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

Running title: Shedding of TFR2

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ABSTRACT
Transferrin receptor-2 is a trans-membrane protein whose expression is restricted to hepatocytes and erythroid cells. Transferrin receptor-2 has a regulatory function in iron homeostasis, since its inactivation causes systemic iron overload. Hepatic transferrin receptor-2 participates to iron sensing and is involved in hepcidin activation, although the mechanism remains unclear. Erythroid transferrin receptor-2 associates with and stabilizes erythropoietin receptors on erythroblast surface and is essential to control erythrocyte production in iron deficiency. We identified a soluble form of transferrin receptor-2 in the media of transfected cells and showed that cultured human erythroid cells release an endogenous soluble form. Soluble transferrin receptor-2 originates from a cleavage of the cell surface protein, that is inhibited by diferric transferrin in a dose-dependent manner. Accordingly, the shedding of the transferrin receptor-2 variant G679A, mutated in the Arginine-Glycine-Aspartic acid motif and unable to bind diferric transferrin, is not modulated by the ligand. This observation links the process of transferrin receptor-2 removal from plasma membrane to iron homeostasis. Soluble transferrin receptor-2 does not affect the binding of erythropoietin to erythropoietin receptor or the consequent signalling and partially inhibits hepcidin promoter activation only in vitro. Whether it is a component of the signals released by erythropoiesis in iron deficiency remains to be investigated. Our results indicate that membrane transferrin receptor-2, a sensor of circulating iron, is released from cell membrane in iron deficiency.
INTRODUCTION

Transferrin receptor-2 (TFR2) is a type II transmembrane glycoprotein homologous to TFR1(1), able to bind diferric-transferrin (holo-TF), although with lower affinity than TFR1. In iron homeostasis TFR2 appears to have regulatory rather than transport functions, since TFR2 mutations in humans(2) and Tfr2 inactivation in mice(3),(4),(5) cause iron overload with low hepcidin(6). TFR2 is mainly expressed in the liver(1),(7) where it is essential for hepcidin control. Its expression is up-regulated during hepatic development but is not modulated by iron; indeed TFR2 mRNA has no detectable iron-responsive elements in its untranslated regions(8).

TFR2 encodes an 801 amino acids protein with a short cytosolic tail that contains a potential internalization signal, a transmembrane domain and a large C-terminal ectodomain. The latter has a protease-associated domain, a peptidase M28 like and a dimerization domain with two RGD motifs, important for protein-protein interaction and for transferrin binding. Mutations of TFR2 cause type 3 hemochromatosis: all reported mutations(2) are rare, often private. Mutations are loss of function including frameshift, premature stop, small deletions and missense mutations prevalently affecting the protein C-terminus, especially the peptidase-like and the dimerization domains(9).

Binding to holo-TF stabilizes TFR2 on cell surface(10) and this interaction redirects TFR2 towards the recycling instead of the lysosomal degradation pathway(4). Experiments in cultured hepatoma cell lines suggest that TFR2, bound to holo-TF, may simultaneously bind HFE. While the HFE- and the TF-binding sites on TFR1 overlap, in TFR2 they are different(10),(11). The HFE-TFR2 complex has been proposed to act as a sensor of circulating iron and as an activator of hepcidin expression(12). However, recent data suggest that the two proteins may have separate functions and also their interaction is controversial(13). Iron overload is more severe in Tfr2−− than in Hfe−− mice and Tfr2-Hfe double knock out mice show the most severe disease(14). Studies in patients indicate that TFR2 plays a prominent and HFE a minor role in hepcidin activation in response to acute increase of transferrin saturation after a single dose of oral iron(15). As a further level of complexity TFR2 gene, which shows two consensus sequences for the erythroid transcription factor GATA-1 in its promoter(12), is expressed in immature erythroid cells(16) where it is a component of the erythropoietin receptor (EPOR) complex(17). The TFR2-EPOR association is required for the efficient transport of EPOR to the cell surface. Although both TFR2-hemochromatosis patients and Tfr2−− mice have no evident hematologic abnormalities, the erythroid progenitors from Tfr2−− young mice appear less sensitive to erythropoietin (EPO) and have increased serum Epo levels. Moreover, TFR2 silencing in human erythroid progenitors delays their terminal differentiation(17).
TFR1 sheds a soluble counterpart (sTFR1), \textit{in vitro} and \textit{in vivo}, that is measurable in serum from normal individuals\cite{18}. TFR1 is highly expressed on the surface of maturing erythroblasts and, when unbound to holo-TF, is released into plasma in high concentration. This process occurs in iron deficiency anemia, in erythropoiesis expansion and in some hematologic malignancies\cite{19}. On the contrary sTFR1 levels are reduced in aplastic and hypoproliferative anemias. Although its function remains unexplained, sTFR1 has been proposed as a marker of iron deficiency and/or of total erythropoietic activity. The shedding of sTFR1 is regulated by its ligand holo-TF and, as recently shown, is mediated by the proprotein convertase subtilisin/kexin type 7 (PCSK7)\cite{20}. Interestingly, \textit{PCSK7} genetic variants are associated with sTFR1 quantitative trait in genome wide association studies\cite{21}.

Here we characterize a previously unrecognized soluble form of TFR2 (sTFR2) that is shed from plasma membrane both in transfected cell lines and in TFR2-expressing erythroid cells. We show that the process of TFR2 release is regulated by the ligand holo-TF, a regulation lost in a TFR2 mutant (TFR2\textsubscript{G679A}), unable to bind the ligand. Because of its low affinity for holo-TF, we suggest that the shedding of TFR2 signals iron deficiency simultaneously in hepatic and erythroid cells.
METHODS

Wild type and mutant constructs

A TFR2 wild type cDNA was cloned in pCMV-TAG4 vector with a FLAG-tag at the C-terminus (TFR2\textsuperscript{WT-C-FLAG}). TFR2 wild type (TFR2\textsuperscript{WT-N-FLAG}) and TFR2\textsuperscript{G679A} constructs in pcDNA3.1(+) have a FLAG-tag at the N-terminus. A “soluble” artificial isoform of TFR2 (sTFR2\#) was generated by amplification of the coding region from nucleotide 313 (Arg105) to nucleotide 2403 (Phe801) and in frame-cloned in pSEC-TAG (B), with a MYC-HIS-tag at C-terminus, using the following oligonucleotides:

sTFR2-KpnI-fw: 5'GGGGTACCCGAGGGTCCTGCCAGGCG-3'
sTFR2-EcoRI-rev: 5'GGAATTCCAGAAGTTGTTATCAATGTCC-3'

Mammalian expressing vectors encoding for wild type dynamin (Dyn\textsuperscript{WT}) and the dominant negative variant Dyn\textsuperscript{K44A} were described in(22). HJV expressing vector and the hepcidin promoter luciferase reporter construct (Hep-Luc) were as described(23). FURIN and PCSK7 expressing vectors were described in Supplemental Methods.

Cell culture and reagents

HeLa, HEK293, CHO-Trvb-0, the erythroleukemic cell line UT7 and the hepatoma cell lines HuH7 and Hep3B culture conditions and reagents are described in Supplemental Methods.

CD34\textsuperscript{+} cells were obtained from human donors who gave informed consent in accordance with the Declaration of Helsinki Principles. Approval for their use was obtained by the La Pitié-Salpêtrière Hospital Institutional Ethic Committee. CD34\textsuperscript{+} cells were purified from the peripheral blood after cytopheresis. CD34\textsuperscript{+} and CD36\textsuperscript{+} cells were isolated by positive selection as described in Supplemental Methods. CD36\textsuperscript{+} cells were cultured in the presence of 2 U/ml EPO, 100 ng/ml Stem Cell Factor and 10 ng/ml Interleukin 3 up to 12 days for erythroid differentiation.

When indicated, cells were treated with different concentrations (0.5, 1.25, 5 and 25\(\mu\)M) of human or bovine diferric-transferrin (H-holo-TF and B-holo-TF, respectively) and human apo-transferrin (apo-TF) (Sigma-Aldrich, St. Louis, MO) in serum-free media; erythroid cells were treated with 25\(\mu\)M of human apo-or holo-TF. Brefeldin-A (BFA, Sigma-Aldrich), a fungal antibiotic that alters the intracellular trafficking by blocking protein maturation across the post-ER compartment, was used at 100-500 ng/mL.
Procedures for pull-down of membrane and soluble TFR2, western blot analysis of conditioned cell culture media and total cell lysates, luciferase reporter assay for sTFR2 effect on hepcidin promoter are described in Supplemental Methods.

**Scatchard analysis**

UT-7 cells were suspended in binding medium (alpha-MEM containing 20 mM Hepes, 10% FCS, 0.1% sodium azide, pH 7.2). Twenty-five μl of the cell suspension, containing 4x10⁶ cells, were incubated with 25 μl of conditioned medium from HEK293 cells transfected with the empty or sTFR2* expressing vectors. Samples were equilibrated at 37°C for 60 min. Twenty-five μl of binding medium alone or 25 μl of a 100-fold excess of unlabeled EPO were added to the binding assay. Then the chosen amount of ¹²⁵I-EPO, in a volume of 25 μl was added, and cells were further incubated for 30 min at 37°C. The content of each well was carefully layered over 900 μl of a cold solution of PBS and centrifuged at 4°C for 10 min at 600 g. Unbound radioactivity was counted and after 3 washes in PBS, cell-bound radioactivity was counted in the pellet. The non-specific binding of ¹²⁵I-EPO determined by adding a 100-fold excess of unlabeled EPO to the binding assay was subtracted from the total binding to give the specific binding.

Two independent experiments have been performed using two different batches of ¹²⁵I-EPO and control conditioned medium or sTFR2*-containing conditioned medium from two independent experiments.
RESULTS

Identification of a soluble form of TFR2 (sTFR2)
To study the cell localization and processing of TFR2, HeLa cells, which do not express endogenous TFR2 (data not shown), were transiently transfected with an expressing vector that encodes for a N-FLAG tagged form of TFR2 (TFR2 WT-N-FLAG). Since TFR1 and other iron-related proteins present on cell membrane release soluble forms, we investigated whether this would also occur with TFR2. In the media of TFR2 transfected cells we identified a single band (sTFR2) of about 80 kDa, which migrated faster than the cell-associated isoforms, suggesting that it was the result of a cleavage mechanism. Since sTFR2 can be detected using the anti-TFR2 antibody, recognizing residues from amino acid 150 to 250, but not with the anti-FLAG antibody when using an N-terminal flagged construct, we concluded that the cleavage occurs at the N-terminal part of the protein, close to the transmembrane domain, as occurs for sTFR1(24). To further confirm this finding, sTFR2 from cells transfected with TFR2 WT-C-FLAG that has a FLAG-tag at the C-terminus, was indeed detected with the anti-FLAG antibody (Figure 1A). The size of sTFR2 is compatible with that of a protein lacking the intracellular and the transmembrane region. Indeed the artificial form of sTFR2 (sTFR2*), generated by cloning the TFR2 ectodomain (amino acids 105-801) downstream of the Ig k-chain leader sequence, migrates as the isoform shed from the full length TFR2 transfected cells (Figure 1A).

sTFR2 is not an artefact due to cDNA overexpression since it is released also by TFR2-expressing cells, as the erythroleukemic cell line UT7 and human primary erythroblasts. Both UT7 cells and primary erythroblasts release a band of sTFR2 of approximately 80 kDa in the culture media, (Figure 1B) compatible with the size of sTFR2 released by TFR2 transfected cells. In the total lysates of erythroid cells, TFR2 migrate as a doublet of about 102 and 92 kDa, as previously observed(17).

sTFR2 is shed from plasma membrane
It was previously reported that the receptor exposed on the cell surface is stabilized in the presence of holo-TF with a maximum effect at 25 µM holo-TF(10). We confirmed the same stabilization both in transfected cells and in TFR2-competent cells. HeLa cells transfected with TFR2 WT-C-FLAG construct were treated with human holo- and apo-TF (25 µM) in serum-free media and were biotinylated to label membrane proteins. sTFR2 was pulled-down using anti-FLAG Sepharose-beads to avoid holo-TF interference with gel migration of concentrated culture media. In these
conditions, as a consequence of holo-TF treatment, we observed a reduction of sTFR2 mirrored by an increase in membrane exposed TFR2 (Figure 2A).

A substantial increase in the expression of membrane TFR2 was also detected in biotinylated plasma membranes from erythroid UT7 cells and primary erythroblasts when cultured in the presence of holo-TF, while endogenous sTFR2 production was decreased (Figure 2B). This confirms that the inverse regulation of membrane and sTFR2 in the presence of the ligand is maintained also in cells expressing endogenous TFR2 (Figure 2A).

We asked whether sTFR2 originates from plasma membrane or is released during receptor internalization. To this aim cell surface proteins were labelled using a cell-impermeable biotin and were treated with holo-TF. sTFR2 was detected in the culture media of transfected cells after streptavidin pull down of biotin-labelled proteins, demonstrating that it originates from membrane exposed TFR2 (Figure S1B).

To further prove the membrane origin of sTFR2, we treated HeLa cells with brefeldin-A (BFA), a fungal antibiotic that alters the intracellular trafficking by blocking protein maturation across the post-ER compartment(25). BFA induced a strong reduction of both membrane-associated and sTFR2 (Figure 2C), consistent with the finding that TFR2 is shed from the cell surface.

To investigate the potential contribution of endocytosis in the release of sTFR2, we exploited the effect of dynamin dead-mutant K44A (Dyn K44A) that blocks the endocytosis process, thus “fixing” the proteins on cell surface(26). The amount of sTFR2 in cells transfected with Dyn K44A was strongly increased compared to that of cells transfected with wild type dynamin (Dyn WT), further strengthening that the cleavage occurs at the cell surface (Figure 2D). Under these conditions, holo-TF was still able to reduce the shedding process (Figure 2E).

**TFR2 shedding is inhibited by holo-transferrin in a dose dependent manner**

TFR2 can bind holo-TF and is highly homologous to TFR1 and the release of sTFR1 is reduced by its ligand(27). For these reasons we investigated whether sTFR2 release was affected by holo-TF in our in vitro system. HuH7 (Figure 3A) and HeLa (Figure S1B-C) cells were transiently transfected with the TFR2 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 biotinylated cell surface proteins and pulled-down sTFR2 in the supernatants using streptavidin beads. As shown in Figure 3A, human holo-TF inhibited the release of sTFR2 in a dose-dependent manner. This inhibition was not mediated by iron, since treatment of TFR2 transfected cells with iron donors (FAC) or iron chelators (DFO) did not modulate sTFR2 concentration in the culture media (Figure S1A).
To understand whether the observed inhibition was a direct effect of the ligand binding, we used the TFR2<sup>G679A</sup>, an artificial variant unable to bind holo-TF since the mutation inactivates the RGD binding domain(28). As shown in Figure 3B, TFR2<sup>G679A</sup> released sTFR2 in high concentration and the process was not modulated by the ligand.

**Shedding of TFR2 occurs independently of TFR1**

To exclude that sTFR2 release might be influenced by binding of holo-TF to its high affinity receptor TFR1 we used two approaches. First, we used bovine holo-TF that specifically binds TFR2, while is unable to bind human TFR1(28). As with human holo-TF, sTFR2 was strongly decreased in the presence of bovine holo-TF both in HeLa (Figure 4A) and in HuH7 cells (not shown). Second, we analysed the shedding process in TFR2 transfected CHO-Trvb-0 cells that lack TFR1(29). We confirmed that these cells did not express TFR1 (Figure S2) and we showed that also in these cells holo-TF inhibited the release of sTFR2 and stabilized TFR2 on cell surface (Figure 4B).

Overall our results demonstrate that the shedding of TFR2 does not require TFR1 and that the inhibition of sTFR2 release directly results from ligand-receptor binding.

**sTFR2 does not affect EPOR expression or EPO binding in erythroid cells and mildly reduces hepcidin promoter activation in hepatoma cells**

Because TFR2-EPOR association is required for the efficient transport of EPOR to the cell surface(17), we studied whether sTFR2 could modify the number of cell surface EPOR or the affinity of EPO for its receptor by Scatchard analysis in UT7 cells. To investigate this issue, we used sTFR2* that is functional (see below) and more efficiently produced than the naturally cleaved TFR2 (Figure 1A). The conditioned medium of HEK293 cells transfected with sTFR2* (Sn sTFR2*) or with empty vector (Sn mock) did not modify significantly the number of EPOR expressed on cell surface nor the affinity for its ligand (Figure 5A). However, BFA treatment decreased the number of EPOR on the plasma membrane (Figure S3). To investigate the activation of the EPO-mediated intracellular signalling, the phosphorylation status of STAT5, AKT and ERK, major targets of this pathway, was assessed in UT7 cells treated or not with sTFR2*. As shown in Figure 5B and quantitated in Figure 5C, the EPO signalling was not significantly affected by sTFR2*. These data were supported by the observation that exogenous sTFR2* did not interact with the EPOR when added to the culture medium (Figure S4).

To evaluate the functional role of sTFR2 in hepatic compartment, we studied the modulation of the hepcidin promoter in HuH7 cells, treated with the conditioned medium of the same hepatoma-
derived cells transfected with TFR2<sup>WT-N-FLAG</sup>. The supernatant of TFR2 transfected cells (SnTFR2) mildly reduced hepcidin activation both in basal condition and in the presence of the BMP co-receptor HJV (Figure S5A). The artificial sTFR2* showed a similar activity when transfected in HuH7 and Hep3B cells, decreasing the HJV-dependent hepcidin activation in a dose dependent manner (Figure S5B-C).

To analyse whether sTFR2 could interfere with BMPs signalling we studied the response of the hepcidin promoter in Hep3B cells in presence of both sTFR2* and BMP6 and we confirmed the same partial inhibitory effect observed both in basal and in HJV-mediated hepcidin activation (Figure S5D). However, an effect of sTFR2 was observed only in vitro on the hepcidin promoter assay.
DISCUSSION

From its discovery more than 10 years ago(1) and its identification as the gene responsible of 
hemochromatosis type 3(2), the function of the second transferrin receptor (TFR2) has remained 
obscure. The role of TFR2 as a component of the liver iron-sensing machinery and hepcidin 
activator is well established, but the molecular mechanisms remain unravelled. The role of TFR2 as 
partner of EPOR in immature erythroid cells has been clearly defined, and our recent results have 
shown that it negatively controls erythropoiesis expansion in iron deficiency(30).

In this paper we expand our knowledge on the structure of this receptor, identifying its soluble form 
(sTFR2). sTFR2 was detected in the medium of different cell lines transfected with TFR2 and most 
importantly was present in the culture media of both erythroid cell lines and primary human 
erythroid cells that endogenously express TFR2(16),(17).

We show that sTFR2 is shed from the membrane-exposed receptor. This conclusion is supported by 
two lines of evidence: 1) the lack of sTFR2 shedding when the receptor does not reach the cell 
surface, as after BFA treatment; 2) the increase of sTFR2 release in the opposite condition, when 
the receptor is stabilized on the membrane by a mutant dynamin.

TFR2 associates with different partners (EPOR in erythroid cells, HJV and HFE in the liver) and 
has likely different functions in the two tissues where is highly expressed. However, the shedding 
mechanism appears to be under a common iron-mediated regulation. Indeed binding of the holo-TF 
ligand to the receptor inhibits sTFR2 release, while in the absence of the ligand binding the release 
is increased both in hepatoma and in erythroid cells, providing a common iron sensing mechanism.

Holo-TF binds to the RGD motif in the TFR2 ectodomain. Importantly, the RGD mutant 
TFR2G679A, unable to bind the ligand(28), is insensitive to the shedding regulation. This 
phenomenon clearly indicates that the shedding process is directly controlled by the receptor-ligand 
molecular interaction and, as previously reported for TFR1, couples the TFR2 shedding process 
with iron deficiency. However, the release of TFR2 does not require TFR1. It is reasonable that the 
shedding of the two receptors occurs separately, since their expression is maximal at different 
maturation stages of the erythroid cells, TFR2 being expressed in more immature erythroid 
precursors than TFRI(16)(17). Also in the liver TfR2 expression increases during mouse 
development in parallel with the decrease of TfR1 expression(8). Furthermore, the cleavage of the 
two receptors is likely operated by different proteases. The consensus cleavage site for PCSK7, the 
proconvertase that cleaves TFR1, is not conserved in TFR2(Figure S6). The observed inhibition of 
TFR2 shedding by the proconvertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone 
(CMK) indicates that a furin-like proconvertase operates the cleavage. However, both furin and
PCSK7, the proteases involved in sHJV(31) and sTFR1(20) cleavage are not involved in TFR2 shedding (Figure S6). Overall, our results indicate that removal of TFR2 from cell surface is not only due to lack of receptor stabilization, but is dependent on an active cleavage process. It is conceivable that membrane stabilization of TFR2 by holo-TF subtracts the receptor to the protease activity by changing its conformation or by hiding the cleavage site. The definition of these molecular mechanisms requires further studies. We cannot exclude that, as previously reported(4), also a decreased endocytosis and TFR2 lysosomal degradation might occur in addition to the reduced shedding in the presence of the ligand.

Different proteins and receptors acquire a distinct function when present either as membrane-bound or soluble forms, as shown for HJV(32),(33),(31). We cannot exclude a dual and opposite role also in case of TFR2. Sensing the increased concentration of the ligand, membrane TFR2 is proposed to be involved in hepcidin activation to reduce the levels of circulating iron(12). Its soluble counterpart might act as an inhibitor to attenuate the signalling pathway, in conditions of reduced iron availability, to increase iron absorption. We consistently showed a modest inhibition of hepcidin promoter activation by sTFR2, but not of endogenous hepcidin in hepatic cell lines (not shown). In addition when iron deficiency is induced in the double knock out Tfr2-/-, Tmprss6 -/- mice hepcidin is inhibited ruling out a significant contribution of sTfr2, that cannot be produced in these animals(34). Thus the decoy function on hepcidin activation is unlikely. However, irrespective of sTFR2 function, removal of membrane TFR2 in iron deficiency would negatively affect hepcidin activation.

An erythroid role for Tfr2 in conditions of low iron has been recently suggested by the observation that iron deficiency caused by ablation of the hepcidin inhibitor Tmprss6 induces erythrocytosis in mice knock out for Tfr2, but not in mice with liver specific Tfr2 deletion (that preserves the Tfr2 erythroid function(34). This event was directly confirmed by the increased erythropoiesis observed in mice that had Tfr2 exclusively deleted in the bone marrow (30).

sTFR2 added as culture media of transfected cells does not seem to modify the affinity of EPO for EPOR or the cell surface expression of the EPOR. Consequently the EPO-mediated signalling appears not to be affected. Preliminary experiments show that the addition of sTFR2 to the culture medium of primary human erythroblasts does not affect their proliferation, survival and terminal differentiation (data not shown). However, irrespective of sTFR2 function, shedding membrane TFR2 in iron deficiency might serve to decrease its interaction with EPOR and to increased EPO sensitivity of erythroid cells, as recently shown in mice with bone marrow specific Tfr2 deletion (30). The lower affinity for holo-TF as compared with TFR1 makes TFR2 an ideal sensor of iron
deficiency in the bone marrow that could anticipate the iron needs for maturing erythroblasts. It is possible that when holo-TF concentration starts decreasing, TFR2 would be shed from the membrane, whereas TFR1, because of its higher affinity, would still be able to bind holo-TF.

In conclusion we provide in vitro evidence of TFR2 shedding, a mechanism regulated by holo-TF, in analogy with the process described for TFR1. Although the in vivo role of sTFR2, as well as that of sTFR1, remains to be clarified, we suggest that while membrane TFR2 in the liver senses excess iron, TFR2 in the bone marrow has the opposite role of sensing iron-deficiency. This would explain why the effect of lack of TFR2 shedding is not evident in TFR2-hemochromatosis where iron-deficiency is never achieved(6),(15).
AUTHORSHIP

Contribution: AP performed experiments, analyzed and discussed data and wrote the manuscript; MV performed experiments, analyzed and discussed data; AN, MR and ML performed experiments and analyzed results; CL, PM and FV design the research and interpreted data; CC and LS design the research, interpreted the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

CONFLICT OF INTEREST: the Authors do not declare any conflict of interest.
REFERENCES


LEGEND TO FIGURES

Figure 1: Identification of a soluble form of TFR2 (sTFR2)

A) HeLa cells were transiently transfected with empty vector (mock), wild type TFR2 FLAG-tagged at the N-terminus (TFR2WT-N-FLAG), wild type TFR2 FLAG-tagged at C-terminus (TFR2WT-C-FLAG) and the artificial sTFR2 (sTFR2*) expressing vectors. Media were replaced 18 hours after transfection with serum-free media. Twenty-four hours later media were collected and concentrated and cells were lysed. Fifty μg of proteins from supernatants and cellular lysates were analysed by western blot, using anti-TFR2 (Abcam) and anti-FLAG antibodies. ♦ = non-specific bands.

B) TFR2 from cell lysates and sTFR2 from supernatant were detected by western blot with an antibody recognizing the C-terminal part of TFR2 (Santa Cruz). Endogenous expression of TFR2 and sTFR2 is shown in UT7 cells (left) and in primary erythroblasts (right). Because human primary erythroblasts cannot survive in albumin-free medium, cell surface biotinylation technique was used to purify sTFR2 from their supernatant.

Lys = total cell lysate, Lys-B = cell lysate pull down with neutravidin agarose beads, Sn= concentrated media, Sn-B = concentrated media pull down with neutravidin agarose beads.

Scales refer to relative molecular mass in kilo Daltons.

Figure 2: sTFR2 is cleaved from plasma membrane

A) HeLa cells were transiently transfected with empty vector (mock) and TFR2WT-C-FLAG coding vector. Eighteen hours after transfection the media were replaced with serum-free media added or not with human holo-transferrin (H-holo-TF, 25 μM) or human apo-transferrin (H-apo-TF, 25 μM). After 24 hours media were collected and concentrated, cells were biotinylated to label membrane protein then lysed. sTFR2 was pull down using anti-FLAG Sepharose-beads. Cellular lysates were precipitated with streptavidin to analyse proteins originating from the cell surface. Total lysates (Lys), biotinylated samples (Lys-B) and concentrated media (Sn) were analysed by western blot using anti-TFR2 (Abcam), anti-FLAG antibody. Anti pan-cadherin, that recognizes a plasma membrane protein, was used to normalize plasma membrane biotinylation and streptavidin pull down.

B) Erythroid cell line UT7 (left panel) and erythroblasts (right panel) were incubated for 24 hours in the presence of 25 μM of human apo- or holo-TF. After biotinylation of cell surface proteins, cell lysates (Lys-B) and supernatant (Sn-B) were precipitated using neutravidin agarose beads. Western blots were performed using an anti-TFR2 antibody (Santa Cruz). Scales refer to relative molecular mass in kilo Daltons.
C) HeLa cells transiently transfected with empty vector (mock) and wild type TFR2 coding vector (TFR2\textsuperscript{WT-N-FLAG}) were treated with brefeldin-A (BFA, 100ng/mL) to block intracellular trafficking and protein export to plasma membrane. After 18 hours cells were incubated with biotin to label membrane proteins and than re-incubated with BFA. Twenty-four hours later media were collected and concentrated, cells lysed (Lys) and both media (Sn-B) and lysates (Lys-B) were subjected to streptavidin pull down and western blot analysis using an anti-TFR2 antibody (Abcam).

D) The cell cultured media and the total lysates of HeLa cells, transiently transfected with wild type TFR2 (TFR2\textsuperscript{WT-N-FLAG}) in combination with Dyn\textsuperscript{WT} and Dyn\textsuperscript{K44A} expressing vectors, were analyzed western blot using anti-TFR2 (Abcam).

E) HeLa cells were transiently transfected with wild type TFR2 (TFR2\textsuperscript{WT-N-FLAG}) and Dyn\textsuperscript{K44A} expressing vectors. After 18 hours cells were incubated with biotin to label membrane proteins and were incubated in serum-free media, treated or not with H-holo-TF (0.5 \(\mu\text{M}\)). After 24 hours media were collected and concentrated and cells were lysed. Both media (Sn-B) and lysates (Lys-B) were precipitated with streptavidin to analyse by western blot proteins originating from the cell surface using an anti-TFR2 antibody (Abcam).

Loading was estimated with anti-\(\beta\)-actin antibody.

The results were confirmed several times in different experiments and western blots of representative ones were shown.

Figure 3. Modulation of sTFR2 release by holo-transferrin

A) HuH7 cells were transiently transfected with empty vector (mock) and wild type TFR2\textsuperscript{WT-N-FLAG} coding vector. Eighteen hours after transfection cells were biotinylated to label membrane protein, incubated in serum-free media and treated with increasing concentrations (0.5, 1.25, 5 and 25\(\mu\text{M}\)) of human diferric-transferrin (H-holo-TF) or human apo-transferrin 25\(\mu\text{M}\) (H-apo-TF) for 24 hours. Cells were then lysed and media concentrated. The supernatant (Sn-B) were precipitated with streptavidin to analyse by western blot proteins originating from the cell surface. Western blots were performed using an anti-TFR2 antibody (Abcam). A densitometric analysis of sTFR2 vs total TFR2 (expressed as % sTFR2) was performed on three independent experiments. The differences of the Holo-TF treatments vs untreated conditions are statistically significant. \(**= p< 0.005\).

B) HeLa cells were transiently transfected with empty vector (mock), TFR2\textsuperscript{WT-N-FLAG} and TFR2\textsuperscript{G679A} coding vectors. Eighteen hours after transfection cells were biotinylated to label membrane protein, were incubated in serum-free media and treated or not with H-holo-TF (5 \(\mu\text{M}\)). After 24 hours cells were lysed, media were collected, concentrated and precipitated with streptavidin. Biotinylated
media (Sn-B) were analysed by western blot technique using an anti-TFR2 antibody (Abcam) to detect the proteins deriving from the cell surface.

Lys= total lysates.

Loading was estimated with anti-β-actin antibody. The results were confirmed several times in different experiments and western blots of representative ones were shown.

**Figure 4: TFR2 shedding is independent of TFR1**

A) HeLa cells were transiently transfected with empty vector (mock) or $TFR2^{WT-C-FLAG}$ vector. Eighteen hours after transfection media were replaced with serum-free media and cells were treated or not for 24 hours with increasing concentrations (0.5, 1.25, 5 μM) of bovine holo-transferrin (B-holo-TF), a ligand specific for TFR2 that does not bind TFR1. Media were collected, concentrated and sTFR2 was pull down using Sepharose-beads anti-FLAG. Immunoprecipitated proteins were analysed by western blot using the anti-FLAG antibody. The results were confirmed in three independent experiments and western blot of representative one was shown.

B) CHO-Trvb-0 ($TFR1$ deficient) cells were transiently transfected with empty vector (mock) and $TFR2^{WT-C-FLAG}$ coding vector. Media were replaced 18 hours after transfection with serum-free media containing or not 1.25 μM of human holo-TF (H-holo-TF), and surface proteins were biotinylated. Media were collected, concentrated and analysed by Western Blot using anti-TFR2 antibody (Abcam). Sn= concentrated media; Lys= total cell lysate, Lys-B = cell lysate pull down with streptavidin-agarose beads.

Loading was estimated with anti-β-actin antibody.

**Figure 5. Effect of sTFR2 in erythroid cells**

A) Scatchard analysis of $^{125}$I erythropoietin (EPO) binding to UT7 cells in the presence conditioned media from sTFR2* (Sn sTFR2*) or empty vectors (Sn mock) transfected HEK293 cells.

B) Growth factor-deprived UT-7 cells were pre-incubated for 1 hour at 37°C with concentrated media from HEK293 cells transfected with empty vector (Sn mock) or sTFR2* (Sn sTFR2*). The cells were then stimulated with 10 U/ml EPO for 10 minutes. Media were concentrated and increasing concentrations of media were added to the UT7 cells. Cellular extracts were analyzed by western blot with anti-p-STAT5, anti-p-AKT and anti-p-ERK antibodies. Anti-STAT5, anti-AKT, anti-ERK were used as loading controls. One representative western blot is shown. ◆ = non-specific band.

C) Western Blots quantification with anti-β-actin antibody is the mean of three independent experiments, SD are shown.
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% CO2). 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-Salpêtrière 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 $\mu$M.

**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 $\mu$l 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 $\mu$l 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$^{WT-C-FLAG}$ 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 $\mu$L of anti-FLAG M2 affinity gel (Sigma-Aldrich) then the resin was resuspended in 20 $\mu$L of Laemmli sample buffer, boiled for 5 minutes and added of 4% v/v $\beta$-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 500$\mu$l 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-TFR2hm (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-β-actin (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
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 TFR2WT 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 (TFR2WT), and treated with the iron chelator deferoxamine (DFO, 100 µM) 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 (TFR2WT-N-FLAG), eighteen hours after transfection media were replaced with serum-starved media and added or not with the iron chelator deferoxamine (DFO, 100 µM) 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 (TFR2WT-N-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 TFR2WT-C-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-β-actin 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-TFR2hm or anti-EPOR antibodies and then analysed by Western blot with anti-Myc, anti-TFR2hm, 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 \textit{TFR2WT-N-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 \textit{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 \textit{HJV} and increasing concentration of \textit{sTFR2*} expressing vectors (10-100 ng). Luciferase activity was detected as described in Material and Methods. In all conditions analysed transfection of \textit{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 \textit{HJV} and \textit{sTFR2*} and treated or not with BMP6 (10 ng/mL). Eighteen hours later luciferase activity was detected as described in Material and Methods. \textit{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 \textit{TFR2WT-N-FLAG}, and \textit{TFR2WT-N-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-β-actin 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$^{WT-N-FLAG}$ 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


SUPPLEMENTARY FIGURES

Figure S1

A

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anti-TFR2

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anti-ßactin

C

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Figure S2

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anti-ßactin
Figure S5

A

Hepatitis promoter luciferase assay
mock Sn mock Sn TFR2
HJV

B

Hepatitis promoter luciferase assay
mock sTFR2
HJV sTFR2

C

Hepatitis promoter luciferase assay
mock sTFR2
HJV sTFR2

D

Hepatitis promoter luciferase assay
HJV BMP6

Figure S6

A

Sn injury
CMK (50 μM)

anti-TFR2
anti-TFR2
anti-β-actin
anti-Furin

B

Sn injury
CMK (50 μM)

anti-TFR2