Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin

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Ferroportin mediates iron export from cells; ferroportin mutations are associated with the iron overloading disorder hemochromatosis. We previously found that the A77D, V162del and G490D mutations inhibited ferroportin activity, but that other disease-associated ferroportin variants retained full iron export capability. The peptide hormone hepcidin inhibits ferroportin as part of a homeostatic negative feedback loop. We measured surface expression and function of wild-type ferroportin and fully active ferroportin mutants in the presence of hepcidin. We found that the Y64N and C326Y mutants of ferroportin are completely resistant to hepcidin inhibition, and N144D and N144H are partially resistant. Hemochromatosis associated ferroportin mutations therefore either reduce iron export ability or produce a ferroportin variant that is hepcidin insensitive. The former mutation type is associated with Kupffer cell iron deposition and normal transferrin saturation in vivo, while patients with the latter category of FPN mutation have high transferrin saturation and tend to deposit iron throughout the liver parenchyma. Ferroportin-linked hemochromatosis may have a variable pathogenesis depending on the causative ferroportin mutant.

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Introduction

Hereditary hemochromatosis is an iron overload disease characterized by excessive body iron that causes tissue damage in the liver, pancreas and heart\(^1\). In Caucasians hemochromatosis is predominantly associated with two mutations in the \textit{HFE} gene\(^2\). Non-\textit{HFE} hemochromatosis is geographically more widespread and results from mutations in other genes involved in iron homeostasis\(^3\). Numerous mutations in the \textit{ferroportin} gene have been reported in hemochromatosis patients from diverse origins\(^3\)\textsuperscript{-20}. Ferroportin transports iron out of cells and is strongly expressed by intestinal enterocytes and liver macrophages (Kupffer cells)\(^{21-23}\). Ferroportin is inhibited by the peptide hormone hepcidin\(^{24,25}\). Hepcidin levels correlate with iron stores, so that as body iron increases hepcidin is induced\(^26\), blocking ferroportin mediated iron transfer from the diet by enterocytes and ferroportin mediated iron recycling from erythrocytes by macrophages\(^3\). This negative feedback loop is thought to maintain iron homeostasis.

We previously found that some hemochromatosis associated ferroportin mutations (A77D, V162del and G490D) showed reduced iron export, but other disease-related mutants Y64N, N144D, N144H, Q248H and C326Y maintained full function \textit{in vitro}\(^27\). We postulated that those functioning FPN mutants might resist negative feedback, and the resulting disturbance in iron homeostasis might lead to hemochromatosis. In this paper we report that some of the hemochromatosis-associated ferroportin mutations are resistant to inhibition to hepcidin, and discuss the implications of this finding for the pathogenesis of hemochromatosis and iron homeostasis in general.
Results

Hepcidin reduces surface expression of wt Ferroportin but not of Ferroportin mutants

Hepcidin causes internalization of ferroportin and so reduction of surface expression\(^2^4\). We wondered if hemochromatosis-associated ferroportin mutants might resist hepcidin-mediated internalization. To test this we transiently transfected 293T cells with constructs encoding c-terminally c-Myc tagged wild-type (wt) human FPN, wt murine FPN, and the human FPN variants Y64N, N144D, N144H, Q248H and C326Y, with or without added hepcidin-25 (the 25 amino-acid form of hepcidin). After 2 days, cells were analyzed for surface expression of the c-Myc tag by flow cytometry (Figure 1A-C). Figure 1A shows a reduction in surface c-Myc expression mediated by hepcidin-25 in cells expressing wt human FPN (compare green histogram to red), consistent with the internalization of murine FPN reported by Nemeth et al\(^2^4\). In contrast hepcidin-25 had no effect on the surface expression of the c-Myc tagged FPN variant C326Y (figure 1B). Figure 1C shows the effect of hepcidin-25 on all FPN mutants analyzed, displayed as the percentage surface expression relative to wt human FPN levels in the absence of hepcidin-25.

Surface wt human and mouse FPN expression was reduced by over a half by hepcidin-25, C326Y and Y64N were resistant to hepcidin-mediated downregulation, N144D and N144H were partially resistant, and Q248H was as susceptible to downregulation as wt FPN. In agreement with Nemeth et al, we found that a preparation of hepcidin-20, the 20 amino acid form of hepcidin, did not reduce surface expression of wt FPN (data not shown)\(^2^4\). We then visualized FPN redistribution by hepcidin-25 using immunofluorescence microscopy. 293T cells transfected with wt human FPN and FPN variants for 24 hours were incubated with cycloheximide (in order to stop \textit{de novo} protein synthesis) with or without added hepcidin-25. Cells were then stained for FPN expression using anti-c-Myc antibody (Figure 1D, green) and cell nuclei were counter-stained using DAPI (Figure 1D, blue). For all FPN variants, without hepcidin-25 added FPN protein was localized to the plasma membranes. Hepcidin-25 caused internalization of wt FPN into discrete vesicles as previously reported\(^2^4\). C326Y and Y64N FPN resisted internalization by hepcidin-25 and remained at the cell surface, while Q248H was
internalized in the same way as wt FPN. The N144 mutants had an intermediate phenotype, with both cell surface and internalized protein apparent.

**Hepcidin inhibits the ferroportin-mediated increase in surface transferrin receptor-1 and reduction in ferritin, but ferroportin mutants are not inhibited.**

Next we evaluated the effect of hepcidin-25 on the function of FPN and disease-associated FPN variants. FPN expression causes cellular iron deficiency and a reduction in the labile iron pool\(^{21}\), leading to reduced ferritin and increased surface expression of transferrin receptor-1 (TfR). We expressed wt FPN and FPN variants in 293T cells with or without added hepcidin-25 and measured surface TfR on transfected cells using two-colour flow-cytometry. As a control, we used cells transfected with CD8, a molecule with no known function in iron transport; hepcidin-25 had no effect on TfR expression by untransfected cells or CD8 transfected 293T cells (data not shown). Figure 2A shows that wt human FPN expression increases surface TfR compared to control CD8 expressing cells (compare red line to filled grey histogram) and that hepcidin-25 inhibits this FPN-mediated TfR increase (green line is similar to filled grey histogram). The Y64N FPN variant similarly increases surface TfR relative to control cells (Figure 2B, red line compared to filled grey histogram), but this increase in TfR is not affected by hepcidin-25 (green line is similar to red line). The effect of hepcidin-25 on all FPN variants is shown in Figure 2C. The Mean Fluorescence Intensity (MFI) of TfR on cells transfected with FPN variants (with or without added hepcidin-25) minus the MFI of TfR on control CD8 transfected cells is given. Wt human and mouse FPN increase TfR levels above those of control cells, but hepcidin-25 blocks this increase; whereas hepcidin-25 did not hinder the increase of TfR mediated by C326Y and Y64N FPN. N144D and N144H are partially susceptible to hepcidin-25 inhibition, while Q248H is blocked by hepcidin-25 to the same degree as wt FPN. Levels of the intracellular iron storage protein ferritin are also reduced by FPN expression. We found that the reduction in ferritin caused by FPN was inhibited by hepcidin-25, but the C326Y mediated ferritin reduction was unaffected by hepcidin-25 (Figure 2D). Thus cellular iron deficiency, caused by ferroportin and leading to increases in TfR1 and lowering of ferritin, is inhibited by hepcidin.
Hemochromatosis associated mutants of ferroportin also cause cellular iron deficiency but are not restrained by hepcidin.

**Hepcidin inhibits ferroportin-mediated changes in cellular iron accumulation and iron release, but ferroportin mutants are not inhibited**

Next we directly measured the effect of hepcidin-25 on iron accumulation by cells. 293T cells were transfected with FPN variants and cultured for 2 days with 40μg/ml \(^{59}\)Fe labeled human transferrin with or without added hepcidin-25 (Figures 3A-B). \(^{59}\)Fe accumulation by control CD8 transfected cells was unaffected by hepcidin-25. Wt human FPN expression caused a marked decrease in iron accumulation that was largely reversed in the presence of hepcidin-25, and this same effect was observed with cells expressing Q248H FPN. In contrast the reduction in \(^{59}\)Fe accumulation by cells expressing Y64N or C326Y was resistant to hepcidin-25, and hepcidin-25 only marginally counteracted the lower \(^{59}\)Fe accumulation displayed by N144D and N144H expressing cells. Finally we directly measured \(^{59}\)Fe release from cells transfected with wt FPN or C326Y FPN with or without added hepcidin. Figure 3C shows that wt and C326Y FPN increased iron release compared to control GFP transfected cells, and that hepcidin inhibited the \(^{59}\)Fe efflux mediated by wt FPN but not C326Y FPN. We conclude from the data shown in Figures 1-3 that wt FPN is internalized and functionally restrained by hepcidin-25 but that the hemochromatosis-associated FPN mutations Y64N, N144D, N144H and C326Y confer total or partial resistance to hepcidin inhibition.
Discussion

In our earlier investigation we found that A77D, V162del and G490D FPN mutants lost iron export function, whereas the other FPN mutants that are the subject of this report maintained efflux ability\(^2\). We postulated that this second set of mutants could cause hemochromatosis if they resisted a natural inhibitor of FPN that was involved in iron homeostasis. We proposed two possible candidate inhibitors, HFE or hepcidin\(^2\). HFE reduces iron export from cell types that naturally express FPN\(^2\), but in our hands HFE does not inhibit FPN under the same experimental conditions as for Figure 3C (data not shown); thus the role of HFE remains enigmatic.

Hepcidin was recently reported to induce FPN internalization and degradation\(^2\). Our results are consistent with these findings, and we show that both murine and human wild-type ferroportin are equally subject to human hepcidin-25 inhibition. We found that Q248H ferroportin was as susceptible to hepcidin as wt FPN; although Q248H has been found in some iron loaded individuals, it is present at high frequency in some African populations and can occur in homozygosity\(^1,11,15\). Q248H may be a polymorphism with a mild effect on FPN function that we could not detect, possibly leading to disease in the presence of modifying factors.

We found that the hemochromatosis-associated FPN mutants Y64N, N144D, N144H and C326Y, which export iron as well as wild-type FPN, had abolished or reduced sensitivity to inhibition by hepcidin. In vivo, a FPN protein that cannot be turned off by hepcidin could lead to higher dietary iron uptake and more iron recycling from red cells by macrophages, resulting in high serum transferrin saturation. We previously showed that the A77D, V162del and G490D FPN mutants had lost iron export ability in vitro\(^2\). Patients with these mutations may have reduced iron export from their macrophages leading to Kupffer cell iron loading without high transferrin saturation; characteristics (along with high serum ferritin) of the ‘ferroportin disease’\(^3\).

To investigate any potential relationship between FPN mutant activity in vitro and in vivo disease phenotype, we searched the literature describing clinical features of hemochromatosis patients with ‘loss of function’ or ‘hepcidin resistant’ FPN mutations. We plotted age versus transferrin saturation (Figure 4A) and age versus serum ferritin (Figure 4B) from the values in the published studies. Figure 4A shows that patients with
the FPN mutations that we have shown to be fully functional but completely hepcidin resistant (Y64N and C326Y) have markedly high transferrin saturation. A second mutation at C326, C326S was recently reported in family members with high transferrin saturation before the age of the twenty\textsuperscript{17}. Although we have not tested C326S, it is at least possible that like C326Y, this FPN variant is also hepcidin resistant. Patients with the FPN variants that have lost iron export function \textit{in vitro}, V162del, G490D and A77D, have generally normal transferrin saturation, although transferrin saturation rises with the age of individuals with A77D. Patients with mutations at N144 (that \textit{in vitro} have active export ability but only partial sensitivity to hepcidin), have an intermediate phenotype, having either high transferrin saturation or transferrin saturation in the normal range. Thus Figure 4A shows that our \textit{in vitro} functional studies categorizing FPN mutants as either loss-of-function or hepcidin resistant have an \textit{in vivo} correlate with transferrin saturation in hemochromatosis patients. The distinction is not so clear when serum ferritin is plotted against age (figure 4B). All individuals with the exception of two Y64N and three C326S patients have raised serum ferritin. Serum ferritin increases with age for all mutations, but may have a higher ‘start-point’ for the loss of function mutations A77D, V162del and G490D.

The loss of function FPN mutations are clearly associated with predominantly macrophage (Kupffer cell) iron loading in the liver \textit{in vivo}\textsuperscript{5-8,12,18}. The pattern of iron deposition in the livers of patients harboring FPN mutations that we have described as hepcidin-resistant is more variable. Heavy iron deposition in both hepatocytes and Kupffer cells was reported in individuals with Y64N FPN\textsuperscript{13}, while iron deposition is predominantly in hepatocytes in C326Y patients and also C326S patients\textsuperscript{17}. There is variation in deposition of iron in patients with mutations at N144 (that confers partial resistance to hepcidin), with iron in either hepatocytes or with a reticuloendothelial distribution\textsuperscript{9,14,19}.

Thus, overall, FPN mutations that have lost iron export capacity \textit{in vitro} are associated generally with normal transferrin saturation (with the exception of older patients with A77D), Kupffer cell iron loading and high serum ferritin even at a young age. In contrast, mutations conferring total hepcidin resistance \textit{in vitro} are linked to markedly high transferrin saturation, a pattern of liver iron deposition that includes and
may be predominantly in hepatocytes, and occasionally lower serum ferritin at least early in disease. However there is variation among family members with the same mutations, perhaps most clearly apparent in patients with mutations at N144 (intermediate phenotype in vitro), which suggest that other factors likely exist that modify the clinical picture.

A final point of interest is that two individuals carrying the loss of function V162del mutation were recently found to have high levels of hepcidin\textsuperscript{32}. Iron overload caused by loss of function FPN mutations (80g of iron was removed from one V162del patient\textsuperscript{8}) has been proposed to result from iron locked in macrophages being withheld from the bone marrow. The erythron then sends compensatory signals to the intestine to upregulate iron absorption. The high hepcidin levels found in the V162del patients raises the possibility that this putative bone marrow-to-intestine signal could be hepcidin independent.

In summary, we have found that some hemochromatosis-associated mutations of FPN confer resistance to inhibition by hepcidin in vitro. These mutations are linked to a clinical phenotype that differs from those patients harboring loss-of-function FPN mutations most notably in terms of higher transferrin saturation. Hepcidin-resistant FPN may be associated with a greater flow of iron through the erythrocyte iron recycling pathway as well as with increased intestinal iron absorption. Patients harboring hepcidin resistant FPN alleles may in consequence be expected to mobilize iron on phlebotomy more readily than those individuals with loss-of-function FPN mutants.
Materials and Methods

Expression vectors
pcDNA3.1 constructs encoding wt human and murine FPN c-terminally tagged with c-Myc-poly-histidine were kind gifts from Dr. A. McKie (King’s College, London) and mutated as described27. A plasmid encoding human CD8 was a kind gift from Dr. G. Gao (Oxford UK), a plasmid encoding GFP was a kind gift from Dr. X. Xu (Oxford UK).

Two-colour flow cytometry
Human embryonic kidney epithelial 293T cells were exposed to DNA-effectene (Qiagen) transfection complexes for 8 hours as described 27, then hepcidin-25 (Peptide Institute Inc., Japan) was added to half the wells at 0.5μM. Two days later cells were harvested and incubated with anti-c-Myc-FITC (Santa Cruz Biotechnologies, CA) or anti-CD8-FITC (Pharmingen) and anti-TfR1-biotin (Pharmingen), mouse anti-rabbit Ig (MR12 clone, Dako) was used as a negative control. Cells were then incubated with streptavidin-phycoerythrin (Sigma) to reveal TfR1, and analyzed using a Becton Dickinson FACScalibur as described27. Transfected cells positive for CD8 or c-Myc were gated, and their surface TfR1 expression measured.

Immunofluorescence
293T cells grown on 8-well chamber slides (BD Falcon) were transfected with wt or mutant FPN. 24 hours later, cycloheximide was added to all wells at 100μg/ml to inhibit protein synthesis and two hours later hepcidin-25 was added to half the wells at 0.5μM. Three hours after adding hepcidin-25, cells were fixed, permeabilized, stained with anti-c-Myc-FITC and processed for imaging as described27.

59Fe accumulation and 59Fe release
For iron accumulation, 293T cells were incubated with DNA-effectene complexes for eight hours, then 40μg/ml 59Fe-labelled human transferrin, prepared as described27,29 was added to the cultures, and hepcidin-25 at 0.5μM was added to half the wells. Forty hours later iron accumulation per million cells was determined as described29. For iron release, 293T cells were pre-loaded with 40μg/ml 59Fe-Tf for 24 hours before being transfected with GFP, wtFPN or C326Y FPN for 15 hours. Cells were then harvested and 59Fe per million cells was determined before reculturing the cells in serum-free medium (Pro293a-
CDM, BioWhittaker) with or without hepcidin-25 at 0.5uM. The percentage $^{59}$Fe export at various timepoints was calculated as $^{59}$Fe in the supernatant at each timepoint divided by cellular $^{59}$Fe at time zero multiplied by 100.

**Ferritin ELISA**

Total cellular ferritin was measured from cell lysates using ferritin ELISA kits (Laguna Niguel, CA) as described$^{27}$. Cells were transfected in the presence of 1mg/ml human holo transferrin (Sigma) with or without hepcidin-25 at 0.5uM. After culturing for 3 days, cells were lysed and the protein content of lysates was measured using the Bradford assay (Bio-Rad, Hercules, CA), and normalized by diluting with lysis buffer as necessary. 20 ul of doubling dilutions were used per well in the ferritin ELISA and compared against provided ferritin standards.
References

Figures:

Figure 1: Downregulation of surface wt FPN-c-Myc but not of mutant FPN-c-Myc by hepcidin-25.
Figure 1: Downregulation of surface wt FPN-c-Myc but not of mutant FPN-c-Myc by hepcidin-25.

A, B: 293T cells were transiently transfected with either c-Myc tagged wt FPN (A) or c-Myc tagged C326Y FPN (B) for two days with or without 0.5uM hepcidin-25 added. Cells were then stained for surface expression of c-Myc and analyzed by flow cytometry. In the case of wt FPN, hepcidin-25 caused a reduction of detectable cell surface c-Myc (green line versus red line in A), but no reduction of C326Y-c-Myc tag by hepcidin-25 was observed (B). The blue filled histogram represents the fluorescence of cells stained with an isotype control antibody (anti-CD8-FITC). C: Quantitation of downregulation of FPN and FPN mutants by hepcidin-25. Cells were transfected with c-Myc tagged FPN and FPN mutants with or without added hepcidin-25 and stained for surface c-Myc then analyzed as described in A, B. The Mean Fluorescence Intensity (MFI) of the different populations was calculated using CellQuest software, and is displayed as a percentage of the MFI of wt human FPN without added hepcidin-25. Hepcidin-25 reduced surface wt human and murine FPN and Q248H surface expression by at least a half, surface C326Y and Y64N was not affected by hepcidin-25, and N144D and N144H were partially downregulated. D: Cells were transfected with c-Myc tagged FPN and FPN mutants for 24 hours, protein synthesis was inhibited with cycloheximide for two hours and then 0.5 uM hepcidin-25 was added to half the wells for three further hours. Cells were then stained for c-Myc expression (green) and cell nuclei were stained by DAPI (blue). Without added hepcidin all FPN variants were localized to plasma membranes (first and third columns from left). In the presence of hepcidin, wt and Q248H FPN were internalized into discrete vesicles, while C326Y and Y64N FPN protein remained at the cell surface and both internalized and surface N144D and N144H were observed (second and fourth columns). These results are representative of three experiments.
Figure 2: Hepcidin-25 inhibits TfR upregulation and ferritin reduction caused by wt FPN but not by mutant FPN.
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A, B: 293T cells were transiently transfected with either c-Myc tagged wt FPN (A) or c-Myc tagged Y64N FPN (B) for two days with or without 0.5uM hepcidin-25 added. Cells were then stained simultaneously for surface expression of c-Myc and TfR and analyzed by flow cytometry. Transfected cells (c-Myc positive cells) were gated on and their TfR expression is displayed relative to the TfR expressed by control CD8 transfected cells. Wt FPN expression caused an increase in TfR expression compared with control cells (compare red line to grey histogram in A) consistent with FPN causing iron deficiency; co-culture with hepcidin-25 reverses the effect of wt FPN (green line is similar to grey filled histogram in (A). In contrast the upregulation of TfR by Y64N (compare red line to grey filled histogram in B) is not counteracted by co-culture with hepcidin-25 (green line is similar to red line in B). The blue filled histogram represents the fluorescence of cells stained with an isotype control antibody (anti-rabbit IgG).

C: Quantitation of inhibition of FPN and FPN mutant mediated upregulation of TfR by hepcidin-25. Cells were transfected with c-Myc tagged FPN and FPN mutants with or without added hepcidin-25 and stained for surface c-Myc and TfR then analyzed as described in A, B. The Mean Fluorescence Intensities (MFI) of the different populations was calculated using CellQuest software, and is displayed with the MFI of the TfR expressed by control CD8 transfected cells subtracted. The increase of TfR expression induced by wt human, wt murine and Q248H FPN is counteracted by hepcidin-25, while the TfR increase mediated by C326Y and Y64N FPN was resistant to hepcidin-25 inhibition, and the raised TfR due to N144D and N144H expression was partially reduced by hepcidin-25.

D: 293T cells were transfected with wild-type FPN or C326Y FPN in the presence of 1mg/ml human holo-Tf in order to increase background ferritin levels, and with or without added hepcidin-25. Cells were transfected with CD8 as a control. After two days cells were analyzed for ferritin levels by ELISA. Cells were lysed in NP40 at 10^7 cells/ml, doubling dilutions were made and 20µl transferred to ELISA plate in triplicate. Graph shows mean total ferritin in ng/10^7 cells (+/- 95% CI). Cells transfected with wtFPN have around 40ng ferritin per 10^7 cells, a three-fold reduction compared to control cells transfected with CD8. Co-culture with hepcidin-25 completely reverses the effect of wtFPN, but hepcidin-
25 has no effect on the reduction of ferritin mediated by C326Y FPN. These results are representative of three experiments.
**Figure 3**

**Figure 3: Effects of hepcidin on Ferroportin and mutant Ferroportin mediated changes in iron accumulation and iron release**

**A, B:** Effect of hepcidin-25 on iron accumulation by FPN and FPN mutant expressing cells. Cells were transfected with FPN and FPN mutants and cultured with 40μg/ml $^{59}$Fe-Tf with or without 0.5 μM hepcidin-25 added for two days, and then cellular $^{59}$Fe accumulation per million cells was determined. Hepcidin did not alter $^{59}$Fe accumulation by control CD8 transfected cells. Expression of FPN and all FPN variants reduced iron accumulation, consistent with enhanced iron export by transfected cells. Hepcidin-25 reversed the effect of wt and Q248H FPN, but did not inhibit C326Y or Y64N FPN, and only marginally increased iron accumulation by cells expressing N144D and N144H FPN. Each point represents the iron accumulation by one aliquot of a million cells. Red bar shows mean $^{59}$Fe accumulation (+/- 95% CI). Asterisk indicates significance compared to CD8 control (p value < 0.001 by Student’s t test).

**C:** Hepcidin-25 inhibits iron release from cells expressing wt FPN but not C326Y FPN. 293T cells were preloaded with $^{59}$Fe then transfected for 15 hours, washed, recultured in serum-free medium and the percentage $^{59}$Fe released of starting was measured over 32 hours. Hepcidin does not affect ‘background’ release from control 293T cells transfected with GFP (or CD8, not shown). Data shown is representative of two or more experiments for each mutant.
Figure 4: Age versus transferrin saturation and age versus serum ferritin in patients with ferroportin-linked hemochromatosis
Figure 4: Age versus transferrin saturation and age versus serum ferritin in patients with ferroportin-linked hemochromatosis

Values for transferrin saturation (A) and serum ferritin (B) were obtained for hemochromatosis patients with the FPN mutants indicated from the specified references. The *in vitro* behavior of the different FPN mutants is also shown. * Data for C326Y obtained by V.V, A. M-C., Y.C. and K.R. † We have not tested the *in vitro* function of C326S or N144T.
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