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Original Contribution

Prion protein functions as a ferrireductase partner for ZIP14 and DMT1

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ABSTRACT

Excess circulating iron is stored in the liver, and requires reduction of non-Tf-bound iron (NTBI) and transferrin (Tf) iron at the plasma membrane and endosomes, respectively, by ferrireductase (FR) proteins for transport across biological membranes through divalent metal transporters. Here, we report that prion protein (PrP^C), a ubiquitously expressed glycoprotein most abundant on neuronal cells, functions as a FR partner for divalent-metal transporter-1 (DMT1) and ZIP14. Thus, absence of PrP^C in PrP-knock-out ($PrP^{-/-}$) mice resulted in markedly reduced liver iron stores, a deficiency that was not corrected by chronic or acute administration of iron by the oral or intraperitoneal routes. Likewise, preferential radiolabeling of circulating NTBI with ^{59}Fe revealed significantly reduced uptake and storage of NTBI by the liver of $PrP^{-/-}$ mice relative to matched $PrP^{+/+}$ controls. However, uptake, storage, and utilization of ferritin-bound iron that does not require reduction for uptake were increased in $PrP^{-/-}$ mice, indicating a compensatory response to the iron deficiency. Expression of exogenous PrP^C in HepG2 cells increased uptake and storage of ferric iron (Fe^{3+}), not ferrous iron (Fe^{2+}), from the medium, supporting the function of PrP^C as a plasma membrane FR. Coexpression of PrP^C with ZIP14 and DMT1 in HepG2 cells increased uptake of Fe^{3+} significantly, and surprisingly, increased the ratio of N-terminally truncated PrP^C forms lacking the FR domain relative to full-length PrP^C. Together, these observations indicate that PrP^C promotes, and possibly regulates, the uptake of NTBI through DMT1 and Zip14 via its FR activity. Implications of these observations for neuronal iron homeostasis under physiological and pathological conditions are discussed.

1. Introduction

Cells require iron for vital metabolic processes, but excess iron is cytotoxic due to its ability to participate in Fenton chemistry [1]. Iron homeostasis is therefore regulated at the systemic, organelle, and cellular levels by the coordinated action of iron uptake, transport, and storage proteins, which are themselves governed by iron regulatory proteins 1 and 2 as well as additional mechanisms [2–4]. Systemic iron homeostasis is maintained mainly by regulating uptake from the duodenum by the peptide hormone hepcidin released by hepatocytes in response to circulating iron [5,6]. Most of the absorbed iron binds tightly to plasma transferrin (Tf), and the rest circulates as non-Tf-bound iron (NTBI) in association with small molecular weight compounds. Normally, 30–40% of plasma Tf is saturated with iron, leaving significant buffering capacity for excess iron that may accumulate in pathological conditions. Additional protection is provided by hepatocytes that can internalize and store large quantities of iron before signs of hepatotoxicity appear [7,8].

Tf iron and NTBI exist in the relatively inert ferric form (Fe^{3+}), and require reduction to the ferrous form (Fe^{2+}) for transport across biological membranes [2,8,9]. Iron-loaded Tf is endocytosed by the transferrin receptor (TfR), and Fe^{3+} iron released in the acidic environment of late endosomes is reduced to Fe^{2+} by membrane-bound ferrireductase (FR) proteins before transport

Abbreviations: Dcytb, duodenal cytochrome *b*; DMT1, divalent metal transporter-1; FAC, ferric ammonium citrate; FR, ferrireductase; GFP, Green-fluorescent protein; ip, intraperitoneal; LIP, labile iron pool; NTBI, non-transferrin-bound iron; PrP^C, prion protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tf, transferrin; TfR, transferrin receptor; ZIP, Zrt Irt-like protein.

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through divalent metal transporters to the cytosol. NTBI, on the other hand, is reduced at the plasma membrane before transport, though recent reports indicate uptake and transport from the endosomes as well [7,8,10,11]. Known FR proteins that function optimally at low pH of the endosomes include the Steap family of proteins, in particular Steap3 that plays a vital role in iron uptake by hematopoietic cells [12].

A prominent plasma membrane FR is duodenal cytochrome *b* (Dcytb) that reduces nonheme Fe^{3+} in the duodenum to Fe^{2+} for uptake by duodenal epithelial cells [13,14]. The FR activity of Steap3 and Dcytb is coupled with the divalent metal transporter-1 (DMT1) that transports reduced iron across the lipid bilayer [15]. Recently, the ZIP family of divalent metal transporters has been described that functions at the plasma membrane and in late endosomes [16,17]. Transported Fe^{2+} joins the cytosolic labile iron pool (LIP), is oxidized by ferritin and stored in its shell, or exported by the coupled action of a ferroxidase such as ceruloplasmin or hephaestin and the iron export protein ferroportin [3,7,18].

Recent reports indicate that prion protein (PrP^C), a mainly neuronal protein implicated in the pathogenesis of prion disorders, promotes cellular iron uptake by functioning as a FR [19–21]. In neuroblastoma cells, PrP^C exhibits FR activity on the plasma membrane, and requires the N-terminal octapeptide repeat region for this activity [19]. Likewise, PrP^C promotes retrieval of iron from the glomerular filtrate via its FR activity, and increases transcellular transport of NTBI from the apical to the basolateral domain of kidney proximal tubule epithelial cells [22]. Deletion of the N-terminal octapeptide repeat region abolishes this function, implicating the FR activity of PrP^C in kidney iron uptake [22]. However, the metal transporter(s) involved in PrP^C-associated iron uptake and transport are not known. Since liver is the principal organ that stores excess circulating iron, we used a combination of PrP-knock-out ($PrP^{-/-}$) transgenic mouse models and HepG2 cells expressing exogenous PrP^C to explore the significance of FR activity of PrP^C and associated divalent metal transporters in hepatic iron uptake. Here, we report that PrP^C promotes the uptake of Fe^{3+} iron by hepatocytes through divalent metal transports ZIP14 and DMT1.

2. Materials and methods

2.1. Animals and ethics statement

Wild-type ($PrP^{+/+}$) and PrP-knock-out mice ($PrP^{-/-}$) [23] were originally obtained from George Carlson and bred on the FVB/NJ background for more than 10 years at the Animal Resource Center (ARC) at Case Western Reserve University (CWRU). Animal protocols and procedures were approved by the IACUC committee at CWRU.

2.2. Materials and cell culture

HepG2 cells (ATCC) were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid encoding DMT1-GFP (-IRE) was from Jerry Kaplan (University of Utah). PrP^C and $PrP^{C\text{-}GFP}$ constructs were prepared by ligating cDNA from previously reported constructs [24] in modified PiggyBac vectors (System Biosciences, Mountainview, CA). HepG2 cells were cotransfected with the PiggyBac vectors and a transposase-expression vector using Lipofectamine LTX (Invitrogen, Carlsbad, CA), and mixed cultures of ~30 stable clones selected with Blasticidin-S were used for all experiments to avoid clonal artifacts. PrP antibody specific for residues 109–112 (3F4) was from Signet Laboratories (Dedham, MA). The specificity of anti-C antibody against C-terminal residues of human PrP (anti-C) has been described previously [25]. Antibodies specific to ferritin and β-actin were obtained from Sigma (Sigma-Aldrich, St. Louis, MO) and Millipore (Bedford, MA), respectively. Wheat germ agglutinin (WGA)–Alexa Fluor-594 was from Molecular Probes (Invitrogen). HRP-conjugated secondary antibodies and ECL detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Radiolabeled $^{59}FeCl_3$ was from Perkin-Elmer (Boston, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Iron uptake in vivo

Age and sex-matched $PrP^{+/+}$ and $PrP^{-/-}$ mice were provided unrestricted access to food and water, and equal numbers of mice from each group were subjected to the following experimental paradigms: (1) Carbonyl iron (200 μg) dissolved in PBS was gavage-fed daily for 1 month. Controls received an equal volume of PBS [26]. (2) Equal counts of ^{59}Fe-citrate or ^{59}Fe-ferritin (purified from mouse liver) [27] containing 11 μg of total iron were injected intraperitoneally (ip). (3) Unlabeled ferric-ammonium citrate (FAC, 70 μg/22 g body weight) was injected ip followed by ^{59}Fe-citrate after 30 min by the same route [28]. A parallel set of animals received ^{59}Fe-citrate without prior injection of FAC. At the indicated times, animals were euthanized and processed as described [20,29]. Liver homogenates and lysed RBCs were resolved by SDS-PAGE and transferred proteins were probed with specific antibodies by Western blotting, or resolved by native PAGE and subjected to autoradiography [20,29]. Serum iron, tissue nonheme iron, and iron content of purified ferritin was quantified by the iron estimation kit (Stanbio Laboratory, Boerne, TX). Serum ferritin was measured using an ELISA assay (Abcam) [20,29].

2.4. Iron uptake in vitro

HepG2 cells stably expressing vector or PrP^C were exposed to 30 μM FAC (Fe^{3+}) or iron ascorbate (Fe^{2+}) for 1–4 h or overnight, and expression of PrP^C and ferritin was evaluated by Western blotting. Short-term experiments were conducted in RPMI medium lacking iron and transferrin. To assess the role of ZIP proteins and DMT1 in PrP^C-mediated iron uptake, stable clones of vector and PrP^C-expressing cells were cotransfected with ZIP8-GFP, ZIP14-GFP, or DMT1-GFP, and cultured for 48 h to allow protein expression. Transfection efficiency was evaluated by flow cytometry, and lysates were processed for Western blotting as above.

2.5. Immunostaining

Monolayers of HepG2 cells expressing $PrP^{C\text{-}GFP}$ were incubated with WGA–Alexa Fluor-594 (1:200) on ice for 30 min, washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (0.5 ng/ml) to visualize the nuclei. Mild fixation was necessary to preserve the GFP signal. Images were captured by confocal microscopy.

3. Results

3.1. Uptake and storage of iron by the liver are impaired in $PrP^{-/-}$ mice

Previous reports indicate a phenotype of iron deficiency in transgenic mice carrying a deletion in *PRNP*, the gene encoding PrP^C ($PrP^{-/-}$) [23], and rescue of this phenotype by inserting *PRNP* in the $PrP^{-/-}$ background [20]. To evaluate whether the underlying cause is reduced availability or impaired uptake, storage, and utilization of circulating iron, age- and sex-matched $PrP^{+/+}$ and $PrP^{-/-}$ mice were fed regular chow or supplemented with 200 μg of carbonyl iron daily

for 1 month [26]. Absorption from the duodenum and storage by the liver were assessed by quantifying levels of serum iron and ferritin, liver nonheme iron, and expression of liver tissue ferritin, the principal iron storage protein of hepatocytes and reticuloendothelial cells (Fig. 1).

Serum iron was significantly lower in $PrP^{-/-}$ mice on normal chow relative to matched controls, but approached control levels when supplemented with excess carbonyl iron (Fig. 1A). However, serum ferritin remained significantly lower in $PrP^{-/-}$ samples despite normal serum iron, indicating a deficiency in iron storage (Fig. 1B). Liver stores of nonheme iron and ferritin expression were reduced in $PrP^{-/-}$ mice, and remained significantly lower than matched controls even after administration of excess iron (Fig. 1C–E). It is notable that despite exposure to excess iron, expression of liver ferritin in $PrP^{-/-}$ mice remained significantly lower than $PrP^{+/+}$ controls on normal chow (Fig. 1E).

These results suggest that the principal cause of iron deficiency in $PrP^{-/-}$ mice is impaired hepatic uptake and storage of circulating iron rather than defective duodenal absorption.

3.2. PrP^C promotes uptake of Tf iron and NTBI by the liver

Since orally introduced carbonyl iron circulates primarily as Tf iron and NTBI that require reduction of Fe^{3+} to Fe^{2+} before hepatic uptake [3,8,9], we considered the possibility that the recently described FR activity of PrP^C on cell models was necessary for the uptake of iron from these sources [19]. Thus, equal amounts of ^{59}Ferric–citrate were injected intraperitoneally in age- and sex-matched $PrP^{+/+}$ and $PrP^{-/-}$ mice. Since plasma Tf is ~30–40% saturated with iron under normal conditions, this procedure radiolabels mainly plasma Tf with ^{59}Fe. Subsequently, uptake of ^{59}Fe by the spleen and femur, organs that utilize mainly Tf iron for hematopoiesis, was quantified at the indicated time points. As expected, a spike in the uptake of ^{59}Fe by newly synthesized red blood cells was noted after 24 h in both mouse lines. However, the counts in $PrP^{-/-}$ samples were significantly lower relative to matched $PrP^{+/+}$ controls (Fig. 2A and B).

To evaluate whether PrP^C influences the uptake of NTBI, a circulating pool of NTBI in $PrP^{+/+}$ and $PrP^{-/-}$ mice was radiolabeled by saturating plasma Tf with excess unlabeled FAC, followed 30 min later by intraperitoneal injection of ^{59}Fe-citrate essentially as described [28]. The mice were euthanized after 6 h, and uptake of ^{59}Fe-NTBI by the liver and pancreas, the principal organs that store and utilize NTBI, respectively, was quantified.

Fractionation of equal aliquots of plasma from untreated (–FAC) and +FAC treated mice on native PAGE followed by autoradiography confirmed ~90% inhibition of ^{59}Fe-labeling of plasma Tf in the latter (Fig. 2C, lanes 1–4 vs 5–8), ensuring radiolabeling of mainly NTBI in +FAC mice [28]. A comparison of ^{59}Fe uptake by the liver and pancreas in the two mouse strains revealed significantly lower levels in $PrP^{-/-}$ samples relative to $PrP^{+/+}$ controls (Fig. 2D). Fractionation of liver homogenates on native PAGE followed by autoradiography confirmed significantly less ^{59}Fe-ferritin in $PrP^{-/-}$ relative to $PrP^{+/+}$ controls (Fig. 2E, lanes 1,2 vs 3,4; arrowhead; Fig. 2F), indicating impaired uptake and/or storage of ^{59}Fe-NTBI in the absence of PrP^C.

These results suggest that PrP^C promotes the uptake of both Tf iron and NTBI, and other iron-modulating proteins are unable to compensate fully for its absence.

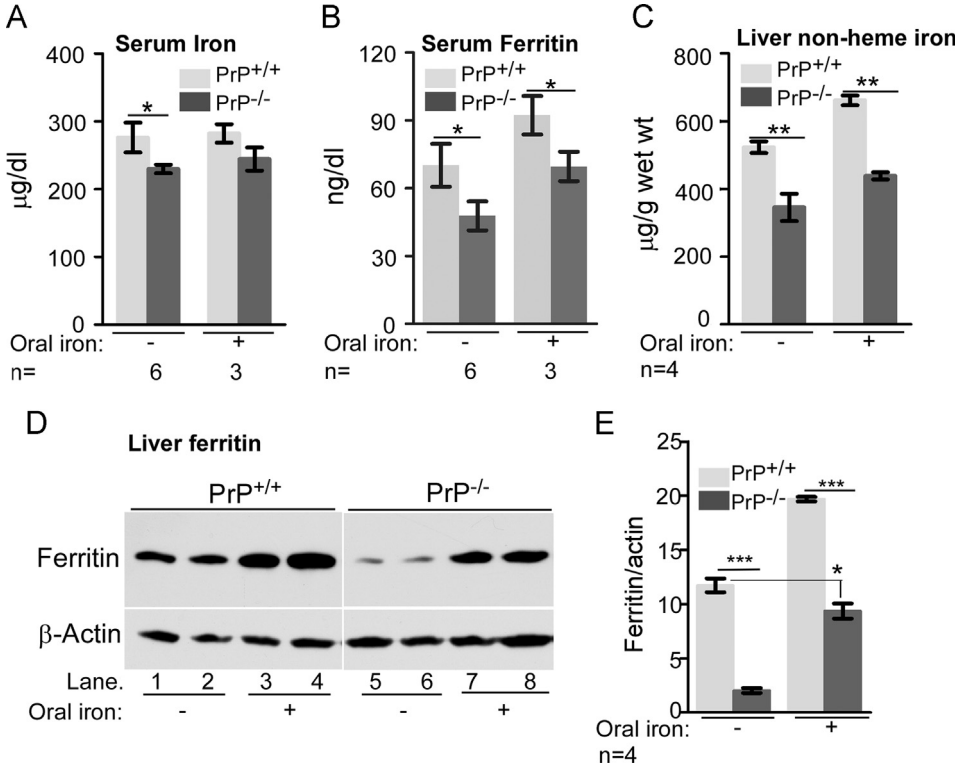

Fig. 1. Uptake and storage of iron by the liver is impaired in $PrP^{-/-}$ mice. (A–C) Serum iron (A) serum ferritin (B), and liver nonheme iron (C) are lower in $PrP^{-/-}$ mice relative to $PrP^{+/+}$ controls. Supplementation with oral iron restores serum iron of $PrP^{-/-}$ mice toward control values, but serum ferritin and liver nonheme iron remain significantly lower than matched controls. (D and E) Ferritin in liver homogenates of $PrP^{-/-}$ mice on regular chow is lower than $PrP^{+/+}$ controls (panel D, lanes 1 and 2 vs 5 and 6, panel E). Iron supplementation upregulates ferritin in both mouse lines (panel D, lanes 3, 4, 7, 8; panel E), but the levels in iron-supplemented $PrP^{-/-}$ mice remain significantly lower than control and iron-supplemented $PrP^{+/+}$ mice (panel D, lanes 7, 8 vs 1–4; panel E). (All samples were fractionated on the same gel that was rearranged using Photoshop). Reaction for β-actin was used to normalize densitometric data in panel E. Values are mean ± SEM of the indicated *n*. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

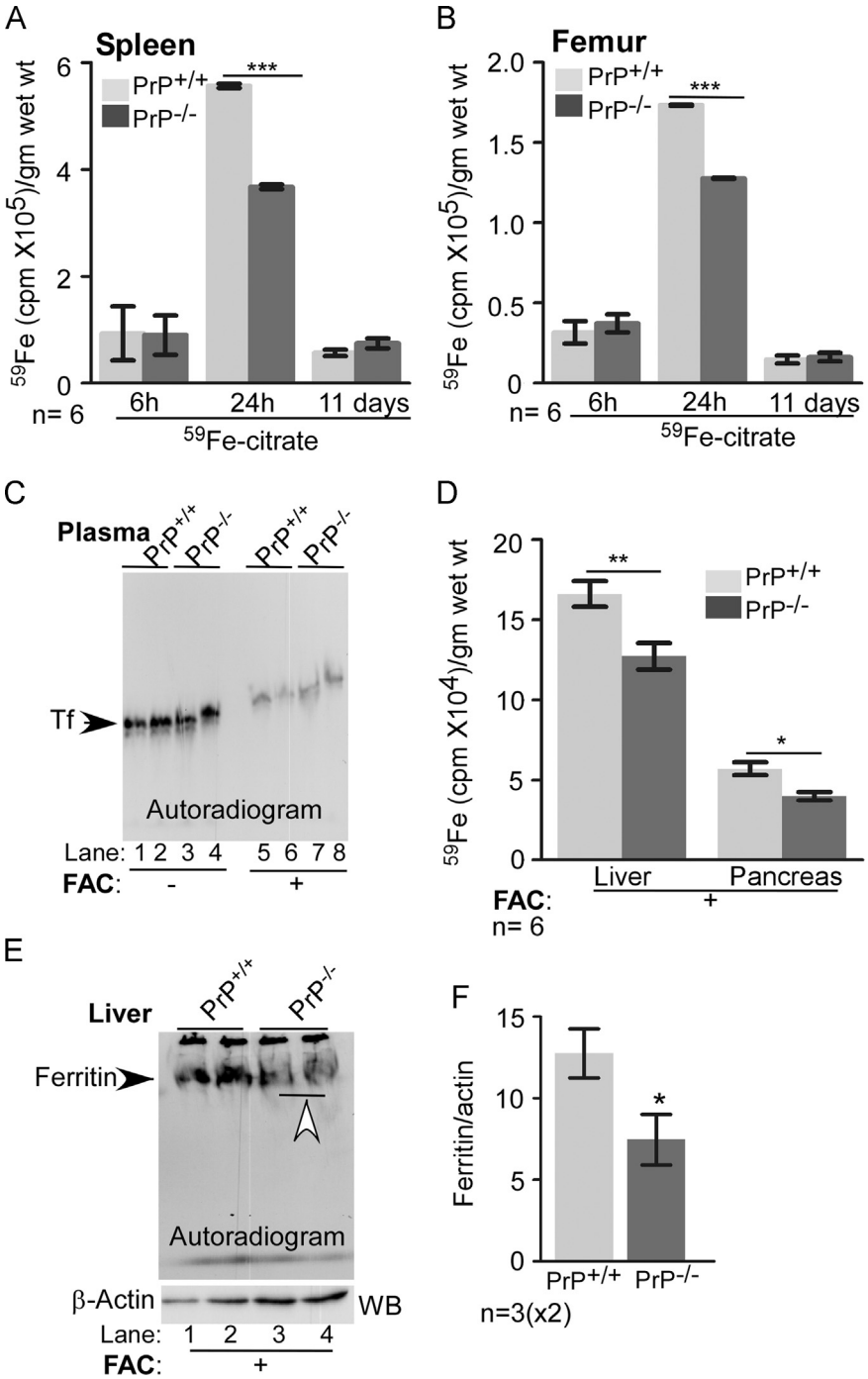

Fig. 2. PrP$^{-/-}$ mice show impaired uptake of Tf iron and NTBI. (A and B) Uptake of ^{59}Fe from intraperitoneally administered ^{59}Fe-citrate by the spleen and femur is maximal after 24 h, and significantly lower in PrP$^{-/-}$ samples relative to PrP$^{+/+}$ controls. (C) Intraperitoneal administration of excess unlabeled FAC prior to ^{59}Fe-citrate inhibits radiolabeling of plasma Tf by ~90% (lanes 1–4 vs 5–8) (D) Uptake of ^{59}Fe-NTBI is significantly lower in the liver and pancreas of PrP$^{-/-}$ mice relative to PrP$^{+/+}$ controls. (E and F) Incorporation of ^{59}Fe-NTBI by liver ferritin (+FAC) is significantly lower in PrP$^{-/-}$ samples relative to PrP$^{+/+}$ controls (lanes 3, 4 vs 1, 2). Western blotting of the same samples and probing for β-actin provides a control for protein loading. Values are mean ± SEM of the indicated n.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.3. Uptake, storage, and utilization of iron from ferritin are increased in PrP$^{-/-}$ mice

To evaluate whether PrP^{C} influences the uptake and storage of an alternative source of iron that does not require the activity of FR proteins, uptake of ^{59}Fe from intraperitoneally introduced ^{59}Fe-ferritin was evaluated since ferritin is endocytosed by the Tim-2 receptor before the release of associated ^{59}Fe in the cytosol [30].

Accordingly, ^{59}Fe-citrate and purified, soluble ^{59}Fe-ferritin associated with equal amounts of total iron and ^{59}Fe counts were introduced intraperitoneally in PrP$^{+/+}$ and PrP$^{-/-}$ mice, and uptake of ^{59}Fe by the liver was quantified at the indicated times (Fig. 3). ^{59}Fe counts in the blood and remaining carcass were quantified to eliminate error due to nonspecific phagocytosis of ^{59}Fe-ferritin in the peritoneal cavity.

Surprisingly, uptake of ^{59}Fe from ferritin was significantly higher in PrP$^{-/-}$ samples relative to PrP$^{+/+}$ controls at all the time points tested (Fig. 3 A). However, uptake of ^{59}Fe from citrate that is likely to associate with plasma Tf and NTBI was significantly lower in PrP$^{-/-}$ mice (Fig. 3B).

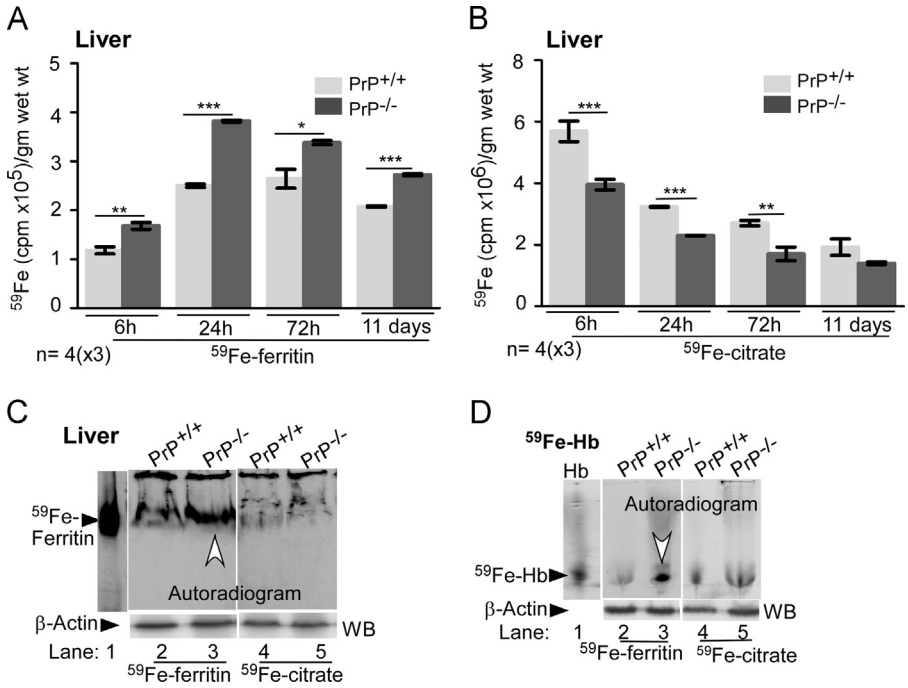

Fig. 3. Uptake, storage, and utilization of intraperitoneally administered ferritin iron is increased in PrP$^{-/-}$ mice. (A and B) Hepatic uptake of ^{59}Fe by PrP$^{-/-}$ mice is higher when exposed to intraperitoneal ^{59}Fe-ferritin, and lower when exposed to equal counts of ^{59}Fe-citrate relative to PrP$^{+/+}$ controls at the indicated time points. Values are mean ± SEM of the indicated *n*.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (C) Autoradiography of 11 day liver homogenate samples fractionated by native PAGE shows significantly more ^{59}Fe in PrP$^{-/-}$ samples exposed to ^{59}Fe-ferritin relative to PrP$^{+/+}$ controls (lanes 2,3), and minimal incorporation in both mouse lines exposed to ^{59}Fe-citrate (lanes 4,5). A representative fluorogram from three independent experiments is shown. (D) Incorporation of ^{59}Fe in hemoglobin is significantly higher in PrP$^{-/-}$ samples exposed to ^{59}Fe-ferritin relative to PrP$^{+/+}$ controls (lanes 2,3), and equivalent in both mouse lines exposed to ^{59}Fe-citrate (lanes 4,5). A representative fluorogram from three independent experiments is shown. (Samples in panels C and D were fractionated on the same gel that was rearranged by Photoshop). Reaction for β-actin confirms equivalent protein loading.

Fractionation of 11 day liver homogenates by native PAGE followed by autoradiography showed significantly higher incorporation of ^{59}Fe in liver tissue ferritin in samples injected with purified ^{59}Fe-ferritin, and much lower levels in both mouse lines exposed to ^{59}Fe-citrate (Fig. 3C, lanes 2–5). Western blotting of the same samples for actin confirmed that the differences were not an artifact of sample loading.

To evaluate whether the stored ^{59}Fe in liver ferritin was used for hemoglobin synthesis, the predominant consumer of systemic iron, lysates prepared from 11 day washed RBCs were fractionated by native PAGE followed by autoradiography. The signal from ^{59}Fe-Hb was much stronger in samples from PrP$^{-/-}$ mice exposed to ^{59}Fe-ferritin relative to matched PrP$^{+/+}$ controls, and from mice exposed to ^{59}Fe-citrate (Fig. 3D, lanes 2–5, arrowhead).

Together, the above observations demonstrate that absence of PrP^C limits hepatic uptake of circulating Tf iron and NTBI that require reduction to Fe^{2+}. Uptake of iron from ferritin that does not require reduction and metabolic processes subsequent to iron uptake, i.e., storage in liver ferritin, and utilization for hemoglobin synthesis are not affected by the absence of PrP^C. Surprisingly, mRNA levels of liver hepcidin, the master regulator of systemic iron homeostasis, did not show a significant difference between PrP$^{-/-}$ samples and matched PrP$^{+/+}$ controls. However, analysis of the same samples for additional iron-modulating proteins revealed significant upregulation of mRNA for divalent metal transporters ZIP8 and ZIP14, probably as a response to the iron deficiency in these mice (supplemental Fig. 1).

3.4. PrP^C promotes NTBI uptake via ZIP14 and DMT1

To determine whether the FR activity of PrP^C is coupled with iron uptake through specific metal transporters, HepG2 cells, a well-characterized hepatoma cell line, was used. Expression of a fusion construct of PrP^C and green-fluorescent protein (PrP$^{C\text{-}GFP}$) [24] revealed expression on the plasma membrane, colocalizing with wheat-germ agglutinin (WGA) that binds to apical and basolateral surface glycoproteins on cultured HepG2 cells (Fig. 4, panels 1–3) [31].

Before proceeding with the identification of PrP^C-associated metal transporters, FR activity of transfected PrP^C in HepG2 cells was confirmed. PrP^C (HepG2-PrP^C) or vector (HepG2-vec)-containing HepG2 cells were exposed to 30 μM FAC (Fe^{3+}) or iron ascorbate (Fe^{2+}), and uptake of iron was measured as a function of ferritin upregulation (Fig. 5A). Expression of PrP^C induced 2.7-fold upregulation of intracellular ferritin, indicating increased uptake of iron from the medium (Fig. 5A, lanes 1 vs 4). Notably, exposure to Fe^{3+} (FAC) induced 3.1-fold upregulation of ferritin in HepG2-PrP^C cells relative to vector controls (Fig. 5A, lane 2 vs 5; Fig. 5B), while exposure to Fe^{2+} from iron ascorbate (Fe^{2+}) caused a similar change in ferritin levels in the two cell lines (Fig. 5A, lane 3 vs 6; Fig. 5B). Since the amount of added Fe^{3+} and Fe^{2+} far exceeded the iron-binding capacity of Tf in the culture medium, these results suggest a facilitative role of PrP^C in the uptake of both Tf iron and NTBI from the medium due to its FR activity.

Subsequently, stable HepG2-vec and HepG2-PrP^C cells were cotransfected with GFP-tagged ZIP8, ZIP14, or DMT1, and uptake of iron from complete medium (with no added iron) was quantified after 24 h as a function of ferritin upregulation. Expression of ZIP8, ZIP14, or DMT1 in HepG2-vec cells caused ~3.2-fold upregulation of ferritin relative to controls, consistent with the iron transport function of these proteins (Fig. 5C, lane 1 vs 2–4, Fig. 5D). However, coexpression of PrP^C with ZIP8, ZIP14, or DMT1 induced a further upregulation of ferritin by 0.8-, 2.8-, and 2.6-fold, respectively, indicating that PrP^C enhances iron uptake via these transporters, mainly ZIP14 and DMT1 (Fig. 5C, lanes 2–4 vs 6–8; Fig. 5D).

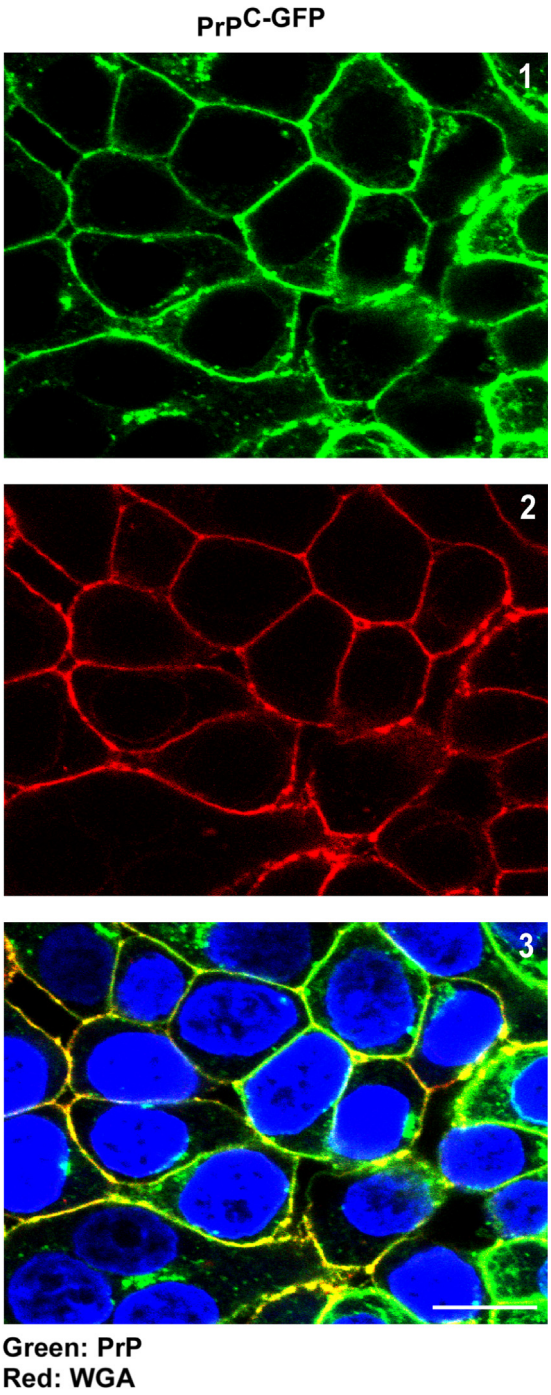

Fig. 4. Distribution of PrP^{C} on HepG2 cells. Confocal imaging of HepG2-$PrP^{C\text{-}GFP}$ cells immunostained with WGA shows mainly plasma membrane localization of $PrP^{C\text{-}GFP}$ (panels 1–3). Scale bar: 10 μM.

Notably, a significant decrease in PrP^{C} expression accompanied coexpression of the iron transporter, particularly ZIP14 and DMT1. The decrease was specific to 3F4-reactive full-length PrP^{C}, including the unglycosylated (27 kDa) and various glycoforms migrating between 30 and 37kDa (Fig. 5C, upper panel; Fig. 5E). On the other hand, anti-C reactive PrP^{C} forms that include N-terminally truncated unglycosylated (18 kDa) and glycosylated forms (18–30 kDa) in addition to full-length PrP^{C} showed a corresponding increase (Fig. 5C, lower panel, lanes 7 and 8). A darker exposure revealed upregulation and cleavage of endogenous PrP^{C} in HepG2-vec cells in the presence of ZIP14 and DMT1 (lower panel, darker exposure, lanes 3 and 4). Since PrP^{C} undergoes physiological recycling from the plasma membrane and is cleaved at residues 111/112 during this process that eliminates the epitope for 3F4 (Fig. 5E) [25], it is likely that expression of ZIP14 and DMT1 upregulates recycling, or triggers cleavage of PrP^{C} by an independent process.

4. Discussion

This report demonstrates that PrP^{C} promotes uptake of NTBI and, to a lesser extent, Tf iron by providing FR activity necessary for the transport of ferric iron through divalent metal transporters ZIP14 and DMT1. This explains reduced hepatic iron stores of $PrP^{-/-}$ mice despite chronic iron supplementation, and enhanced uptake of ferric iron by HepG2 cells expressing exogenous PrP^{C}. Coexpression of PrP^{C} with ZIP14 and DMT1 augments ferric iron uptake further, suggesting that PrP^{C} functions as a FR partner for these transporters. In contrast, PrP^{C} does not influence hepatic uptake and storage or utilization of ferritin-bound iron *in vivo*, or of iron ascorbate (Fe^{2+}) by HepG2 cells *in vitro*, sources that do not require reduction of associated iron for uptake. These observations underscore the functional significance of PrP^{C} as a FR, and the inability of other FR proteins to compensate fully for its absence.

As reported previously [20] and expanded here, absence of PrP^{C} results in systemic iron deficiency in $PrP^{-/-}$ mice as evidenced by significantly lower levels of serum iron, serum ferritin, liver nonheme iron, and liver ferritin relative to $PrP^{+/+}$ controls. Although chronic exposure to excess oral iron restored serum iron in $PrP^{-/-}$ mice to control levels, serum ferritin, liver nonheme iron, and liver ferritin remained significantly lower than iron-supplemented $PrP^{+/+}$ mice, suggesting impaired hepatic uptake and storage of iron as the principal cause of iron deficiency, not insufficient absorption from the duodenum.

It is notable that impairment of hepatic iron uptake in $PrP^{-/-}$ mice is specific to NTBI and Tf iron that require reduction to the ferrous form for transport through divalent metal transporters [7,9]. Uptake of ^{59}Fe-Tf by the spleen and bone marrow, main hematopoietic organs that utilize Tf-bound iron, and ^{59}Fe-NTBI by the liver and pancreas, principal organs that store and utilize NTBI, was significantly lower in $PrP^{-/-}$ mice relative to $PrP^{+/+}$ controls, despite systemic iron overload [28]. On the other hand, uptake, storage, and utilization of ^{59}Fe from iron-loaded ferritin that utilizes receptor-mediated uptake pathway [30] were increased in $PrP^{-/-}$ mice, highlighting the iron deficiency of these mice, and implicating PrP^{C} in the uptake of ferric iron, not ferritin iron that is stored and used effectively in its absence. It is likely that the relatively mild iron deficiency in $PrP^{-/-}$ mice is due to the participation of PrP^{C} in the uptake of mainly NTBI from the plasma membrane, not Tf iron that can utilize alternative FR proteins such as Steap3 in late endosomes. Consistent with this assumption, transcription of liver hepcidin, the master regulator of systemic iron homeostasis, was not altered in $PrP^{-/-}$ mice, though ZIP8 and ZIP14 showed significant upregulation (supplemental Fig. 1).

In vitro observations on HepG2 expressing exogenous PrP^{C} support the *in vivo* observations in $PrP^{-/-}$ mice. Thus, expression of PrP^{C} in HepG2 cells increased the uptake of Fe^{3+} from FAC, and had minimal influence on Fe^{2+} from iron ascorbate, leaving little doubt that PrP^{C} is a functional FR on the plasma membrane of these cells. However, this is an unusual function for a glycosylphosphatidylinositol-linked protein that is unlikely to utilize reducing equivalents from the cytosol because of linkage to the outer leaflet of the plasma membrane. It is likely that PrP^{C} utilizes extracellular reducing agents for its FR activity such as conversion of ascorbate released from cells to dehydroascorbate through the intermediate ascorbyl free radical [32]. We believe

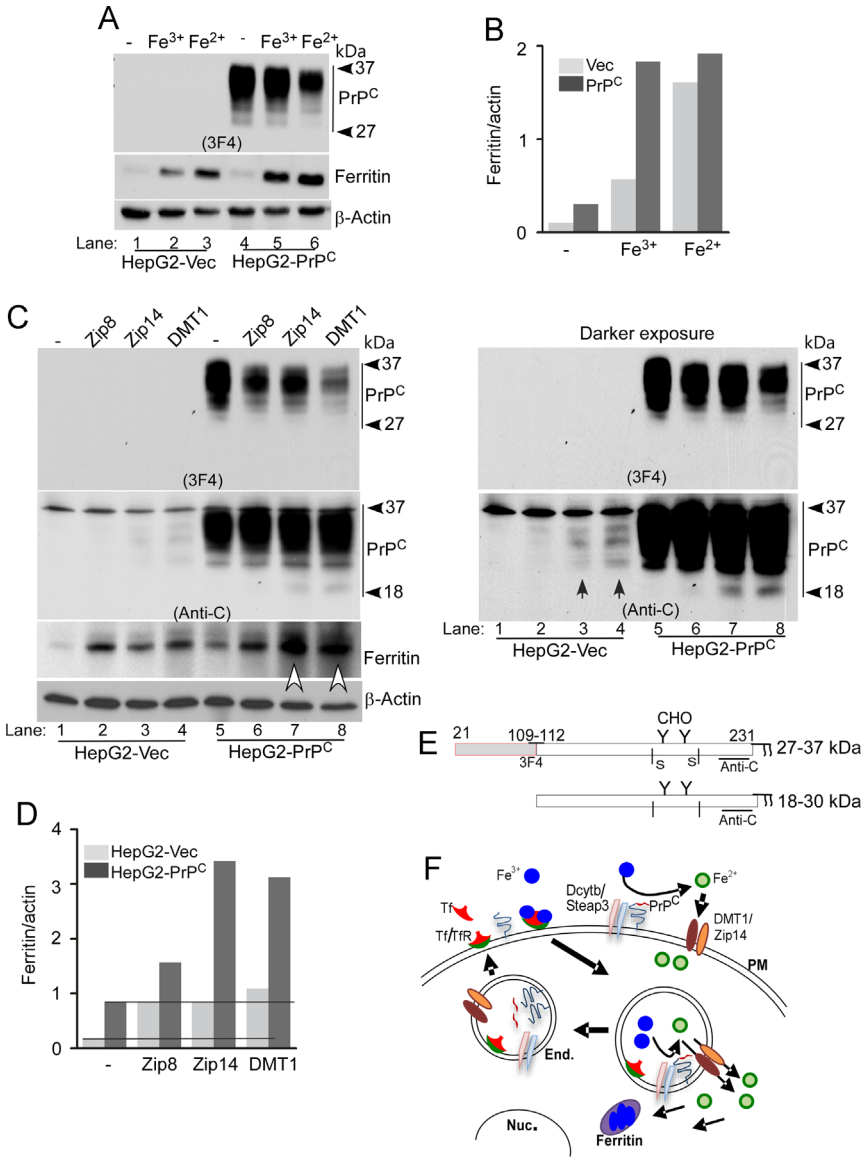

Fig. 5. PrP^C functions as a FR partner for ZIP14 and DMT1. (A and B) Expression of PrP^C in HepG2 cells upregulates ferritin by 2.7-fold relative to vector-expressing controls (A, lane 1 vs 4; B). Exposure to FAC (Fe^{3+}) causes 3.1-fold upregulation of ferritin in HepG2-PrP^C cells relative to HepG2-vector controls (A, lane 2 vs 5; B). Uptake of iron from iron ascorbate (Fe^{2+}) is similar in HepG2-vector and HepG2-PrP^C cells (lane 3 vs 6; B). A representative gel from three independent experiments and corresponding densitometric analysis is shown. (C and D) Transfection of HepG2-vector cells with ZIP8, ZIP14, or DMT1 upregulates ferritin by ~3.2-fold (C, lane 1 vs 2–4, D). Expression of ZIP14 and DMT1 in HepG2-PrP^C cells causes a further upregulation of ferritin by 2.8- and 2.6-fold, respectively (C, lanes 5,6 vs 7,8; D). Surprisingly, 3F4-reactive PrP^C is downregulated in the presence of ZIP8, ZIP14, and DMT1 (C, upper panel, lane 5 vs 6–8, darker exposure), while the anti-C-reactive N-terminally truncated forms of PrP^C increase in abundance especially in the presence of ZIP14 and DMT1 (C, lower panel, lane 5 vs 6–8, darker exposure; E). Notably, anti-C-reactive truncated forms of endogenous PrP^C are visible even in vector-transfected cells coexpressing ZIP14 and DMT1 (lower panel, darker exposure, lanes 3, 4). A representative gel from four independent experiments and corresponding densitometric analysis are shown. (E) Schematic representation of epitopes for 3F4 and anti-C. (F) Proposed function of PrP^C as a FR. PrP^C at the plasma membrane and endosomal membrane reduces Fe^{3+} to Fe^{2+}, which is then transported across the lipid bilayer through ZIP14 and DMT1. This process results in cleavage of PrP^C at residues 111/112 and loss of the N-terminal FR domain. PrP^C lacking the N-terminal domain is transported back to the plasma membrane, precluding further FR activity. Tf iron is endocytosed by the Tf/TfR complex, and Fe^{3+} released from Tf in acidic endosomes is reduced to Fe^{2+} by PrP^C and other membrane-bound FR proteins such as Dcytb and/or Steap3. Fe^{2+} transported to the cytosol joins the cellular labile iron pool, and excess is oxidized to Fe^{3+} and stored in cytosolic ferritin. PM, plasma membrane; End, endosome; Nuc, nucleus, Tf/TfR, transferrin/transferrin receptor.

that PrP^C itself functions as a ferrireductase based on the following observations: (1) uptake of Fe^{3+} is reduced in the liver and kidney of $PrP^{-/-}$ mice, organs involved in storage and reuptake of iron from the glomerular filtrate, respectively, and likely to express a distinct set of proteins on the plasma membrane [this study; 22], and (2) exogenous expression of PrP^C on HepG2 cells, proximal kidney tubule epithelial cells [this study; 22], neuroblastoma cells [19], and K562 cells, a hematopoietic cell line [unpublished observations], increases FR activity on the plasma membrane and in cell lysates, indicating a primary role of PrP^C in iron uptake. The increase in FR activity due to PrP^C is sensitive to heat, pH, and is saturated at a specific concentration of PrP^C and Fe^{3+} [19], suggesting an enzymatic nature. However, the possibility that PrP^C interacts with a ubiquitously expressed FR via its octapeptide repeat region cannot be ruled out from our data. Further studies are necessary to characterize the FR activity of PrP^C fully.

It is notable that coexpression of PrP^C with ZIP14 or DMT1 increased uptake of Fe^{3+} from the medium, indicating a close functional interaction. Interestingly, expression of ZIP14 and DMT1 upregulated endoproteolytic cleavage of PrP^C, a physiological process that increases the proportion of N-terminally cleaved, C-terminal fragment of PrP^C relative to the full-length form [25].

Since PrP^C is expressed abundantly on neurons, it possibly functions as a FR partner for ZIP14 and DMT1 on the neuronal plasma membrane and endosomes, facilitating the uptake of NTBI and Tf iron by these cells. Concomitant cleavage of the N-terminus necessary for its FR activity is likely to regulate iron uptake, a phenomenon unique to PrP^C and absent in other FR proteins such as Dcytb and Steap3 abundant on the plasma membrane and endosomes, respectively, of specific cell types (Fig. 5F). A detailed understanding of the relationship between iron transport and endoproteolytic cleavage of PrP^C is required for defining the contribution of PrP^C to cellular iron metabolism.

Interestingly, PrP and the Zip family of proteins share an evolutionary link [33,34], and certain members of the Zip family are expressed in close proximity with PrP^C at the plasma membrane [35] and are altered during prion infection [36]. ZIP14 is most abundant in the liver, pancreas, and heart and, like PrP^C, is localized at the plasma membrane where it mediates the uptake of NTBI [16,17,37]. Recent reports indicate localization of ZIP14 in late endosomes and lysosomes in addition to the plasma membrane, suggestive of a role in the transport of Tf iron across the endosomal membrane [38]. ZIP8, on the other hand, is most abundant in the lung, testis, and kidney [39]. Impaired uptake of ^{59}Fe-NTBI by the liver and pancreas of $PrP^{-/-}$ mice therefore suggests that PrP^C is the principal FR partner for ZIP14 on the plasma membrane of these organs. Although physical interaction of PrP^C with the metal transporters is not necessary for the coupled reduction and transport of Fe^{3+} iron, limited colocalization of PrP^C with ZIP14 was evident in HepG2 cells by confocal microscopy (unpublished observations).

In conclusion, this report provides convincing evidence that PrP^C functions as a FR partner for ZIP14 and DMT1, and possibly regulates the uptake of NTBI and Tf iron by shedding its N-terminal FR domain during recycling from the plasma membrane. Since DMT1 and ZIP14 mediate the transport of other cations including copper and zinc, aggregation of PrP^C to the disease-associated PrP-scrapie form during prion disease pathogenesis is likely to alter the homeostasis of other metals in addition to the reported effect on brain iron homeostasis [29,40]. In addition, these observations have significant implications for Alzheimer's disease where brain metal imbalance is a prominent feature, and PrP is believed to play an important pathogenic role [41–44].

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.03.037.

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