TFR2, HFE, AND HJV IN THE REGULATION OF BODY IRON HOMEOSTASIS

By

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TABLE OF CONTENTS i

List of Figures ii
Acknowledgements iv
Abbreviations v

Abstract: 1

Chapter 1: Introduction 5
- Abstract and Introduction 6
- Binding partners, regulation, and trafficking of TFR2 9
- Disease-causing mutations in TFR2 12
- Hepcidin regulation 12
- Physiological function of TFR2 15
- Current TFR2 models 18
- Summary 19
- Figure 21

Chapter 2: The cytoplasmic domain of TFR2 is necessary for HFE, HJV, and TFR2 regulation of hepcidin 22
- Abstract 23
- Capsule & Introduction 24
- Materials and Methods 26
- Results 32
- Figures 41
- Discussion 49

Chapter 3: Lack of functional TFR2 results in stress erythropoiesis 53
- Introduction 54
- Materials and Methods 55
- Results 57
- Figures 60
- Discussion 65

Chapter 4: Conclusions and future directions 67

Appendices
- Appendix A: Coculture of HepG2 cells reduces hepcidin expression 71
- Appendix B: $Hfe^{-/-}$ macrophages handle iron differently 80
- Appendix C: Both ZIP14A and ZIP14B are regulated by HFE and iron 91
- Appendix D: The cytoplasmic domain of HFE does not interact with ZIP14 loop 2 by yeast-2-hybris 99

References 105

LIST OF FIGURES

Figure Abstract 1: Body iron homeostasis.

Figure 1.1: Models of Tf/TFR2-induced upregulation of hepcidin transcription.

Figure 2.1: Tfr2, Hfe, and Hjv all participate in Tf-dependent hepcidin expression in hepatocytes.

Figure 2.2: The TFR2/HJV complex is enhanced by HFE.

Figure 2.3: Both HFE and HJV interact with Tfr2 (1-250), but not with Tfr2 (1-117).

Figure 2.4: Truncated TFR2 constructs are able to dimerize.

Figure 2.5: The cytoplasmic domain of Tfr2 is necessary for Tf-sensitivity in hepatocytes.

Figure 2.6: The cytoplasmic domain of Tfr2 is necessary for hepcidin induction in mice.

Figure 2.7: The cytoplasmic domain of Tfr2 is necessary for normalization of iron homeostasis in mice.

Figure 2.8: Tf-sensing requires either intact Tfr2 or a combination of Tfr2ΔCD and Tfr2 245X.

Figure 3.1: Tfr2 mutant mice have enlarged spleens and increased spleen cell numbers.

Figure 3.2: Tfr2 mutant spleens show no differences in hematopoietic subsets or lineage.

Figure 3.3: Tfr2 mutant spleens have mild stress erythropoiesis.

Figure 3.4: Tfr2 mutant mice have a higher reticulocyte count.

Figure 3.5: Red blood cells from Tfr2 mutant mice do not have increased CD81 protein.

Figure A1: Co-culture of HepG2 cells and T6 Stellate cells does not increase hepcidin expression.

Figure A2: Co-culture of HepG2 cells and T6 Stellate cells does not restore Tf-sensitivity.

Figure A3: Co-culture of HepG2 cells with SK Hep-1 reduces hepcidin expression.

Figure A4: Co-culture of HepG2 cells with SK Hep-1 or SK Hep-1 and macrophage-conditioned media reduce hepcidin expression.

Figure B1: Macrophages from *Hfe*$^{-/-}$ mice have higher ferroportin levels.

Figure B2: Macrophages from *Hfe*$^{-/-}$ mice respond differently to Red Blood Cell treatment.

Figure B3: Hepcidin increases in macrophages treated with heme, but not iron.

Figure B4: *Hfe*$^{-/-}$ macrophages respond to heme treatment differently than WT macrophages.

Figure C1: Both ZIP14A and ZIP14B are down-regulated by HFE expression.

Figure C2: Both Zip14A and ZIP14B are regulated by iron.

Figure C3: ZIP14 has some mRNA regulation by iron.

Figure D1: Experimental results of Yeast-2-hybrid..

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LIST OF ABBREVIATIONS

TFR2: Transferrin receptor 2
HFE: Hereditary hemochromatosis protein
HJV: Hemojuvelin
FPN: Ferroportin
ZIP14: Zrt- and Irt-like protein 14
DMT-1: Divalent metal transporter 1
HH: Hereditary Hemochromatosis
Y2H: Yeast-2-Hybrid
BMP: Bone morphogenetic protein
DcytB: Duodenal cytochrome B
Tf: Transferrin
TLR-4: Toll-like receptor-4
IRE: Iron response element
PACs-1: Phosphofurin acidic cluster sorting protein-1
ER: Endoplasmic reticulum
STAT: Signal transducer and activator of transcription
SMAD: SMA + Mothers against decapentaplegic
iSMAD: inhibitory SMAD
pSMAD: phosphorylated SMAD
ActRIIa: Activin type IIA receptor
Alk2 and 3: Activin receptor-like kinase-2 and 3
MHC-I: Major histocompatibility complex-I
WT: Wild type
RBC: Red blood cell
Epo: Erythropoietin
EpoR: Erythropoietin receptor
ProE: Proerythroblast
Ery A,B,C: Erythroblast A,B,C
BFU-E: Erythroid burst forming unit
CFU-E: Erythroid colony forming unit
VCAM-1: Vascular cell adhesion molecule-1
BMPER: BMP binding endothelial regulator
SIH: Salicylaldehyde isonicotinoyl hydrazone
HLA-B7: Human leukocyte antigen-B7

ABSTRACT

Iron homeostasis in the body is a tightly regulated process. While iron is necessary for transport of oxygen through the body, erythropoiesis, and numerous cellular processes, too much iron is toxic to cells. Hereditary hemochromatosis (HH) is a disease that results in the accumulation of iron in the liver, heart, pancreas, and joints leading to diseases such as cirrhosis, heart failure, type II diabetes, and arthritis. HH causes the mis-regulation of body iron homeostasis and results in the absorption of too much dietary iron. Multiple tissues are involved iron homeostasis such as the intestine (which absorbs dietary iron), the liver (which senses the levels of iron in the blood and stores iron), and the macrophages of the spleen and liver (which recycle iron from senescent red blood cells) (Abstract Figure 1). All of these organs work together to ensure that enough iron (20-30mg) is available for daily erythropoiesis, while preventing the accumulation of too much iron. The majority (85-95%) of daily iron need is met by the efficient recycling of red blood cells by spleen and liver macrophages. These macrophages phagocytose senescent red blood cells, break down heme, and transport the resultant iron through the only known iron exporter, ferroportin (FPN), into the bloodstream where it is chaperoned by transferrin (Tf). The remainder of the daily iron need (1-2 mg) is met by absorption of dietary iron and needs to be very tightly regulated. Dietary iron is brought in through the apical side of enterocytes and either transported into the bloodstream on the basolateral side, when body iron is low, or stored in the enterocyte by ferritin when the body has enough iron. Iron that is stored in the enterocyte is usually lost when enterocytes are sloughed off, and excreted. The amount of FPN located on the

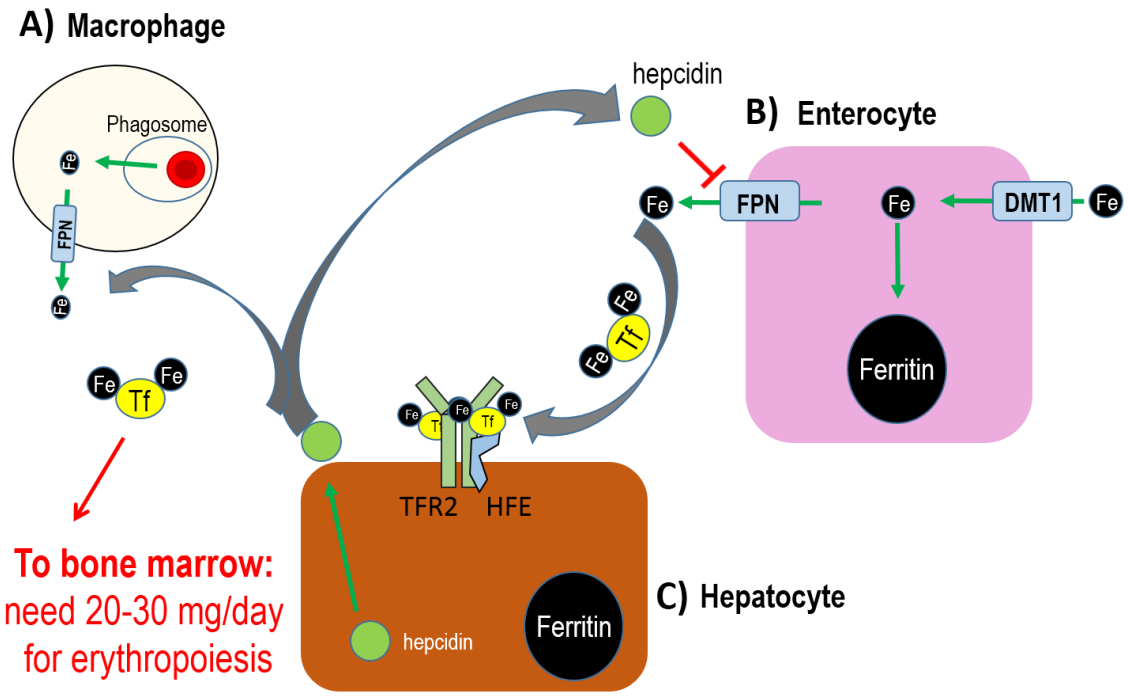
basolateral side of the enterocyte determines how much iron is transported into the bloodstream. FPN protein levels are regulated by hepcidin, a peptide hormone produced by the liver. Hepcidin binds to FPN and induces its internalization and degradation, making hepcidin a negative regulator of body iron uptake.

Hepcidin expression is finely tuned in response to increases in iron-loaded transferrin (Tf-saturation). As the Tf-saturation increases, hepcidin is upregulated through the BMP-signaling pathway. Two proteins that are mutated in HH, HFE (the HH protein) and TFR2 (transferrin receptor 2) are hypothesized to be the Tf-saturation sensors. Mutations in either of these proteins result in loss of Tf-sensitivity, through a reduction in BMP-signaling, though how they intersect with the BMP-signaling pathway is unknown.

In chapter 2 of this thesis we found that TFR2 and HFE interact with the BMP co-receptor, HJV, providing a link between BMP-signaling and Tf-sensing. HFE appears to enhance the interaction between TFR2 and HJV, providing a possible mechanism for the role of HFE in Tf-sensing. In addition, we found that all three members of the complex (TFR2, HFE, and HJV) are required for the sensing of Tf-saturation, indicating the possibility that the complex is involved in this process. Using a series of Tfr2 constructs packaged in adeno-associated virus and injected into Tfr2-deficient mice, we found the cytoplasmic domain of Tfr2 is necessary, indicating that Tf-sensing leads to modulation of BMP-signaling in the liver through intracellular domain interactions involving TFR2. These results provide an important link between Tf-saturation sensing and BMP-signaling, as well as further characterizing the functions of HJV, HFE, and TFR2 in Tf-sensing.

While the primary function of TFR2 appears to be its role in sensing Tf-saturation, work in chapter 3 of this thesis shows that TFR2 is also involved in erythropoiesis. TFR2 expression is limited to the liver and erythropoietic progenitors, and its function in these erythropoietic progenitors is unknown. Work in the lab found that spleens from TFR2 mutant mice were slightly enlarged and that this effect was not due to iron overload or a result of lack of TFR2 in the liver. In this study we show that TFR2 mutant mice show signs of stress erythropoiesis as evidenced by enlarged spleens and increased BFU-e (erythroid burst forming) and CFU-e (erythroid colony forming) assays. In addition, TFR2 mutant mice also have an elevated reticulocyte count, indicating increased erythropoiesis and consistent with stress-erythropoiesis. While a mechanism for the role of TFR2 in stress-erythropoiesis remains elusive, we provide interesting evidence that TFR2 influences erythroid maturation.

In the appendix of this thesis, I further explored Tf-sensing by attempting to use a co-culture system to make a hepatoma cell line Tf-sensitive. In addition I tested the hypothesis that HFE-dependent autocrine secretion of hepcidin by macrophages would reduce FPN levels and increase intracellular iron. I also tested the effect of HFE on two isoforms of ZIP14, an iron transporter, as HFE has an effect on cellular iron loading. This work showcases the complexity of Tf-sensitivity as well as the multi-functional roles of the proteins involved.



Abstract Figure 1: Body iron homeostasis. Different cell types coordinate to ensure proper iron homeostasis. A) 85-95% of iron for erythropoiesis is provided by macrophages, which phagocytose red blood cells, break down the heme, and transport iron into the bloodstream to be chaperoned by Tf. B) The remaining iron is provided by enterocytes, which bring iron through the apical side through the transporter DMT-1. Iron can then be either stored in ferritin or transported through the basolateral side by FPN. C) Iron from either macrophages or enterocytes is loaded onto Tf and can be sensed by TFR2/HFE on hepatocytes, causing an increase in hepcidin expression. Hepcidin can then travel through the bloodstream to reduce FPN expression on both macrophages and enterocytes.

CHAPTER 1

Introduction

The role of hepatic transferrin receptor 2 in the regulation of iron homeostasis in the body.

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Abstract

Fine tuning of body iron is required to prevent diseases such as iron-overload and anemia. The putative iron-sensor, transferrin receptor 2 (TFR2), is expressed in the liver and mutations in this protein result in the iron-overload disease Type III hereditary hemochromatosis (HH). With the loss of functional TFR2, the liver produces about two-fold less of the peptide hormone hepcidin, which is responsible for negatively regulating iron uptake from the diet. This reduction in hepcidin expression leads to the slow accumulation of iron in the liver, heart, joints, and pancreas and subsequent cirrhosis, heart disease, arthritis, and diabetes. TFR2 can bind iron-loaded transferrin in the bloodstream, and hepatocytes treated with transferrin respond with a two-fold increase in hepcidin expression through stimulation of the BMP-signaling pathway. Loss of functional TFR2 or its binding partner, the original HH protein (HFE), results in a loss of this transferrin-sensitivity. While much is known about the trafficking and regulation of TFR2, the mechanism of its transferrin-sensitivity through the BMP-signaling pathway is still not known.

Introduction

Iron is a necessary element for organisms, playing a role in vital processes such as the electron transport chain, the distribution of oxygen throughout the body by hemoglobin, and as a cofactor in numerous enzymatic reactions. Despite its importance, excess iron can be very toxic to the cell. Its participation in the Fenton reaction results in the formation of free radicals, which can wreak havoc by

oxidizing lipids, cleaving proteins, and damaging DNA and RNA. Because of this duality, cells and organisms have evolved exquisite control mechanisms to ensure that the proper amount of iron is present, that excess iron is stored in non-toxic forms, and that within the body iron is chaperoned both outside and within cells.

The body needs 20-30 mg of iron per day for erythropoiesis, the vast majority of this iron is acquired through the efficient recycling of red blood cells by macrophages[17]. The remainder is met by the dietary absorption of ~2mg of iron per day[17]. The iron importer, divalent metal transporter 1(DMT-1) is located on the apical side of enterocytes in the small intestine and transports dietary iron, which has been reduced to ferrous iron by the ferrireductase, Dcytb, into the cell [2, 41, 73]. Once in the cell, iron that is not exported can be stored in the iron storage protein, ferritin, which is a 24-subunit protein with a hollow core that can oxidize and store up to 4500 atoms of ferric iron [58]. Enterocytes are quickly turned over by sloughing into the lumen of the intestine. Thus, enterocyte iron stored in ferritin is lost if not mobilized beforehand. Iron transport into the bloodstream is accomplished through the basolateral iron exporter, ferroportin (FPN)[1, 24, 72], which facilitates the transport of ferrous iron and its subsequent oxidation by hephaestin and loading into transferrin (Tf) the iron transport protein in plasma [77, 111]. This process is at the crux of body iron regulation; amount of FPN on the basolateral membrane determines whether iron is transported into the body and FPN levels are regulated by the peptide hormone, hepcidin.

Hepcidin is regulated in and secreted by hepatocytes in the liver. This 25 amino-acid peptide is the result of two cleavages of the 87 amino acid precursor, a

prepropeptide cleavage of the signal sequence, and a propeptide cleavage by furin [104]. Serum and urine levels correspond to mRNA levels, indicating that in many circumstances hepcidin is regulated at the level of transcription[55]. Serum hepcidin binds FPN and stimulates the internalization and subsequent degradation of FPN, thus making it a negative regulator of total body iron influx[77].

In addition to its role as a master regulator of dietary iron absorption, hepcidin plays a role in immunity. Macrophages can also secrete hepcidin in response to inflammation through the TLR-4 signaling pathway[66, 80]. Increased hepcidin prevents both absorption of iron from the gut, and the release of iron from iron-recycling macrophages, restricting the use of iron by invading pathogens. High levels of hepcidin in diseases of chronic inflammation cause the anemia of chronic disease (ACD). In contrast, abnormally low levels of hepcidin expression leads to iron overload. Genetic mutations that reduce hepcidin expression result in the disease, HH.

Of the four types of HH, three are the result of low levels of hepcidin expression; Type I, (HFE mutation), Type II a & b (hemojuvelin (HJV) and hepcidin mutations respectively), and Type III (TFR2 mutation). Type IV is a result of mutations in ferroportin itself, making it insensitive to hepcidin regulation. Type I & III mutations cause a slow accumulation of iron over the individual's lifetime and a disease phenotype starting in adulthood, while Type II a & b mutations have the more severe phenotype of Juvenile Hemochromatosis. Left untreated, HH leads to iron accumulation in the liver, heart, pancreas, and joints leading to cirrhosis, arrhythmias, diabetes, and arthritis[13, 94, 116]. The slow onset of adult HH clearly

shows that fine tuning of the daily influx of iron is necessary for normal iron homeostasis.

TFR2 is the putative "iron sensor" that fine-tunes this system, based on its ability to bind and be stabilized by iron-bound Tf[48, 49, 88]. In addition, its mutation leads to low hepcidin expression[52, 76], as well as an inability to respond to acute iron-loading[38].

Binding partners, regulation, and trafficking of TFR2

The structure of TFR2 and its known binding partners provide clues to its function. The interesting features of TFR2 start in its structural similarity to the originally characterized transferrin receptor 1 (TfR1). They are both type II membrane proteins that function as a disulfide-linked dimer and share 66% homology in their ectodomain[54]. Both TfR1 and TFR2 bind transferrin and many of the amino acids involved in the binding of TfR1 to transferrin are conserved in TFR2[37]. TfR1 binds iron-loaded transferrin (holo-Tf) with a KD of 1.1nM and is responsible for the endocytosis of holo-Tf into acidic compartments. This iron is then released from Tf, reduced, and transported across the endosomal membrane by the metal transporters DMT-1 and Zip14[22, 86, 117, 125]. While TFR2 is capable of iron-uptake, its binding affinity for Tf is 25-fold less than that of TfR1[54, 117]. This difference in affinity may enhance the role of TFR2 as an iron sensor, allowing it to be sensitive to changes in Tf saturation in the blood. The lower affinity of TFR2 does not seem to diminish its ability to endocytose iron. In TRVb cells lacking both TfR1 and TFR2, transfection of TFR2 increased Tf- mediated ^{55}Fe uptake to similar levels

as transfected TfR1[54]. TfR1 is expressed in many tissues whereas TFR2 expression is limited to the liver and erythropoietic progenitors[97]. The limited expression of TFR2 may explain why deletion of TfR1 is embryonic lethal[64]. While both TfR1 and TFR2 bind and endocytose Tf, their different affinity for Tf and different expression patterns suggest different functions.

Other differences exist which explain the inability of TFR2 to replace TfR1. TfR1 and TFR2 are differentially regulated by iron and holo-Tf. Iron response elements (IRE's) on the 3' TfR1 mRNA account for the rapid turnover of TfR1 mRNA under high iron conditions, which functions to reduce iron import [78]. While TfR1 mRNA levels respond quickly to iron levels it is a relatively stable protein with a turnover of ~24 hours. Therefore, the response of cells to high intracellular iron by downregulation of TfR1 is relatively slow. In contrast, TFR2 lacks the IRE's for the regulation of its mRNA by intracellular iron and at the protein level, turns over much faster. The binding of Tf to TFR2 regulates both its stability and its trafficking within cells[48, 49]. In the presence of holo-Tf, TFR2 levels are increased by redirection of TFR2 to the recycling endosomes, which increases its stability[16, 49, 88]. These differences are the result of very distinct cytoplasmic domains. The TfR1 and TFR2 cytoplasmic domains both have a YXXΦ-based endocytic motif for clathrin-mediated endocytosis, but share little else. In addition to the YXXΦ motif, TFR2 also has a phosphofurin acidic cluster sorting (PACS-1) motif and coprecipitates with the PACS-1 protein[16]. This motif is most likely responsible for the Tf-dependent recycling of TFR2 from endosomes to the cell surface[16]. Human TFR2 is glycosylated at three sites: 240, 339, and 754. This glycosylation is necessary for the Tf-induced

stabilization of TFR2, but does not affect its ability to bind Tf or its trafficking to the cell surface [124]. Despite their structural similarity and ability to bind Tf, the differences in Tf-induced stability and the cytoplasmic domains of TfR1 and TFR2 indicate that they both handle and are affected by Tf differently.

In addition to functional differences in Tf handling, TfR1 and TFR2 appear to interact with the original hereditary hemochromatosis protein (HFE) through alternate domains. TfR1 and HFE interact through the helical domain of TfR1 and the α1 & α2 domains of HFE [7]. Tf and HFE compete with each other for binding to TfR1 because they have overlapping binding sites[36, 37]. TFR2 and HFE interact through the TFR2 stalk region between residues 104 and 250 and the HFE α3 domain[14, 21]. The binding sites of HFE and Tf do not appear to overlap in TFR2[14]. This lends itself to the hypothesis that Tf-binding to TfR1 releases HFE, making it available to functionally interact with TFR2. Coprecipitation studies indicate that TFR2 and HFE interact readily, however, TFR2/HFE interaction remains controversial as coprecipitation of endogenous Tfr2 from liver lysates expressing myc-tagged Hfe did not yield positive results [14, 93]. However, in terms of functionality, it appears that both TFR2 and HFE are needed for Tf-sensing[30]. In addition to the binding of HFE and Tf, a recent report has found an interaction between TFR2 and the BMP co-receptor, HJV, which is an interesting link between the TFR2/HFE complex and BMP-signaling [21]. The ability of HFE, TFR2, and HJV to form a complex in vitro, coupled with the fact that mutations in any one of these proteins causes HH suggests a role for this complex in the regulation of hepcidin.

This is consistent with the different roles of TfR1 and TFR2. TfR1 regulates cellular iron uptake and TFR2 senses iron levels and regulates body iron uptake.

Disease-causing mutations in TFR2

TFR2 mutations result in the disease HH. Unlike HFE HH, which seems to have for the most part risen from a single amino acid HFE mutation and spread throughout Europe, TFR2 HH is far rarer and is the result of various mutations. The first reported TFR2 mutation, the truncation mutant Y250X, was found in two unrelated Sicilian families [10]. Since then, a variety of other TFR2 mutations have been found in Italian patients[8, 35, 68, 83]. Because the most common mutation in HFE is not present in the Japanese population, Japanese patients with HH most frequently have mutations in TFR2[43, 45, 59]. Many mutations in TFR2 identified to date fail to give insight into TFR2 function because most mutations result in misfolded proteins that remain in the endoplasmic reticulum (ER)[113], where they are presumably degraded by the ER quality control pathway. Two interesting point mutations, Q890P and M172K (predicted to disrupt Tf binding and HFE binding respectively)[70, 89], fail to reach the cell surface in analogous mouse mutations (Q685P and M167K)[113]. TFR2 mutations continue to be found in patients around the world[46, 62, 121]

Hepcidin regulation

Hepcidin is a primary regulator of total body iron homeostasis and disease-causing TFR2 mutations cause HH through a reduction in hepcidin transcription.

The transcription of hepcidin is regulated by iron, bone morphogenetic proteins (BMPs), inflammation, hypoxia, and erythropoietic activity. The hepcidin promoter has two BMP response elements (RE), the distal BMP RE2 and the proximal RE1[102, 106]. Within the proximal BMP RE1 lies a STAT3 binding site that is responsible for upregulation of hepcidin in response to inflammation[106]. Interestingly, response of hepcidin to inflammation requires the BMP-binding element in RE1 be intact as well as the STAT binding site, indicating that BMP signaling may be required to keep the chromatin open for STAT binding [12, 115] Within the distal BMP RE2, lies a hepatocyte-specific Hepatocyte Nuclear Factor 4 (HNF4α) binding site as well as bZIP (basic leucine zipper domain) and COUP (chicken ovalbumin upstream promoter transcription factor) motifs indicating that hepcidin transcription relies on a set of transcription factors, including ones that are tissue specific [102]. Stimulation of hepcidin in response to BMP signaling and HJV expression requires that both BMP RE1 and BMP RE2 be intact. The distal BMP RE2 is required for hepcidin response to iron levels[102, 103]. While hepcidin can respond to inflammation and hypoxia, it seems that regulation of hepcidin expression requires BMP-signaling.

While BMP's 2, 4, and 6 are all expressed in the liver and are capable of stimulating hepcidin expression in hepatocytes and hepatoma cell lines, BMP-6 is the only one that is positively regulated by iron [50], and it is expressed mainly in the endothelial cells of the liver [26, 56, 122]. Deletion of BMP-6 results in severe iron-overload, confirming its role in iron homeostasis[3, 74]. BMP-6 also interacts with the BMP co-receptor HJV[3]. This indicates that while other BMP's may have an effect on basal hepcidin expression, it is BMP-6 that is regulated by iron, and functions as a

regulator of iron homeostasis. As of yet, how BMP-6 is regulated by iron is not known. Deletion of BMP-6 has little effect on bone formation, with only a slight delay in sternal ossification[95], indicating that BMP-6 may function primarily as a regulator of iron homeostasis.

BMP-signaling requires binding of BMP's to BMP-receptors. BMP-receptors are arranged as a tetramer of two type I and type II receptors. The type II receptors phosphorylate the type I receptors upon ligand binding, and the phosphorylated receptor can then phosphorylate and activate intracellular receptor-associated SMADs (R-SMADs), which then bind the co-SMAD, SMAD4 to then enter the nucleus and regulate transcription [61, 67, 96, 118]. In the liver, HJV binds to the BMP type II receptor ActRIIa to enhance BMP-signaling and hepcidin expression[119]. Two BMP type I receptors are involved in hepcidin regulation, Alk2 and Alk3 [4, 99, 119]. The Alk3 receptor is necessary for basal hepcidin expression in mice and deletion of Alk3 has a more severe iron-overload phenotype than Alk2, however, Alk2 seems to be necessary for the response of hepcidin to iron and HJV[99]. Therefore, the ligand BMP-6, the BMP co-receptor HJV, and BMP receptors ActRIIa, Alk2, and Alk3 all make up the liver BMP-signaling pathway.

The BMP-signaling pathway can also be modulated by the inhibitory SMADs (iSMADs), SMAD6 and SMAD7. These iSMADs are part of a negative feedback loop and are induced by BMP signaling. They inhibit BMP-signaling by binding to BMP receptors (which inhibits SMAD phosphorylation), recruiting ubiquitin ligases (to induce degradation of the receptors), or they can enter the nucleus and disrupt

binding of phosphorylated SMADs to target genes[44, 51, 123]. In keeping with their role as negative-feedback loop inhibitors of BMP-signaling, SMAD6 and SMAD7 are co-regulated with hepcidin and SMAD7 is upregulated in response to iron[50, 110]. In addition, SMAD7 can modulate signaling by directly binding the promoter region and inhibiting hepcidin expression[75].

Deletion of HFE, HJV, or TFR2 results in a reduction of phosphorylated SMADs 1/5/8, indicating that reduced hepcidin expression is mediated through the BMP-signaling pathway[4, 19, 20]. Presumably, HJV regulates BMP-signaling through enhancing binding of BMP ligand to BMP-receptors and promoting the assembly of the BMP-signaling complex[4]. The severe phenotype of HJV knockout mice and juvenile hemochromatosis patients is in keeping with the important role of HJV as a BMP co-receptor and with the importance of the BMP-signaling pathway in basal hepcidin transcription. HFE and TFR2 mutations are far less severe, indicating that they are involved in fine-tuning of iron levels. How HFE and TFR2 modulate hepcidin expression through the BMP-signaling pathway is not understood.

Physiological function of TFR2

TFR2 has been hypothesized to be the Tf-sensor since the discovery of its disease-causing mutations[10, 28, 90]. Wild type mouse primary hepatocytes, when treated with holo-Tf, will respond within 24 hours by a 2-fold upregulation of hepcidin expression and wild type mice injected with iron will also see this increase in hepcidin levels [52, 65, 84, 85]. This mirrors the ~2-fold increase in urinary hepcidin seen in humans who were challenged with iron and had a corresponding increase in

Tf-saturation[38, 65]. In contrast, Tfr2 mutant mouse primary hepatocytes do not respond to treatment, indicating a role of Tfr2 in Tf-sensitivity[31]. In addition, deletion of the Tfr2 binding partner, Hfe, also results in loss of Tf- sensitivity, indicating that it may be the TFR2/HFE complex that is involved in iron sensing[31]. In human patients with TFR2 HH, urinary hepcidin levels do not respond to iron challenge, and HFE HH patients have a blunted hepcidin response, indicating that both molecules are needed in order to modulate iron uptake in response to dietary iron[38]. The two-fold hepcidin response to holo-Tf in primary hepatocytes is physiological, as HFE HH patients only have a two-fold difference in hepcidin levels and the disease results in the slow accumulation of iron over the lifetime of the individual[105].

While mutations in TFR2 and HFE both result in a slow disease progression, loss of TFR2 appears to be more severe than loss of HFE. There is a reported case of juvenile hemochromatosis resulting from TFR2 mutation and serum hepcidin levels are lower in TFR2 HH patients[38, 76, 81]. Because of the scarcity of TFR2 HH patients in contrast to HFE HH patients, it is hard to compare severity of HFE and TFR2 mutations. Tfr2 mutant mice of the same genetic background as *Hfe*$^{-/-}$ mice have higher iron accumulation than *Hfe*$^{-/-}$ mice[112]. Mice and humans lacking both Tfr2 and Hfe have a more severe phenotype than either single mutation[20, 81, 112], indicating that either one or both proteins may have alternate functions, or that the complex may be able to partially function with one member missing. Transfection of HFE into cell lines that do and do not express TFR2 decreases iron uptake indicating that HFE

almost certainly has another function than that of the TFR2/HFE complex [11, 91, 114]. Other responses that can be attributed to TFR2 remain unknown.

The existence and role of the Tfr2/Hfe complex is not without controversy. While Tfr2 and Hfe immunoprecipitate readily in transfected cells, one report was unable to confirm interaction with endogenous Tfr2 in primary hepatocytes expressing myc-tagged Hfe transgene[93]. In addition, reports differ as to whether Hfe overexpression in Tfr2 mutant mice can increase hepcidin levels and reduce iron accumulation in mice[30, 93]. These results are further complicated by the ability of Hfe to affect iron uptake, as chronic higher iron stores lead to increased BMP-6 expression independently of either HFE or TFR2. BMP-6 expression is not however, dependent on Tf-saturation. Tf-deficient mice have high iron stores, despite being anemic [101]. In keeping with their high tissue iron levels, these mice have increased BMP-6 levels while hepcidin levels are still below normal[5, 6], indicating that Tf is a necessary part of the BMP-signaling pathway that leads to hepcidin expression. Tf-deficient mice that are treated with Tf increase hepcidin expression, however, this increase in hepcidin expression is attenuated when Hjv is also deleted [6], indicating that the hepcidin response to Tf requires HJV. Experiments in isolated primary hepatocytes get around the complication of BMP-6 expression, because BMP-6 is expressed in the endothelial cells. In primary hepatocytes, both Hfe and Tfr2 are needed for a hepcidin response to holo-Tf, indicating that, at least in regards to blood iron-sensing, both proteins are required.

While the mechanism by which TFR2 affects hepcidin expression through the BMP-signaling pathway remains nebulous, its ability to bind Tf and its requirement

for the hepcidin response to holo-Tf makes it likely that TFR2 is the Tf- sensor. That it also interacts with HFE, and that HFE is also required for the response of hepcidin to holo-Tf provides a strong indication that TFR2 senses iron as part of a TFR2/HFE complex, and that this complex formation is important to body iron homeostasis. The further binding of both HFE and TFR2 to the BMP co-receptor, HJV, provides a possible link between the TFR2/HFE Tf-sensing complex and the BMP-signaling complex, and further research is needed to ascertain the functionality of this complex.

Current TFR2 models

The controversy regarding the TFR2/HFE complex, coupled with the new report of a TFR2/HFE/HJV complex lends itself to three possible models for the Tf-sensitive regulation of hepcidin by TFR2 (Figure 1). First, if TFR2 and HFE do not functionally interact, then Tf/TFR2, HFE, and the BMP-signaling complex affect p-SMAD levels independently of one another. Support for this model lies in the increased severity of the Tfr2-Hfe double knockout mouse [112] and the failed interaction of the myc-tagged Hfe transgene with endogenous Tfr2[93]. Second, if reports of TFR2/HFE and TFR2/HFE/HJV interactions are functionally significant, then the TFR2/HFE complex could interact with the BMP-signaling complex upon Tf-binding, thereby affecting pSMAD levels. Support for this model lies in the reports of TFR2/HFE interaction [14, 21, 40], the requirement of both Tfr2 and Hfe for Tf-sensitivity [30, 31], and the recent report of a TFR2/HFE/HJV complex [21]. The third model proposes that HJV interacts with both the TFR2/HFE complex and the BMP-

signaling complex, and both of these complexes affect pSMAD levels independently. While the functional significance of the TFR2/HFE complex or the TFR2/HFE/HJV complex may still be up for debate, TFR2 plays an important role in regulating hepcidin levels in response to holo-Tf through the BMP-signaling pathway.

Summary

TFR2 plays an important role in the fine-tuning of body iron uptake and loss of TFR2 function leads to Type III HH. TFR2 senses changes in blood-iron levels through its interaction with holo-Tf. While it is similar in structure to the iron-endocytosis protein, TfR1, it has a lower affinity for Tf, an alternate binding site for HFE, and is differentially trafficked and regulated. These differences, along with the tissue expression pattern of TFR2, indicate that the function of TfR1 is to bind and endocytose iron for cellular purposes, while the function of TFR2 is to sense blood iron levels. TFR2 is able to regulate body iron uptake in response to blood iron levels by modulating hepcidin expression through the BMP-signaling pathway. The formation and functional significance of the TFR2/HFE complex remains controversial, but both proteins are necessary for Tf-sensitivity. The interaction of TFR2/HFE with the BMP co-receptor, HJV, may provide an interesting link between TFR2, HFE, and the BMP-signaling pathway.

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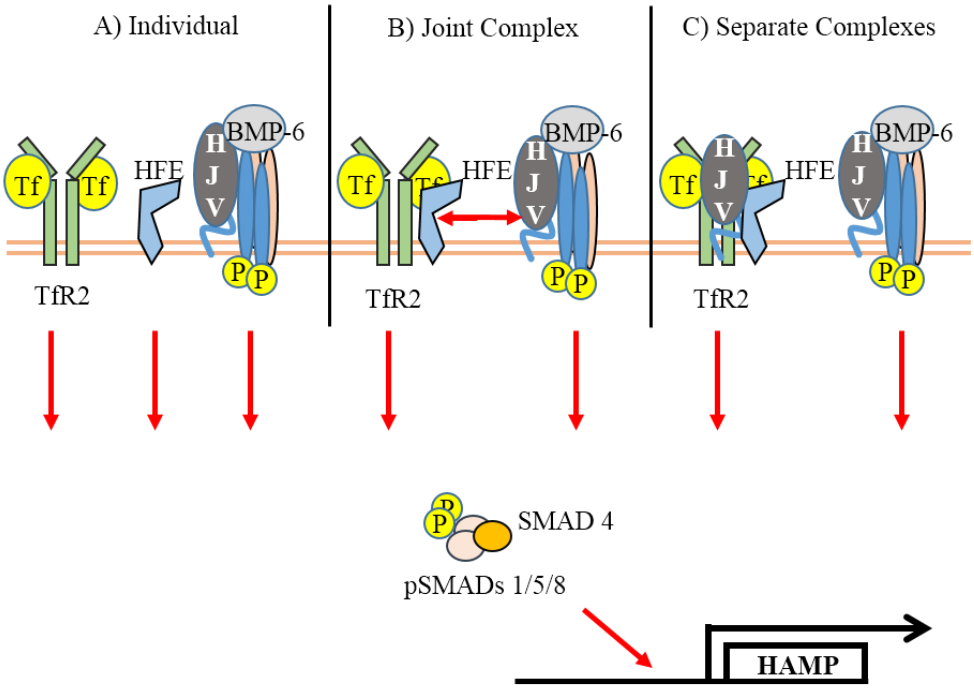

Figure 1: Models of Tf/TFR2-induced upregulation of hepcidin transcription. A) TFR2 and HFE act independently to increase pSMAD induction of hepcidin transcription. B) Tf induces the formation of a large complex between TFR2/HFE/HJV/BMPR's to enhance pSMAD. C) Separate complexes composed of Tf/TFR2/HFE/HJV and HJV/BMP-6/BMPR's signal to enhance pSMAD.

CHAPTER 2

The cytoplasmic domain of TFR2 is necessary for HFE, HJV, and TFR2 regulation of hepcidin

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Running Title: The cytoplasmic domain of TFR2 and the TFR2/HFE/HJV complex

ABSTRACT

Iron homeostasis in the body is regulated in part by the peptide hormone, hepcidin. Hepcidin, in turn, can be regulated through the Bone Morphogenic Protein (BMP)-signaling pathway in liver hepatocytes. The hemochromatosis protein (HFE), and its binding partner, transferrin receptor 2 (TFR2), are postulated to be sensors of iron-bound transferrin (holo-Tf) in the blood, and mutation in either protein results in a reduction in BMP-signaling, lowered hepcidin expression, and the slow accumulation of iron leading to hereditary hemochromatosis. While the link between mutation of TFR2 or HFE and reduced BMP-signaling (through reduction of phosphorylated SMADs 1/5/8) has been established, how the Tf-sensing TFR2/HFE complex affects BMP-signaling is unknown. A recent report found an interaction between TFR2, HFE, and the BMP co-receptor, hemojuvelin (HJV), providing an interesting link between the Tf-sensing complex and BMP-signaling. We wanted to explore the functionality of this complex, and determine how this complex affects BMP-signaling. Using transfection of primary hepatocytes, we determined that all three members of the complex are required for hepcidin response to holo-Tf and that Tfr2 and Hjv play a larger role in signaling than Hfe. Using *Tfr2*$^{245X/245X}$ mice injected with adeno-associated virus, expressing Tfr2 constructs under a hepatocyte-specific promoter, as well as transfection of primary hepatocytes, we show that the cytoplasmic domain of Tfr2 is necessary for Tfr2 function and Tf-sensitivity. The results provide evidence that TFR2, HFE, and HJV are all required for Tf-sensing, and that Tf-sensing is transmitted through the cytoplasmic domain of TFR2.

CAPSULE:

Background: Iron-sensing requires the proteins transferrin receptor-2 (TFR2) and hereditary-hemochromatosis protein (HFE), which alter hepatocyte BMP-signaling through unknown mechanisms.

Results: The TFR2/HFE complex interacts with the BMP-coreceptor, hemojuvelin (HJV). Signaling is through the TFR2 cytoplasmic domain.

Conclusion: The cytoplasmic domain of TFR2 is critical for the response of hepatocytes to transferrin and requires all three complex members.

Significance: HJV may link Tf-sensing and BMP-signaling.

INTRODUCTION

Iron homeostasis within the body is controlled through the uptake of dietary iron, because in mammals the excretion of iron does not appear to be regulated. The ability to sense iron levels in the body is important in maintaining optimal iron homeostasis. Lack of function of key proteins such as HFE, a MHC1-related protein, hemojuvelin (HJV), or transferrin receptor 2 (TFR2) results in the iron overload disease, hereditary hemochromatosis (HH). Most of the evidence shown to date indicates that these membrane proteins participate in regulation of hepcidin, a peptide hormone secreted by hepatocytes. Hepcidin negatively regulates iron levels in the body and plasma by binding to ferroportin (FPN) causing FPN internalization and degradation. FPN is responsible for iron efflux from the basolateral side of enterocytes as well as from macrophages that recycle iron from senescent red blood

cells. Thus hepcidin both decreases iron uptake into the body as well as plasma iron levels[77]. Under normal conditions, the iron in plasma is bound to transferrin (Tf). Hepcidin levels are finely tuned and are responsive to changes in Tf- saturation[38]. The mechanisms involved in this process are not completely understood.

TFR2, which is mutated in type III HH, binds HFE, which is mutated in type I HH[10, 31, 40]. Chronically lower hepcidin expression and the inability to respond to an acute influx of iron are characteristics of these forms of HH[52, 85]. In addition, primary hepatocytes from *Hfe*$^{-/-}$ and Tfr2 deficient mice do not upregulate hepcidin in response to iron-bound Tf (holo-Tf) compared to wild type (WT) primary hepatocytes[31, 65]. TFR2 binds holo-Tf [54, 117], is stabilized by holo-Tf [48, 49], and is required for hepcidin response to holo-Tf [31], making it the likely iron sensor. The TFR2/HFE complex could be the Tf-sensing complex both because they interact with each other and because they are both required for Tf-sensing[31]. The current hypothesis is that TFR1 can sequester HFE, and upon Tf binding, HFE is released, making it available to bind TFR2 and form the Tf-sensing complex. The function of the complex is controversial because the interaction has not been detected in vivo in mice [93] and evidence in mice shows that mutations or knockout of both genes result in a more severe phenotype than the single mutations[112]. Although Tf-sensing only results in a 2-4 fold increase in hepcidin levels in response to acute iron, these changes are physiologically relevant because they directly translate to decreased iron absorption, making hepcidin regulation important in normal iron homeostasis.

Hepcidin is transcriptionally regulated through the canonical bone morphogenetic protein (BMP)-signaling pathway via phosphorylation of SMADs

1/5/8[102, 106]. Mutations in either TFR2 or HFE reduce phosphorylated SMAD levels, leading to lowered hepcidin levels[19, 20]. While the effect of TFR2 or HFE mutation is reduced BMP-signaling[19, 20], how they accomplish this is unknown. A recent report showed an interaction between the TFR2/HFE complex and the BMP co-receptor, HJV, in transiently transfected cells[21]. This provides an interesting link between Tf-sensing and BMP-signaling but does not address the physiological significance of the interaction. In order to determine whether the TFR2/HFE/HJV interactions could play a functional part in Tf-sensing, we used primary hepatocytes to study the role of all three proteins in Tf-sensing. We found that all three proteins were necessary for the response of hepcidin to Tf. In addition, we studied the role of the cytoplasmic domain of Tfr2 both in primary hepatocytes and in vivo. We found that the cytoplasmic domain of Tfr2 is necessary for the stimulation of hepcidin expression, leading to the conclusion that Tf-sensing is transmitted through the membrane to the cytoplasmic domain of Tfr2, where it increases BMP-signaling.

MATERIALS AND METHODS

Reagents and Antibodies- Rabbit anti-hTFR2 was used in immunoblots as described previously [48], as well as for immunoprecipitation of TFR2. M2 Anti-FLAG (Sigma, St Louis) 100 ng/mL, rabbit anti human-c-myc antibody 0.2 ng/mL (sc-789 Santa Cruz Biotechnology, Santa Cruz). Anti-mouse Trueblot HRP (Rockland, Gilbertsville), anti-Rabbit Trueblot-HRP (Rockland, Gilbertsville), anti-rabbit Alexa Fluor 680 (Life Technologies, Grand Island). Rabbit polyclonal antibody to mouse Tfr2 (25257) was generated using the Tfr2 ectodomain. Holo-transferrin (human plasma, low

endotoxin) was used to treat hepatocytes (Calbiochem Cat# 616424). Chemiluminescence was detected using Pierce ECL western blotting substrate (Thermo, Waltham) and X-ray film (Midsci, St Louis). Fluorescence was detected using LI-COR fluorescence imaging apparatus (LI-COR Inc.). Collagen was used at 0.5 mg/mL (BD Biosciences, San Jose). Streptavidin-agarose (Solulink, San Diego) was used to bind biotinylated cell-surface proteins (EZ-Link Sulfo NHS-SS-Biotin, Thermo, Waltham).

Constructs- The generation of plasmid pcDNA3.1 encoding human TFR2 was described previously[48]. The plasmid, pCMV-9 3XFLAG-HJVwith an N-terminal triple FLAG tag was a gift from Dr. Jodie Babitt at Harvard University. The generation of pcDNA3.1 HFE-FLAG was described previously[14]. TFR2 1-250-mycX was cloned into pcDNA 3.1 using primers encoding a c-terminus myc-tag and a stop codon and HindIII/NotI cut sites. TFR2 1-117-mycX was cloned in to pcDNA3.1 using primers containing BamHI/NotI cut sites as well as a myc tag and stop codon. HFE-myc was created using pCDNA3.1 HFE as a template and Quikchange lightning to insert the myc tag at the C-terminus. MN502A1encoding eGFP was cloned by amplification from eGFP pCDNA3.1 with primers containing XbaI/EcoRI cut sites and ligated into MN502A1. FLAG-Hfe MN502A1 was generated using pCDNA3.1 mouse Hfe-FLAG is a template[30], and PCR primers with XbaI/EcoRI cut sites and ligated into MN502A1. Tfr2 MN502A1 plasmid was generated using pCDNA3.1 mTfr2 [30] as a template and primers with XbaI/EcoRI cut sites and ligated into MN502A1. Tfr2 ΔCD, Tfr2 245X, and Tfr2 117X MN502A1 all used pcDNA3.1 mTfr2 as a template and used primers with XbaI/EcoRI cut sites to ligate into MN502A1. Primers for Tfr2 250X and Tfr2

117X also had a stop codon in the primer sequence. All MN502A1 plasmids were induced to form minicircles as per manufacturer's protocol (System Biosciences minicircle DNA vector technology).

The generation of full-length Tfr2 AAV2/8 was described previously[30]. Y23A Tfr2 was made using site-directed mutagenesis of pCDNA3.1 Tfr2 and the following primers: For: 5'-ccctctcagaccatcgccagacgcgtggaagg-3' and Rev: 5'-ccttccacgcgtctggcgatggtctgagaggg-3'. Construct was then amplified by PCR to generate a 5' KPNI site and a 3' NHEI site using the following primers: For: 5'-aataaggtaccatggagcaac-3' and Rev: 5'-ataatgctagctcaaaagttat-3' and cloned into the AAV2/8 vector. The Tfr1/Tfr2 chimera was generated by inserting a HindIII site between the Tfr2 cytoplasmic domain and the transmembrane domain using the following primers: For: 5'-gcagcaggtcgaaagcttaaggctgccccc-3' and Rev: 5'-gggggcagccttaagctttcgacctgctgc-3'. The Tfr1 cytoplasmic domain was amplified by PCR with HindIII sites at each end using the following primers: For: 5'-aataaaagcttatgatggatcaagccagatc-3' Rev: 5'-atattaagctttcttccattaaacctcttggg-3'. The construct was then amplified with a 5' KPNI site and a 3' NHEI site using the following primers: For: 5'-aataaggtaccatgatggatc -3' and Rev: 5'-ataatgctagctcaaaagttat-3' and cloned into the AAV2/8 vector. The ΔCD Tfr2 construct was generated by inserting a HindIII site and a start site just before the transmembrane domain using the following primers: For: 5'-ctgggctgcagcaggtaagcttatgcgaaaggctgcc-3' and Rev: 5'-ggcagcctttcgcataagcttacctgctgcagcccag-3'. The construct was then amplified using primers encoding a 5' KPNI site and a 3' NHEI site using the following primers: For:

5'-aataaggtaccatggagcaac-3' and Rev: 5'- ataatgctagctcaaaagttat-3' and cloned into the AAV2/8 vector. The previously described AAV2/8 vector contains a thyroid-hormone globulin gene hepatocytes-specific promoter and 2 copies of the α1-microglobulin/bikunin enhancer sequence[30]. Virus was produced at the OHSU viral core.

Cell Culture- TRVb cells were grown in F-12 media supplemented with 2 mg/mL dextrose and 5% FBS. Primary hepatocytes were isolated using a two-step perfusion process and type I collagenase (Worthington Biochem, Lakewood) as described previously[31]. Hepatocytes were filtered through a nylon mesh (100 μM) and washed 2X in DMEM 10% FBS and centrifuged at 200 x g for 3 mins. The hepatocytes were resuspended in DMEM 10% FBS then seeded in collagen-coated 12-well plates at 250,000 cells/well. After 2-4 hours, the medium was changed to hepatocyte maintenance media supplemented with Singlequot and EGF (Lonza, Basel). The next morning, hepatocytes were transfected. Four hours after transfection, holo-Tf (2mg/mL) was added to cells and hepatocytes were then harvested 24 hrs. later.

Transfections- TRVb cells were transfected using 3 μg DNA and 9 μL FugeneHD in a 6-well dish per manufacturer's protocol. Primary hepatocytes were transfected using 1 μg minicircle DNA, 1 μL PLUS reagent, and 4 μL Lipofectamine LTX in a 12-well dish per manufacturers protocol.

Immunoprecipitations- Sigma anti-FLAG M2 affinity gel was used to isolate constructs containing FLAG. Myc constructs were isolated using Sigma EZview red anti-c-myc affinity gel (Sigma, St. Louis). TFR2 was immunoprecipitated using rabbit

anti-hTFR2 and protein A agarose beads (Thermo, Waltham). All beads were prewashed three times with NETT (20mM Tris, 100mM NaCl, 1mM EDTA, 0.5% Triton X100) and incubated with cell lysates from one 6-well for 2 hours at 4°C. The beads were washed three times with 1mL NETT. Pellets were then heated to 95°C in 2X-Lamelli buffer (10% SDS, 1M Tris-Cl, 0.02% Bromophenol Blue, 5% BME) for 5 min and centrifuged. The resultant supernatants were subjected to denaturing SDS-polyacrylamide gel electrophoresis SDS-PAGE gels.

Biotinylation experiments- Transfected cells in a six-well plate were washed 3X with ice-cold PBS+ (PBS, 1mM $MgCl_2$, 0.5mM $CaCl_2$) and incubated in 2mL for 30 minutes in PBS, 0.25mg/mL EZ-Link Sulfo NHS-SS-Biotin while rocking on ice. Wells were then washed 5X with 2mL of quench solution (PBS, 100mM Glycine). Cells were then lysed using NETT buffer and lysates were then incubated for 2 hours with 50µL streptavidin beads. Pellets were then heated to 95°C in 2X-Lamelli with 2-mercaptoethanol for 5 min and centrifuged. The resultant supernatants were then run on SDS-PAGE gels.

Mice- *Hfe*$^{-/-}$ mice and *Hjv*$^{-/-}$ mice were on the 129/SvEvTac background. TFR2$^{245x/245x}$ mice were on an FVB/NJ (FVB) background. Both mutant and wild-type mice were bred and maintained in the Laboratory Animal Facility of Oregon Health & Science University. Eight-week-old male mice were injected with 2×10^{11} genome equivalents/mouse or with 5×10^{11} genome equivalents/mouse by intraperitoneal injection. Two weeks later, they were euthanized using mouse cocktail (ketamine 7.5 mg, xylazine 1.5 mg, and acepromazine 0.25 mg/mL). Blood was collected by

cardiac puncture. Serum was collected from clotted samples after 24 hours at 4° and centrifugation at 4°C. Liver tissue was harvested and stored in liquid nitrogen for assays.

Iron analysis- Non-heme iron, serum iron and Tf-saturations were measured as described previously[30].

qPCR- RNA was harvested and extracted using Trizol reagent (Life Technologies, Carlsbad) per manufacturer's protocol. RNA was DNAse treated using recombinant DNAse and then re-precipitated and washed with 70% ethanol. cDNA was made with 2 μg mRNA using MMLV-reverse transcriptase kit (Invitrogen) per manufacturer's protocol. qPCR was then performed using SYBER-green and validated primers on an ABI ViiA7 qPCR machine. For Tf-treated primary hepatocytes, all experiments were checked for expression of IL-6. Experiments with detectable levels (less than 30 cycle numbers by qPCR) of IL-6 were discarded. Verified qPCR primers were as follows: (hepcidin, For: 5'-caccaacttccccatctgcatcttc-3' and Rev: 5'- gaggggctgcaggggtgtagag-3'), (Hfe, For: 5'-tctgggacagcaagtgcctac-3' and Rev: 5'-ggcatccagtggttggttgt-3'), (Id1, For: 5'- accctgaacggcgagatca-3' and Rev: 5'-tcgtcggctggaacacatg-3'), (Tfr2, For: 5'-gagttgtccaggctcacgtaca-3' and Rev: 5'-gctgggacggaggtgactt-3'), (Hjv, For: 5'-tctgtggacaggctcactccc-3' and Rev: 5'-gccgcgtgcagagagcgta-3'), and (actin, For: 5'-ctgcctgacggccaggt-3' and Rev: 5'-tggatgccacaggattccat-3').

RESULTS

Tfr2, Hfe, and Hjv all participate in Tf-dependent hepcidin expression in hepatocytes- We wanted to determine what proteins were involved in the sensing of iron levels in the body and the transferring of this information to regulate hepcidin expression. Lack of functional HFE, HJV, or TFR2 results in low hepcidin levels leading to the iron overload associated with HH. Primary hepatocyte cultures were chosen because they respond to holo-Tf[52, 65, 84] . Isolated hepatocytes afford the opportunity to examine Tf-sensing, independently of changes in BMP6, which is also sensitive to iron and expressed in other cell types in the liver [26, 56, 122].Hepatocytes were isolated from WT, *Hfe*$^{-/-}$, *Hjv*$^{-/-}$, and *Tfr2*$^{245X/245X}$ mice and hepcidin expression was measured by qPCR after 24 hrs. in the absence or presence of holo-Tf. WT hepatocytes of either FVB/NJ or 129/SvEvTac strains of mice responded to Tf with a 2-4 fold increase in hepcidin expression as previously reported [31], while *Hfe*$^{-/-}$ and *Tfr2*$^{245X/245X}$ hepatocytes did not (Figure 2.1A). Interestingly, hepatocytes from *Hjv*$^{-/-}$ mice also were unable to respond to Tf-treatment, indicating that Hjv may be necessary for Tf-sensing (Figure 2.1B). Since deletion of any one of these three proteins results loss of Tf-sensitivity, we tested whether over expression of one of the proteins could compensate for the loss of another. In *Hjv*$^{-/-}$ hepatocytes, only transfection of Hjv increased basal levels of hepcidin and rescued Tf-sensitivity (Figure 2.1C). Similarly, only transfection of Hfe into *Hfe*$^{-/-}$ hepatocytes increased basal hepcidin levels and showed Tf-sensitivity (Figure 2.1D). This indicates that all three proteins are necessary for Tf-sensitivity.

Earlier studies indicated that Hfe might be a limiting protein in the sensing of iron[30]. Primary WT hepatocytes were transfected with plasmids encoding Tfr2, Hjv, Hfe, or enhanced green fluorescent protein (GFP) as a control (Figure 2.1D). The expression of Tfr2 in WT mice increased Tfr2 protein levels (Figure 1B-D). Unfortunately endogenous levels of Hfe and Hjv were not detectable in the primary hepatocytes (results not shown). Tfr2 transfection could be detected by western blot, as well as Hjv (Figure 2.1B-D), and its cleaved form could be detected when expression levels were high (Figure 2.1C, D). Unfortunately, Hfe-FLAG was unable to be detected by western blot in primary hepatocytes, although qPCR data showed it was expressed (data not shown). In WT primary hepatocytes, overexpression of Tfr2 did not affect hepcidin levels (Figure 2.1D). Overexpression of either Hjv or Hfe increased basal levels of hepcidin, however, overexpression of Hfe resulted in the loss of Tf-sensitivity (Figure 2.1D). These results indicate that Hjv may be limiting, and that Tf-sensitivity may rely on the stoichiometry of Tfr2, Hfe, and Hjv.

Since all three members appear to be necessary, hepcidin expression might be induced by overexpressing two or more of the proteins simultaneously in WT primary hepatocytes. Hepatocytes were co-transfected with combinations of Hfe, Hjv and Tfr2 to test this hypothesis. Co-transfection of *Hfe* and *Hjv* resulted in a 5-fold increase in basal hepcidin levels and loss of Tf-sensitivity, while co-transfection of Tfr2 and Hjv resulted in a 10-fold increase in basal hepcidin levels and maintenance of Tf-sensitivity (Figure 2.1E). This indicates that increased levels of theTfr2/Hjv protein complex upregulate signaling while still maintaining Tf-sensitivity. Transfection of all three constructs increased basal hepcidin levels 13-

fold and resulted in a loss of Tf-sensitivity (Figure 2.1E). Taken together, transfection of primary hepatocytes shows that all three proteins participate in Tf-sensitivity and that the stoichiometry of these molecules may have a functional purpose.

The TFR2/HJV interaction is enhanced by HFE- A possible explanation for the necessity of all three proteins being responsible for Tf-dependent stimulation of hepcidin expression may be that they form a complex. This possibility was tested in a Chinese hamster cell line, TRVb, that does not contain functional or detectable transferrin receptors[71]. TRVb cells were transiently transfected with TFR2, HFE-myc, and 3X FLAG-HJV. C-terminal epitope-tagged Hfe and 3XFLAG-Hjv are biologically active in mice (unpublished data)[30]. Both HFE and HJV co-precipitate with TFR2 in triply transfected cells. Anti-TFR2 does not immunoprecipitate either HJV or HFE in the absence of TFR2 demonstrating the specificity of the antibody. In a reciprocal immunoprecipitation TFR2 and HFE co-precipitate with HJV (Figure 2.2A). Since HFE is known to interact with TFR2[14], we tested whether HFE interacts with HJV in the absence of TFR2. HJV co-precipitates with HFE in the absence of TFR2 (Figure 2.2B). TFR2 interacted with HJV in the absence of HFE (Figure 2.2B). Even though TFR2 interacted with HJV in the absence of HFE, more TFR2 was immunoprecipitated by HJV with the expression of HFE (Figure 2.2C). Taken together, the co-precipitation data shows that TFR2, HJV, and HFE all form a complex, each protein is capable of interacting with one another, and the TFR2/HJV complex is enhanced by HFE binding. The increase in TFR2 immunoprecipitation by HJV after HFE transfection is particularly interesting because it ascribes a possible

function to HFE; release of HFE from TfR1 could make HFE available to form a complex between TFR2 and HJV and promote signaling.

Both HFE and HJV interact with truncated TFR2 (1-250), but not TFR2 (1-117) and truncated TFR2 constructs are able to dimerize- We used truncated versions of TFR2, TFR2 (1-250) and TFR2 (1-117) (Figure 2.3A), to determine if these residues were also important for the binding of HJV. As expected, FLAG-HFE was able to immunoprecipitate TFR2 (1-250), but not TFR2 (1-117) (Figure 2.3A). Interestingly, 3XFLAG-HJV was also able to immunoprecipitate TFR2 (1-250) and not TFR2 (1-117) (Figure 3B). These results indicate that TFR2 binds both HFE and HJV in a similar region spanning from the transmembrane domain to residue 250 (104-250).

TFR2 is a disulfide-bonded dimer[54], and TFR2 has a similar domain to that of the dimerization domain present in TfR1[54]. The truncated TFR2 constructs (TFR2 (1-250) and TFR2 (1-117)) do not contain this dimerization domain but they do contain the cytoplasmic domain, the transmembrane domain and a portion of the ectodomain that contains intersubunit disulfide bonds. To test whether full length TFR2 could interact with the truncated versions of TFR2, TRVb cells were transiently transfected with full-length TFR2 and truncated TFR2 constructs. Full length TFR2 was able to co-precipitate with both truncated versions of TFR2, indicating the presence of a dimerization domain in cytoplasmic domain, the transmembrane, or short ectodomain domain of TFR2 (Figure 2.4A). We then looked for evidence of dimerization by the formation of intersubunit disulfide bonds on a non-reducing gel. On a reducing gel, TFR2 (1-250) migrates as two bands

running at roughly 40 and 35 kD and three bands at roughly 95, 75, and 65 kD on a non-reducing gel (Figure 2.4B) indicating that TFR2 (1-250) is able to form intersubunit disulfide bonds. TFR2 (1-117) runs at 15 kD on a reducing gel and 25 kD on a non-reducing gel (Figure 2.4B), indicating that TFR2 (1-117) is also capable of dimerization. Co-transfection of full-length TFR2 and TFR2 (1-250) resulted in the appearance of three bands consistent with dimerization (Figure 2.4C). Likewise, co-transfection of full-length TFR2 and TFR2 (1-117) resulted in the appearance of a band around 110 kD, consistent with dimerization (Figure 2.4C). Taken together, these results indicate that TFR2 contains an additional dimerization domain and that truncation mutants are still able to dimerize.

The cytoplasmic domain of Tfr2 is necessary for hepcidin induction in hepatocytes- The TFR2/HJV/HFE complex could function to sense Tf through extracellular interactions (affecting assembly of the BMP-HJV complex or altering the cell-surface amount of HJV) and/or intracellular interactions that could indirectly affect SMAD-signaling. HJV is a BMP co-receptor that binds BMP and facilitates the binding of BMP to BMP receptors[4]. HJV is subjected to regulation by matriptase 2, a protease that cleaves and releases HJV from the cell surface thereby downregulating hepcidin expression. We initially examined whether altered Hjv cell surface levels or changes in Hjv cleavage after Tf-treatment occurred, and were unable to detect any differences (data not shown). A complex could function by binding to the BMP receptors and increase signaling. Alternately, the binding of Tf to a complex could transmit a signal across the membrane. The fact that HJV is a GPI-linked protein with no cytoplasmic domain[60], and HFE lacking its cytoplasmic

domain can increase hepcidin expression[92], led us to examine the role of the cytoplasmic domain of TFR2 in the stimulation of hepcidin expression. A series of mutated Tfr2 constructs were made. The Y23A Tfr2 mutation eliminated the motif responsible for clathrin-mediated endocytosis of Tfr2[48]. The ΔCD mutation eliminated the cytoplasmic domain of Tfr2, and the Tfr1/Tfr2 chimera substituted the cytoplasmic domain of Tfr2 with the cytoplasmic domain of Tfr1. Previous data indicated that in human cell lines, once endocytosed, TfR1 recycles to the plasma membrane whereas TFR2 is directed to the multivesicular body along a degradative pathway[48]. Furthermore, the TfR1/TFR2 chimera was stable and acted similarly to TfR1 in its transit within the cell[15]. WT Tfr2 and Y23A Tfr2 transfected into hepatocytes were able to rescue Tf-sensitivity, while Tfr1/Tfr2 and ΔCD Tfr2 were not (Figure 2.5A), indicating that the cytoplasmic domain of Tfr2 is necessary for transmitting the Tf signal. The protein levels of the different forms of Tfr2 were similar to or above that of the WT Tfr2 (Figure 2.5B), demonstrating that lack of expression was not the cause of lack of signaling. These results also indicated that endocytosis mediated by Tfr2 was not necessary because the mutated endocytic signal for Tfr2 was equally capable of sensing Tf and substitution of a functional Tfr1 cytoplasmic domain with an endocytic signal was not sufficient to restore signaling.

The cytoplasmic domain of Tfr2 is necessary for hepcidin induction in mice- AAV2/8 (AAV) encoding WT Tfr2, Tfr2 with a deleted cytoplasmic domain (ΔCDTfr2), a Tfr1/Tfr2 chimera with a Tfr1 cytoplasmic domain and Tfr2 transmembrane and extracellular domains (Tfr1/Tfr2), or a Tfr2 endocytosis

mutant (Y23A Tfr2) were used to test the role of the cytoplasmic domain of Tfr2 in *Tfr2*$^{245X/245X}$ mice and to determine their roles in affecting iron levels in vivo. The constructs were expressed in hepatocytes using AAV with a hepatocyte-specific promoter as described previously[30]. After two weeks mice were sacrificed. The expression of WT Tfr2 increased hepcidin expression similar to that of WT mice (Figure2. 6A). Expression of Y23A Tfr2 was also able to increase hepcidin expression (Figure 2.6A), indicating that Tfr2-mediated endocytosis is not necessary for induction of hepcidin. Expression of Tfr1/Tfr2 or ΔCD Tfr2 were unable to rescue hepcidin expression (Figure 2.5A), indicating that specifically, the Tfr2 cytoplasmic domain is necessary for Tfr2 function and the intact cytoplasmic domain of Tfr1 does not substitute. Id1 mRNA levels, a downstream indicator of BMP-signaling mirrored those of hepcidin (Figure 2.6B), indicating that this response correlated with BMP-signaling. The protein levels of the Tfr2 constructs were at least as high as the WT Tfr2 construct, which was sufficient for restoring hepcidin expression, except for ΔCD Tfr2 (Figure 2.6C&D). To ensure that the inability of ΔCD Tfr2 to rescue hepcidin expression was not due to inadequate protein expression levels, the amount of virus injected was increased from $2x10^{11}$ to $5x10^{11}$ genome equivalents/mouse. A higher level of ΔCD Tfr2 was still unable to rescue hepcidin expression to the same extent as WT Tfr2 or the Y23A Tfr2 (Figures 2.6E-G). Since hepcidin mRNA increased, we tested whether the constructs were able to normalize liver iron levels, Tf-saturation, and serum iron levels within two weeks. WT Tfr2 and Y23A Tfr2 were able to lower iron in the liver, Tf-saturation, serum iron, and total iron binding capacity (TIBC) while Tfr1/Tfr2 and ΔCD Tfr2

were not (Figure 2.7). Taken together, these results indicate that the cytoplasmic domain of TFR2 is necessary for signaling but the endocytic motif within the cytoplasmic domain is not necessary.

Tf-sensing requires either intact Tfr2 or a combination of Tfr2ΔCD and Tfr2 245X- The dimeric nature of Tfr2 led us to test whether the ecto-domain of Tfr2 (Tfr2ΔCD) could functionally interact with Tfr2 245X to sense Tf. In *Tfr2*$^{245X/245X}$ mice, truncated Tfr2 245X acts as a null mutation. Tfr2 mRNA is 32-fold lower than in WT mice, presumably due to nonsense-mediated decay. Consistent with the low mRNA levels, Tfr2 245X protein cannot be detected[27]. Tfr2 245X is analogous to the TFR2 1-250 truncation in humans, which causes the most common form of HH that is due to mutations in TFR2. Since Tfr2 245X is still capable of binding both Hfe and Hjv, we tested whether Tfr2 245X was capable of rescuing Tf-sensing in *Tfr2*$^{245X/245X}$ mouse primary hepatocytes, using a minicircle vector encoding Tfr2 245X without the remaining Tfr2 message that could be responsible for nonsense-mediated decay. Tfr2 245X did not rescue Tf-sensitivity (Figure 8A) but the protein could be detected (Figure 2.8A), indicating that Tf-binding to Tfr2 is likely necessary for Tf-sensing. Interestingly, co-transfection of Tfr2 245X and ΔCD Tfr2 resulted in rescue of Tf-sensitivity (Figure 2.8A), indicating that dimerization between these two non-functional truncation mutants restores Tfr2 function. Western blots indicated that transfection of Tfr2 245X alone resulted in detectable protein, however Tfr2 245X levels were higher with the co-transfection of ΔCD Tfr2, indicating that dimerization with Tfr2 245X may stabilize Tfr2 245X protein (Figure 2.8B). The analogous human mutation TFR2 250X does not traffic to the cell surface[113]. In order to determine

whether ΔCD Tfr2 facilitated the appearance of Tfr2 245X at the cell surface, TRVb cells transfected with ΔCD Tfr2 and Tfr2 245X were cell-surface labeled with a non-membrane permeable biotinylation reagent. Co-transfection of both constructs resulted in both the increased levels of Tfr2 245X, and an increased percentage of Tfr2 245X at the cell surface (Figure 2.8C). In addition, cotransfection of ΔCD Tfr2 and Tfr2 245X decreased ΔCD Tfr2, consistent with the endocytic sequence on the cytoplasmic domain of Tfr2 245X being responsible for its internalization and degradation (Figure 2.8C). Taken together, these results indicate that Tf-sensing requires both the ecto-domain and the cytoplasmic domain of TFR2, and that this signal can be transmitted in trans by the dimerization of ΔCD Tfr2 and Tfr2 245X.

FIGURES

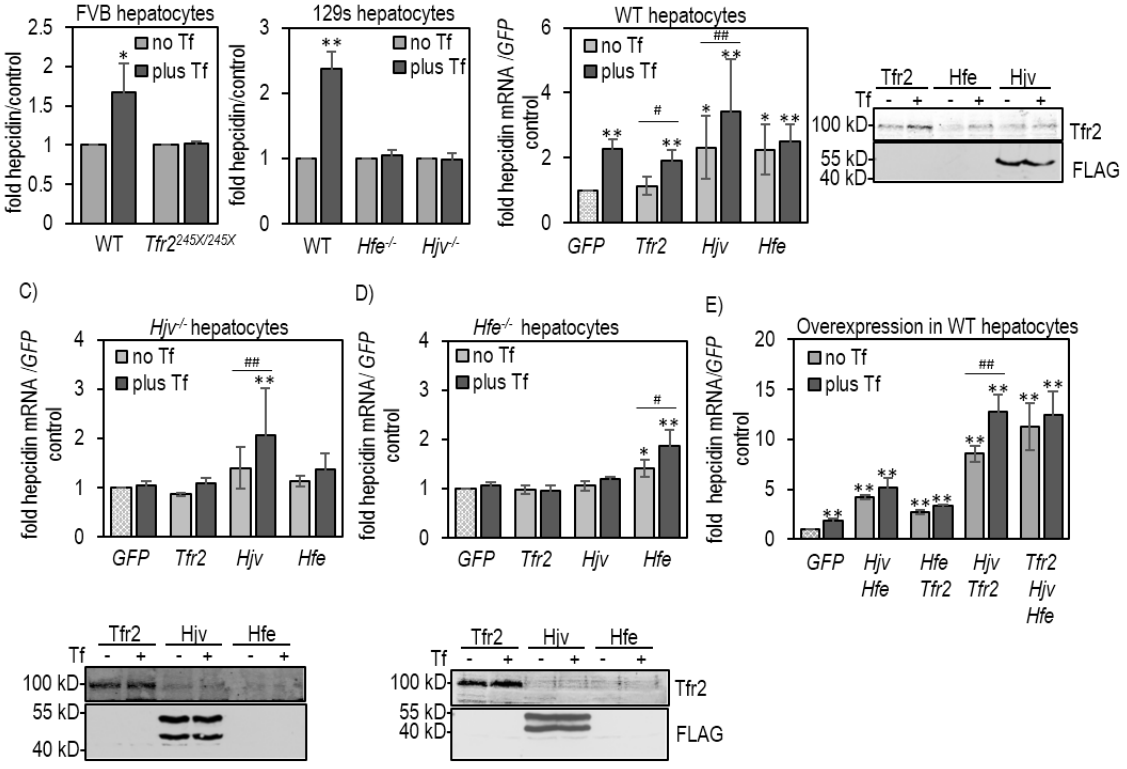

Figure 2.1: Tfr2, Hfe, and Hjv all participate in Tf-dependent hepcidin expression in hepatocytes. Primary hepatocytes (n=5 mice) were isolated and transfected as described in Experimental Procedures. They were treated with or without 24 μM holo-Tf and incubated for 24 hours before harvest. Statistics were performed using pairwise T-TESTs on ΔCT's. * = p< 0.05 from GFP control, ** = p< 0.01 from GFP control, # = p< 0.05, and ## =p< 0.01 A) Comparison of hepcidin mRNA Tf response in WT FVB, *Tfr2*$^{245X/245X}$, WT 129s, *Hjv*$^{-/-}$, and *Hfe*$^{-/-}$ primary hepatocytes. Results are shown as fold change of hepcidin/actin from non Tf-treated control. B) Comparison of hepcidin mRNA Tf response in WT 129s primary hepatocytes transfected with GFP, Tfr2, Hjv, or Hfe. Results are shown as fold change of hepcidin/actin from non Tf-treated GFP-transfected control. C) Comparison of hepcidin mRNA Tf response in *Hjv*$^{-/-}$ 129s primary hepatocytes transfected with GFP, Tfr2, Hjv, or Hfe. Results are shown as fold change of hepcidin/actin from non Tf-treated GFP-transfected control. D) Comparison of hepcidin mRNA Tf response in *Hfe*$^{-/-}$ 129s primary hepatocytes transfected with GFP, Tfr2, Hjv, or Hfe. Results are shown as fold change of hepcidin/actin from non Tf-treated GFP-transfected control. E) Comparison of hepcidin mRNA Tf response in WT 129s primary hepatocytes transfected with combinations of GFP, Tfr2, Hjv, or Hfe. Results are shown as fold change of hepcidin/actin from non Tf-treated GFP-transfected control.

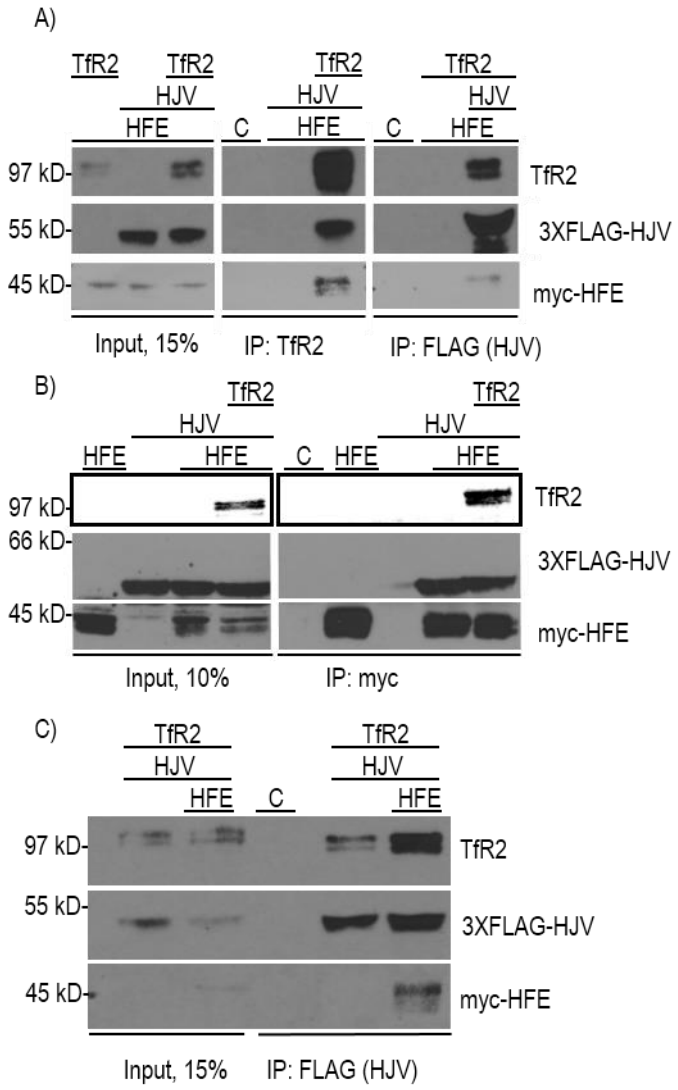

Figure 2.2: The TFR2/HJV complex is enhanced by HFE. TRVB cells were transiently transfected with combinations of TFR2, 3X FLAG HJV, and myc-tagged HFE. All cells transfected with HFE were also transfected with mouse β2-microglobulin. All immunoprecipitations were repeated at least 3 times. C=beads and antibody only. All inputs and immunoprecipitations (IPs) are the same exposure (except in B) on the same blot. A) HFE and HJV co-precipitates with TFR2. TFR2 and HFE co-precipitated with HJV in the absence of HFE. B) 3XFLAG-HJV co-precipitates with HFE-myc in the presence or absence of TFR2. HFE-myc and 3XFLAG-HJV were detected as described in A. C) The presence of HFE-myc increases the amount of TFR2 that co-immunoprecipitates with 3XFLAG HJV. In all of the experiments TFR2 was immunoprecipitated with rabbit anti-TFR2. HFE-myc was immunoprecipitated with anti-myc beads and 3XFLAG HJV was immunoprecipitated with M2 anti-FLAG beads. TFR2 was detected with rabbit anti-TFR2 and Trueblot HRP anti-rabbit in A) and C) and with a fluorescent anti-rabbit secondary in B). 3XFLAG-HJV was detected with HRP anti-FLAG.

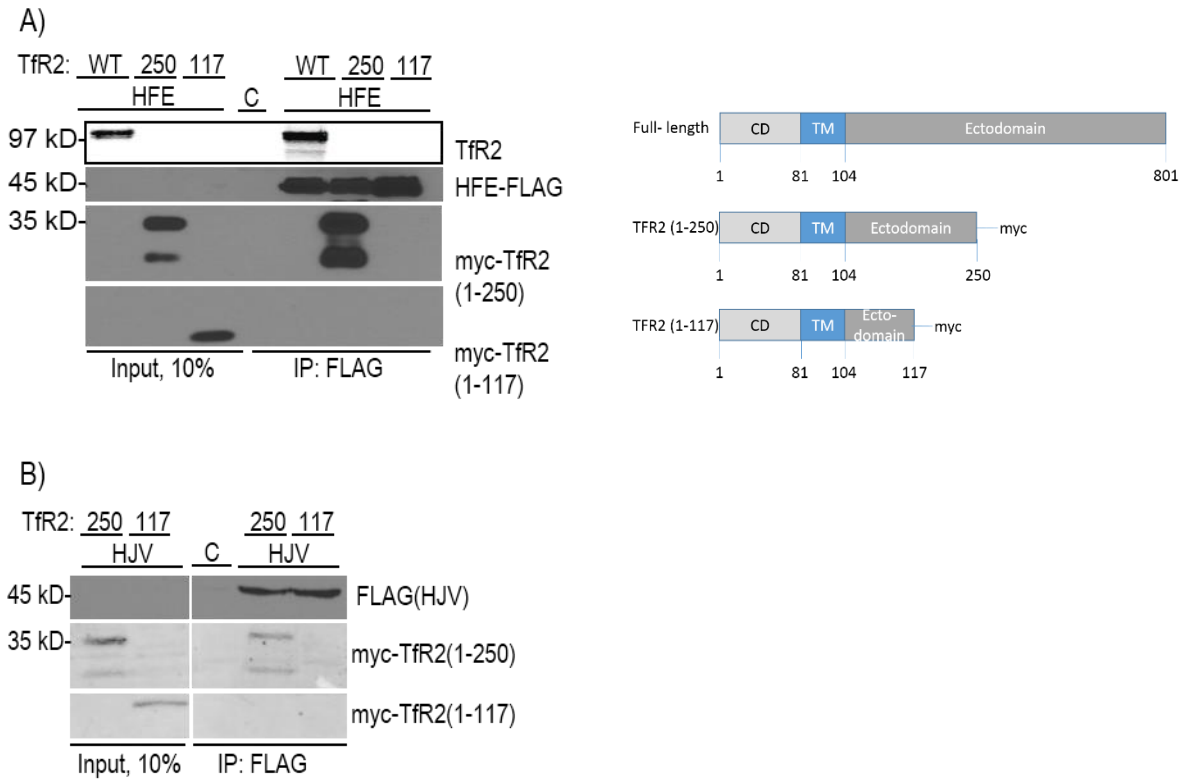

Figure 2.3: Both HFE and HJV interact with Tfr2 (1-250), but not with Tfr2 (1-117). TRVB cells were transiently transfected with A) HFE-FLAG, β2 microglobulin, and TFR2, or TFR2 (1-250)-myc or (1-117)-myc. TFR2 was detected using a rabbit anti-TFR2 antibody and a fluorescent anti-rabbit. HFE-FLAG was detected using anti-FLAG HRP. Myc-TFR2 was detected using a rabbit anti-myc and Trueblot HRP anti-rabbit. B) 3XFLAG-HJV and TFR2, or myc-tagged TFR2 (1-250) or (1-117). Input and IP are same exposure with a lane deleted. Myc was detected using anti-rabbitαmyc antibody and a fluorescent anti-rabbit secondary for LICOR. FLAG epitope was detected using anti-FLAG HRP antibody.

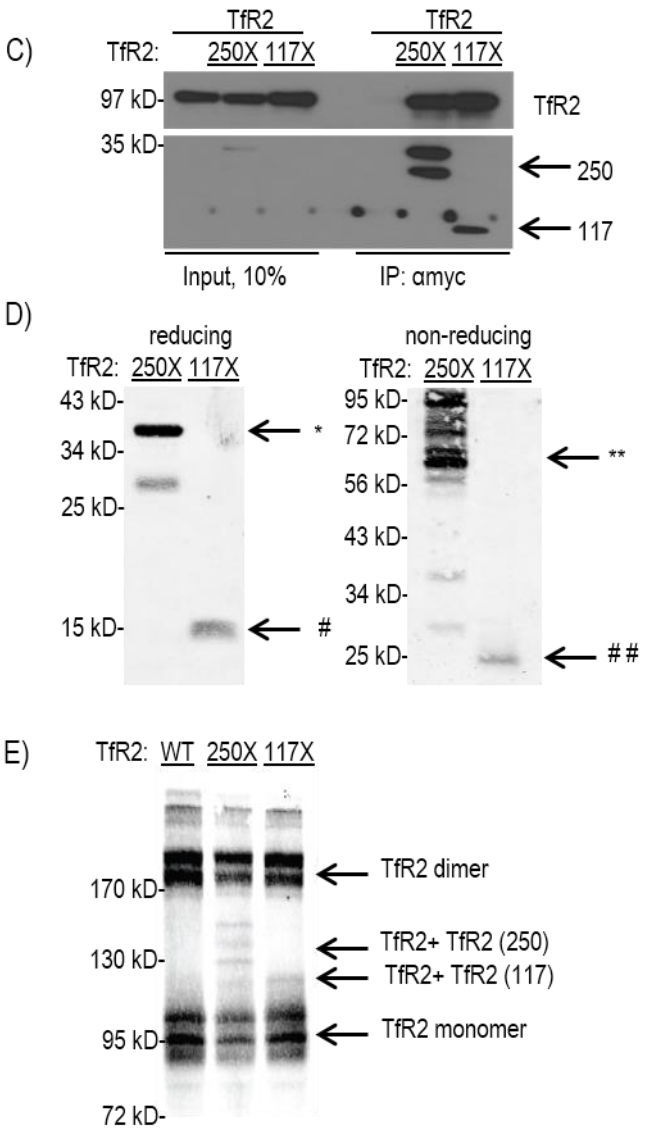

Figure2.4: Truncated TFR2 constructs are able to dimerize A)Full length TFR2 co-precipitates with truncated forms of TFR2. TRVb cells were transfected with TFR2 and TFR2 (1-250)-myc or TFR2 (1-117)-myc and immunoprecipitated using anti-myc beads. TFR2 was detected using a rabbit anti-TFR2 and Trueblot HRP anti-rabbit. TFR2-myc constructs were detected using a rabbit anti-myc and Trueblot HRP-anti rabbit. B) Western blots of TRVb cell lysates transiently transfected with TFR2 (1-250) or TFR2 (1-117) and subjected to electrophoresis under reducing or non-reducing conditions. Myc-TFR2 constructs were detected using a rabbit anti-myc and a fluorescent anti-rabbit. C) Western blot of TRVb cells transiently transfected with TFR2 and Tfr2 (1-250)-myc or TFR2 (1-117)-myc and subjected to electrophoresis under non-reducing conditions. TFR2-myc constructs were detected using rabbit anti-myc and a fluorescent anti-rabbit. TFR2 was detected using a rabbit anti-TFR2 and a fluorescent anti-rabbit. All western blots were repeated at least 3 times. C=beads and antibody only. * denotes TFR2 (1-250), ** dimerized TFR2 (1-250). # denotes TFR2 (1-117), ## dimerized TFR2 (1-117).

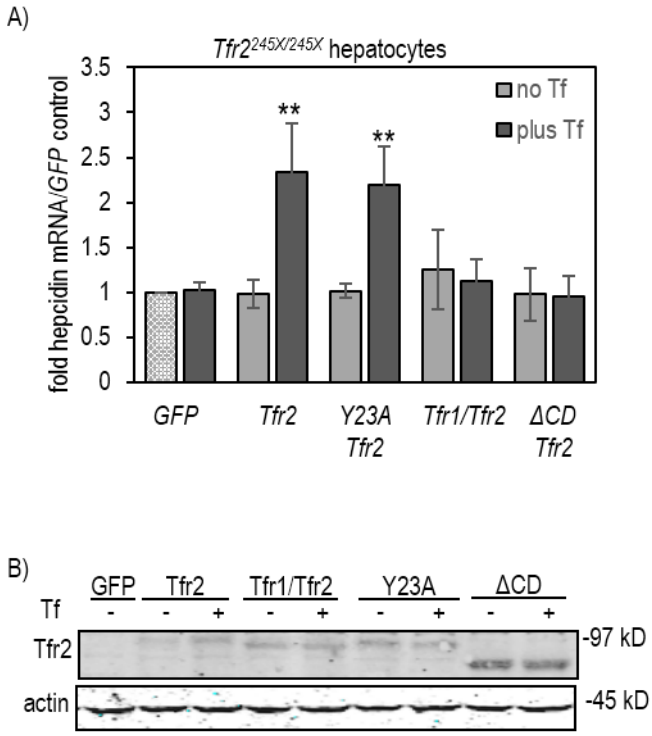

Figure 2.5: The cytoplasmic domain of Tfr2 is necessary for Tf-sensitivity in hepatocytes. Primary hepatocytes from *Tfr2*$^{245X/245X}$ FVB mice (n=4) were isolated and transfected as described in Experimental Procedures. They were treated with or without 24 μM holo-Tf and incubated for 24 hours before harvest. Statistics were performed using pairwise T-TESTs on □CT's. A) qRT-PCR of hepcidin mRNA Results are shown as fold change of hepcidin/actin from non Tf-treated GFP transfected control. B) Western blot of lysates from hepatocytes transfected with GFP, Tfr2 and Tfr2 mutant minicircles.

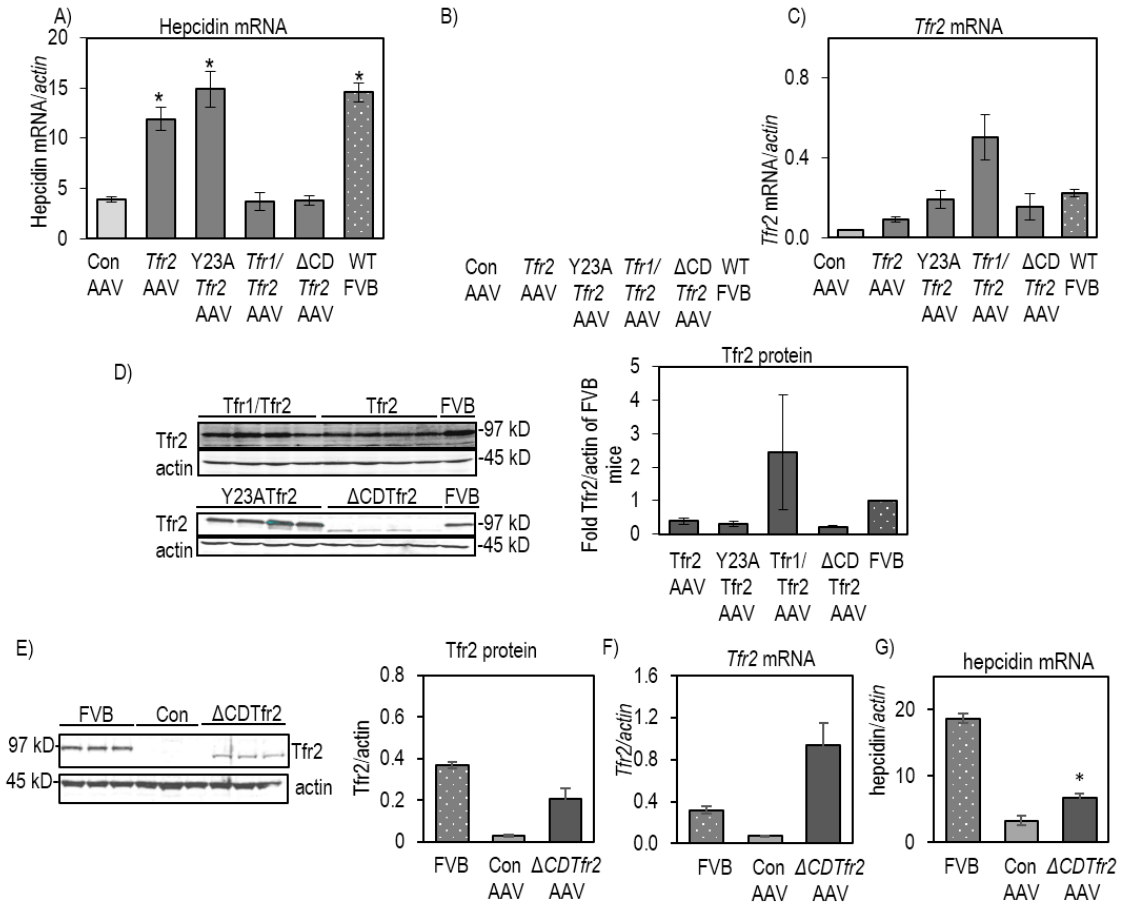

Figure 2.6: The cytoplasmic domain of Tfr2 is necessary for hepcidin induction in mice. Eight-week old male *Tfr2*$^{245X/245X}$ mice were injected with 2X10^{11} genome equivalents/mouse (n=5) and sacrificed 2 weeks later. Age/sex matched control vector WT mice were used as controls. A) qRT-PCR of hepcidin mRNA normalized to β-actin from mouse livers. B) qRT-PCR of ID1 mRNA normalized to β-actin. C) qRT-PCR of Tfr2 mRNA normalized to β-actin D) Western blot of liver extract (μg) using rabbit anti-Tfr2 and mouse anti-actin antibodies. Fluorescent anti-rabbit and anti-mouse were used to detect Tfr2 and actin bands respectively. E) Western blot of liver extract (50 μg) injected with 5X10^{11} genome equivalents/mouse. Tfr2 and actin were detected as described in panel D. F) qRT-PCR of Tfr2 mRNA normalized to β-actin in mice injected with 5X10^{11} genome equivalents/mouse. G) qRT-PCR of hepcidin mRNA normalized to β-actin from mouse livers in mice injected with 5X10^{11} genome equivalents/mouse.

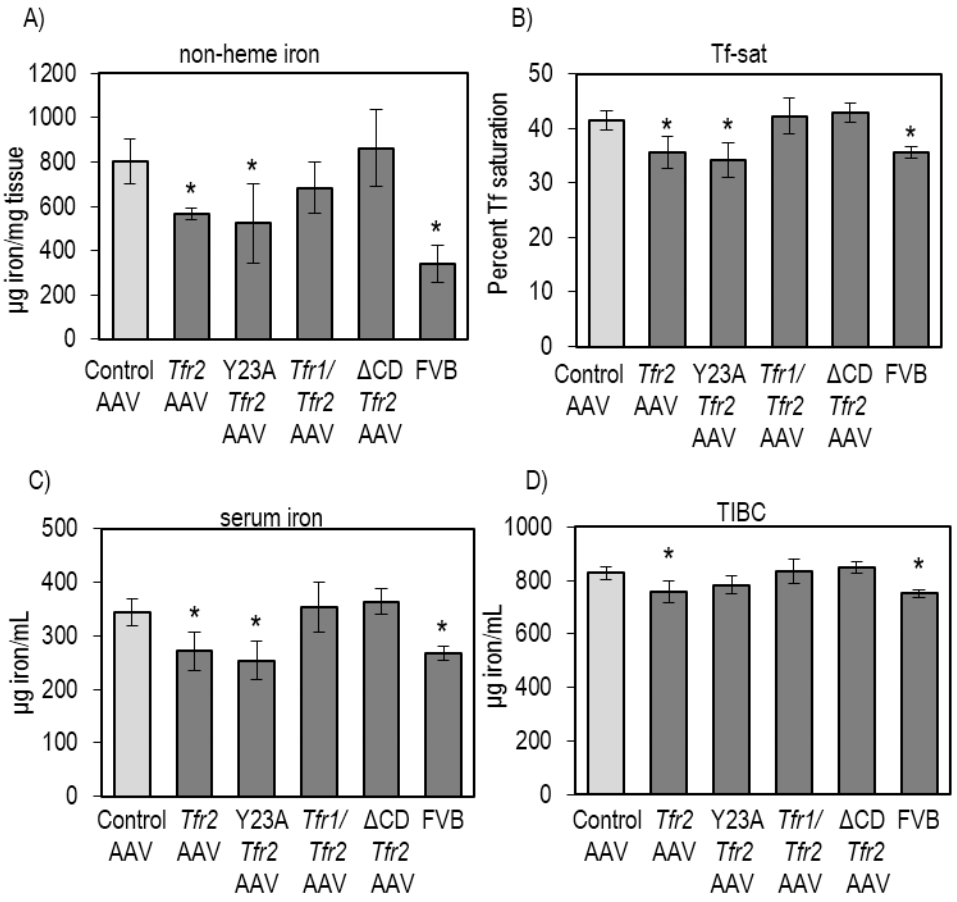

Figure 2.7: The cytoplasmic domain of Tfr2 is necessary for normalization of iron homeostasis in mice. Eight-week old male *Tfr2*$^{245X/245X}$ mice were injected with 2X10^{11} genome equivalents/mouse (n=5) and sacrificed 2 weeks later. Age/sex matched control vector WT mice were used as controls. A) Liver iron. B) Tf-saturation was measured from serum. C) Serum iron. D) Total Iron binding capacity (TIBC).

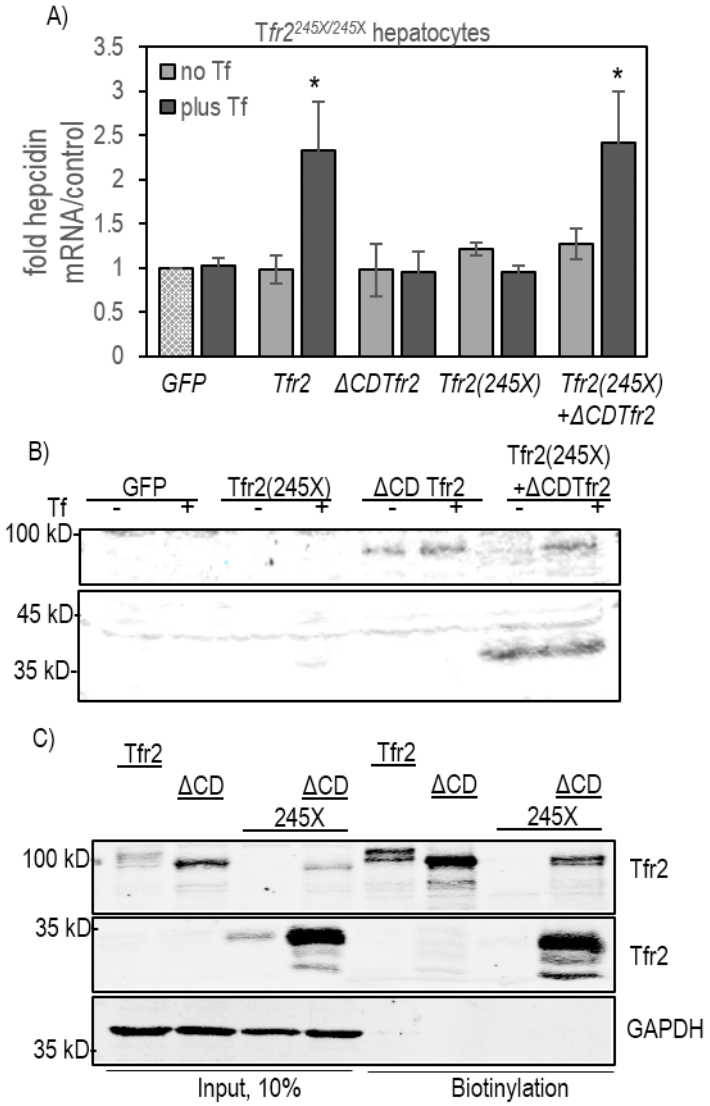

Figure 2.8: Tf-sensing requires either intact Tfr2 or a combination of Tfr2ΔCD and Tfr2 245X. A) qRT-PCR of hepcidin mRNA from transfection of primary hepatocytes from *Tfr2*$^{245X/245X}$ mice (n=4) with Tfr2, Tfr2ΔCD, Tfr2 245X, or Tfr2245X + Tfr2ΔCD and treated for 24 hours with or without holo-Tf. B) Western blot of cell lysates from transfected hepatocytes detected using rabbit anti-Tfr2 and fluorescent anti-rabbit. C) Tfr2ΔCD increases the ability of Tfr2 245X to traffic to the cell surface. TRVb cells were transiently transfected with Tfr2, Tfr2ΔCD, or Tfr2ΔCD and Tfr2 245X. After 24 hrs. they were labeled with a cell impermeable biotinylation reagent, solubilized and incubated with streptavidin beads. Eluates of the strepavidin beads were detected on western blots using rabbit anti-Tfr2 for Tfr2 245X and Tfr2ΔCD and fluorescent anti-rabbit antibody.

DISCUSSION

Sensing of acute change in iron levels plays an important role in iron homeostasis. HFE and TFR2 participate in Tf-sensing through the BMP-signaling pathway, but how the transferrin-sensing complex was connected to the BMP-signaling complex has previously been unknown. Our data is in agreement with previous data[21], showing that TFR2 and the BMP co-receptor HJV form a complex, and that this interaction is strengthened by HFE. The previous experiments were done in Huh7 cells, which are known to express endogenous TFR2, making it impossible to definitively discern whether HJV and HFE can interact in the absense of TFR2. Our experiments were done in TRVb cells, which do not express TFR2, confirming that HFE and HJV can interact on their own. In addition, the previous work used the Huh7 cell line for the interaction of TFR2 with truncation mutants. We were concerned that truncation mutants may be able to dimerize with full-length TFR2, enabling HFE and HJV to immunoprecipitate with the truncation mutants. We performed our experiments in TRVb cells, to eliminate the possibility of dimerization with the full-length TFR2, and our results were still in agreement. There is still the possibility that TRVb cells express some form of HFE, which could interact with HJV, allowing the co-precipitation of TFR2 by HJV when no human HFE is present. However, this is unlikely as TRVb cells have never been shown to express any HFE, and if they did, Hamster HFE and β2-microglubinlin should be sufficiently different from human HFE.

The interaction of TFR2, HFE, and HJV provides both a link between the Tf-sensing TFR2/HFE complex and the BMP-signaling pathway as well as a possible

mechanism for HFE function, in that HFE enhances the interaction of TFR2 with HJV. This makes physiological sense as well. TFR2 and HJV can still interact without HFE, and mutations in Tfr2 and Hjv are more severe independently and both completely inactivate hepcidin response to iron challenge in vivo, while Hfe mutants still mildly respond to iron[85]. Tf-sensitivity also seems to be, at least in part, regulated by the availability of Hfe. We have shown that overexpression of Hfe in primary hepatocytes raises basal levels of hepcidin, but reduces Tf-sensitivity. This is in agreement with the hypothesis that release of HFE from TfR1 makes HFE available to interact with TFR2, promoting Tf-sensitivity. However, mice with mutations in both Hfe and Tfr2 have been shown to have a more severe phenotype than either single mutation[112], indicating possible alternative roles for Hfe or Tfr2 other than Tf-sensing. Indeed, transfection of HFE into cell lines that lack TFR2 results in decreased iron uptake[11, 91, 114]. Since lack of HFE can increase iron loading in cells, and is normally expressed in a wide variety of cells, this could add to the severity of the Tfr2/Hfe double knockout mouse.

We also have shown that Hjv plays a role in Tf-sensitivity in primary hepatocytes, and that all three members of the Tfr2/Hjv/Hfe complex are required for Tf-sensitivity. The requirement of Hjv for Tf-sensitivity is in agreement with a previous study[85], which found that *Hjv*$^{-/-}$ mice given acute iron challenge lacked a hepcidin response, while *Hjv*$^{-/-}$ mice that were chronically loaded with iron could still adjust hepcidin levels. The difference in acute and chronic loading is most likely due to the upregulation of BMP-6 in endothelial cells of the liver. Our experiments in primary hepatocytes explored Tf-sensitivity without the complication of BMP-6

upregulation. Our data shows that Hjv plays a role in Tf-sensitivity, which strongly suggests that the TFR2/HFE/HJV complex is functionally relevant.

Despite the ability of TFR2 and HFE to co-immunoprecipitate in vitro, there is still controversy as to the interaction of TFR2 and HFE in vivo. Immunoprecipitation of endogenous Tfr2 with a myc-tagged Hfe transgene, lacking the cytoplasmic domain, failed to confirm in vitro immunoprecipitations[92]. In addition, another group failed to see an interaction of HFE and TFR2 using a proximity ligation assay [87]. However, this assay used an antibody to the cytoplasmic domain of TFR2 coupled with an antibody to the extracellular domain of HFE, making these results hard to interpret. In addition, they failed to address how TfR1 and TFR2 could co-localize and TfR1 and HFE could co-localize without TFR2 and HFE being able to co-localize [87]. Adding to the controversy of TFR2/HFE binding is the fact the HFE almost certainly has another function, and its alternate functions are unknown.

This work also provides insight into the mechanistic function of TFR2. We show that truncated versions of TFR2 are able to dimerize, indicating that TFR2 has an additional dimerization domain. While the cytoplasmic domain is necessary for Tf-dependent hepcidin activation, the extracellular portion of TFR2 is necessary for Tf-binding. How the cytoplasmic domain of TFR2 interacts with BMP-signaling is unknown. The TFR2/HFE/HJV complex could directly interact with the BMP-receptors, bringing the cytoplasmic domain of TFR2 in close proximity to the cytoplasmic domain of the BMP-receptors. The interaction of TFR2/HFE with HJV provides a key to the intersection of Tf-sensing and BMP-signaling, but how the

cytoplasmic domain of TFR2 functions to affect hepcidin expression remains to be seen.

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CHAPTER 3

Lack of functional TFR2 causes stress erythropoiesis

INTRODUCTION

Unlike the mostly ubiquitous expression of TfR1, TFR2 expression is restricted to the liver and erythropoietic progenitors[53, 57, 97]. Its expression in the liver regulates body iron homeostasis through the upregulation of hepcidin expression in response to changes in blood Tf saturation, however, the purpose of its erythropoietic expression is unknown. A former postdoc in the lab, Juxing Chen, noticed that Tfr2 mutant mice had larger spleens, which can be a sign of stress erythropoiesis, which is a state of increased red blood cell production in response to erythropoietin secretion. Because of this difference in spleen size, along with the known expression of TFR2 in erythroid precursors, Juxing performed experiments to determine the role of TFR2 in erythropoiesis. She found that the larger spleen size of Tfr2 mutant mice was due to a larger number of cells in the spleen, ruling out fibrosis or edema. She determined that this effect was not due to iron-overload by using Tfr2 mutant mice fed a low iron diet or mutant mice where Tfr2 expression was rescued in the liver (normalizing body iron levels) using adeno-associated virus. In addition, mice with a liver-specific deletion of Tfr2 did not have the enlarged splenic phenotype. These experiments confirmed that the enlarged spleens were due to lack of erythropoietic expression of Tfr2, and not iron overload associated with lack of Tfr2 expression in the liver. Juxing also determined that red blood cells (RBC's) from Tfr2 mutant mice had a higher turnover using a biotinylation assay. Faster turnover could be due to either an intrinsic effect of the RBC's, or increased pressure on RBC's going through an enlarged spleen.

Devorah Goldman and I further characterized the erythropoietic defects in *Tfr2*^{245X245X} mice using flow cytometry and a stress erythropoiesis assay.

MATERIALS AND METHODS

Nucleated cell number count- Spleens cells were dissociated using a 70 μM cell strainer and resuspended in PBS. Nucleated cells were counted using Turk's stain (1% gentian violet, 6% acetic acid).

Lineage analysis- 1X10^6 cells from spleen or bone marrow of 5, 10 week-old male mice were used for each condition. Proerythroblast, early basophilic erythroblast, late basophilic erythroblast, and polychromatic/orthochromatic erythroblasts, were determined by staining with CD71-FITC, Ter119-PE, c-KIT-APC, Propidium Iodide (PI), and Hoechst. Proerythroblasts were determined as Ter119-mid, CD71-high. Ter119+ cells were gated and early basophilic cells were forward scatter (FSC)-high, CD71-high. Last basophilic cells were FSC-low, CD71-high. Polychromatic/orthochromatic erythroblasts were FSC-low, CD71-low. Nucleated Lymphoid and myeloid cells were determined by staining with Gr1-FITC, B220/CD3-PE, Ly5.2-APC, PI, and Hoechst. All cells were then discriminated by PI-negative (Live), Hoechst-High (nucleated), and singlets (doublet discriminator). Flow cytometry was performed at the OHSU Flow core and analyzed using FlowJo software.

Flow cytometry antibodies- CD71-FITC (1:100), Ter119-PE (1:100), c-kit-APC (1:100), Mac1Gr1-FITC (1:200), B220/CD3-PE (1:200), Ly5.2-APC (1:100) CD81-FITC (1:500), Hamster anti-mouse CD81 Alexa488 10μL/test (AbD serotec, Raleigh).

Reagents- Propidium Iodide 3µg/mL, Hoechst 10µg/mL, Methocult M3234 and M3334 (StemCell technologies, Vancouver), Erythropoietin (Cell Signaling, Beverly),

Colony assays- For stress BFU-E assay, 10^6 cells were mixed with 3U (30 ng) Erythropoietin in Methocult M3234 medium and plated onto scored 60 mm dishes. Colonies were then counted after 5 days of incubation. For CFU-E assay, 3.5 X 10^5 cells were mixed with Methocult M3334 and plated onto scored 60 mm dishes and counted after 2 days of incubation.

Reticulocyte count- Whole blood was obtained from mice by cardiac puncture in EDTA tubes and spun down at 8000Xg for 10 minutes. Serum and white blood cells were removed by aspiration and RBC's were washed 3X in HBSS. 10^6 cells were then stained with 1 mL of 0.1ng/mL Thiazole orange for 30 minutes and flow cytometry was performed on a BD FACS calibur and analyzed by FlowJo software.

CD81 detection- Red blood cell western blot: 500 µL of blood was harvested by cardiac puncture and centrifuged at 8000xg in EDTA-coated tubes for 10 minutes. Serum and white blood cells were removed by aspiration and RBC's were washed 3X with PBS. RBC's were then lysed in 1mL of 5mM sodium phosphate and vortexed. RBC "ghosts" were then pelleted at 16,000xg and washed several times with PBS. The pellet was then dissolved in 500 µL NETT and 50µg of protein was then resolved on a 12% SDS PAGE gel. CD81 protein was detected using goat polyclonal C-20 sc7102 (Santa Cruz Biotechnology, Santa Cruz) at 0.2 ng/mL and anti-goat 680 secondary using LICOR. Flow cytometry: washed RBC's were incubated with Hamster anti-mouse CD81 Alexa488 10µL/test (AbD serotec, Raleigh) for 1 hour.

Isolated splenocytes were used a positive control. Cells were then washed and detected on a BD FACS Calibur and analyzed using FlowJo software.

RESULTS

Tfr2 mutant spleens show no differences in hematopoietic subsets or lineage by flow cytometry

To determine whether Tfr2 mutant mice are undergoing stress erythropoiesis, we isolated spleen and bone marrow cells from wild type (WT) and *Tfr2*$^{245X/245X}$ mutant mice. Tfr2 mutant mice have increased spleen size (while having similar body weight) and spleen cell number, in agreement with Juxing's data (Figure 3.1A&B). To quantify subsets of erythroid precursors, we used a flow cytometry assay and the markers CD71 (Tfr1) and Ter119 (a marker of erythroid differentiation). CD71 is expressed early in differentiation, and its expression wanes as differentiation progresses. The proerythroblast (ProE) stage is CD71 high, and is starting to express Ter119. We did not find any significant difference in this population between WT and Tfr2 mutant splenocytes or bone marrow (figure 3.2A&B). The early (EryA) and late (EryB) basophilic erythroblast, as well as the polychromatic/orthochromatic erythroblasts (EryC) can be quantified by plotting forward scatter and CD71 expression of Ter119+ cells, as the cells reduce their size and lose expression of CD71. No significant difference in EryA, B, or C in the spleen (Figure2A) or in EryA or B in bone marrow (Figure 3.2B) was found, although a slight difference was seen in EryC in bone marrow. In addition, we saw no significant differences in the percent of lymphoid or myeloid cells (Figure 3.2C).

This flow data is not suggestive of classical stress erythropoiesis, where a buildup of erythroid precursors is often observed, in Tfr2 mutant spleens.

Tfr2 mutant spleens have mild stress erythropoiesis

Another more traditional way of detecting stress erythropoiesis is through colony-formation assays *in vitro*[47]. Splenocytes can be grown in a semi-solid medium in the presence of erythropoietin (EPO) and erythroid progenitors of two types will form colonies. Burst forming units (BFU-E) form large colonies in 5 days[47]. Stress BFU-E's are specific to the spleen and only require EPO for growth[63]. More differentiated colony forming units (CFU-E), will form small colonies in 2 days[100]. Both of these colony types can increase dramatically in the spleen during stress erythropoiesis[9, 42]. We looked for differences in both stress BFU-E and CFU-E in WT and Tfr2 mutant spleens. We found that Tfr2 mutant mouse spleens had both higher stress BFU-E and CFU-E colonies (Figure 3.3 A&B), indicating the presence of stress erythropoiesis.

Another marker of stress erythropoiesis is an increase in circulating reticulocytes, since the presence of reticulocytes is a measure of erythropoietic activity[23]. Reticulocytes have extruded their nucleus, but still have some organelles and residual RNA. Thiazole Orange can bind to this residual RNA and differentiate the reticulocytes from red blood cells using a flow cytometry assay. We found that peripheral blood from Tfr2 mutant mice had a higher reticulocyte count than WT mice, indicating that these mice have increased erythropoietic activity (Figure 3.4).

Red blood cells from Tfr2 mutant mice do not have increased CD81 protein.

Lack of functional Tfr2 in mice causes a mild stress erythropoiesis, but the mechanism by which this happens is unknown. A recent paper described the rise and fall of the tetraspanins CD81 and CD82 during erythropoiesis[98]. They showed that CD81 and CD82 bound to the only integrin expressed during erythropoietic differentiation, α4β1, and enhanced its ability to bind to VCAM-1, expressed on macrophages[98]. This interaction, between CD81, α4β1, and VCAM1 facilitates the interaction of erythroblasts with macrophages and the extracellular matrix, which is important for erythropoiesis[98]. From unpublished data in the lab, we know that TFR2 interacts directly with the tetraspanin CD81. Both TFR2 and CD81 seem to be expressed during the same window of differentiation. I hypothesized that TFR2 may decrease CD81 protein levels in erythroblasts by helping downregulate CD81 during differentiation. TFR2 mutant erythroblasts would then have more CD81 present on mature RBC's, making them interact more readily with macrophages, and leading to the higher turnover of RBC's seen by Juxing. If Tfr2 mutant mouse RBC's turn over faster, it is possible that this could lead to the mild stress erythropoiesis that we saw in Tfr2 mutant mice. In order to test this hypothesis, I measured CD81 expression on RBC's from Tfr2 mutant and WT mice. I found no differences in the very low expression of CD81 on RBC's by either western blot or flow cytometry (Figure 3.5 A&B), indicating that Tfr2 is not responsible for regulating the protein level of CD81 in maturing erythroblasts.

FIGURES

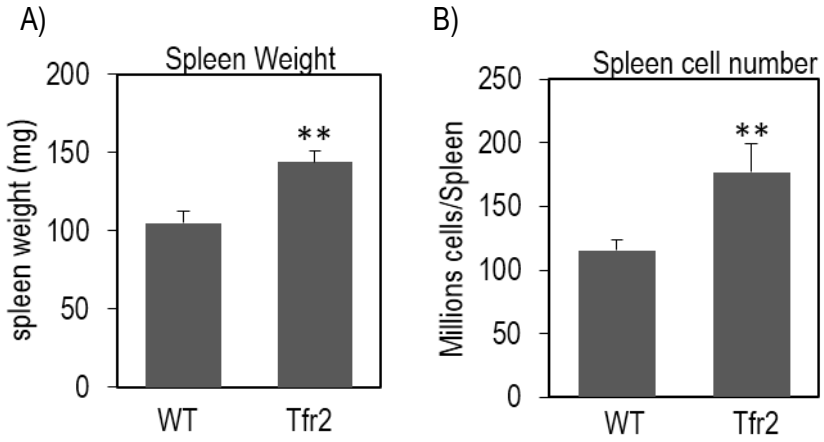

Figure 3.1: Tfr2 mutant mice have enlarged spleens and increased spleen cell numbers. Spleens from WT and Tfr2 mutant mice (n=5). A) Spleen weight B) nucleated cell number. Statistics done using paired students T Test **= $p < 0.01$.

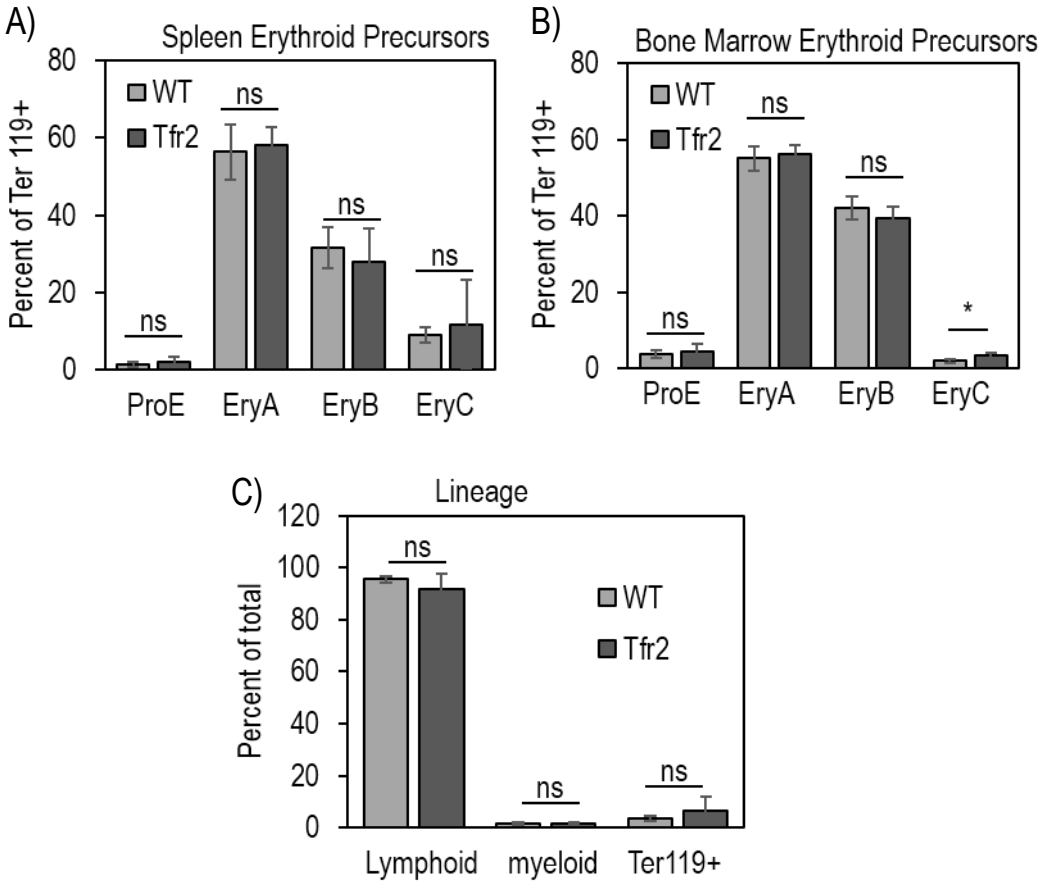

Figure 3.2: Tfr2 mutant spleens show no differences in hematopoietic subsets or lineage. Flow cytometry was used to look at hematopoietic subsets and lineage from spleens and bone marrow of n=5 WT and *Tfr2*$^{245X/245X}$ mice. Percentage of proerythroblast (ProE), early basophilic erythroblast (EryA), late basophilic erythroblast (EryB), and polychromatic/orthochromatic erythroblasts (EryC) from A) spleen and B) bone marrow. C) Percentage of nucleated Lymphoid (B220+), myeloid (Mac1Gr1+), and erythropoietic (Ter119+) cells in spleen. Statistics done using paired students T Test, ns=not significant, *= $p < 0.05$.

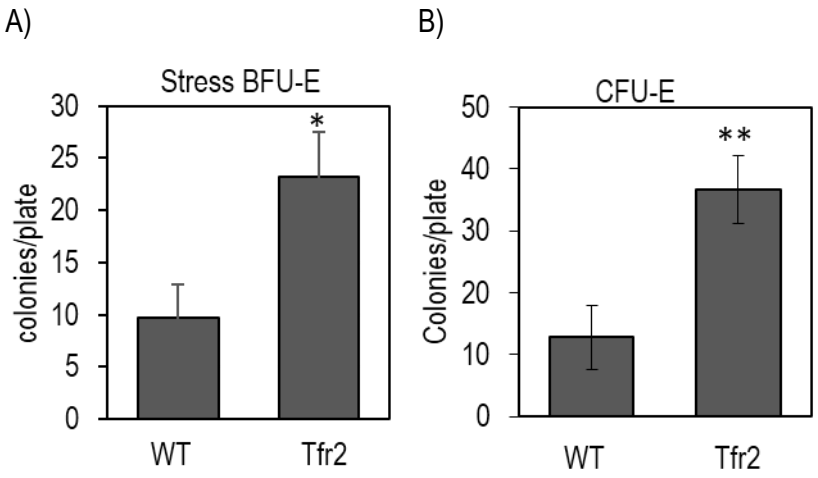

Figure 3.3: Tfr2 mutant spleens have mild stress erythropoiesis. Average colonies per plate grown under A) stress BFU-E or B) CFU-E condition. Graphs are the result of n=5 mice, plated in triplicate. Statistics done using paired students T-Test, *= $p< 0.05$, **=$p< 0.01$.

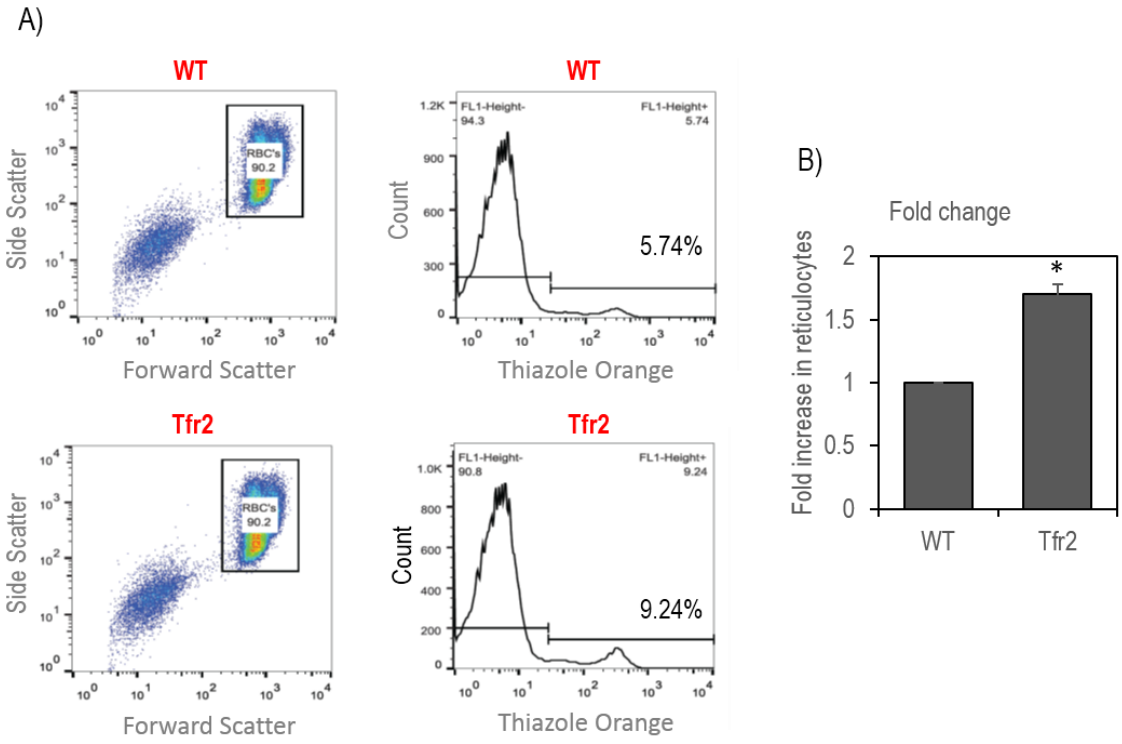

Figure 3. 4: Tfr2 mutant mice have a higher reticulocyte count. A) Representative **f**low cytometry assay of WT and Tfr2 mutant mouse blood for percent reticulocytes. B) Quantification of fold increase of Tfr2 mouse reticulocytes (n=3) *=p< 0.05.

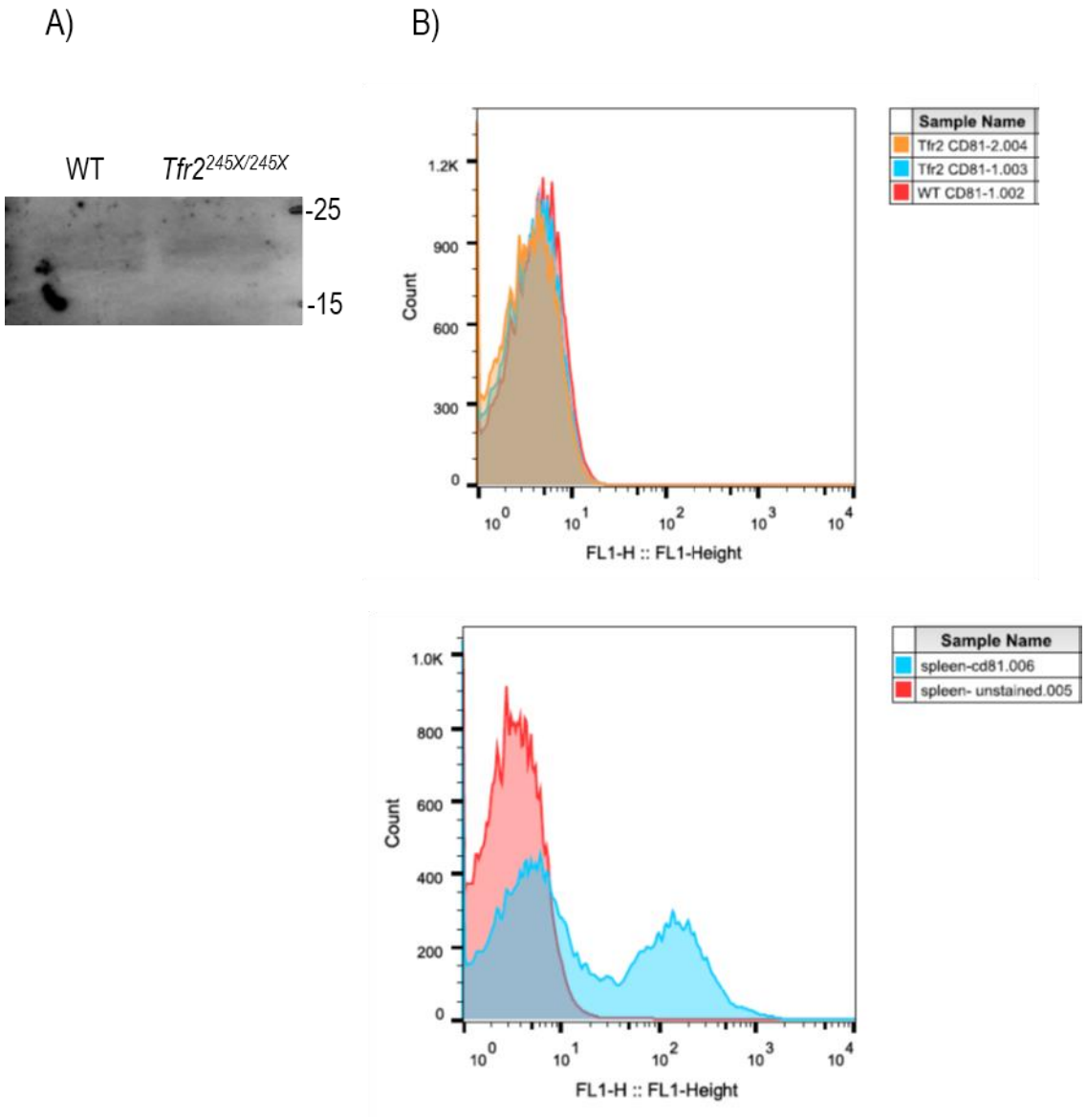

Figure 3. 5: Red blood cells from Tfr2 mutant mice do not have increased CD81 protein. CD81 expression in red blood cells from WT and Tfr2 mutant mice (n=2 mice) by A) western blot of 50ug protein or B) Flow cytometry.

DISCUSSION

TFR2 expression in hematopoietic precursors is functionally significant, as Tfr2 mutant mice have larger spleens, faster RBC turnover, and show signs of mild stress erythropoiesis. Sorting of erythropoietic precursors by flow cytometry did not yield clear signs of stress erythropoiesis. However, the increased spleen size is indicative of a mild erythropoietic defect, which may be too subtle for this assay. A recent paper[29], was able to detect an accumulation of ProE and EryA progenitors by a flow cytometry assay in purified human erythroid progenitors that had been cultured for 5-12 days and had TFR2 knocked down by shRNA. It is likely that culture conditions are able to exacerbate differences in erythroid differentiation between WT and Tfr2 mutant cells. For example, we did see evidence of stress erythropoiesis using the canonical stress BFU-E and CFU-E assay, which also requires culture. It is a mild phenotype, however, as both stress BFU-E and CFU-E can increase by a thousand-fold during stress erythropoiesis[100]. In keeping with the enlarged spleen and induction of stress BFU-E and CFU-E, we also saw an increase in circulating reticulocytes, consistent with a mild stress erythropoiesis.

Unpublished data in the lab has found a direct interaction between TFR2 and the tetraspanin CD81. The involvement of CD81 in the erythropoietic niche and its ability to promote the interaction between differentiating erythrocytes and macrophages[98] led me to explore whether TFR2 mutants may have a higher level of CD81 protein left on mature erythrocytes. I did not find any evidence of this. However, the expression pattern of CD81, which is the only tetraspanin expressed during erythropoiesis and coincides with TFR2 expression, is curious. Interactions

between erythroid cells and macrophages are important to erythroid proliferation[120]. Further studies are warranted to explore any impact of TFR2/CD81 binding on erythropoiesis.

A recent paper has found a physical interaction between TFR2 and the erythropoietin receptor (EpoR), and has evidence that TFR2 is required to efficiently bring EpoR to the cell surface[29]. This Tfr2/EpoR interaction may be a mechanism by which lack of TFR2 results in inefficient erythropoiesis, but, other options such as CD81 interaction and BMP-signaling should also be explored. TFR2 modulates BMP-signaling in the liver, it could possibly modulate BMP-signaling during erythropoiesis, as BMP-signaling has been shown to be important during this process[63]. Taken together, we present good evidence that Tfr2 is required for efficient erythropoiesis.

Chapter 4:

Conclusions and future directions

The interaction of TFR2, HFE, and HJV and its role in transferrin-sensitivity

TFR2 and HFE have long been thought to be the iron sensors in the body. Until now, no clues existed as to how deletion of either protein resulted in a reduction in BMP-signaling. The interaction between TFR2, HFE and the BMP co-receptor HJV provides a possible mechanism to link BMP-signaling and Tf-sensitivity. In Chapter 2 of this thesis, we showed that TFR2, HFE, and HJV proteins interact by co-immunoprecipitation (Figure 2.2). We showed that this interaction is functional, as all three members of the complex are required for Tf-sensitivity (Figure 2.1). In addition, TFR2 and HJV may play a larger role in Tf-sensitivity than HFE and that HJV may be limiting (Figure 2.1). While the TFR2/HJV interaction appears to be important for Tf-sensing, we still do not understand how HJV binding to TFR2 and HFE contributes to signaling. HJV could either bring the TFR2/HFE complex in contact with the BMP-signaling complex, or TFR2/HFE/HJV form a separate complex (Figure 1.1). Future experiments of Hjv should focus on the function of this interaction.

This work also confirmed that TFR2 was a main component of Tf-sensitivity, in that it is responsible for relaying the signal (holo-Tf binding) into the cell through its cytoplasmic domain. We showed that the cytoplasmic domain was required for both Tf-sensitivity (Figure 2.5) and for iron homeostasis (Figure 2.6-7). The TFR2 cytoplasmic domain is very different from the cytoplasmic domain of TfR1, and it is conserved across many species. Future work on the function of the TFR2 cytoplasmic domain should focus on resolving the residues of the cytoplasmic

domain involved in signal transduction, and exploring what intracellular proteins may be involved in transmitting the signal from the TFR2 cytoplasmic domain to the BMP-pathway.

Interestingly, the endocytic sequence of the Tfr2 cytoplasmic domain was not required for transferrin-sensitivity (Figure 2.5). TFR2 protein levels are regulated by holo-Tf, and the endocytic sequence is responsible for the rapid internalization of TFR2 in the absence of holo-Tf. The apparent functionality of the Tfr2 endocytosis mutant shows that signaling does not require the endocytosis of TFR2 . Mice with the mutant endocytic motif (Figure 2.7) resolved their iron-overload in only two weeks, similarly to the expression of wild type protein in Tfr2 mutant mice. Since protein levels of the endocytosis mutant were much higher than the wild type, it would be interesting to see whether continued expression of this mutant for a long period could over-stimulate this signaling pathway and lead to anemia.

Chapter 2 also showed that TFR2 could form heterodimers with different TFR2 truncation mutants, indicating that TFR2 has an additional dimerization domain (Figure 2.4). The biggest surprise was that two non-functional Tfr2 mutants (ΔCD Tfr2 and Tfr2 245X) could come together to form a functional heterodimer that is Tf-sensitive (Figure 2.8). In addition, this hetero-dimerization appears to be sufficient to bring Tfr2 245X, which is normally localized in the ER, to the cell surface (Figure 2.8). This experiment confirms that the extracellular portion of TFR2 is required for proper trafficking of the protein to the cell membrane, while the cytoplasmic domain is required for downregulation of the protein by endocytosis.

Understanding the interaction of TFR2, HFE, and HJV is an important step in resolving the mechanism of Tf-sensitivity. Future work will focus on how the cytoplasmic domain of TFR2 relays Tf-sensing to the BMP-signaling pathway and how HJV plays a role in this process.

Lack of functional TFR2 leads to a mild stress erythropoiesis

TFR2 expression in the liver is important for total body iron homeostasis because of its ability to upregulate hepcidin in response to high serum iron levels. However, how TFR2 affects erythroid maturation is unknown. In chapter 4 of this thesis, we provide evidence that Tfr2 mutant mice have a mild stress erythropoiesis phenotype as evidenced by larger spleens (Figure 3.1), an increased presence of stress BFU-E and CFU-E's in the spleen (Figure 3.3), and a higher reticulocyte count (Figure 3.4). This stress erythropoiesis phenotype is mild, but detectable. While we provide evidence of stress erythropoiesis, we were unable to provide a mechanism. Possible further research could include exploring how the interaction of TFR2 and CD81 or TFR2 and the erythropoietin receptor could affect erythropoiesis. In addition, it is possible that TFR2 could modulate BMP-signaling in erythropoiesis, since it modulates BMP-signaling in the liver.

APPENDIX A

Coculture of HepG2 cells reduces hepcidin expression

RATIONALE

Tf-sensing is an important process by which hepatocytes can sense increases in Tf-bound iron in the blood and respond with an increase in hepcidin expression to lower body iron uptake. Mutations that disrupt this pathway result in iron-overload. Tf-sensing can be observed in primary hepatocytes, which are difficult to culture, difficult to transfect, and dedifferentiate rapidly. Tissue culture hepatocyte cell lines would be a desirable model to study Tf-sensing, however, no hepatocyte cell line to date has been able to recapitulate the Tf-sensing seen in primary hepatocytes, and instead have they an opposite phenotype of lowering hepcidin levels when treated with iron or holo-Tf[34]. Hepcidin expression in cultured cells is also ~400 fold lower than in primary hepatocytes[31]. Differences between primary hepatocytes and tissue culture lines could be a result of hepatocyte de-differentiation, or a lack of physical or paracrine signaling between different liver cell types. Endothelial cells, for instance, are known to upregulate BMP-6 in response to iron loading, resulting in increased hepcidin expression[26]. Thus, we hypothesized that paracrine factors from different cell types may be necessary to keep hepatocytes differentiated to be Tf-sensitive. We wanted to test whether if we co-cultured tissue culture hepatocytes with other cell types, if we could increase hepcidin expression, restore Tf-sensitivity, and create a model system to study Tf-sensing.

METHODS

Cell Lines- HepG2, a human hepatoma cell line, SK Hep-1, a human liver endothelial carcinoma cell line, and RAW264.7, a mouse macrophage cell line were obtained from ATCC. HSC-T6, an immortalized rat liver stellate cell line[107].

Co-culture of HepG2 and T6 stellate cells- HepG2 and T6 Stellate cells were co-cultured at a ratio of 1:10 in 10 cm dishes with DMEM 10% FBS for 48 hours. Cells were then trypsinized and differentially centrifuged at 200 RPM 3 times (Hepatocytes are heavier and are in the pellet). A small amount of the pelleted hepatocytes were replated and stained using a stellate-specific antibody, OC21C6 (a kind gift from the Grompe lab), and anti-rat 586 secondary. Very few stellate cells were observed in culture, with the few that were observed having invaded clumps of HepG2 cells.

Coculture of HepG2 and SK Hep-1- HepG2 and SK Hep-1 cells were co-cultured at a ratio of 1:10 in 10 cm dishes with DMEM 10% FBS for 48 hours. Cells were then trypsinized and differentially centrifuged 3X at 200 RPM.

Coculture of HepG2 and SK Hep-1 using a transwell- A transwell insert was inverted in a 10 cm dish and HepG2 cells were seeded and allowed to adhere in an incubator for 2 hours at 37° 5% CO_2. The transwell was then inverted and inserted into 6-well plates containing 3 mL DMEM 10% FBS or conditioned media from RAW 264.7 for 3 days in DMEM 10% FBS. SK Hep-1 cells were then seeded in the upper chamber.

qPCR - See chapter 2.

RESULTS AND CONCLUSIONS

In the liver, hepatocytes make physical contact with stellate cells in the space of Disse, which is the space between endothelial cells and hepatocytes in the liver sinusoids. To determine if physical contact with stellate cells affects hepatocyte hepcidin expression, or expression of the Tf-sensors HFE and TFR2 (which have low expression levels in cell lines), we co-cultured the hepatoma cell line, HepG2, with the rat stellate cell line, HSC-T6. Because of the size difference between hepatocytes and stellate cells, separating the cell types after culture was a fairly easy process using differential centrifugation. After centrifugation, Stellate cell and hepatocyte populations were at least 95% pure, as observed by microscopy after plating and staining with a stellate specific antibody (data not shown). We measured hepcidin, HFE, and TFR2 mRNA expression in HepG2 cells and found that hepcidin expression was decreased by co-culture (Figure 1A), indicating that co-culture may have a negative effect on hepcidin expression. TFR2 expression had a slight increase, while HFE expression was unaffected (Figure A1B). Since HepG2 cells have the opposite phenotype of primary hepatocytes when treated with iron of holo-Tf, we looked to see the effect of co-culture on iron treatment. Co-culture of HepG2 cells with T6 stellate cells resulted in lower hepcidin levels in iron treated, apo-Tf treated, and holo-Tf treated conditions (Figure A2), indicating that stellate cell co-culture does not restore Tf-sensitivity and has a negative effect on hepcidin expression.

Endothelial cells of the liver line the sinusoids play an important role in hepcidin expression in response to chronic iron-loading through increased BMP-6 expression[26, 56]. We co-cultured HepG2 cells with the liver endothelial cell line SK-

Hep1, and found that hepcidin expression was drastically lowered by co-culture (Figure A3), indicating that endothelial cells also have a negative effect on HepG2 hepcidin expression. In addition to the important effect of endothelial cells on hepcidin expression in liver hepatocytes, macrophages play an important role in iron homeostasis through the recycling of iron in red blood cells. To explore both the effect of endothelial cells and macrophage secretion, we co-cultured HepG2 cells separated by a media-permeable transwell and added RAW 264.7 cell macrophage-conditioned media. We found that both endothelial cell co-culture and macrophage conditioned media reduced hepcidin expression inHepG2 cells (Figure A4), indicating that both endothelial cells and macrophage-conditioned media have a negative effect on hepcidin expression.

Studying iron homeostasis and Tf-sensitivity in primary hepatocytes can be difficult due to their rapid de-differentiation, short life in culture, and difficulty of transfection. A cell culture model that is Tf-sensitive would be desirable and avoid the use of animals in simple experiments. While other cell types no doubt play an important role in the differentiation state of hepatocytes, these co-culture experiments show that other factors are necessary to keep hepatocytes in their differentiated state and allow Tf-sensitivity. In addition, it seems that other cell types may actually secrete factors that inhibit hepcidin expression. The endothelial cell secreted BMPER is a potent inhibitor of BMP-signaling[79] and could be a possible culprit. Further co-culture experiments are warranted, and other possibilities should be explored, including extracellular matrix support and differentiation factors.

FIGURES

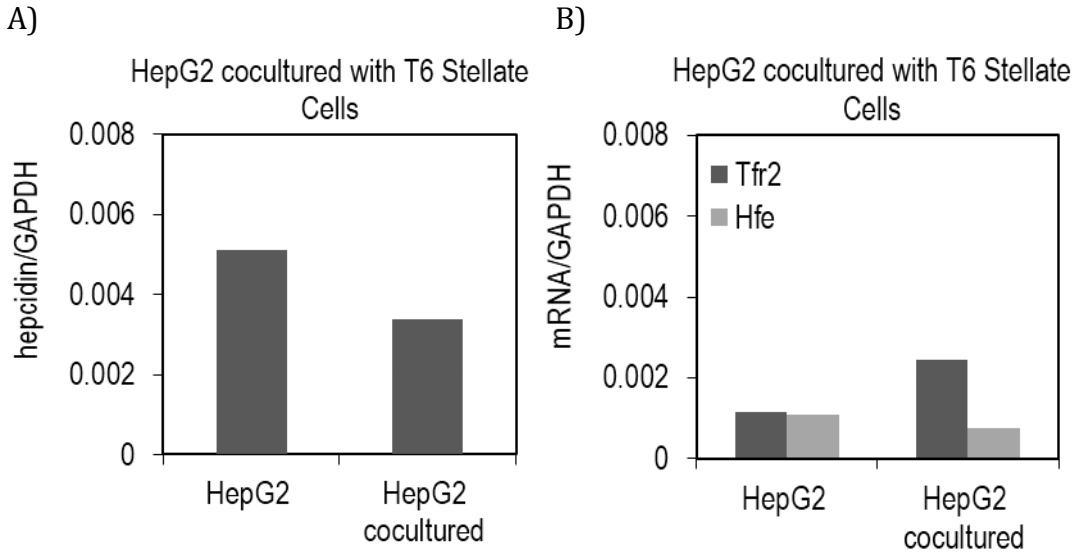

Figure A1: Co-culture of HepG2 cells and T6 Stellate cells does not increase hepcidin expression. qPCR (n=2) of A) Hepcidin and B) TFR2 and HFE mRNA expression in HepG2 cells cultured with and without T6 Stellate cells.

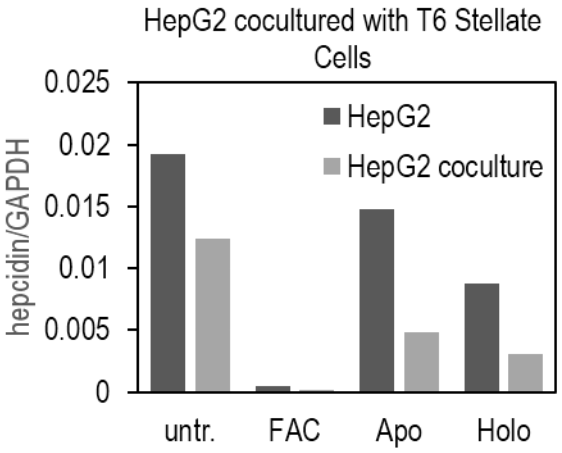

Figure A2: Co-culture of HepG2 cells and T6 Stellate cells does not restore Tf-sensitivity. Hepcidin mRNA expression in HepG2 cells cultured with and without T6 Stellate cells and treated with 50µg/mL ferric ammonium citrate, 2mg/mL Apo-Tf, or 2mg/mL holo-Tf overnight (n=2).

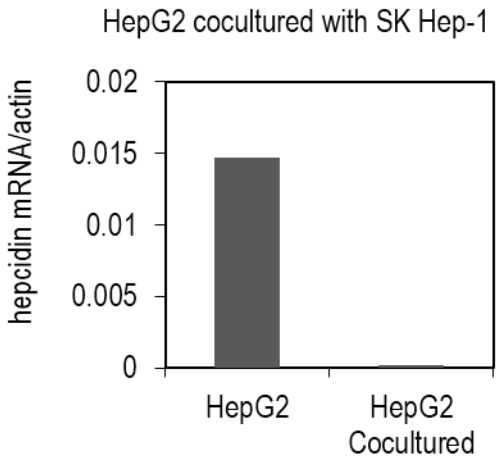

Figure A3: Co-culture of HepG2 cells with SK Hep-1 reduces hepcidin expression. Hepcidin mRNA expression of HepG2 cells cultured with and without SK Hep-1 cells

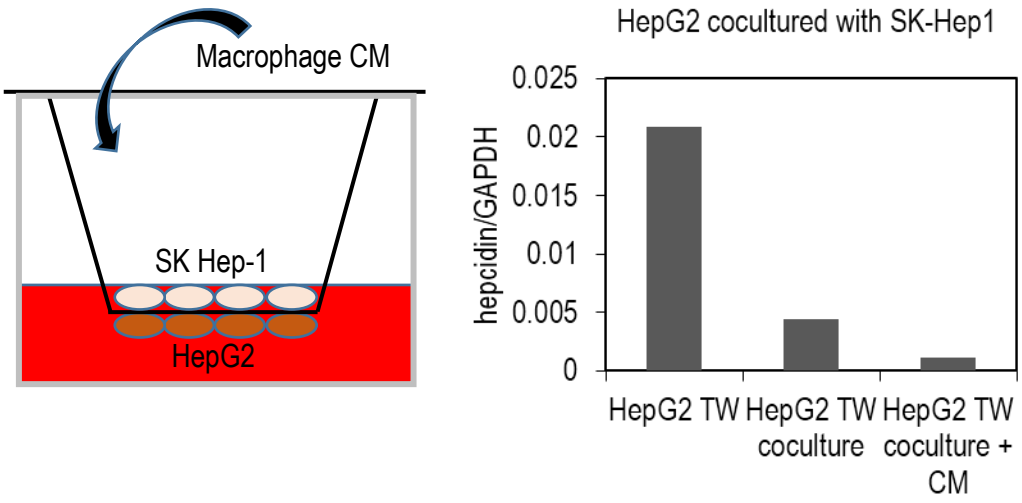

Figure 4: Co-culture of HepG2 cells with SK Hep-1 or SK Hep-1 and macrophage-conditioned media reduce hepcidin expression. Hepcidin mRNA expression of HepG2 cells cultured with and without SK Hep-1 cells or RAW 264.7-conditioned media.

APPENDIX B

Hfe$^{-/-}$ macrophages handle iron differently

RATIONALE

Body iron homeostasis is affected by HFE deletion through the inability of hepatocytes to sense Tf-bound iron and respond with an increase in hepcidin expression. This causes an abnormally high level of iron efflux from intestinal epithelia into the blood and subsequent loading of excess iron in organs and tissues. While the participation of HFE in this liver signaling pathway seems to be its major role, HFE can also affect cellular iron homeostasis through unknown mechanisms. Indeed, most cell types that are transfected with HFE will decrease iron loading, including cells that are deficient in both TfR1 and TFR2[11]. Interestingly, macrophages are reported to have an alternate phenotype[33].

Macrophages play a critical role in iron homeostasis. Senescent erythrocytes are phagocytosed in both the spleen (by red pulp macrophages) and liver (by Kupffer cells), which recycle the iron and export it for either storage in the liver, or formation of new erythrocytes in the bone marrow. While the hepatocytes and other cells begin to iron-overload as a result of HFE deletion, Kupffer cells and red pulp macrophages, which are normally iron rich become iron poor[33]. This paradox has been hypothesized to be a result of low serum hepcidin levels[109]. Macrophages, along with enterocytes, express high levels of FPN. Low serum hepcidin levels (as a result of HFE deletion in the hepatocytes) would result in higher levels of FPN and reduced intracellular iron. However, some experiments point to a direct role for HFE in macrophages. Studies using tissue culture macrophages have shown that HFE increases both iron import and export[25]. Studies using bone marrow transplants between WT and *Hfe*$^{-/-}$ mice found that *Hfe*$^{-/-}$ macrophages were still iron-poor when

transferred into WT mice[69]. In another paper, *Hfe*$^{-/-}$ mice were transplanted with WT livers (because of macrophage turnover, this gave them *Hfe*$^{-/-}$ Kupffer cells and WT hepatocytes). This reversed the iron overload of the liver, but failed to reverse the iron-poor phenotype of the Kupffer cells or spleen[33].

Because of the direct effect of HFE on macrophage iron in both tissue culture and *in-vivo* macrophages, the high level of FPN in macrophages, and the regulation at the protein level of FPN by hepcidin, I hypothesized that HFE-dependent autocrine secretion of hepcidin by macrophages reduces membrane FPN levels and increases intracellular iron.

METHODS

Macrophage Differentiation- Flushed femurs from 1 mouse were added to 4, 35mm petri dishes and treated for 7 days in differentiation media (RPMI 20% FBS, PenStrep, 20uM βME, 2mM L-Glutamine, and recombinant mouse 100ng/mL CSF-M) for 7 days. After 7 days, cells were either continued for 3 days in differentiation media (Mo), plus 50 ng/mL recombinant mouse IFNɣ (M1), or plus 10ng/mL recombinant mouse IL-4 (M2). RBC macrophages were Mo macrophages treated for 4 or 8 hours with aged red blood cells.

Antibodies and western blots- Monoclonal mouse anti-Tfr1 3B82A1 [108]was used at 1:1,000. Mouse anti-horse serum ferritin (Sigma, St Louis) was used at 1:10,000. Mouse antibodies were detected with anti-mouse 800 (Life Technologies, Grand Island). Rabbit anti-mouse Fpn antisera was a gift from Dr. David Haile was used at 1:10,000 and was detected with anti-rabbit Alexa Fluor 680 (Life Technologies,

Grand Island). Cells were lysed in NETT and run on 10% SDS PAGE gels. Fluorescence was detected using LI-COR fluorescence imaging apparatus (LI-COR Inc.)

Aging of Red Blood Cells- Expired human red blood cells from the OHSU blood bank were washed 3X with PBS and resuspended in HBSS with 50μM β-Lapachone (Sigma, St. Louis). Cells were then incubated overnight at 4°C. In the morning, cells were washed 3X w/PBS and resuspended in HBSS before being added to macrophages.

qPCR- RNA was harvested and extracted using Trizol reagent (Life Technologies, Grand Island) per manufacturer's protocol. RNA was DNAse treated using recombinant DNAse and then re-precipitated and washed with 70% ethanol. cDNA was made with 2 μg mRNA using MMLV-reverse transcriptase kit (Invitrogen) per manufacturer's protocol. qPCR was then performed using SYBER-green and validated primers on an ABI ViiA7 qPCR machine. . Verified qPCR primers were as follows: (hepcidin, For: 5'-caccaacttccccatctgcatcttc-3' and Rev: 5'-gaggggctgcaggggtgtagag-3'), (Tfr2, For: 5'-gagttgtccaggctcacgtaca-3' and Rev: 5'-gctgggacggaggtgactt-3').

RESULTS AND CONCLUSIONS

Different populations of macrophages are known to handle iron differently. M1 macrophages have limited iron recycling, because they are differentiated toward an inflammatory pathway[18]. Alternatively activated M2 macrophages, on the other hand, have a higher intracellular labile iron pool, higher HFE expression, and have

both higher import and export of iron consistent with their function of scavenging cells and recycling metals[18]. Since these different populations express varied levels of iron metabolism genes, we wanted to know how Hfe deletion would affect the expression of iron import (Tfr1), export (FPN), and storage (ferritin) proteins. We explored the effect of Hfe deletion in different populations of mouse macrophages isolated from WT and *Hfe*$^{-/-}$ mice, using undifferentiated macrophages (Mo), inflammatory macrophages (M1), alternative macrophages (M2), and macrophages that had been treated with senescent red blood cells (RBC) to mimic the splenic macrophages and Kupffer cells. Because of the regulation of Tfr1 mRNA stability by iron, as a result of its 3' IRE, we can monitor intracellular iron Tfr1 protein levels. Alternatively, ferritin translation is inhibited by a 5' IRE in the absence of iron, allowing us to monitor intracellular iron levels and cellular iron storage. Macrophages from *Hfe*$^{-/-}$ mice resulted in higher Fpn and Tfr1 levels in all subsets of macrophages (Figure B1), consistent with the iron-poor phenotype of *in vivo. Hfe*$^{-/-}$ macrophages. Differences were most pronounced in RBC macrophages, indicating that HFE may play a role in iron recycling after erythrophagocytosis. Higher Fpn levels of Hfe macrophages could be due to the loss of autocrine secretion of hepcidin, so we looked at hepcidin mRNA in WT and Hfe macrophages treated with RBC's. Hepcidin in WT macrophages treated with RBC's increased roughly 2-fold, while *Hfe*$^{-/-}$ macrophages did not (Figure B2A), indicating that macrophages may secrete hepcidin after RBC ingestion. Interestingly, Tfr2 mRNA also increased with RBC treatment (Figure B2B). We hypothesized that Tfr2 levels could be increasing, allowing a complex to form between Hfe and Tfr2, leading to increased

hepcidin expression. This experiment was replicated 4 times, and the results were significant. However, later experiments, with different sources of blood, had varied outcomes and I could not determine why the results were varied.

RBC's are broken down in the phagosome and both iron and heme are transported into the cytoplasm. We wanted to know if iron treatment or iron chelation could also affect hepcidin and Tfr2 levels. WT macrophages treated with iron or the cell-permeable iron chelator, SIH, did not alter hepcidin or Tfr2 levels (Figure B3A and B), indicating that iron loading in macrophages does not affect hepcidin expression. However, WT macrophages treated with a heme timecourse did show a slight increase in hepcidin mRNA at 2 hours, and a preceding increase in Tfr2 expression at 0.5 hours (Figure B3C and D), indicating that hepcidin may be regulated by heme in macrophages. We then looked at the effect of heme treatment on iron storage and transport proteins in WT and *Hfe*$^{-/-}$ macrophages. *Hfe*$^{-/-}$ macrophages started out with higher Tfr1 and Fpn and lower ferritin, confirming that Hfe macrophages have lowered intracellular iron store (Figure B4). However, while WT macrophages increased Tfr1, Fpn, and ferritin levels in response to hemin treatment, *Hfe*$^{-/-}$ macrophages decreased Tfr1, Fpn, and ferritin levels (Figure B4). This altered response to heme treatment suggests that *Hfe*$^{-/-}$ macrophages handle heme differently, however, a mechanism for this difference remains elusive.

Macrophages perform an important function in body iron homeostasis, as they provide the majority of iron for erythropoiesis. HFE has an effect on both body and cellular iron homeostasis, and the mechanism by which it affects cellular iron homeostasis is entirely unknown. Low intracellular iron levels in *Hfe*$^{-/-}$ macrophages

could provide a clue to the role of HFE in cellular iron homeostasis. These experiments reiterate that *Hfe*$^{-/-}$ macrophages have higher iron efflux (as suggested by higher Fpn levels), a higher iron influx and (as suggested by higher Tfr1 levels), and lower iron stores (as demonstrated by lower ferritin levels). While it was tempting to trust my initial mRNA data (showing an increase in hepcidin and Tfr2 in WT macrophages) after RBC treatment, I had trouble repeating this phenomena with later batches of blood. In addition to technical problems, the increase in hepcidin mRNA is probably negligible, as hepcidin expression in macrophages is ~1000-fold lower than in the liver, and hundreds- fold less than cultured hepatocytes. This small change in expression is unlikely to have an autocrine effect. The time-course of heme treatment (Figure B4), does show a very interesting difference in the response of macrophage iron transporters and storage proteins to *Hfe*$^{-/-}$ macrophages. While the mechanism of the low-iron phenotype seen in *Hfe*$^{-/-}$ macrophages remains elusive, a clear difference exists in the handling of recycled iron in *Hfe*$^{-/-}$ macrophages.

FIGURES

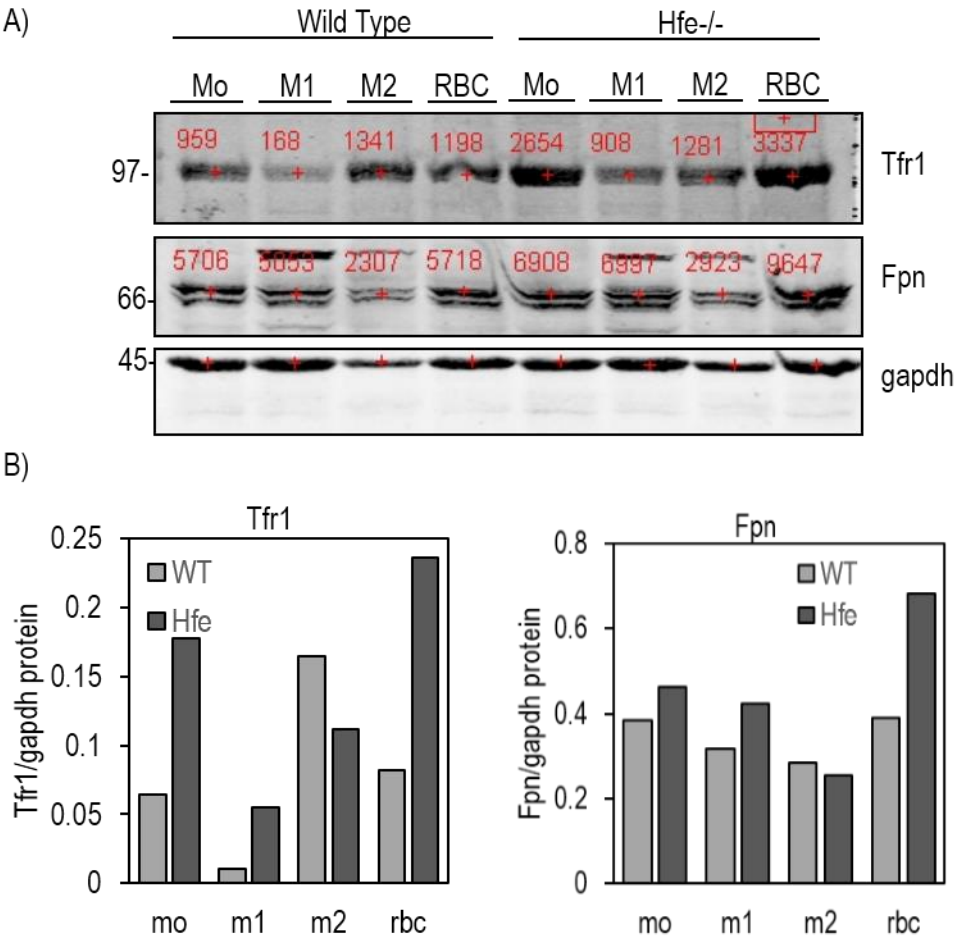

Figure B1: Macrophages from Hfe mice have higher ferroportin levels. A) Western blot (n=2) of 50µg cell lysates from wild type and *Hfe*$^{-/-}$ mouse bone marrow derived macrophages (BMDM's) differentiated into Mo, M1, and M2 lineages or treated with 30,000 β-Lapachone treated red blood cells for 8 hours. B) Western blot quantification.

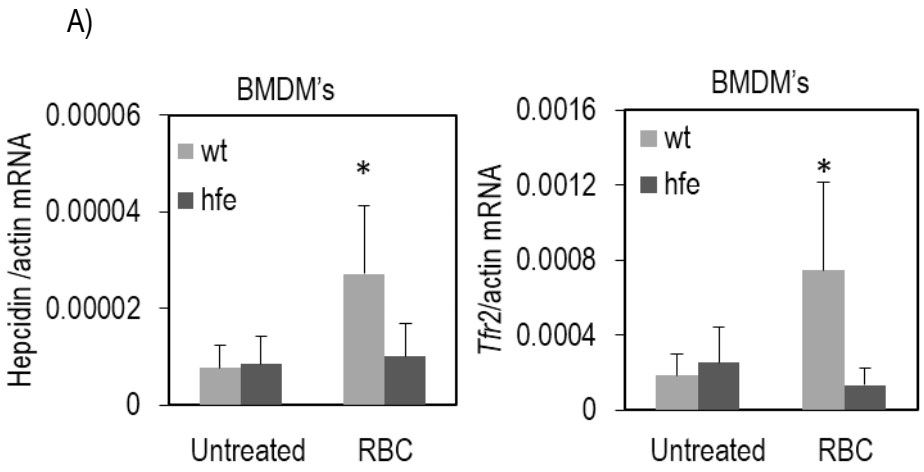

Figure B2: Macrophages from Hfe mice respond differently to red blood cell treatment. qPCR of hepcidin (A) and Tfr2 (B) mRNA from wild type and *Hfe*$^{-/-}$ mouse bone marrow derived macrophages (BMDM's) differentiated into Mo, M1, and M2 lineages or treated with 30,000 β-Lapachone treated red blood cells for 4 hours (n=4).

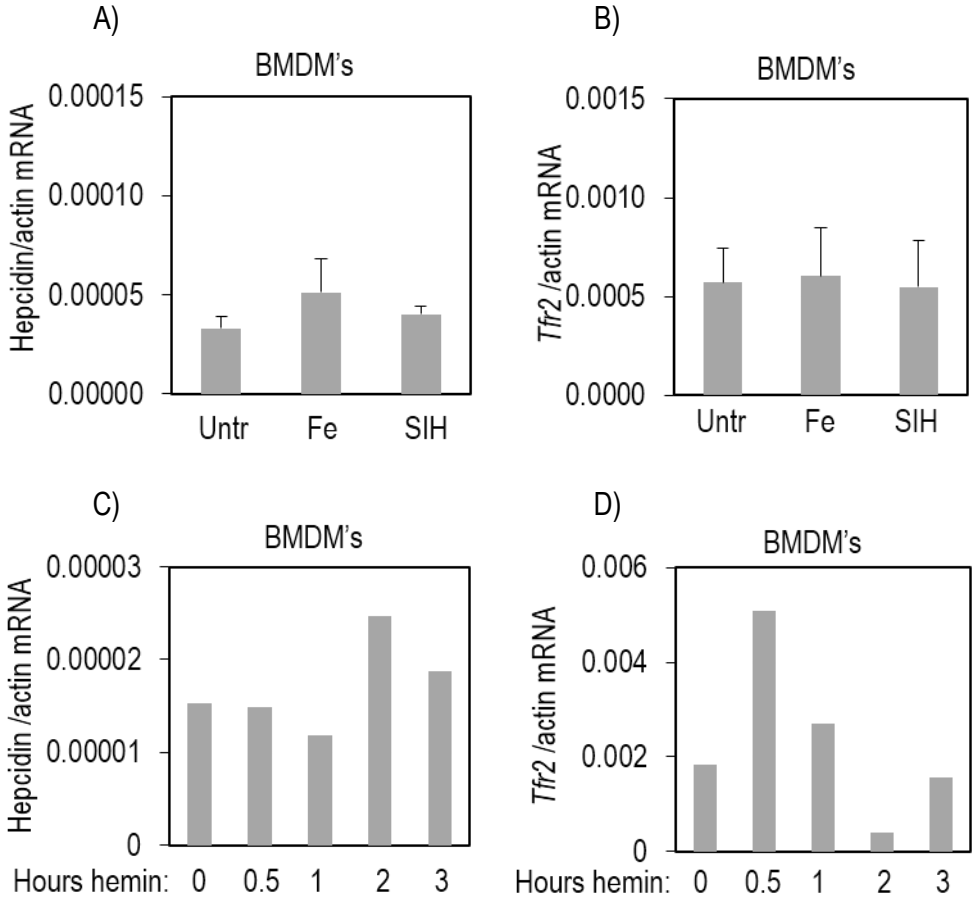

Figure B3: Hepcidin increases in macrophages treated with heme treatment, but not iron treatment. qPCR of hepcidin and Tfr2 from WT Bone Marrow Derived Macrophages that were treated with 5μg/mL ferric ammonium citrate or 50μM SIH for 4 hours (n=3). qPCR of A) hepcidin or B) Tfr2 mRNA. 10 μM Heme timecourse for 0, 0.5, 1, 2, or 3 hours (n=1). qPCR of C) hepcidin or D) Tfr2 mRNA

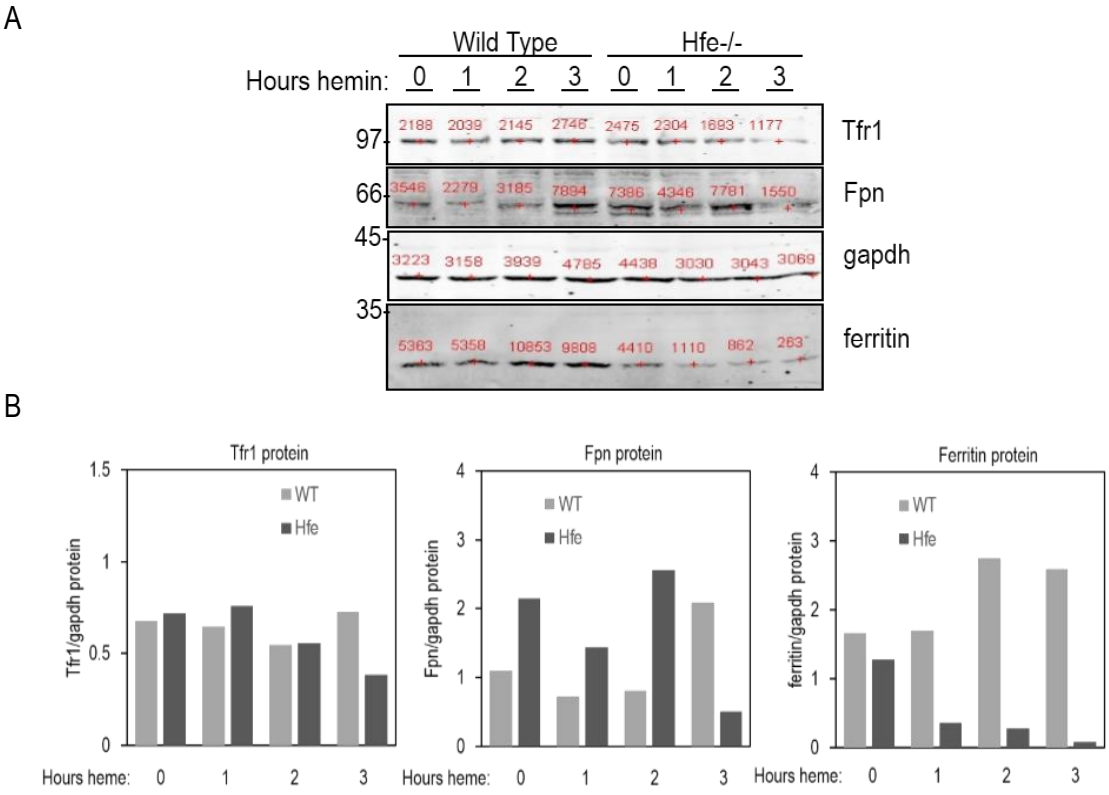

Figure B4: *Hfe*$^{-/-}$ macrophages respond to heme treatment differently than WT macrophages. Western blot of 50µg cell lysates from wild type and *Hfe*$^{-/-}$ mouse bone marrow derived macrophages treated with 10uM Heme for 1, 2, or 3 hours (n=1).

APPENDIX C

Both ZIP14A and ZIP14B are regulated by HFE and iron

RATIONALE

ZIP14 is a multi-transmembrane metal transporter that can transport several different metals, including zinc, iron, manganese, and cadmium[82]. In addition to its ability to transport iron into the cell, it is also regulated by iron at the protein level (article in press), and by HFE expression[32], presumably due to the effect of HFE expression on lowering intracellular iron. ZIP14 has four isoforms; one isoform has an alternate 5'UTR and one has a truncated 3' end. Of interest to us is ZIP14A and B which have several different amino acids in the first and second transmembrane domain. ZIP14A and ZIP14B have a different expression patterns and different affinities for metals such as cadmium and manganese[39]. We hypothesized that ZIP14 A and ZIP14B may also have an alternate affinity for iron, and differential sensitivity to HFE.

MATERIALS AND METHODS

Antibodies- M2 Anti-FLAG (Sigma, St Louis) 100 ng/mL, rabbit anti human-c-myc antibody 0.2 ng/mL (sc-789 Santa Cruz Biotechnology, Santa Cruz), mouse anti actin, 1:10,000 (MAB1501 Chemicon, Temecula),
anti-rabbit Alexa Fluor 680 (Life Technologies, Grand Island), anti-mouse 800 (Rockland, Gilbertsville).

Plasmids- The generation of pcDNA3.1 HFE-FLAG was described previously[14]. pCMV6-myc-ZIP14A and ZIP14B were ordered from Origene.

Western blot detection- Chemilumenesce was detected using Pierce ECL western blotting substrate (Thermo, Waltham) and X-ray film (Midsci, St Louis)

Fluorescence was detected using LI-COR fluorescence imaging apparatus (LI-COR Inc.).

qPCR- See chapter 2. Validated primers- ZIP14 A&B: (F: 5'-gtaaaccttgagctgcacttagc-3', R: 5'-tgcagccgcttcatggt-3'). GAPDH: (F: 5'-ctgcaccaccaactgcttagc-3', R: 5'-gggtggcagtgatggcat-3')

RESULTS AND CONCLUSION

ZIP14A protein levels are reduced by HFE overexpression[32]. In order to determine if ZIP14B protein levels were also affected by HFE expression, HEK293 cells were transfected with ZIP14A or ZIP14B, with or without HFE co-transfection. Both ZIP14A and ZIP14B protein levels were decreased by HFE overexpression (Figure C1A), indicating that the alternate sequence in ZIP14B does not affect regulation by HFE. In order to ensure that differences in ZIP14 protein levels were not due to cotransfection issues, a stable HEK293-ZIP14B cell line was made. Transfection of HFE was still able to reduce ZIP14B protein expression (Figure C1B).

HFE expression is known to decrease intracellular iron. We wanted to know whether iron treatment or iron chelation could also affect ZIP14 protein levels. HEK293 cells were transfected with ZIP14A and treated with iron or the iron chelator Desferal overnight. Treatment with iron caused a slight increase in ZIP14A protein expression, while iron chelation caused a large decrease in ZIP14A protein expression (Figure C2A). Treatment of HEK293 cells expressing stable ZIP14A showed similar results (Figure C2B). To determine if ZIP14B was also regulated by iron, HEK293 cells stably overexpressing ZIP14B were also treated with either iron

or the iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH), overnight. Treatment with iron caused an increase in ZIP14B protein expression, while iron chelation caused a decrease in ZIP14B protein expression (Figure C2C), indicating that ZIP14B is regulated similarly to ZIP14A. Since ZIP14A and ZIP14B are both regulated at the protein level by iron, it is likely that the alternate residues of ZIP14B are not involved in iron regulation of ZIP14 protein levels.

Several proteins in iron homeostasis are regulated at the mRNA level by iron through the presence of hairpin Iron Response Elements (IRE's) in either the 5' or 3' untranslated regions. With the regulation of ZIP14 protein by iron, we wanted to know whether it was regulated at the protein level or the mRNA level. Treatment of HepG2 cells with iron or Desferal resulted in a slight increase in endogenous ZIP14 mRNA levels with iron treatment and a slight decrease in ZIP14 mRNA levels with iron chelation (Figure C3A), indicating that there may be some regulation of ZIP14 at the mRNA level. In order to determine whether the 5' or 3' untranslated regions could play a role in the regulation of ZIP14 mRNA by iron, HEK293 cells stably expressing ZIP14A plasmid (which does not contain the untranslated regions of ZIP14) were also treated with iron or iron chelation. These cells also had a small increase in ZIP14 mRNA with iron treatment and a decrease in ZIP14 mRNA with iron chelation (Figure C3B), indicating that ZIP14 mRNA regulation by iron is not the result of regulation by IRE's in untranslated regions.

ZIP14B has a different expression pattern, affinity for some metals, and is the most different isoform from ZIP14A. Despite these differences, they both appear to be down-regulated by HFE overexpression and by iron chelation. Because of this, it

is unlikely that any of the residues that are different between ZIP14A and ZIP14B are involved in its regulation by HFE or iron chelation. While there appears to be some regulation of ZIP14 by iron at the mRNA level, it is unlikely to account for the large changes seen at the protein level. Both ZIP14A and ZIP14B protein are regulated by cellular iron.

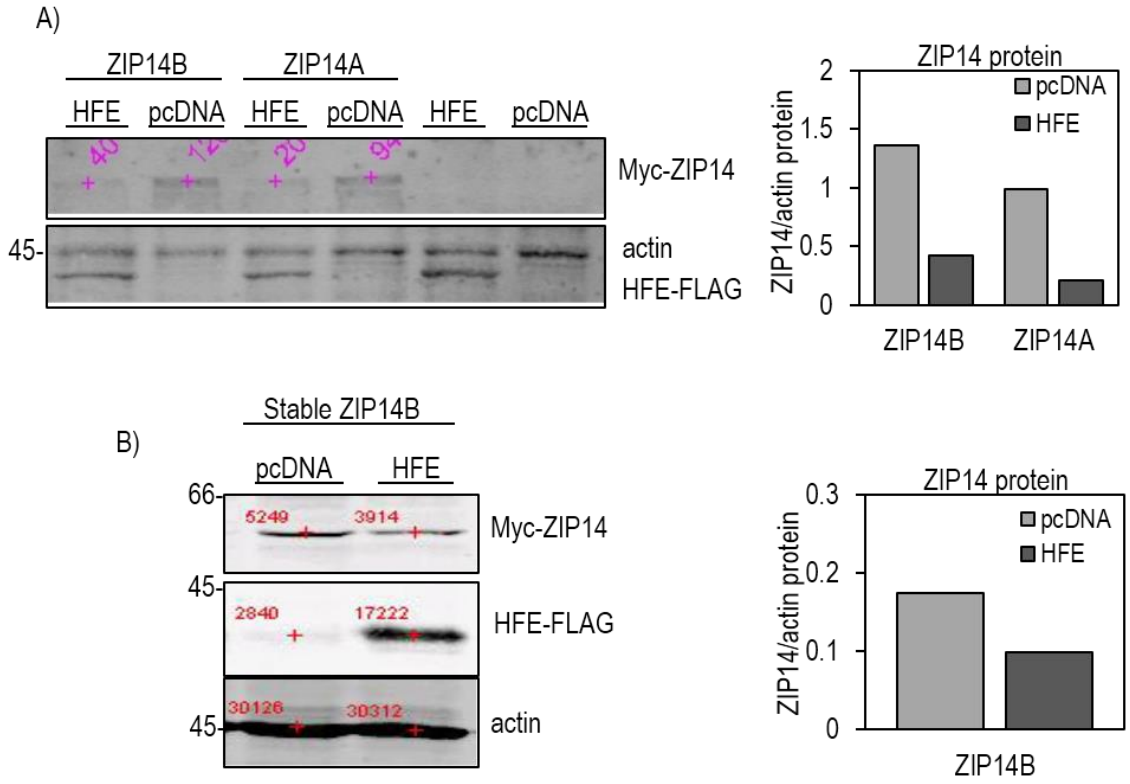

Figure C1: Both ZIP14A and ZIP14B are down-regulated by HFE expression. A) Western blot of Hek293 cells transfected with ZIP14A or ZIP14B and cotransfected with either pcDNA control or HFE-FLAG (n=2) B) Western blot of Hek293 cells stably expressing ZIP14B and transfected with either HFE-FLAG or pcDNA3.1 as a control. (n=1) 45ug of protein was used for each blot and blots were visualized using fluorescent secondary antibodies and LICOR.

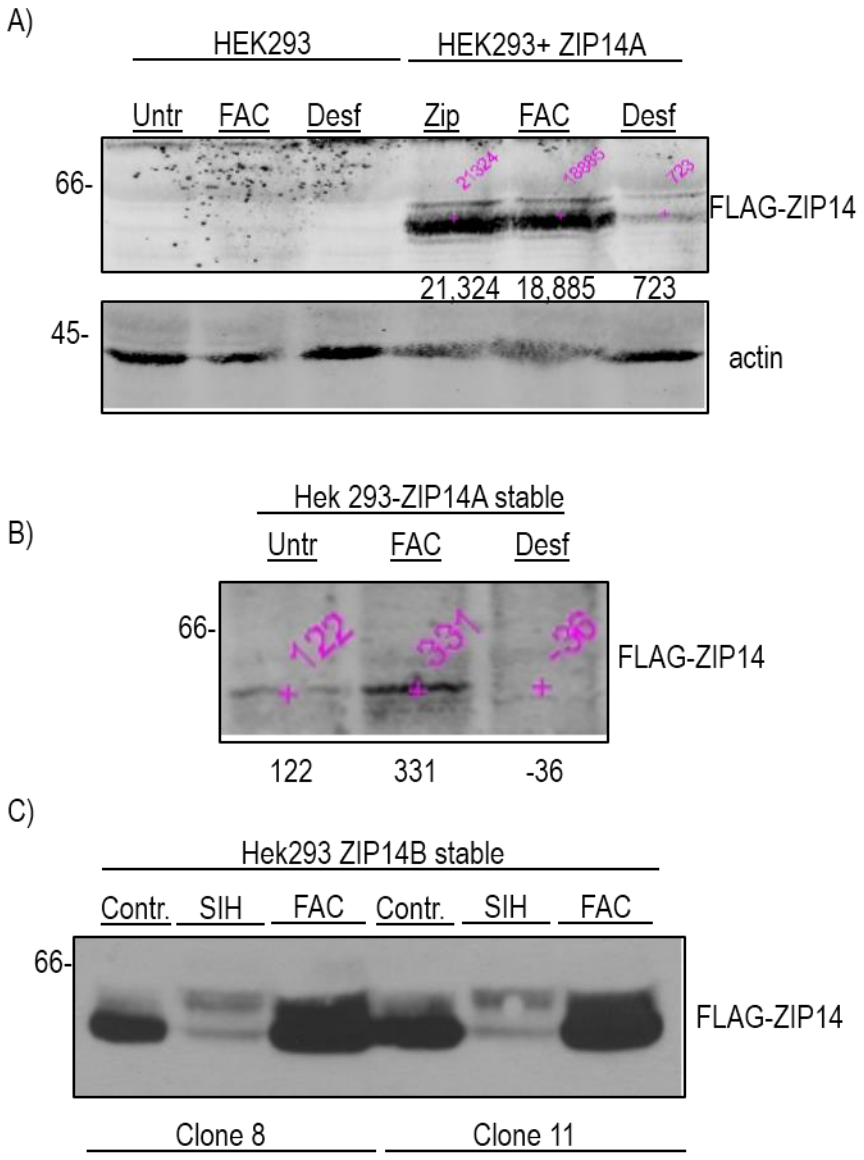

Figure C2: Both Zip14A and ZIP14B are regulated by iron. A) Western blot of HEK293 cells transfected with ZIP14A and treated with 50 μM Ferric Ammonium Citrate (FAC) or 75 μM Desferal overnight. 45 μg of protein was used and blot was visualized using fluorescent secondary antibodies and LICOR. B) HEK293 cells stably transfected with ZIP14A and treated with 50 μM FAC or 75 uM Desferal overnight. 45 μg of protein was used and blot was visualized using fluorescent secondary antibodies and LICOR C) HEK293 cells stably transfected with ZIP14B and treated with 50 μM FAC or 100 μM salicylaldehyde isonicotinoyl hydrazone (SIH) overnight. 90 μg of protein was used and blot was visualized using HRP antibody and chemiluminescence.

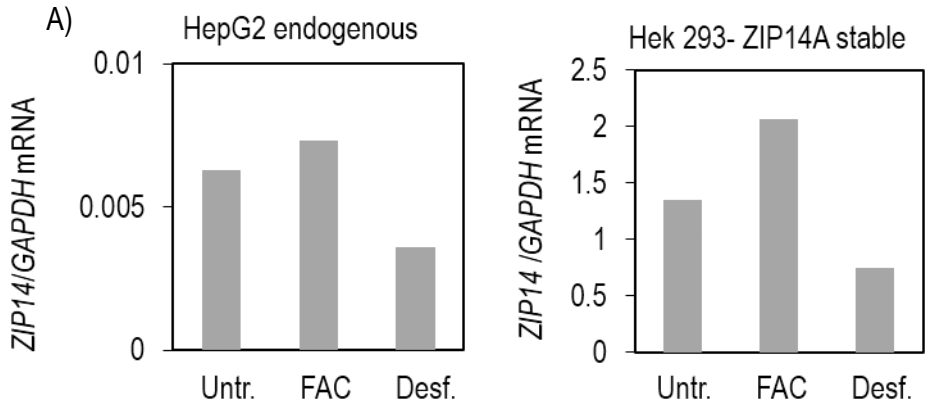

Figure C3: ZIP14 has some mRNA regulation by iron. A) ZIP14 mRNA by qPCR from HepG2 cells (n=1) or from B) Hek293-ZIP14A stable cell line (n=1) that were treated with 50 μM Ferric Ammonium Citrate (FAC) or 75 μM Desferal overnight.

APPENDIX D

The cytoplasmic domain of HFE does not interact with ZIP14 loop2 by Yeast-2-Hybrid

RATIONALE

The main function of HFE is its role in body iron homeostasis through the signaling pathway in the liver leading to hepcidin expression. However, HFE is also able to decrease cellular iron in cells that do not express either TfR1 or TFR2 through unknown mechanisms[11, 91, 114]. HFE overexpression has been shown to down-regulate the iron importer, ZIP14[32], which is also regulated by intracellular iron levels (results in press). ZIP14 is a multi-transmembrane protein with four intracellular and four extracellular domains. We wanted to know if HFE could physically interact with ZIP14, providing a possible mechanism by which HFE could alter intracellular iron levels. We used a yeast-2-hybrid (Y2H) assay to look for a physical interaction between the cytoplasmic domain of HFE and the largest predicted intracellular domain of ZIP14, loop 2 (114 amino acids).

MATERIALS AND METHODS

Plasmids- pGBK-T7-TFR2-CD was cloned from pCDNA3-TFR2 using primers that insert EcoRI and PstIR cut sites on either side of the cytoplasmic domain, to clone into pGBKT7 using primerset: (F: 5'- ccgaattcggatggagcggcttt-3', and R: 5'- ttctgcagcctccgtcctgct-3'). pACT2-CD81 (clone1, amino acids 4-386) and pACT2-SMAD6 (clone55, amino acids 441-783) were from positive clones from TFR2-CD Y2H of cDNA library. pGBK-T7-HFE-CD was cloned from pCDNA3.1-FLAG-HFE using primers that insert EcoRI and BamHI cut sites on either side of the cytoplasmic domain (amino acids 329-348), to clone into pGBKT7 using primerset: (F: 5'- catggaggccgaattcttaaggaagagg-3', and R: 5'- gcaggtcgacggatccctcacgttcag-3'). pGAD-

T7-ZIP14 loop2 was cloned from pCMV6-ZIP14A (from ORIGENE) using primers that insert NdeI and BamHI cut sites on either side of loop2 (amino acids 327-351), to clone into pGAD-T7 using primerset: (F: 5'-gagtggcatatgaagcagaaaa-3', and R: 5'-ggttaaggatccgccgatatca-3'). pGBK-T7-HLA-B7-CD was cloned from pCDNA3-HLAB7 using primers that insert EcoRI and BamHI cut sites on either side of the cytoplasmic domain (amino acids 331-361), to clone into pGBKT7 using primerset: (F: 5'- ggccgaattcaggaggaagagttca -3', and R: 5'- cgacggatccagctgtgagagacac -3'). pGBK-T7-HFE-CD (RKR-AAA) was cloned using site directed mutagenesis of pGBK-T7-HFE-CD with Quikchange (Agilent Technologies, Santa Clara) and primerset: (F: 5'- gcatatggccatggaggccgaattcttagcggcggcgcagggttcaagaggagc-3' and R: 5'-gctcctcttgaaccctgcgccgccgctaagaattcggcctccatggccatatgc-3'). pGBK-T7-HLA-B7-CD (RRK-AAA) was cloned using site directed mutagenesis of pGBK-T7-HLA-B7-CD and primerset: (F: 5'- tgtggtcgctgctgtgatgtgtgcggcggcgagttcaggtggaaaaggaggg -3' and R: 5'- ccctccttttccacctgaactcgccgccgcacacatcacagcagcgaccaca -3'). pGAD –T7-T, pGBKT7-53, and pGBKT7-53 were provided in the Matchmaker Y-2-Hybrid system (Clontech, Mountainview)

Y2H- Yeast-2-hybrid was performed using the Matchmaker (Clontech, Mountain View) system and protocols. pACT2-SMAD6, pACT2-CD81, pGAD-T7-ZIP14 loop2, and pGAD –T7-T (T-antigen positive control) plasmids were transformed into yeast strain AH109. pGBK-T7-TFR2-CD, pGBK-T7-HFE-CD, pGBK-T7-HLA-B7-CD, pGBK-T7-HFE-CD (RKR-AAA), pGBK-T7-HLA-B7-CD, pGBKT7-53 (p53 positive control), and pGBKT7-Lam (Lamin-c negative control) were transformed into yeast strain Y187, according to Matchmaker protocols. Yeast strains were mated according to

Matchmaker protocols, selected on SD –Leu/-Trp plates, and replica-plated onto SD –Leu/-Trp/-Ade/-His, where they were allowed to grow at 30° C until colonies formed (5-10 days).

RESULTS AND CONCLUSIONS

In order to determine if there was a physical interaction between the intracellular domains of HFE and ZIP14, the cytoplasmic domain of HFE (HFE-CD) was cloned into the Y2H vector pGBKT7 and the 114 amino acid intracellular domain of ZIP14 loop 2 was cloned into pGADT7. In another Y2H performed in the lab, the cytoplasmic domain of TFR2 (TFR2-CD) was used as bait and had shown a positive interaction with both CD81 and SMAD6 (an iSMAD in BMP-signaling) of a cDNA library. Clones from this screen were used as both a positive control and to confirm this earlier result, which would be an important step to understanding how the cytoplasmic domain of TFR2 intersects with BMP-signaling. Y2H of HFE-CD and ZIP14 loop2 resulted in tiny colonies, which were very different from the large colonies seen in the supplied positive controls (a combination of Large T-antigen and p53), or in the TFR2-CD paired with CD81 or SMAD6 (Table D1). All negative controls had no colonies, so we did not know whether to count the HFE-CD/ZIP14 loop2 colonies as a positive or a negative result. To ensure that this result was specific to HFE, we cloned the cytoplasmic domain of the closest-related MHC- class 1 protein, HLA-B7, which is not involved in iron homeostasis, into the Y2H vector pGBKT7. Surprisingly, this combination also resulted in tiny colonies (Table D1), indicating that the result of HFE-CD and ZIP14 loop2 was most likely a false positive.

We calculated the isoelectric points of the constructs and found that ZIP14 loop2 had an isoelectric point of 5.68 (making it negatively charged in the cell), while HFE-CD and HLAB7-CD had isoelectric points of 10.83 and 9.1 (making them positively charged in the cell). We hypothesized that the different charges between ZIP14 loop 2 ant HFE-CD/HLAB7-CD could be causing a weak association and a false positive. We looked at the sequences for both HFE and HLA-B7, and found that both molecules had a positively charged sequence (RKR and RRK) in their cytoplasmic domain. We mutated the RKR/RRK sequences in both HFE-CD and HLA-B7-CD to AAA, giving them an isoelectric point of 6.8 and 4.21 respectively. This combination resulted in the absence of any colonies (Table D1), indicating that the RKR/RRK sequence in the cytoplasmic domain of HFE and HLA-B7 was responsible for the false positives. Taken together, these results indicate that the Y2H approach did not show an interaction between the cytoplasmic domain of HFE and the cytoplasmic loop2 of ZIP14.

Plasmids	Purpose	Result	Plasmids	Purpose	Result
pGBKT7-HFE-CD+ pGADT7- ZIP14 Loop2	Experimental	Tiny colonies	pGBKT7-TfR2-CD+ pACT2-CD81-CD	Experimental/ Positive control	Large colonies
pGBKT7-HFE-CD (RKR-AAA)+ pGADT7- ZIP14 Loop2	Experimental	No colonies	pGBKT7-Lam pACT2-CD81-CD	Negative Control	No colonies
pGBKT7-HLAB7-CD+ pGADT7- ZIP14 Loop2	Experimental	Tiny colonies	pGBKT7-TfR2-CD+ pACT2-SMAD6	Experimental/ Positive control	Large colonies
pGBKT7-HLAB7-CD (RKR-AAA)+ pGADT7- ZIP14 Loop2	Experimental	No colonies	pGBKT7-Lam pACT2-SMAD6	Negative Control	No colonies
pGBKT7-Lam pGADT7- ZIP14 Loop2	Negative Control	No colonies	pGBKT7-53+ pGADT7-T	Positive Control	Large colonies

Table D1: Experimental results of Yeast-2-hybrid. Plasmid combinations used and results of Y2H. TFR2-CD and CD81 or SMAD6 (n=2). HFE-CD and Zip14 loop 2 (n=4). HLA-B7-CD and Zip14 loop2 (n=2). HFE-CD (RKR-AAA) and Zip14 loop2 (n=1). HLA-B7-CD (RKR-AAA) and Zip14 loop2 (n=1).

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