Wild-type and mutant ferroportins do not form oligomers in transfected cells

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Ferroportin [FPN; Slc40a1 (solute carrier family 40, member 1)] is a transmembrane iron export protein expressed in macrophages and duodenal enterocytes. Heterozygous mutations in the FPN gene result in an autosomal dominant form of iron overload disorder, type-4 haemochromatosis. FPN mutants either have a normal iron export activity but have lost their ability to bind hepcidin, or are defective in their iron export function. The mutant protein has been suggested to act as a dominant negative over the wt (wild-type) protein by multimer formation. Using transiently transfected human epithelial cell lines expressing mouse FPN modified by the addition of a haemagglutinin or c-Myc epitope at the C-terminus, we show that the wtFPN is found at the plasma membrane and in Rab5-containing endosomes, as are the D157G and Q182H mutants. However, the delV162 mutant is mostly intracellular in HK2 cells (human kidney-2 cells) and partially addressed at the cell surface in HEK-293 cells (human embryonic

kidney 293 cells). In both cell types, it is partially associated with the endoplasmic reticulum and with Rab5-positive vesicles. However, this mutant is complex-glycosylated like the wt protein. D157G and G323V mutants have a defective iron export capacity as judged by their inability to deplete the intracellular ferritin content, whereas Q182H and delV162 have normal iron export function and probably have lost their capacity to bind hepcidin. In co-transfection experiments, the delV162 mutant does not co-localize with the wtFPN, does not prevent its normal targeting to the plasma membrane and cannot be immunoprecipitated in the same complex, arguing against the formation of FPN hetero-oligomers.

Key words: endoplasmic reticulum, ferroportin, glycosylation, haemochromatosis, iron transport, oligomer.

INTRODUCTION

Iron homeostasis in mammals relies on the continuous recycling of iron by macrophages following degradation of senescent red blood cells and on iron absorption from the diet by duodenal enterocytes to compensate for minimal daily losses. This continuous exchange of iron between body compartments requires several iron transport molecules important for iron translocation through biological membranes. Ferroportin [FPN; also known as IREG1 (iron-regulated transporter 1) or MTP1 (metal transporter protein 1)], the product of the *Slc40a1* (solute carrier family 40, member 1) gene, is an iron exporter mostly expressed in tissue macrophages and at the basolateral side of duodenal enterocytes and placental cells [1–4]. Conditional knockout of FPN in mice at the post-natal stage suggests that it is the sole iron exporter in mammals, since FPN-deficient animals exhibit iron retention within enterocytes and macrophages [5] and rapidly become anaemic. Functional studies in *Xenopus* oocytes or in transfected HEK-293 cells (human embryonic kidney 293 cells) have shown that FPN overexpression increases iron export and creates an iron-deficient phenotype with reduced cellular ferritin content [1,6]. Transfection of FPN in macrophages also increases iron export following incubation with opsonized ⁵⁹Fe-labelled red blood cells [7]. Recent studies have shown that hepcidin, a soluble peptide that regulates iron homeostasis, can bind to FPN in transfected epithelial cells and induce its internalization and subsequent degradation [8]. Moreover, hepcidin can also act on native FPN in macrophages by inducing its internalization and degradation [9],

and can block iron recycling following phagocytosis of opsonized red blood cells [10]. More evidence for the essential role of FPN as an iron export protein arises from human pathology. Heterozygous mutations in the FPN gene result in an autosomal dominant iron overload condition (type-4 haemochromatosis) with rather heterogeneous phenotypes. At least 12 point mutations leading to an amino acid replacement and one codon deletion have been described so far (see [11] for a review and Figure 1 for positions of the mutations). Important variability has been reported in the phenotypic expression of the disease according to the mutation. Some mutations (A77D, delV162 and G490D) are responsible for mild patterns of iron loading with moderately elevated serum ferritin levels, normal transferrin saturation and a restricted pattern of iron overloading limited to macrophages [12–16], while other mutations (Y64N, N144H, N144D, N144T and C326S) induce high levels of transferrin saturation and iron accumulation predominantly in parenchymal cells [17–21]. It has been proposed that the mutations in the first group result in loss-of-function alleles, while the other mutations are thought to have preserved transport capacities but fail to bind hepcidin and act as gain-of-function mutations [6,22]. This defect in negative feedback regulation of some FPN mutants is thought to contribute to increased intestinal iron absorption and hepatocyte iron loading. In addition, some evidence has been provided that FPN is multimeric and that mutant FPN can multimerize with normal FPN and have a dominant negative effect [22]. These observations suggest that FPN contains several functional domains important either for membrane targeting or for iron recycling and export activity.

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; ER, endoplasmic reticulum; FCS, fetal calf serum; FPN, ferroportin; GFP, green fluorescent protein; HA, haemagglutinin; HEK-293 cell, human embryonic kidney 293 cell; HK2 cell, human kidney-2 cell; PFA, paraformaldehyde; PI, protease inhibitor; PNGase F, peptide N-glycosidase F; TM, transmembrane domain; wt, wild-type.

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Figure 1 Predicted membrane topology of mouse FPN and positions of FPN mutations reported in human type-4 haemochromatosis

Two models are proposed for mouse FPN, with nine or ten (right inset) TMs, based on a multiple sequence alignment performed with the ProteinPredict server. The alternative model for the N-terminal end of the molecule as proposed by Liu et al. [23] is also shown (left inset). Mutations studied in the present paper are indicated (black symbols) as well as positions of previously reported mutations [12–21, 26] (grey symbols) and the loop (amino acids 224–304) used as peptide to raise the anti-FPN antibody. Y indicates putative glycosylation sites.

Several models have been proposed for FPN based on computer-assisted structural predictions [2, 3, 13] or on epitope mapping and site-directed mutagenesis ([23] and Figure 1). The mechanism of iron transport via FPN is not clear and we are unable to say at the present time whether it is occurring by ion exchange or by facilitated transport, as has been described for DMT1 (divalent metal transporter 1), a proton-dependant Fe^{2+} transporter [24]. It is widely accepted that cellular export of iron by FPN and loading on to serum transferrin require ferroxidase activity, served by multicopper oxidases such as hephaestin [25] in the enterocyte or its soluble homologue, ceruloplasmin, in all other cell types, suggesting that FPN exports Fe^{2+} . Thus several of the mutations could alter a functional binding site for regulatory proteins involved in iron export, including the hepcidin-binding site.

The main objectives of the present paper were (i) to contribute to our knowledge of FPN topology using two different antibodies, (ii) to study the intracellular distribution and iron export function of both normal and mutated FPNs and (iii) to re-evaluate the multimer model. We have restricted our functional studies to the delV162 mutations and to three missense mutations (D157G, Q182H and G323V) that we have initially described [26].

MATERIALS AND METHODS

Antibodies

Production and purification of rabbit polyclonal anti-mouse FPN antibody were described previously [4]. Monoclonal anti-HA (haemagglutinin) and polyclonal anti-c-Myc antibodies were obtained from Covance. Organelle-specific antibodies were: mouse anti-BiP/GRP78 (where BiP is immunoglobulin heavy-chain binding protein and GRP78 is 78 kDa glucose related protein) (Stressgen) for ER (endoplasmic reticulum), and rabbit anti-Rab5 for early and recycling endosomes (Stressgen). Mouse anti-(human H ferritin) antibody was a gift from Dr Paolo Santambrogio

(Milano, Italy), Alexa Fluor® 546- or Alexa Fluor® 488-labelled goat anti-mouse and anti-rabbit IgG antibodies (both highly cross-absorbed) (Molecular Probes) were used as secondary antisera.

Plasmid constructs

Mouse FPN coding sequence was modified by in-frame addition of an HA epitope tag at the C-terminus. This was carried out by PCR amplification from mouse bone marrow-derived macrophages using oligonucleotide mFPN5 (5'-CGGGAATTCTCG-GTTCCTCTCACYCCTGTCAACAAG-3') containing the EcoRI restriction site and mFPN-HA (5'-CCCGCTCGAGCTAGCTA-GCGTAATCTGGCAGTCATATGGATATACAACAGATGTA-TTCGGTTGATTTTCATCTG-3') containing an XhoI site and the HA sequence (boldface indicates restriction sites and underlined type indicates HA sequence). The PCR product was digested with EcoRI and XhoI and inserted into the corresponding sites of pcDNA3. FPN mutations naturally occurring in patients with dominant type-4 haemochromatosis were introduced by site-directed mutagenesis in the HA-tagged protein using the QuikChange® site-directed mutagenesis kit (Stratagene). The wtFPN-c-Myc (where wt is wild-type) and wtFPN constructs were created by removing the 3'-end of the wtFPN-HA coding sequence using an internal EcoRV restriction site and XhoI, and subcloning the PCR amplification product obtained with oligonucleotides mFPN-EcoRV (5'-CGGGATATCCGTTCTAGGTT-TGTGAATGTGGAGCCAGTGTC-3') and mFPN-c-Myc (5'-CCCGCTCGAGCTACAAGTCCTCTTCAGAAATGAGCTT-TTGCTCTACAACAGATGTATTTCGGTTGATTTTCATCTG-3') for the wtFPN-c-Myc construct and oligonucleotide mFPN3 (5'-CCCGCTCGAGCTACAACAGATGTATTTCGGTTGATT-TTCATCTG-3') for the wtFPN construct. (Boldface indicates respectively EcoRV and XhoI restriction sites and underlined type indicates c-Myc sequence.)

CMV-loxP-Hygro-loxP-FPN-c-Myc (where CMV is cytomegalovirus) construct was obtained by subcloning the mouse wtFPN-c-Myc cDNA into the CMV-loxP-Hygro-loxP-LacZ construct kindly provided by Dr Frederic Jaisser (Collège de France, Paris, France). wtFPN-c-Myc construct was isolated from the pcDNA3 vector by EcoRI and XbaI digestion and subcloned after blunt ligation into the BamHI site of the CMV-loxP-Hygro-loxP-LacZ construct.

Cell culture and transfections

HEK-293 cell line was maintained in DMEM (Dulbecco's modified Eagle's medium)/Ham's medium containing 10% (v/v) FCS (fetal calf serum; Invitrogen). On the day before transfection, plates were seeded at 50% confluency. Cells were transfected using Effecten (Qiagen) following the manufacturer's standard method. In co-transfection assays, the total amount of plasmid was the same as for single transfections.

Human kidney epithelial cell line [HK2 (human kidney-2)] was maintained in RPMI 1640 medium containing 10% FCS (Invitrogen). On the day before transfection, plates were seeded at 50% confluence. Cells were transfected using Lipofectamine™ Plus (Invitrogen) following the manufacturer's standard method. Transiently transfected cells were analysed approx. 48 h post-transfection.

The RCCD2mIN 8 clone, constitutively expressing the MerCreMer-inducible recombinase, kindly provided by Dr Frederic Jaisser, was cultured on Petri dishes coated with rat type I collagen. Selection pressure was maintained throughout the culture by growing the cells in the presence of Geneticin (G418; 200 $\mu\text{g/ml}$) in a defined medium that contained 1:1 Ham's F-12/DMEM (Invitrogen) with 14 mM NaHCO_3 , 20 mM Hepes

buffer (pH 7.4), 10 units/ml penicillin–streptomycin, 2 mM glutamine, 5 μ g/ml transferrin, 50 nM sodium selenite, 50 nM triiodothyronine, 10 μ g/ml epidermal growth factor and 2 % FCS (Invitrogen). The RCCD2mIN 8 clone was stably transfected with CMV-loxP-Hygro-loxP-FPN–c-Myc construct, following the method described by Ouvrard-Pascaud et al. [27]. Briefly, 4×10^5 cells were seeded in six-well plates 24 h before transfection in G418-free medium. For each well, 1 μ g of linearized CMV-loxP-Hygro-loxP-FPN–c-Myc construct was used and cells were transfected using Lipofectamine™ Plus (Invitrogen), following the manufacturer's standard method. Cells were incubated in 300 μ g/ml hygromycin (Invitrogen) 2 days after transfection and were then seeded at very low density. Stably transfected cells were selected in the presence of 300 μ g/ml hygromycin, and hygromycin-resistant cells were isolated and amplified. FPN expression was analysed by immunofluorescence following treatment or not with 100 nM 4-hydroxytamoxifen (Sigma) for 20 h. Clone 14 was found to have the highest level of expression and was retained for further use.

Immunofluorescence

Cells grown on coverslips were fixed with methanol for 10 min at -20°C or with 4 % (w/v) PFA (paraformaldehyde) for 20 min at 4°C and, where indicated, permeabilized with 0.1 % Triton X-100. Cells were then washed with PBS, and blocked with 1 % BSA and 10 % (v/v) heat-inactivated goat serum in PBS. Incubations with the antibodies were performed in the blocking solution [7 % (w/v) skimmed milk in TBST (0.15 % Tween 20 in Tris-buffered saline)], in humid chamber, for 1 h at room temperature (20°C), using the following dilutions: rabbit anti-FPN, 1:50 to 1:100; mouse anti-HA, 1:250; rabbit anti-c-Myc, 1:50 to 1:100; mouse anti-BiP, 1:100; rabbit anti-Rab5, 1:100; and mouse anti-H ferritin, 25 μ g/ml. After three rinses with PBS, 0.5 % BSA and 0.01 % Triton X-100, cells were incubated for 1 h at room temperature with Alexa Fluor® 546- or/and Alexa Fluor® 488-labelled goat anti-mouse and anti-rabbit IgG antibodies, diluted at 1:200 in blocking solution. The stained cells were mounted in Prolong™ anti-Fade Solution (Molecular Probes). Fluorescence imaging was performed using either an epifluorescence microscope LEICA DM-IRM with a $\times 40$ immersion oil objective or a confocal microscope (Zeiss LSM 510), with a $\times 60$ oil immersion objective, using the multitracking-scanning procedure to avoid cross-talk between channels.

Glycosidase treatments

Cells grown in 100 mm dishes were washed with cold PBS, scraped into PBS/2 mM EDTA, using a rubber policeman and centrifuged at 200 g in a refrigerated microcentrifuge for 5 min. Cell pellets were then homogenized in 250 μ l of lysis buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 10 mM EDTA and 1 % Triton X-100) supplemented with a PI (protease inhibitor) cocktail (Sigma). The lysate was centrifuged at 400 g in a microcentrifuge at 4°C for 10 min to eliminate nuclei and unbroken cells. The post-nuclear supernatant fractions were used for deglycosylation, adapting manufacturer conditions: initial denaturation step was performed at room temperature for 30 min. Proteins (20 μ g) were then treated with 500 units of PNGase F (peptide N-glycosidase F) or endo H (endoglycosidase H; New England Biolabs), for 90 min at room temperature or 60 min at 37°C . Reactions were stopped by adding 1 vol. of $2.5\times$ Laemmli buffer to the samples. Proteins were run on an SDS/10 % polyacrylamide gel and blotted on to a PVDF membrane (Millipore). Ponceau Red staining confirmed similar gel loading and similar transfer of proteins to membranes. The membrane was probed with anti-HA

antibody (1:300) in blocking solution, and proteins were visualized by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase and SuperSignal West Pico Chemiluminescence substrate (Pierce).

Non-denaturing gel electrophoresis

Microsomal fractions were prepared following the previously described method [24]. Briefly, cells were washed with cold PBS, scraped into PBS/2 mM EDTA, using a rubber policeman and centrifuged at 200 g in a refrigerated microcentrifuge for 5 min. Cell pellets were then homogenized in 250 μ l of lysis buffer (10 mM Tris/HCl, pH 7, and 1 mM MgCl_2) supplemented with a PI cocktail (Sigma) by 20 passages through a 25-gauge needle. The lysate was centrifuged at 400 g in a microcentrifuge at 4°C for 10 min to eliminate nuclei and unbroken cells. The post-nuclear supernatant fraction was ultracentrifuged for 45 min at 60000 rev./min using a TLA100 rotor in a TLA Beckman centrifuge. Membrane pellets were resuspended in TNE buffer (100 mM NaCl, 10 mM Tris/HCl, pH 7, and 10 mM EDTA) containing PIs and stored at -80°C until use. Protein concentrations of microsomal fractions were determined by the Bradford assay (Bio-Rad). For the analysis under non-denaturing conditions, Triton X-100 was added to the microsomal fractions at 1 % final concentration to solubilize membrane-bound proteins. Total cell extracts were obtained following the method described above for glycosidase treatment. Samples containing either 10 μ g of microsomal proteins or 50 μ g of total cellular proteins were run in parallel on a 6 % polyacrylamide gel that did not contain SDS or 2-mercaptoethanol, and blotted on to membrane, and FPN was revealed using mouse anti-HA antibodies (1:400).

Immunoprecipitation

Cell lysates from double-transfected HEK-293 cells were obtained as described for glycosidase treatment but using a different lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, and 1 % Triton X-100). To avoid the possible disruption of weak protein interactions and membrane-bound complexes by SDS, an ionic detergent, the immunoprecipitation assay was performed with and without 0.2 % SDS. Protein concentration of the samples was determined by the Bradford assay (Bio-Rad) and adjusted to 2 mg/ml. Cell lysates (200 μ g; 100 μ l) were cleared by addition of 1 μ l of non-immune mouse serum and 50 μ l of a 1 % Protein G–Sepharose beads (Amersham) suspension previously equilibrated in the same extraction buffer. FPN in the cleared lysates was then immunoprecipitated by adding 1 μ l of mouse anti-HA antibody for 2 h at 4°C , followed by overnight incubation with 10 μ l of 1 % equilibrated Protein G–Sepharose. Supernatant was saved for further analysis. Beads were then extensively washed with the lysis buffer before solubilization in $2.5\times$ Laemmli buffer (30 min, room temperature). Total extracts (12.5 μ g), immunoprecipitates from 200 μ g cell lysates and supernatant (18.5 μ g) were run in parallel on an SDS/10 % polyacrylamide gel, blotted on to membrane and the membranes were probed with either mouse anti-HA antibody (1:400) or rabbit anti-c-Myc antibody (1:2000) to detect FPN.

Flow cytometry analysis

FPN–HA-transfected cells were removed from plates by incubation with PBS containing 2 mM EDTA and washed with 2 % FCS in PBS. Cells were incubated with monoclonal anti-HA antibody and then washed and incubated with Alexa Fluor® 488-labelled anti-mouse IgG antibody. To evaluate transfection efficiency, transfected cells were fixed with 2 % PFA at 4°C and

permeabilized with 0.1 % Triton X-100/PBS/3 % FCS in order to observe both surface and intracellular protein expression. Cells were incubated with monoclonal anti-HA antibody and then were washed and incubated with Alexa Fluor® 488-labelled anti-mouse antibody. The cell staining was analysed using the program Expo TM32 ADC for the Becton Coulter Epics XL-MCL.

Ferritin measurements

Transfected cells (2×10^6) were lysed with 1 % Triton X-100 buffer (containing 40 mM KCl and 25 mM Tris/HCl, pH 7.4) at 4 °C. Protein content of lysates was measured using the Bradford assay (Bio-Rad). Total cellular ferritin was measured in cell lysates using N Latex Ferritin kit (DadeBehring), an improved ELISA system with polystyrene particles coated with antibodies. Antigen-antibody aggregates scatter a beam of light that is proportional to the concentration of the relevant antigen in the sample.

Statistical analysis

Values are means $\pm$ S.E.M. and comparison between means was made using unpaired Student's *t* test.

RESULTS

FPN topology and plasma membrane targeting in epithelial cell lines

FPN topology is not clearly determined and several models have been proposed varying in the number of TM (transmembrane domain) regions from 9 to 12. Hydropathy analysis and prediction of TMs based on a multiple sequence alignment and performed on the ProteinPredict server (<http://www.embl-heidelberg.de/predictprotein/predictprotein.htm>) predict a model with nine or ten TMs (Figure 1, and right inset). However, these models differ at the N-terminal end from the one proposed by Liu et al. [23], based on epitope tagging and cysteine mutagenesis (Figure 1, left inset).

To get insight into the membrane topology of FPN and its localization in epithelial cells, we transfected HK2 cell line with three different constructs in pcDNA3, expressing either the native mouse FPN, or the mouse FPN fused at the C-terminus to an HA (FPN-HA) or a c-Myc (FPN-c-Myc) epitope. First of all, we compared the cellular localization of the native FPN with that of the tagged FPN using a specific polyclonal anti-mouse FPN antibody, raised against the putative third intracellular loop of FPN [4]. Indirect immunofluorescence staining of FPN in -20°C methanol-fixed HK2 cells shows that all three proteins are found at the cell surface, as well as in vesicular structures (Figure 2A, panels a–c). A similar cell surface targeting was also observed when the FPN-HA construct was transiently transfected into HEK-293 cell line (Figure 2A, panel d). In addition, using an inducible Cre-lox system [27] in an RCCD2 rat cortical collecting duct cell line that forms tight polarized monolayers with high transepithelial resistance, overexpressed FPN-c-Myc was found to localize at the basolateral membrane of the cells (Figure 2A, panels e and f).

All together, our observations are in agreement with the basolateral localization of FPN observed in enterocytes and indicate that the presence of a small tag at the C-terminus of FPN does not interfere with the proper targeting of the protein in epithelial cells.

We then probed FPN topology by epitope accessibility and indirect immunofluorescence. HK2 cells transfected with the FPN-HA construct were fixed with 4 % PFA and indirect immunofluorescence studies were performed in the absence or in

Figure 2 Expression and membrane topology of differentially tagged FPNs in epithelial cell models

(A) Indirect immunofluorescence was performed in transiently transfected HK2 (a–c) or HEK-293 (d) cells, and in stably transfected RCCD2 cells (e, f). HK2 cells were transfected with FPN-HA (a), FPN-c-Myc (b) or native FPN (c) and FPN was detected with a specific polyclonal anti-mouse FPN antibody (a–c) followed by the Alexa Fluor® 488-conjugated anti-rabbit antibody. HEK-293 (d) cells were transiently transfected with FPN-HA and incubated with a monoclonal anti-HA antibody followed by Alexa Fluor® 488-conjugated anti-mouse antibody. RCCD2 cells stably expressing FPN-c-Myc were allowed to polarize and were incubated with anti-FPN polyclonal antibody followed by Alexa Fluor® 488-conjugated anti-rabbit antibody. Images (e) and (f) show X–Y and X–Z sections respectively. Scale bar, 10 μm . (B) FPN topology was determined by epitope accessibility using immunofluorescence on HK2 cells transfected with FPN-HA. Prior to labelling, 4 % PFA fixed cells were permeabilized with 0.1 % Triton X-100 (d–f) or were left intact (a–c). Staining was performed with polyclonal anti-mouse FPN antibody (a, d), with monoclonal anti-HA antibody (b, e) or with monoclonal anti-BiP antibody (c, f). Scale bar, 10 μm . (C) FACS analysis of live FPN-HA-transfected HEK-293 cells using mouse monoclonal anti-HA antibody and Alexa Fluor® 488-labelled anti-mouse IgG antibody.

the presence of Triton X-100. In non-permeabilized cells, there was no staining with the anti-FPN antibody (Figure 2B, panel a), whereas intracellular staining was clearly seen in the presence of Triton X-100 (Figure 2B, panel d), suggesting that the loop encompassing amino acids 224–304 is intracytoplasmic. To determine the localization of the C-terminus, we used the anti-HA antibody. Under conditions of non-permeabilization, FPN staining was clearly visible at the cell surface (Figure 2B, panel b), whereas

no intracellular staining of the ER was detected using the anti-BiP antibody as a negative control (Figure 2B, panel c). In the presence of Triton X-100, we clearly detected intracellular staining of BiP (Figure 2B, panel f), and FPN detected with the anti-HA antibody was found at the plasma membrane and in additional intracellular vesicles (Figure 2B, panel d). In addition, FACS analysis of live cells using the anti-HA antibody allowed us to detect the population of transfected cells (Figure 2C). Overall, our observations favour a model with the C-terminal end of the molecule extending towards the extracellular medium, and the loop between TM 4 and TM 5 recognized by the anti-FPN antibody in an intracellular localization. However, we cannot differentiate between the two models for the first half of the molecule, so that the exact number of TMs remains elusive (Figure 1, left inset).

Subcellular localization of wtFPN and FPN mutants in two epithelial cell models

To identify the vesicular structures stained for FPN in HK2 and HEK-293 cells transfected with the FPN constructs, we double-stained with a series of antisera to organelle-specific markers (Figures 3A and 3B, in green). We used anti-BiP for the ER and anti-Rab5 to identify early and recycling endosomes, and FPN was identified with the polyclonal anti-FPN antibody. In Figure 3(A), it can be seen that the intracellular vesicles containing wtFPN did not co-localize with the ER, whereas there was a partial co-localization with the anti-Rab5 staining in both cell models (Figure 3A, insets in panels f and l). In addition, there was no significant overlap between wtFPN–HA and marker of the Golgi complex (Golgin 97), but in contrast, there was an extensive overlap with aminopeptidase N, a membrane-anchored hydrolase specific to proximal cell terminal differentiation (see Supplementary Figure 1A at <http://www.BiochemJ.org/bj/396/bj3960265add.htm>).

To examine the effects of FPN mutations on intracellular localization and function, a series of mutants were transiently expressed in both cell lines. We tested three missense mutations D157G, Q182H, G323V and the delV162 mutant. Transfected cells were fixed and stained with anti-HA antibody. For the two missense mutations D157G and Q182H, there was no obvious mislocalization of the protein, which appeared at the plasma membrane and in a juxtanuclear intracellular vesicular compartment (see Supplementary Figure 1B). Therefore these mutations do not appear to disturb intracellular routing of the protein. In contrast with the wt protein, a fraction of the delV162 mutant (and of G323V, not shown) was found in vesicular compartment which accumulated preferentially in a juxtanuclear region and co-localized with markers of the ER (BiP). This cellular pool of mutant FPN associated with the ER was much more pronounced in HK2 cells (Figure 3B, panels a–c) than in HEK-293 cells (panels g–i), where a larger fraction of the mutant protein was able to reach the plasma membrane. In both epithelial cell lines, a small proportion of the mutant protein was found associated with early and recycling endosomes (Rab5; Figure 3B, panels d–f and j–l).

N-glycosylation pattern of wild-type and mutant FPN

Since the delV162 mutant was found partially associated with the ER (Figure 3B), we wanted to compare its glycosylation pattern with that of the normal protein. Supranuclear extracts were prepared from transfected HEK-293 cells and immunoblotted with anti-HA antibody. The wtFPN and delV162FPN yielded two immunoreactive bands around 67 and 55 kDa (Figure 4A). The upper band probably corresponds to the fully N-glycosylated protein (white arrowhead in Figure 4A), which shows a shift in

Figure 3 Intracellular localization of wtFPN and FPN mutants in HK2 and HEK-293 cells

HK2 (a–f) and HEK-293 (g–l) cells transiently expressing FPN–HA were double-labelled with organelle-specific antibodies and with monoclonal anti-HA or polyclonal anti-mouse FPN antibody, depending on the origin of the paired antibody. FPN staining is represented in red and the intracellular markers in green: BiP (a–c, g–i) and Rab5 (d–f, j–l). Co-localization is represented by the yellow colour. (A) In both cell models, normal FPN shows partial overlap with Rab5 but no co-localization with BiP. (B) delV162FPN mutant is partially retained in intracellular compartments co-localizing with BiP and Rab5 markers, with partial targeting at the plasma membrane in HK2 cells, but more pronounced in the HEK-293 cell model (white arrows). Scale bar, 10 μ m.

mobility following PNGase F treatment and migrates as a 62 kDa protein, the predicted molecular mass for the native mouse FPN (black arrowhead in Figure 4A). The nature of the smaller 55 kDa band is not clear but could correspond to a cleaved fragment of the protein. This additional band is also seen in Western-blot analysis of tissues [4] or cultured cells [7]. FPN was resistant to endo H, which specifically cleaves oligosaccharides of the high-mannose and hybrid forms, but not complex carbohydrate structures processed in the Golgi apparatus. Similar results were obtained at both room temperature and 37°C. No differences were observed between the electrophoretic mobility of the wtFPN and of the delV162FPN, either before or after glycosidase treatments. These results suggest that the transfected FPNs, both the wt and the delV162 mutant, are post-translationally modified by N-linked glycosylation.

Figure 4 Western-blot analysis of FPN glycosylation and of the occurrence of FPN multimer formation

(A) Supranuclear protein extracts prepared from HEK-293 cells transfected with wtFPN-HA or with delV162FPN-HA were denatured and incubated for 90 min at room temperature (RT) or for 60 min at 37 °C in the absence (C) or presence of endo H or PNGase F as indicated. Aliquots of 10 μ g of untreated (C) or 20 μ g of treated protein extracts were separated by SDS/PAGE (10% gel) and analysed by immunoblotting with anti-HA antibody. The white arrowhead indicates position of complex-glycosylated FPN and the black arrowhead of native FPN. No differences were found between wtFPN and delV162FPN. (B) Electrophoretic mobility of FPN under non-denaturing conditions: supranuclear extracts (TE) from HEK-293 cells transiently transfected with wtFPN-HA were obtained with a lysis buffer preserving native structures. Proteins from the microsomal fractions (MBP) were solubilized by adding 1% Triton X-100 final concentration prior to electrophoresis. Aliquots of 50 μ g of protein from TE and 10 μ g of microsomal fractions were analysed under non-denaturing conditions on a 6% (w/v) native polyacrylamide gel, followed by immunoblotting with anti-HA antibody. (C) Co-immunoprecipitation studies of double-transfected HEK-293 cells. Aliquots of 12 μ g of protein from total extracts (TE), immunoprecipitates obtained with the anti-HA antibody from 200 μ g of protein from total extracts (IP), or 18.5 μ g of protein from the supernatant remaining after immunoprecipitation (SN), obtained from control non-transfected HEK-293 cells (lanes 1–3 and 7–9) or from transfected cells expressing wtFPN-HA and wtFPN-c-Myc (lanes 4–6 and 10–12) were run in parallel on an SDS/10% polyacrylamide gel and immunoblotted (WB) with either anti-HA (lanes 1–6) or anti-c-Myc (lanes 7–12) antibodies. Immunoprecipitation of wtFPN-HA does not pull down wtFPN-c-Myc. The positions and sizes (in kDa) of molecular mass markers are indicated on the left. The asterisk denotes a non-specific band, inconsistently observed, probably resulting from cross-reaction with the mouse IgG. (D) Co-immunoprecipitation studies of double-transfected HEK-293 cells. Aliquots of 12 μ g of protein from total extracts (TE) and immunoprecipitates obtained with the anti-HA antibody from 200 μ g of protein from total extracts (IP), obtained from HEK-293 cells transfected with wtFPN-c-Myc and delV162FPN-HA, were run in parallel on an SDS/10% polyacrylamide gel and immunoblotted with either anti-HA (lanes 1–6) or an anti-c-Myc antibody (lanes 7–12). The wtFPN-c-Myc was not immunoprecipitated with the delV162FPN-HA protein.

Co-expression of wt and mutant FPN in the same cells

To assess directly the possible formation of oligomers by FPN, as previously suggested [22], we analysed the electrophoretic mobility of FPN in native gel electrophoresis (Figure 4B). We solubilized FPN from supranuclear protein extracts or from microsomal fractions of wtFPN-HA-transfected HEK-293 cells. We tested several detergents at various concentrations but only 1% Triton

X-100 was able to solubilize FPN and to give a significant signal after blotting and probing the membrane with the anti-HA antibody. Under these conditions, FPN migrates as a very-high-molecular-mass complex above 400 kDa, and the size of the complex varies between total cell extract (Figure 4B, TE) and microsomal fraction (Figure 4B, MBP). The existence of high-molecular-mass structure migrating as a poorly resolved band changing in size between two different membrane preparations is

suggestive of aggregate formation due to the high hydrophobicity of the transmembrane protein rather than existence of a multimer of definite composition.

To confirm this hypothesis, we then performed co-immunoprecipitation experiments in cells co-expressing the HA- and c-Myc-tagged FPN (Figures 4C and 4D). Since SDS can help to solubilize membrane proteins but is known to disrupt weak protein interactions, we performed the incubation with the antibody in the absence of SDS (Figure 4C) but identical results were obtained when we repeated the immunoprecipitations in the presence of SDS (see Supplementary Figure 2A at <http://www.BiochemJ.org/bj/396/bj3960265add.htm>). Figure 4(C) shows Western-blot analysis of total cell extracts (TE) shows that both wtFPN-HA (lane 4) and wtFPN-c-Myc (lane 10) are present in co-transfected cells, whereas immunoprecipitation of cell proteins with the anti-HA antibodies pulls down most of the wtFPN-HA molecules (lane 5) with almost nothing remaining in the supernatant (lane 6), but does not precipitate any wtFPN-c-Myc protein (lane 11), with most of the protein remaining in the supernatant (lane 12). The minor band seen in the immunoprecipitates probed with the anti-c-Myc antibody was inconsistently present (lane 11, asterisk) and probably represents cross-reactivity with immunoglobulin heavy chain (close to 50 kDa). The immunoprecipitation of non-transfected cells with the anti-HA antibody did not yield any immunoreactive bands (Figure 4C, lanes 1–3 and 7–9).

The formation of hetero-oligomers between the wt and a mutant FPN has been proposed as a mechanism accounting for the dominant phenotype of FPN mutations. To test this hypothesis, we co-transfected a wtFPN-c-Myc construct with a delV162FPN-HA construct and repeated the co-immunoprecipitation experiments (Figure 4D). Similarly, the wtFPN-c-Myc was not present in the immunoprecipitates obtained with the anti-HA antibody (Figure 4D, lane 11), in contrast with the delV162FPN-HA (Figure 4D, lane 5), although both proteins were present in the initial cell extracts (Figure 4D, lanes 2 and 8). Under those experimental conditions, we were not able to show the formation of hetero-oligomers between the two differentially tagged FPN molecules.

In addition, dual immunofluorescence studies revealed that there was no overlap in the subcellular localization of the wtFPN-c-Myc and the delV162FPN-HA proteins when they were both expressed in HK2 cells (Figure 5). In these cells, as previously observed (Figure 3B), the delV162 mutant was preferentially associated with an intracellular perinuclear compartment and did not alter the targeting of the normal protein at the plasma membrane (Figures 5a–5c). Similarly, the G323VFPN-HA mutant was found associated with endomembranes and its co-expression with the wtFPN-c-Myc in the same cell did not prevent targeting of the wtFPN at the plasma membrane (see Supplementary Figure 2B). In HEK-293 cells, although both wtFPN and delV162FPN proteins reached the plasma membrane (Figures 5d–5f), merging of the two signals revealed only partial co-localization (Figure 5f, inset). Finally, when the cells were co-transfected with the two differently tagged wtFPNs (HA and c-Myc), we saw that both proteins were distributed at the plasma membrane, with only partial co-localization (Figure 5i, inset).

Together, these observations tend to indicate that FPN proteins are not interacting together in the same cellular compartment and therefore do not support the existence of hetero-oligomers between the different FPN molecules.

Effect of normal and mutant FPN on cellular ferritin content

To assess the effect of the mutations on the iron export activity of the FPN molecule, and to further evaluate the possibility that the FPN mutants exert a dominant-negative effect on the wtFPN func-

Figure 5 Intracellular distribution of wtFPN and delV162FPN in co-transfection experiments

HK2 (a–c) or HEK-293 (d–f) cells were transiently co-transfected with wtFPN-c-Myc and delV162FPN-HA, and HEK-293 cells were co-transfected with wtFPN-c-Myc and wtFPN-HA (g–i). Cells were stained with antibodies against the c-Myc tag (green) and the HA tag (red). Co-expression of delV162FPN does not alter wtFPN distribution in both cell models. The two tagged wtFPNs are also normally distributed and show punctual co-localization (represented by the yellow colour) in the co-expression model. Insets in (c, f, i) are magnifications of the indicated areas. Scale bar, 10 μ m.

tion, we used the ferritin content of the transfected cells as an indirect measurement of the intracellular iron content. We first evaluated the direct relationship between reduced ferritin content and overexpression of FPN by dual fluorescent labelling experiments. wtFPN-HA- or delV162FPN-HA-transfected HEK-293 cells were labelled with the anti-FPN antibody and with an anti-H ferritin antibody (Figure 6A). It can be seen that the cells expressing either wtFPN or delV162FPN (middle panels) contain almost no detectable ferritin, as compared with the adjacent non-transfected cells (left panels). These observations suggest that FPN overexpression efficiently depletes the cellular ferritin content and that the mutant delV162FPN has retained a significant iron export function when expressed in a cell system.

To quantify more precisely the export function of the various FPN molecules, HEK-293 cells were transfected with the series of FPN-HA constructs, either alone or in combination with the wtFPN construct, and collected 48 h after transfection for FACS analysis using the anti-HA antibody or for intracellular ferritin assay using an ELISA. The FACS analysis was performed on cells fixed with 4% PFA and permeabilized with Triton X-100. The results show that the efficiency of transfection is very similar between the different constructs (Figure 6B), with the exception of the D157G transfectants, allowing us to assign differences in ferritin content between the transfected cells to differences in iron export activity of the transfected protein. The ferritin content of the different transfectants shows that the wtFPN

Figure 6 Effect of FPN expression on the cellular ferritin content

(A) Double staining was performed with the polyclonal anti-(human H ferritin) antibody (a, d) and with the mouse anti-FPN antibody (b, e) on HEK-293 cells double-transfected with wtFPN-HA or delV162FPN-HA. Merging of the two images shows that cells expressing normal (c) or mutated (f) FPN have much lower ferritin content as compared with adjacent non-transfected cells (arrows). Scale bar, 10 μ m. (B) Transfection efficiency of HEK-293 cells transiently transfected with wtFPN-HA or with a mutant FPN-HA was analysed by flow cytometry. Cells were fixed with 4% PFA and permeabilized with Triton X-100, which permits us to observe both surface and intracellular protein expression. The percentage of transfected cells expressing the FPN-HA construct did not vary significantly between wt and mutant FPN constructs, with the exception of the D157G mutant. (C) Ferritin content of the different transfectants in HEK-293 cells was assayed by ELISA and is expressed as a percentage of the ferritin values in the mock-transfected cells. Results are the means $\pm$ S.E.M. for three separate experiments. Lysates of 2×10^6 transfected cells were used for ferritin measurements. Bar graph on the left side represents transfections of 5 μ g of each individual plasmid, whereas the bar graph on the right side represents double transfections of 2.5 μ g of wtFPN-HA together with 2.5 μ g of pcDNA3, G323VFPN or delV162FPN. When each plasmid is transfected separately, wtFPN expression caused a 4-fold reduction in cell ferritin content when compared with mock-transfected cells. G323V and D157G mutations had no effect on the cell ferritin content. Q182H mutant was fully functional as seen from the 3–4-fold reduction in ferritin content, identical with the effect of the expression of the wtFPN. delV162FPN had an intermediate effect on ferritin content but the difference from the ferritin content in the wtFPN-transfected cells was not statistically significant ($P = 0.07$). * Indicates that the difference in ferritin content between FPN-transfected and mock-transfected cells is statistically significant ($P < 0.01$); ** indicates that the difference in ferritin content between mutant FPN-transfected and wt FPN-transfected cells is statistically significant ($P < 0.01$). The double transfection experiments showed that the mutant proteins do not exert a dominant-negative effect on the function of the wtFPN.

results in a statistically significant ($P < 0.01$) 3–4-fold reduction in ferritin content as compared with mock-transfected cells (Figure 6C). G323V and D157G FPN mutants had no effect on the cell ferritin content, suggesting that these mutants have impaired iron export activity. In contrast, the Q182H mutant is fully functional since its expression also leads to a 3–4-fold reduction in ferritin content ($P < 0.01$) as compared with the mock-transfected cells, identical with the effect of the expression of the wtFPN. Interestingly, the delV162FPN induces a 2-fold reduction in ferritin content and although the difference from either mock-transfected or wt transfected cells is not statistically significant, this suggests that this mutant has retained a functional iron export activity, in agreement with the immunofluorescence experiments. Finally, we co-transfected a fixed amount of wtFPN–HA with the same amount of either pcDNA3, G323VFPN–HA or delV162VFPN–HA. The mutant proteins did not alter the transport activity of the wtFPN, arguing against a dominant-negative effect of the mutant FPN.

DISCUSSION

Since the first description of FPN in 2000 [1–3], rapidly followed by the identification of its role in type-4 haemochromatosis [12,18], its exact topology has remained elusive and various controversial data have been reported on the structure–function relationships of the various FPN mutants. In the present paper, we re-evaluate some of these critical issues to gain further insight in the physiopathology of type-4 haemochromatosis. After showing that the presence of a small tag (HA or c-Myc) at the C-terminal end of FPN does not alter membrane localization of the protein in both non-polarized (HK2 and HEK-293) and polarized (RCCD2) epithelial cells, we used anti-HA antibodies to determine the orientation of the C-terminal end of the molecule. The HA tag of the transfected wtFPN–HA molecule was accessible to anti-HA antibody under fixative, non-permeabilizing conditions, and was also accessible to the antibody in live HEK-293 transfected cells, as shown by FACS experiments. These observations corroborate earlier studies where FACS analysis was also performed to detect FPN surface expression via tag labelling [6], and suggest that the C-terminus is orientated towards the extracellular medium. Furthermore, the loop encompassing amino acids 234–304 was only detected on fixed and permeabilized cells under the conditions required to detect BiP, an exclusive intracellular protein, which confirmed the cytoplasmic orientation of the epitope. Therefore we favour the model with nine TMs as proposed by the ProteinPredict server-based program, although it disagrees with the results obtained by Liu et al. [23] showing an extracellular orientation for the C-terminus. However, their study was performed in COS cells where the protein might adopt a different conformation than in epithelial cells. Our model also places the four putative N-linked glycosylation sites (NXS/T) of the mouse FPN sequence (Asn¹⁶⁶, Asn¹⁷⁴, Asn⁴³⁷ and Asn⁵⁶⁶) facing the outer cellular medium (Figure 1). However, an external orientation for the C-terminus is intriguing since the last three amino acids of the molecule, SVV, match a consensus type I PDZ motif (S/T-X-L/V/I/M). These motifs are known to mediate basolateral sorting of transmembrane proteins by interactions with clathrin adaptor AP-1B complex [28]. FPN is found at the basolateral membrane of duodenal enterocytes and we could also observe basolateral targeting in stable transfectants of a rat cortical collecting duct cell line. However, it is conceivable that an N-terminal determinant is required for normal targeting of FPN, as has been shown for the kidney anion-exchanger 1 [29].

Our predicted model, based on the alignment of conserved FPN sequences from various species, also differs from that of Liu et al.

[23] for the first half of the molecule. Although we have no experimental evidence, it is tempting to speculate that the loop between amino acids 144 and 203 is extracellular (see Figure 1) and represents the hepcidin-binding site, since mutations affecting amino acids 144, 162 and 182 all result in a loss of ability to bind hepcidin [6,22].

Our experiments show that the wtFPN in HK2 and in HEK-293 transfected cells is distributed both at the plasma membrane and in vesicular structures. The intracellular compartment consists of Rab5-positive vesicles and of vesicles of unknown nature, differing from the ER and the Golgi. Rab5 was demonstrated to be one of the regulatory components both of the transport machinery involved in membrane organization and recycling [30,31] and in the protein quality control at early endosomes [31,32]. FPN colocalization with Rab5-positive vesicles suggests a phenomenon of membrane recycling/trafficking from plasma membrane to intracellular endomembranes, and the absence of ER retention is consistent with the observation that normal FPN is entirely complex-glycosylated.

A normal cellular distribution was found for the missense FPN mutants D157G and Q182H, whereas the delV162 and the G323V (not shown) mutants were found partially associated with the ER and with Rab5-positive vesicles, especially in HK2 cells. In HEK-293 cells, membrane targeting was more pronounced. Association of delV162FPN with the ER could reflect misfolding of the protein. There are multiple examples of mutations in ion transporters that result in protein misfolding, ER retention and sometimes targeting to lysosomes for degradation. These mutant proteins retained in the ER are usually unable to mature to their fully glycosylated forms. However, the delV162FPN, as well as the wtFPN, is insensitive to endo H treatment, which removes only the high-mannose oligosaccharides that have been added in the ER and have not been processed to complex oligosaccharides in the Golgi apparatus. The observation that in HEK-293 cells the delV162FPN shows a bimodal distribution, with localization at the plasma membrane and in intracellular vesicles consisting of ER and recycling endosomes while being fully glycosylated, suggests that the mutant protein exits from the ER, is normally processed in the Golgi apparatus, transits to the cell surface and is then recycled from the plasma membranes to endosomes and ER. The resident time of the mutant protein at the plasma membrane might be shorter than that of the wt protein.

When the iron export function of FPN is evaluated by measuring intracellular ferritin content, we found that the delV162FPN–HA mutant has an intermediate transport activity as compared with the wtFPN, which might result from the partial association of the mutant FPN with endomembranes. Immunofluorescent labelling of both FPN and ferritin in transfected cells clearly showed that the delV162FPN-transfected cells have much lower ferritin content than the adjacent non-transfected cells. Two groups have already analysed the iron export capacity of the delV162 mutant and found it to be non-functional [6,22]. Reasons for this discrepancy could be due to differences between experimental approaches. In our experiment, we have measured ferritin levels under basal situation, while the data in the literature were obtained on transfected cells loaded for 2 days with iron-saturated transferrin [6] or for 2 days with ferric ammonium citrate [22] prior to ferritin measurements, possibly altering membrane targeting of the mutant FPN. Iron has been shown to trigger redistribution of FPN from intracellular vesicles to the plasma membrane in macrophages [9] and, although this phenomenon has not been documented in epithelial cells, it is conceivable that cellular iron loading increases the processing or slows the recycling of the wt protein but not that of the mutated protein. Using the ferritin assay as a marker of iron depletion, we believe that the G323V and the

D157G FPN mutants are non-functional since they failed to reduce the ferritin content of transfected HEK-293 cells, whereas the Q182H and the delV162FPN mutants reduced the cellular ferritin to levels similar to those observed with wtFPN transfection, indicating a full transport activity of these proteins. These mutants might also fall into the category of those FPN mutants that, although retaining functional activity, are resistant to hepcidin [22,33].

Finally, it has been recently proposed that FPN is a multimeric protein, and that FPN mutants act as dominant negatives [22]. However, under our experimental conditions and by using different approaches, we were not able to demonstrate oligomerization of the FPN molecules. First, in HK2 cells, where most of the delV162FPN mutant is found in intracellular vesicles, there was no overlap between the localization of the wtFPN and the delV162FPN when both proteins were expressed in the same cell, showing that the mutant did not induce intracellular retention of the wt protein. In co-transfected HEK-293 cells, the two proteins were found at the plasma membrane, with little co-localization. Similarly, wtFPN-HA and wtFPN-c-Myc were mostly found in different plasma membrane regions. Secondly, biochemical analyses showed that FPN can indeed be found in large complexes in non-denaturing gels but the molecular mass of these complexes is poorly defined and changes with experimental conditions, and the complexes tend to dissociate during the migration, arguing against the presence of a stable multimer with a definite composition. This is in agreement with a recent paper showing by sucrose gradient sedimentation and cross-linking experiments that native FPN is a monomer [34]. Thirdly, co-immunoprecipitation studies failed to show the existence of FPN heteropolymers. The FPN-c-Myc was found in the supernatant of the immunoprecipitation performed with the anti-HA antibody, under conditions where all the FPN-HA had been precipitated. Similarly, Drakesmith et al. [33] failed to co-immunoprecipitate two differentially tagged FPNs co-transfected in HEK-293 cells. In the work by De Domenico et al. [22], the authors were able to co-immunoprecipitate FPN-FLAG and FPN-GFP (green fluorescent protein) proteins. The presence of GFP at the C-terminal end of the molecule might have changed the membrane topology and favoured the formation of polymers. The authors also argued that multimer formation of FPN would explain the dominant-negative effect of the mutant over the wt proteins, while haploinsufficiency would not in itself induce intracellular iron retention. However, in double transfection experiments, when we replaced pcDNA3 by either G32VFPN or delV162FPN mutant, there was no change in ferritin content, arguing against a dominant-negative effect of the mutant over the wt protein. We would like to propose an alternative mechanism, although not mutually exclusive with the multimer model, whereby the iron export activity of the wt protein would be reduced in the presence of the mutant protein. Two papers have shown that urinary hepcidin levels are increased in patients with the heterozygous delV162FPN mutation [15,35], probably as a result of the progressive hepatic iron overload. This increase in hepcidin expression could trigger internalization and degradation of the wtFPN expressed from the normal allele. This negative feedback loop would contribute to the aggravation of the phenotype of macrophage iron overload. Absence of nonsense mutations in type-4 haemochromatosis has also been proposed as an argument in favour of a dominant-negative effect of the mutations. However, absence of nonsense mutations is not unique; for instance, heterozygous mutations of SCN1A, the voltage-gated sodium channel α subunit 1, responsible for generalized epilepsy with febrile seizures, are exclusively missense mutations [36]. Nonsense and frameshift mutations of the same gene are

responsible for a much more severe phenotype. It is possible that nonsense or frameshift FPN mutations will be found associated with more severe or early onset macrophage iron overload disorders. Interestingly, a Japanese patient has been described recently with a severe iron overload associated with a heterozygous mutation in the 5'-untranslated region of the FPN mRNA which did not affect FPN function [37]. Clearly, additional experiments are required to solve these controversial issues and to elucidate the consequences of the molecular defects of FPN for macrophage iron recycling, since most of the studies performed so far have been conducted in epithelial cells.

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