT\text{-}N\text{-}FLAG}$), eighteen hours after transfection media were replaced with serum-starved media and added or not with the iron chelator deferoxamine (DFO, 100μM) or the iron donor ferric ammonium citrate (FAC, 100 μM) or human diferric transferrin (H-holo-TF, 1,25 μM). Twenty-four hours later media were collected and

concentrated and cells were lysed. Fifty μg of proteins from media (Sn) and cellular lysates (Lys) were analysed by western blot, using an anti-TFR2 antibody (Abcam).
B) HeLa cells were transiently transfected with empty vector (mock) and wild type *TFR2* coding vector (*TFR2*$^{WT\text{-}N\text{-}FLAG}$). Eighteen hours after transfection cells were biotinylated to label membrane protein and were incubated in serum-free media treated or not with increasing doses of human holo-transferrin (H-holo-TF). After 24 hours media were collected and concentrated, cells were lysed and both media (Sn-B) and lysates (Lys-B) were precipitated with streptavidin to analyse by western blot proteins originating from the cell surface. Anti-TFR2 antibody (Abcam) was used to detect TFR2.
C) HeLa cells were transiently transfected with *TFR2*$^{WT\text{-}C\text{-}FLAG}$ cDNA expressing vector and then treated with increasing concentrations of human holo-TF. To avoid holo-TF interference with gel migration of concentrated culture media, we pulled-down sTFR2 using anti-FLAG Sepharose-beads taking advantage of the FLAG tag at the C-terminus. As shown in Figure 2A, human holo-TF inhibits the release of sTFR2 in a dose-dependent manner. Anti-FLAG antibody was used to detect TFR2.

Figure S2. CHO-Trvb-0 cells lack TFR1
Western blot analysis of TFR1 expression in CHO-K1 and CHO-Trvb-0 cells showing lack of expression of TFR1 in the latter cell line. Fifty μg of proteins from cellular lysates (Lys) of both cell lines were analysed by standard western blot technique using anti-TFR1 and anti-βactin antibodies.

Figure S3. Modulation of ^{125}I-EPO binding to EPOR in UT7 by sTFR2* and BFA treatment. ^{125}I-EPO binding in the absence of sTFR2* (-sTFR2*), in the presence of sTFR2* (+sTFR2*) or in the presence of 5 μg/ml of BFA. Measurement was performed in triplicate and expressed as % of 125 I-EPO binding. *: $P < 0.05$. ns: not significant.

Figure S4. sTFR2 does not bind to the erythropoietin receptor (EPOR)
HEK293 cells were transfected with empty vector (mock), Prolactin Receptor (PrlR), EPOR, or TFR2 and co-transfected with EPOR and TFR2. Forty-eight hours after transfection, cells were preincubated for 1 hour at 37°C with (+) or without (-)

sTFR2*. Cellular extracts were immunoprecipitated by IgG (IP cont), by anti-PRLR, by anti-$TFR2^{hm}$ or anti-EPOR antibodies and then analysed by Western blot with anti-Myc, anti-$TFR2^{hm}$, anti-EPOR and anti-PRLR antibodies.

Figure S5. sTFR2 mildly inhibits hepcidin promoter *in vitro* in hepatoma cells

A) HuH7 cells cultured in 100 mm Petri dishes were transiently transfected with the empty vector (mock) or $TFR2^{WT\text{-}N\text{-}FLAG}$ construct. Cell cultured media were replaced with serum-free DMEM and 24 hours later used (Sn mock and Sn TFR2) for conditioning HuH7 cells transfected with the Hep-Luc vector in combination with empty vector or the *HJV* construct, to follow the luciferase activity. The medium containing sTFR2 (Sn TFR2) shows a significant inhibitory effect on hepcidin promoter in basal and HJV stimulated condition.

B and C) HuH7 (B) and Hep3B (C) cells were co-transfected with the Hep-Luc vector in the presence/absence of *HJV* and increasing concentration of *sTFR2** expressing vectors (10-100 ng). Luciferase activity was detected as described in Material and Methods. In all conditions analysed transfection of sTFR2* causes a significant inhibitory effect on hepcidin promoter.

D) Hep3B cells cells were co-transfected with the Hep-Luc vector in the presence/absence of *HJV* and *sTFR2** and treated or not with BMP6 (10 ng/mL). Eighteen hours later luciferase activity was detected as described in Material and Methods. sTFR2* causes a significant inhibitory effect on hepcidin promoter even in presence of the strong activator BMP6.

The experiments were performed three times in triplicate. Statistical significance is indicated above the bars: * = $p< 0.05$; ** = $p< 0.005$.

Figure S6. Shedding of TFR2 is inhibited by CMK even when furin or PCSK7 are overexpressed

A) HeLa cells transiently transfected with $TFR2^{WT\text{-}N\text{-}FLAG}$, and $TFR2^{WT\text{-}N\text{-}FLAG}$ +PCSK7 or +furin cDNA expressing vectors were treated with the proconvertases inhibitor (CMK) (50 μM) Both furin and PCSK7 are inhibited by CMK. After 24 hours media were collected and concentrated and cells were lysed. Western blot analyses were performed on media (Sn) and lysates (Lys) using an anti-TFR2 (Abcam), anti-βactin and anti-Furin antibodies.

B) Sequence alignment of TFR1 and TFR2. Grey box indicates the region of PCSK7 consensus cleavage site identified in TFR1 that is not conserved in TFR2.

C) HeLa cells transiently transfected with empty vector (mock), $TFR2^{WT\text{-}N\text{-}FLAG}$ and $TFR2^{G679A}$ were treated with CMK (50 μM). After 24 hours media were collected and concentrated and cells were lysed. Western blot analyses were performed on media (Sn) and lysates (Lys) using an anti-TFR2 antibody (Abcam).

UT = untreated cells.

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY FIGURES

Figure S1

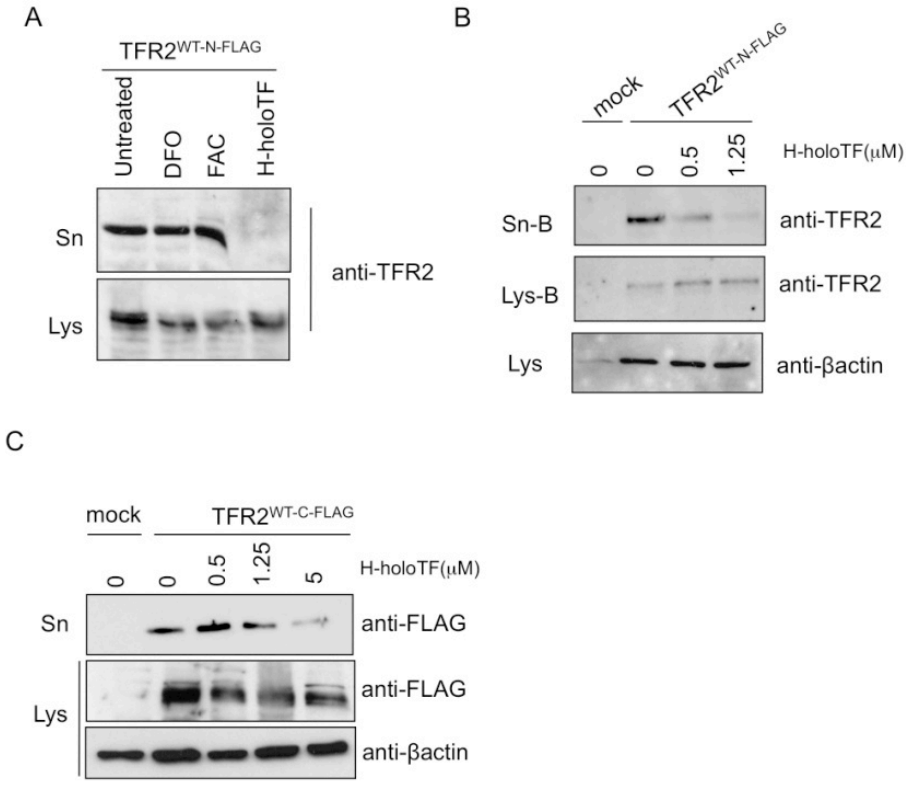

Figure S2

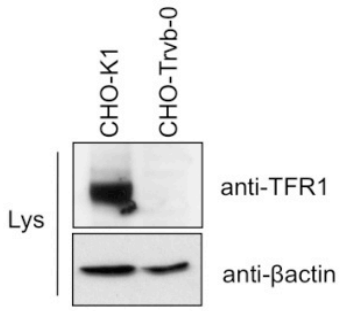

Figure S3

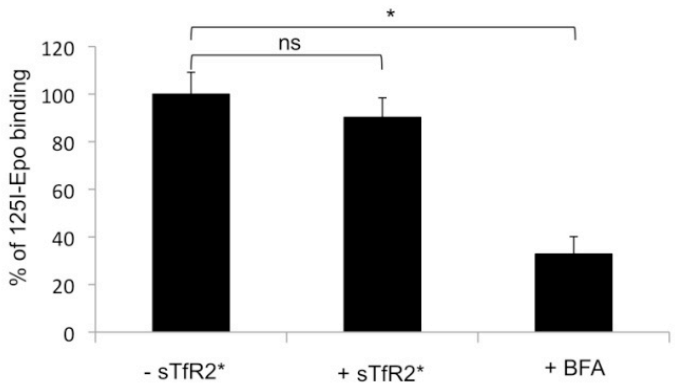

Figure S4

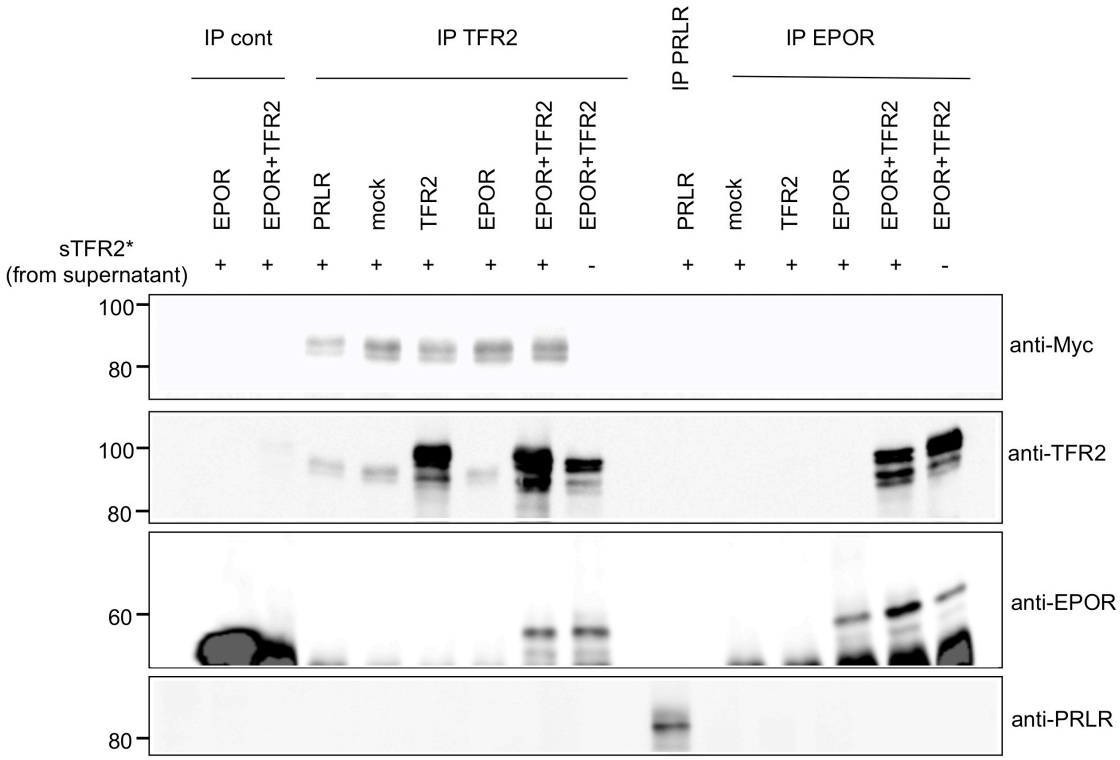

Figure S5

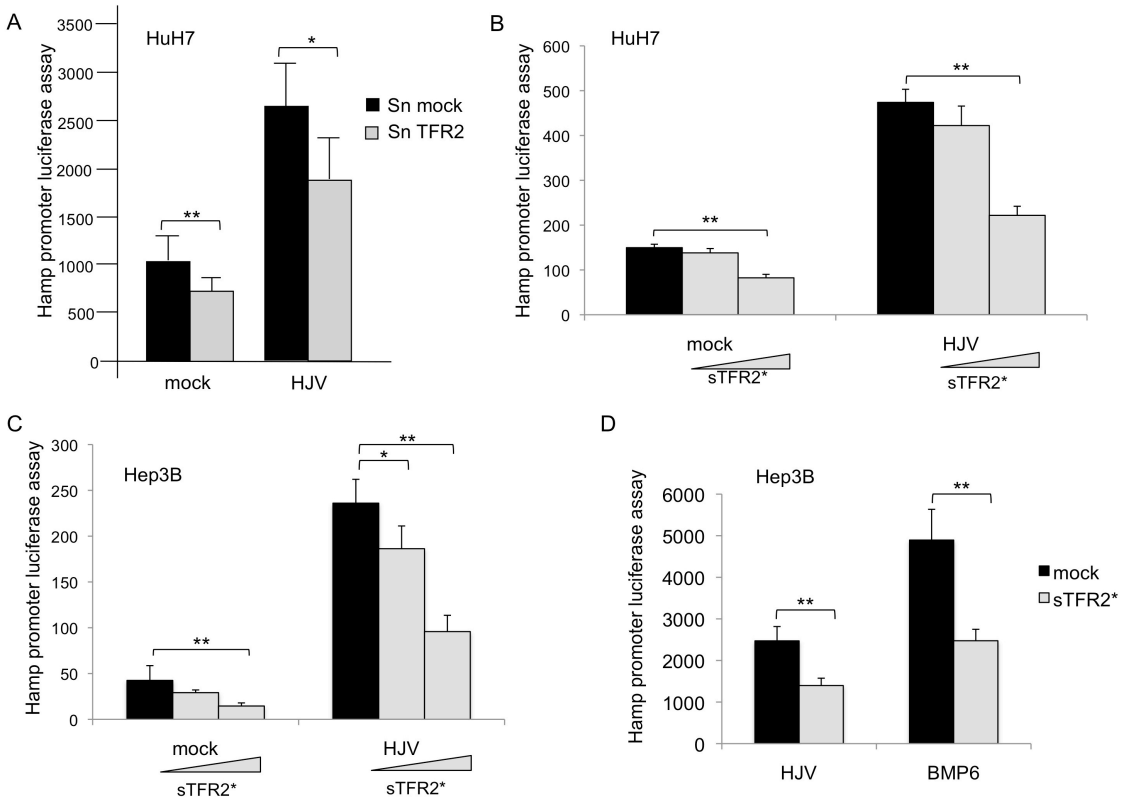

Figure S6