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Hepcidin Mediates Transcriptional Changes in Ferroportin mRNA in Differentiated Neuronal-Like PC12 Cells Subjected to Iron Challenge

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Abstract

Ferroportin is the only known iron exporter, and its regulation seems to be controlled at both transcriptional, post-transcriptional, and post-translational levels. The objective of the current work was to investigate how cellular iron status affects the expression of the ferroportin gene *Fpn* under the influence of hepcidin, known to post-translational lower the available ferroportin protein. Nerve growth factor-beta (NGF-β)-differentiated PC12 cells, used as a model of neuronal cells, were evaluated in terms of their viability and expression of ferroportin after inducing cellular iron overload with ferric ammonium citrate (FAC) or hepcidin, iron deficiency with deferoxamine (DFO), or hepcidin in combination with FAC or DFO. Ferritin mRNA was significantly upregulated following treatment with 20 mM FAC. The viability of the differentiated PC12 cells was significantly reduced after treatment with 30 mM FAC or 1.0 μM hepcidin, but when combining FAC and hepcidin treatment, the cells remained unaffected. The expression of *Fpn* was concurrently upregulated after treatment with FAC in combination with hepcidin. Fifty millimolar DFO also increased *Fpn*. Together, these data point towards a transcriptional induction of *Fpn* in response to changes in cellular iron levels. Epigenetic regulation of *Fpn* may also occur as changes in genes associated with epigenetic regulation of *Fpn* were demonstrated.

Keywords Ferroportin · Iron · Neurodegeneration · Hepcidin · Epigenetic

Abbreviations

ARE	Antioxidant response element
BSA	Bovine serum albumin
Cy5	Cyanine-5
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DFO	Deferoxamine
Dmt1	Divalent metal transporter 1
DIV	Days in vitro
FAC	Ferric ammonium iron (III) citrate
FCS	Fetal calf serum
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fpn	Ferroportin gene expression
Ftl	Ferritin light chain
Fth	Ferritin heavy chain
Hdac1	Histone deacetylase 1
IRE	Iron-responsive element
IRP	Iron regulatory protein
NGF-β	Nerve growth factor beta1
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
Phf8	PHD finger protein 8
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of the mean
Tet1	Ten-eleven translocation methylcytosine dioxygenase 1
Tubb4b	Class IV β-tubulin
UTR	Untranslated region

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Introduction

Iron participates in multiple physiological functions within the brain, such as mitochondrial respiration, myelination of axons, and neurotransmitter synthesis [1, 2]. Iron deposition increases with age in the brain [1, 3–5], but the deposition may lead to iron-induced cellular oxidative stress, which can

induce further release from iron-containing proteins, e.g., ferritin, heme, and other proteins with iron-sulfur clusters [6]. To scavenge intracellular iron excess, neurons and glia can induce ferritin expression but another principle mechanism for handling iron excess could rely on induction of ferroportin to enhance cellular iron export [3, 7–10].

Ferroportin encoded by the *SLC40A1* gene, located on chromosome 2 at position 2q32, is a 571 amino acid long protein containing 12 transmembrane regions, 21–23 amino acids each, although some controversy regarding its structure remains [7–9]. Ferroportin is highly expressed by cells associated with iron transport, e.g., hepatocytes, enterocytes, and macrophages [3, 10–12], but it is also expressed in adipocytes and neurons [3, 10, 13]. The regulation of ferroportin expression is multilayered and varies between cell types and tissues with potential regulation at transcriptional, post-transcriptional, and post-translational levels [14]. Increased mRNA transcription of ferroportin is mediated by a so-called antioxidant response element (ARE) found in the promoter sequence. AREs are bound by transcription regulator protein, either BACH1, which leads to gene repression, or NRF2 (also known as NFE2L2), which leads to gene activation. Heme causes BACH1 degradation, allowing Nrf2 to activate the transcription of ferroportin [12, 15]. Transcription of the ferroportin gene *Fpn* can be induced by zinc and copper [7, 16], as well as hypoxia and heme [7]. Concerning the role of iron for the possible induction of *Fpn* transcription in neurons and the involvement of hepcidin degradation of ferroportin in the brain has not been studied.

The translational regulation governs the stability of iron-related ferroportin mRNA [17] through interaction between iron regulatory proteins (IRP) and iron responsive elements (IRE) [3]. The IRPs only bind to IRE when intracellular iron concentration is low, like high concentration results in IRPs binding to 4Fe-4S cluster [1]. Ferroportin mRNA has an IRE at the 5′UTR, which means that the translation of ferroportin mRNA is reduced when intracellular iron concentration is low [1, 12]. Thus, the IRE/IRP mechanisms only allow for inhibition of the translation of ferroportin mRNA, and not its induction [1, 12]. Post-translational regulation of ferroportin is modulated by hepcidin [3, 18, 19], which binds to a domain on the extracellular loop of ferroportin resulting in phosphorylation, internalization, and degradation of the ferroportin and hepcidin complex in lysosomes [3, 10, 19]. A recent study also provided evidence that ferroportin expression is modulated by methylation of the *Fpn* promoter in breast cancer cells [20], thereby confirming the role of epigenetic regulation of ferroportin gene expression.

The objective of the present work was to examine *Fpn* mRNA expression in NGF-β differentiated PC12 cells after inducing iron overload with ferric ammonium iron (III) citrate (FAC) and iron deficiency with deferoxamine (DFO), both under the influence of hepcidin. Our data demonstrates upregulation of *Fpn* after exposure for FAC, but the upregulation was even stronger in combination with hepcidin. We also report on an upregulation of *Fpn* after DFO exposure although the response was blunted after simultaneous exposure with hepcidin.

We also report that both FAC and DFO exposure show changes in genes associated with epigenetic regulation of *Fpn*, i.e., histone deacetylase 1 (HDAC1), the ten-eleven translocation (TET), and PHD finger protein 8 (PHF8). HDAC1 is known to have neuroprotective properties as well as being involved in learning and cognitive restoration [21]. Histone acetylation promotes transcription by providing binding sites for various proteins involved in gene activation, whereas deacetylase operates in the opposite manner, repressing transcription [22–24]. TET protein family dioxygenases are able to promote demethylation by catalyzing the conversion of 5mC to 5hmC [22, 25, 26]. Among three known isoforms, TET1 is dominantly expressed in the brain [25], and the TET proteins might serve as stress sensors, modulating their activity in response to changes in, e.g., cellular iron levels and hypoxia [25]. The third epigenetic regulator PHF8 is a Jumonji-containing histone demethylase, which is moderately expressed in microglia, astrocytes, and neurons [25]. Phf8 is hypoxia-regulated and its expression is therefore likely altered after any form of cellular stress [25].

Materials and Methods

Cell Culture

PC12 cells derived from rat adrenal medulla pheochromocytoma were cultivated in collagen IV-coated (Sigma, cat#C5533) 75-cm^2 flasks (Cellstar) with RPMI 1640 medium (Gibco, cat#61870010) supplemented with 10% horse serum (Sigma-Aldrich, cat#H0146) and 5% FCS (Gibco, cat#10106-169). The cells were cultured for 7 days with 5% CO_2 and 95% air at 37 °C using an incubator. After 7 days, equal to approximately 90% confluence, the cells were trypsinized with premixed 1:1 trypsin-EDTA-solution and seeded into 12-well plates at a density of 20.000 cells/cm^2 for later isolation and purification of RNA and DNA, in 24-well plates on coverslips with 50.000 cells/cm^2 for immunocytochemistry, and 96-well plates with 100.000 cells/cm^2 for WST-1 cytotoxicity. The cells were differentiated by the addition of NGF-β (NGF-β recombinant, Sigma-Aldrich, rat cat# N2513) to the media in a final concentration of 50 ng/mL [27]. The state of differentiation was followed by simple phase-contrast microscopy (Zeiss AxioCam Erc5s) every 24 h. After 6 days in vitro (DIV), an estimated 80–90% of the cells were differentiated into cells with a neuronal-like phenotype. The differentiation of the cells was repeated five times, all resulting in the same degree of differentiation.

Immunocytochemistry

To characterize the differentiated PC12 cells immunocytochemistry was performed. The cells were fixed for 5 min in 4% paraformaldehyde (PFA) at room temperature. They were then permeabilized for 5 min in 3% bovine serum albumin (BSA) supplemented with 0.3% Triton X-100 in phosphate buffer saline (PBS) (Europa Bioproducts, cat#EQBAH62), followed by a subsequent blocking period of 30 min in plain 3% BSA solution. The primary antibodies, mouse anti-β tubulin IV (Sigma, cat#T7941), and rabbit anti-SLC40A1 (Alpha Diagnostics, cat#MTP11-S) were diluted in 1% BSA (1:200), added to the cells, and incubated on a belly dancer for 60 min at room temperature. The cells were washed three times with 1% BSA solution to remove excess unbound antibodies. The secondary antibodies, donkey anti-mouse IgG (Invitrogen, cat# A-21202) conjugated with Alexa Fluor 488, and goat anti-rabbit IgG (Invitrogen, cat#A11037) conjugated with Alexa Fluor 594 were diluted in 1% BSA solution (1:200). The cells were incubated for 30 min at room temperature and washed three times in 1% BSA. The cells were additionally incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, cat#D9542) diluted in PBS (1:1000) for 10 min at room temperature followed by a single washing in PBS. The cells were mounted on glass slides in fluorescent mounting medium (DAKO, cat#S3023) and examined (Zeiss Axio Observer Z1 microscope and ZEISS LSM 800 equipped with Airyscan).

WST-1 Cytotoxicity Assay

Cell viability was determined using a conventional WST-1 cytotoxicity assay to identify the appropriate range of concentrations of various experimental situations (see below). Ten microliters of the Cell Proliferation Reagent WST-1 (Roche Diagnostics, cat#05015944001) was added to each well and incubated at 37 °C and 5% CO_2 for 2 h. The plate was prepared with controls containing differentiated cells that received no treatment, as well as control wells containing only media. Absorbance (OD_{450}) was measured at 450 nm with a reference wavelength at 620 nm (Tecan Sunrise) using the Magellan2 software. The absorbance values were normalized to the average value of the control group receiving no treatment, which was 100% viable. Control wells containing only media without cells were used to subtract background.

Treatment of PC12 Cells

Six days after treatment with NGF-β, the cells underwent various treatment with iron overloading or chelating compounds, some in combination with hepcidin. Iron overload was induced by FAC (ferric ammonium iron (III) citrate, Sigma-Aldrich) where the concentrations ranged from 0 to 230 mM. Hepcidin (Peptides International cat# PLP-3769-PI) was added in concentrations of 0–2.3 μM. The cells were also treated with 0–20 mM FAC in combination with 1.0 μM hepcidin to investigate how the combination of excess iron and hepcidin would affect the cells. The cells were also subjected to iron deficiency through treatment with DFO (Deferoxamine mesylate salt, Sigma-Aldrich) in concentrations ranging from 0 to 70 mM. In this case, the culture medium was pre-treated for 24 h before; it was added to cells to allow DFO to chelate iron of the medium. The cells were furthermore treated with a combination of 0–2.3 mM DFO and 2.3 μM hepcidin. The selection of these various concentrations was based on data from the WST-1 cytotoxicity assay.

RT-qPCR

The purification of DNA and RNA after iron challenge was carried out using an AllPrep DNA/RNA Mini Kit (QIAGEN, cat#80204) according to the manufacturer's instructions. The concentration and purity of RNA were assessed using a NanoDrop Spectrophotometer (ND-1000, Fisher Scientific). Five hundred nanograms of RNA was treated with DNase I enzyme (Thermo Scientific, cat#EN0521) to remove potential genomic DNA contamination. Complementary DNA (cDNA) was synthesized using a Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, cat#K1652). One hundred nanograms of each sample was used in the following PCR reaction. Probe-based qPCR was carried out for analysis of *Fpn* using a Brilliant III Probe Mastermix (Agilent Technologies, cat#600880). The optimal concentration of probe was determined to be 150 nM and the optimal concentration of each primer was furthermore determined and depicted in Table 1. The thermal profile was as follows: hot start at 95 °C for 3 min followed by 40 amplification cycles with 95 °C for 5 s and 60 °C for 10 s. The *Fpn* expression was normalized to *Actb* and *Rpl29* expression.

Analysis of Ferritin H (*Fth*) and Ferritin (*Ftl*) as well as epigenetic markers *Tet1*, *Hdac1*, and *Phf8* was carried out by RT-qPCR and normalized with *Actb* expression. Each qPCR reaction was performed by mixing 2.5 ng cDNA and 10 pmol of each primer (Table 2) with the Maxima SYBR Green qPCR Mastermix. Each sample was performed in triplicates, while non-reversed RNA and water served as negative controls. The qPCR reaction was 95 °C for 3 min, 40 cycles of 95 °C for 5 min, and 60 °C for 10 min, followed by one cycle of 95 °C for 30 s, 65 °C for 30 s and 95 °C. Data were normalized to *Actb*. All qPCR reactions were performed using AriaMx Real-Time PCR system (Agilent Technologies).

Statistics

The data were analyzed by one-way ANOVA with Dunnett's multiple comparisons post hoc test, except for the

Table 1 Primers used for probe-based qPCR as well as their concentration

Target	Forward primer	Conc.	Reverse primer	Conc.	Probe	5′end
Slc40a1	GGTCCTTACTGTCTGCTA	350	TCTGCTAATCTGCTCCTG	300	TTCTCCTGCTACGACAACAA TCCA	Cy5
Actb	CTGGAGAAGAGCTA TGAG	350	GATGGAATTGAATGTAGTTT C	350	CACTATCGGCAATGAGCGGTTC	FAM
Rpl29	GACTGGGGATAACTGAGC	350	AGCTGTTTCCTGAGACTC	400	TGCCTGTAACCTTGCTCTGATGT	HEX

comparisons of FAC and DFO treatment with or without hepcidin, which was as analyzed by a two-way ANOVA with a Tukey's multiple comparisons post hoc test. Significance levels were $*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p = 0.0001–0.001$, and $****p < 0.0001$.

Results

Differentiation of PC12 Cells

Prior to differentiation, the PC12 cells displayed a round morphology without apparent neurites (Fig. 1a). Treatment with 50 ng/mL NGF-β for 24 h revealed that the PC12 cells had started to display an altered morphology seen as the appearance of cell bodies with multipolar shape and extensions with the similarity of minor neurites (Fig. 1b). After 3 days, the extensions became even more visible and several cells resembled neurons, while other cells maintained the morphology of undifferentiated PC12 cells (Fig. 1c). After 6 days, the neurites looked mature, forming an intermingling network (Fig. 1d). The treatment with NGF-β for 6 days was considered sufficient for differentiation of PC12 cells as approximately 80% of the cells had obtained the intended morphological characteristics, and no further increase in the appearance of the number of differentiated neurons in spite increasing the incubation time (not shown).

Immunocytochemistry

PC12 cells were immunolabeled with the neuronal marker β-tubulin IV (Tubb4b) in order to assess the level of neuronal differentiation [28]. To verify ferroportin expression in differentiated PC12 cells, the cells were also immunolabeled with rabbit anti-SLC40A1. Tubb4b was seen within the cytosol in well-differentiated PC12 cells, but absent from non-differentiated cells (Fig. 2a, d). The Tubb4b protein localized both to the neurites and the perinuclear cytosol without labeling of the cellular nuclei in differentiated PC12 cells (Fig. 2b, e). The ferroportin protein also displayed a higher appearance near the cellular membrane compared to the labeling of the perinuclear cytosol (Fig. 2b, e). Well-differentiated PC12 cells revealed higher expression of ferroportin than cells lacking neuronal morphology (Fig. 2b).

Treatment with FAC, DFO, and Hepcidin

The cells were treated with hepcidin using concentrations from 0.1 to 2.3 μM (Fig. 3a). The viability of the differentiated PC12 cells after hepcidin treatment showed a continuous decline reaching 84.28 ± 6% and 79.17 ± 8.9% at 0.3 and 0.4 μM hepcidin respectively (Fig. 3a). The viability was further decreased to 78.8 ± 1.4% at 1 μM hepcidin and continued to decline at higher concentrations.

To investigate the effects of iron overload induced by excessive extracellular iron, the cells were treated with FAC in concentrations ranging from 10 to 230 mM (Fig. 3b). The viability of the differentiated PC12 cells decreased at 20 mM to reach 12.85 ± 6.8% when added with 30 mM FAC. The cells showed a continuous decline in cell viability at 40 mM and 50 mM to 8 ± 1.33% and 3.8 ± 0.41% respectively. The viability stabilized at around 4% with FAC concentrations of 50–230 mM; hence, some cells remained viable even after 230 mM FAC (not shown). Based on these observations, the following concentrations of FAC were chosen for further molecular analyses: 10 mM (no effect), 20 mM (~ 10% cell

Table 2 Primers used for RT-qPCR

Target	Forward primer	Reverse primer
Fth	GACCACCGCGTCTCCCTCGC	CAGGGCCACATCATCCCGGTC
Ftl	TGACGTGGCTTTGG AAGGCG	GATGGCTTCTGCACATCCTGG
Hdac1	GCTGAGGAGATGACCAAG	GTGGACAACTGACAGAAC
Phf8	CCAGAAAGCAAAGCTCAA	GCACTGTCTACCTTCTTC
Tet1	GAGTCTTCACCATGACAC	GGACATGAATTCTTAGAACT ATC

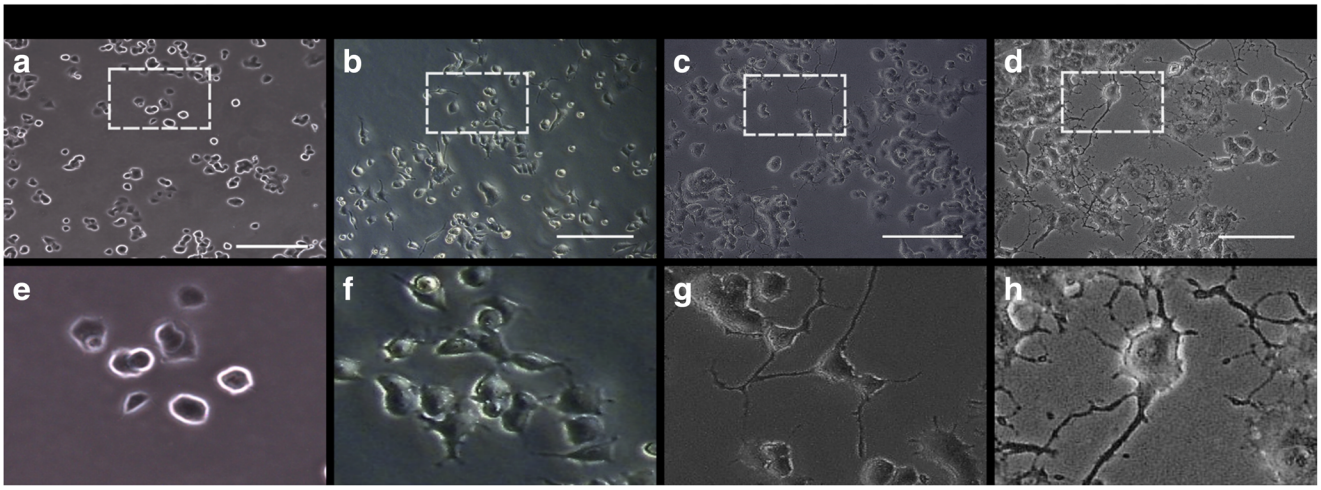

Fig. 1 **a**–**d** Growth properties of PC12 cells before (**a**) and after receiving 50 ng/mL NGF-β for 1, 3, and 6 days DIV (**b**–**d**). Eighty to ninety percent of the cells display differentiated morphology after 6 DIV. **e**–**h** Areas marked with respective squares in **a**–**d** shown in higher magnification obtained by computed enlargement. The PC12 cells differentiate into cells with morphological changes corresponding to neurons with polygonal cell bodies and extended cellular processes with several branches sharing the morphology of mature neurites. Scale bar = 200 μm

death), and 30 mM (~ 90% cell death). FAC treatment was further combined with 1 μM hepcidin, thereby assuming that inhibition of iron export from the cells would lead to intracellular iron overload and resulting toxicity. However, when combining 20 mM FAC with 1 μM hepcidin treatment no significant change in cell viability was detected (Fig. 3c), indicating a putative protective effect of hepcidin.

In cells treated with the iron chelator DFO to reduce extracellular iron, significant effects on viability could be observed when treatment with DFO was increased in ranges towards 70 mM (Fig. 3d). A significant decrease in cell viability was observed at 50 mM, 83.33 ± 4.8%, and at 60 and 70 mM, the viability was further decreased to 79.59 ± 7% and 76.94 ± 11.16%, respectively (Fig. 3d). The highest concentration of

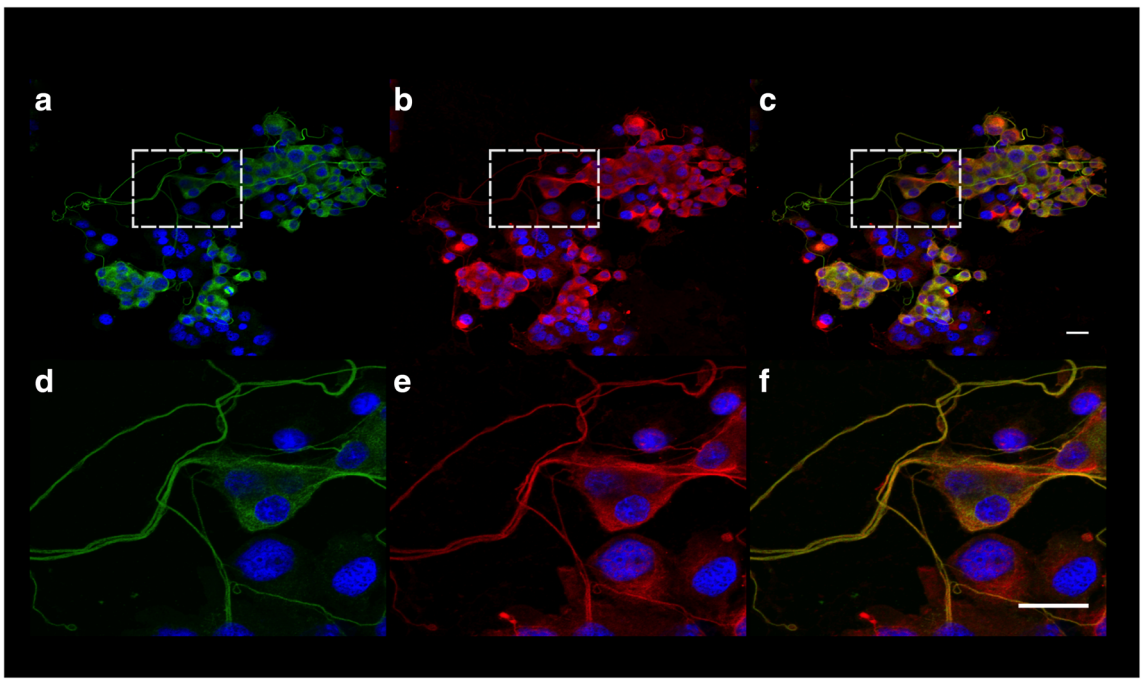

Fig. 2 **a**–**b** Confocal images of differentiated PC12 cells after 6 days of treatment with NGF-β revealing protein expression and cellular localization of Tubb4b (**a**, **d**) (green) and ferroportin (**b**, **e**) (red). Nuclei are counterstained with DAPI (blue). **d**–**e** Areas marked with respective squares in **a**–**c** shown in higher magnification obtained by computed enlargement. The extended processes of the differentiated PC12 cells are clearly seen in these high-power magnifications. **c**, **f** Merged photos. Scale bar = 20 μm

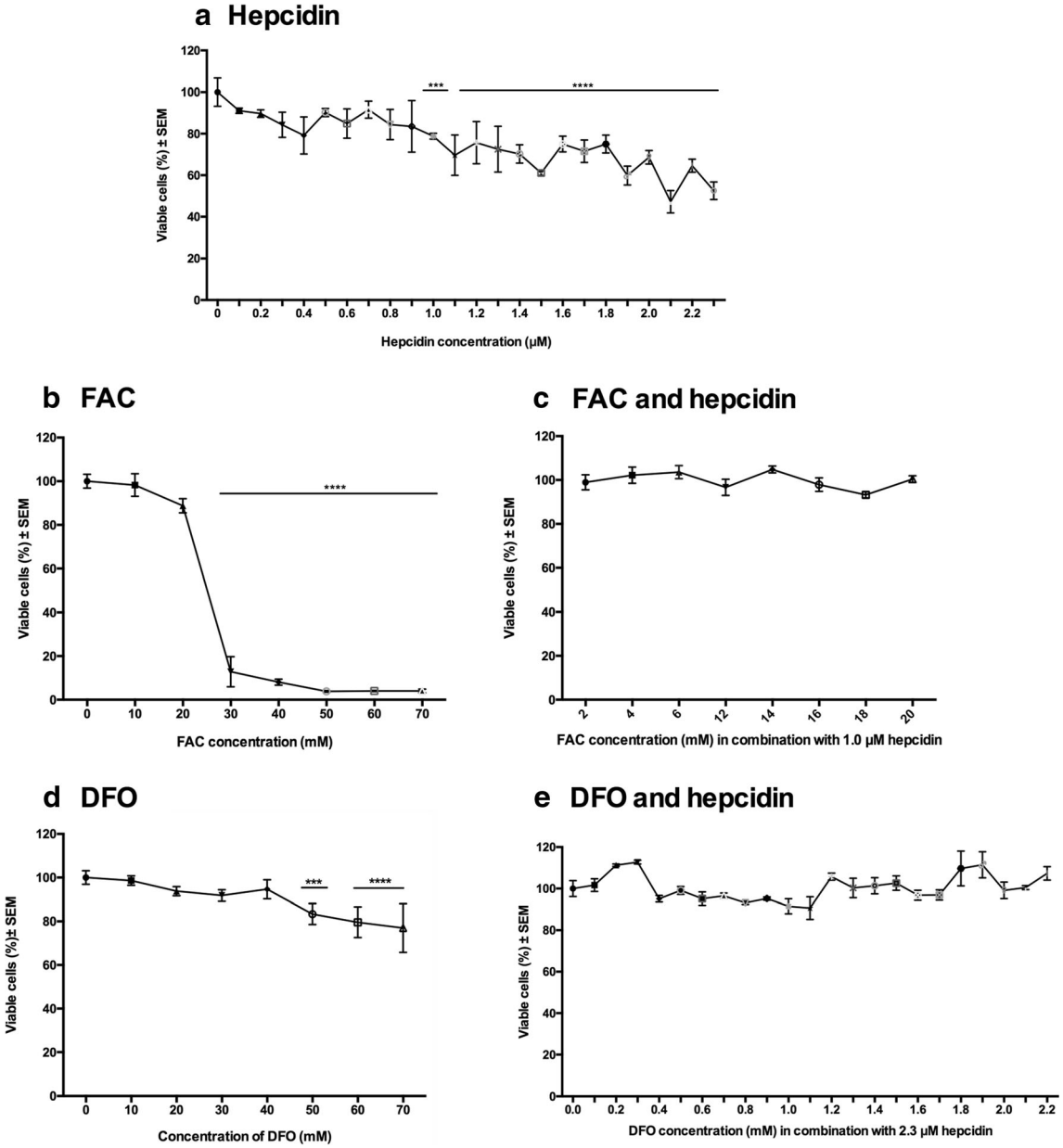

Fig. 3 Graphs illustrating the percentage of cell viability as a function of the concentration of hepcidin (**a**), FAC (**b**), FAC in combination with hepcidin (**c**), DFO (**d**), and DFO in combination with hepcidin (**e**) after 24-h treatment. *X*-axis: concentration. *Y*-axis: percentage viable cells. **a** Hepcidin lowers the viability at doses above 1.0 μM. **b** FAC lowers viability at doses higher than 20 mM. **c** Combining FAC up until 20 mM with 1.0 μM does not affect viability. **d** DFO lowers the viability at doses higher than 40 mM. **e** Combining DFO in doses up to 2.2 mM with hepcidin results in unaltered viability showing that chelation of available iron repeals the negative influence of hepcidin on viability. Data are presented as mean ± SEM. Significance: $*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p = 0.0001–0.001$, $****p < 0.0001$

DFO (40 mM) before the cells were affected was chosen for further the analyses on mRNA expression and examined in combination with 2.3 μM hepcidin, to study whether chelation of iron could reduce the cytotoxicity effect of hepcidin (Fig. 3e). DFO had a positive effect on cell viability with concentrations ranging from 0.1 to 2.2 mM when combined with 2.3 μM hepcidin (Fig. 4b).

Expression of Ferroportin mRNA in PC12 Cells

The relative gene expression of *Fpn* generally increased with increasing concentration of FAC, and when 20 mM FAC was combined with 1.0 μM hepcidin, the increase in *Fpn* expression was significantly (Fig. 4a) and approximately 90–100-fold compared to FAC alone ($p = 0.0025$) (Table 3). This indicated that hepcidin and not iron in the extracellular environment was essential for upregulation of *Fpn* expression.

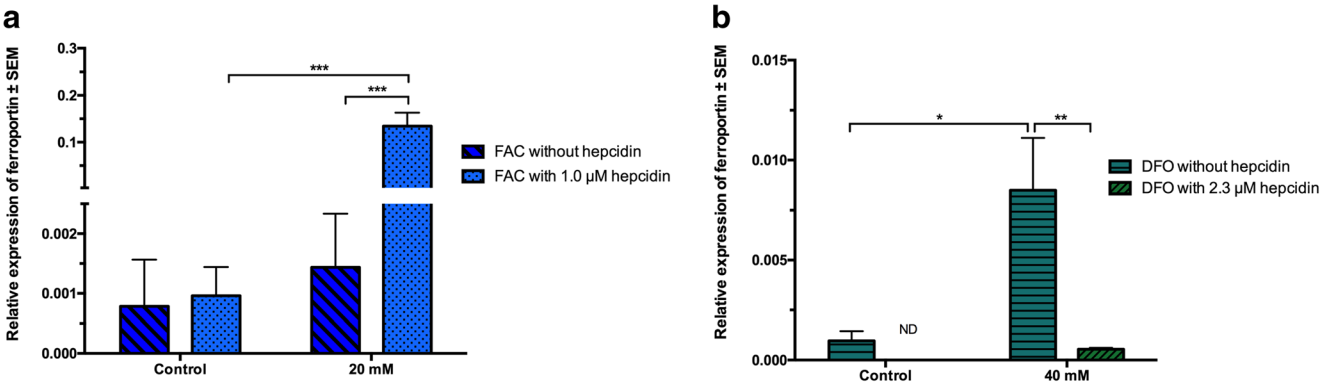

Fig. 4 Expression of *Fpn* mRNA following treatment with FAC (**a**) or DFO (**b**) with or without hepcidin. The expression of *Fpn* after treatment with FAC/DFO is normalized to that of the control group receiving no treatment, whereas the expression of Fpn after treatment with FAC/DFO in combination with hepcidin is normalized to the group receiving 1.0 μM hepcidin or 2.3 μM hepcidin, respectively. **a** FAC or hepcidin alone does not increase expression of *Fpn*. In contrast, combining FAC and hepcidin leads to significant increase in *Fpn* mRNA expression compared to the remaining groups. **b** *Fpn* is not detectable after treatment with 2.3 μM hepcidin alone. In contrast, DFO treatment without hepcidin leads to a significantly higher expression. Combining DFO treatment with 2.3 μM hepcidin does not lead to significant changes in *Fpn* mRNA expression. Note that compared to that of cells added with FAC, the increase in *Fpn* mRNA expression following treatment with DFO is far-fold lower. Data are presented as mean ± SEM. Statistics: $*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p = 0.0001–0.001$, $****p < 0.0001$, ND, not detectable

In cells treated with the iron chelator DFO, the culture medium was pre-treated for 24 h and then it was added to cells for DFO to chelate iron found in the medium. The relative gene expression of *Fpn* was measured subsequently, and the expression of *Fpn* after treatment with DFO was in general much lower than after treatment with FAC, but a significantly higher expression was observed after treatment with 40 mM DFO compared to the control group (Fig. 4b, Table 4). There was no significant effect on *Fpn* expression when combining DFO 40 mM with 2.3 μM hepcidin. However, when comparing treatment with 40 mM DFO + 2.3 μM hepcidin, it was obvious that the combined treatment of DFO and hepcidin decreased *Fpn* expression approximately 20-fold (Fig. 4b, Table 4).

Expression of Ferritin mRNA After Iron Challenge

The relative expression of *Fth* and *Ftl* was investigated after iron challenge with 20 mM FAC and 20 mM FAC+ 1.0 μM hepcidin in order to estimate the iron index of the cells. There was observed a significant 2-fold increase in *Fth* expression after treatment with 20 mM FAC, and 4-fold increase after treatment with 20 mM FAC + 1.0 μM hepcidin (Fig. 5). *Ftl* expression was likewise altered by iron challenge. *Ftl* expression displayed a 4-fold increase after treatment with 20 mM FAC and 6-fold increase after treatment with 20 mM FAC + 1.0 μM hepcidin (Fig. 5). The treatment with iron resulted in significant increase in

Table 3 The expression of *Fpn* mRNA after treatment with various concentrations of FAC in combination with a fixed concentration of hepcidin. Values below 1 indicate downregulation of *Fpn* mRNA expression, and values above 1 the reverse.

Treatment group	Ratio (treatment/control)	Effect on *Fpn* mRNA	Significance
FAC 10 mM	0.889	(↓)	n.s
FAC 20 mM	1.49	(↑)	n.s
FAC 30 mM	5.97	(↑)	n.s
FAC 6 mM + 1.0 μM hepcidin	14.122	(↑)	n.s
FAC 20 mM + 1.0 μM hepcidin	95.14	↑	***
FAC 20 mM + 1.0 μM hepcidin/FAC 20 mM	93.92	↑	***

The expression of Fpn after treatment with FAC is normalized to that of the control group receiving no treatment, whereas the expression of Fpn after treatment with FAC in combination with hepcidin is normalized to the group receiving 1.0 μM hepcidin. Effects presented in brackets display tendency without significance. Combining 20 mM FAC and 1.0 μM hepcidin upregulates the expression of Fpn, approximately times 90. A comparison between the treatments with 20 mM FAC and 20 mM FAC in combination with 1.0 μM hepcidin, measured as the ratio of expression, demonstrates a significant increase mediated by hepcidin to the two situations with identical concentrations of FAC. Significance: ***p = 0.0001—0.001, n.s. not significant.

Table 4 The expression of *Fpn* mRNA after treatment with 40 mM DFO in combination with 2.3 μM hepcidin.

Treatment group	Ratio (treatment/control)	Effect on *Fpn* mRNA	Significance
DFO 40 mM	8.81	↑	*
DFO 40 mM + 2.3 μM hepcidin	0.42	↓	n.s.
DFO 40 mM/DFO 40 mM +2.3 μM hepcidin	20.63	↑	**

Values below 1 indicate a downregulation of Fpn mRNA expression, and values above 1 the reverse. The expression of Fpn after treatment with DFO is normalized to that of the control group receiving no treatment, whereas the expression of Fpn after treatment with DFO in combination with hepcidin is normalized to the group receiving 2.3 μM hepcidin. Forty millimolar40 mM DFO upregulates Fpn significantly. Forty millimolar40 mM DFO combined with 2.3 μM hepcidin results in downregulation of Fpn mRNA. A comparison between the treatments with 40 mM DFO and 40 mM DFO + 2.3 μM hepcidin, measured as the ratio of expression, reveals strong upregulation of Fpn mRNA in the single treatment group without hepcidin with an approximately 20 times higher expression. Significance: *p = 0.01—0.05, ** p = 0.001—0.01, n.s. not significant.

both *Fth* and *Ftl*, but simultaneous treatment with iron and hepcidin increased the *Fth* and *Ftl* expression even further, suggesting that hepcidin can mediate an even stronger response than iron loading alone.

Expression of Epigenetic Regulators in Differentiated PC12 Cells

Due to the complicated nature of *Fpn* regulation, we investigated the possibility of epigenetic regulation of *Fpn* by testing the expression of *Hdac1*, *Tet1*, and *Phf8* in PC12 cells after a series of treatments with FAC or DFO with hepcidin as described above.

The expression level of histone deacetylase *Hdac1* was significantly increased after treatment with 1.0 μM hepcidin ($p = 0.0044$), but decreased when combining 20 mM FAC with 1.0 μM hepcidin (Fig. 6a). The expression of *Tet1*, involved in DNA demethylation, was significantly decreased after treatment with 1.0 μM hepcidin ($p = 0.0044$) and 20 mM FAC + 1.0 μM hepcidin ($p = 0.0034$) (Fig. 6a). The expression of *Tet1* was slightly upregulated after treatment with 20 mM FAC alone, suggesting that the downregulation of *Tet1* was dependent on hepcidin. Furthermore, the expression of *Phf8* was significantly decreased after treatment with 1.0 μM hepcidin ($p = 0.0007$) and 20 mM FAC + 1.0 μM hepcidin ($p = 0.0011$) (Fig. 6a), suggesting that alteration in *Phf8* was also dependent on hepcidin. *Hdac1* expression was significantly lower after treatment with 40 mM DFO in combination with 2.3 μM hepcidin ($p = 0.0011$) (Fig. 6b). *Tet1* expression was significantly higher after treatment with 40 mM DFO ($p = 0.0015$) (Fig. 6b) and the expression of *Phf8* was significantly decreased after treatment with 2.3 μM hepcidin ($p = 0.0084$) (Fig. 6b). These results suggest that multiple epigenetic mechanisms have been initiated as a response to iron challenge, further emphasizing the importance of identifying the transcriptional factors involved in transcriptional activation of *Fpn*.

Discussion

Iron Loading and Ferritin Expression After Iron Challenge

Cellular iron overload was induced by excessive iron in the extracellular environment and by inhibiting the export of iron from cells with hepcidin. As the iron content of the extracellular environment increases, it is more likely that the cells import more iron than required. Once this scenario becomes toxic for some cells, they may undergo ferroptosis, an iron

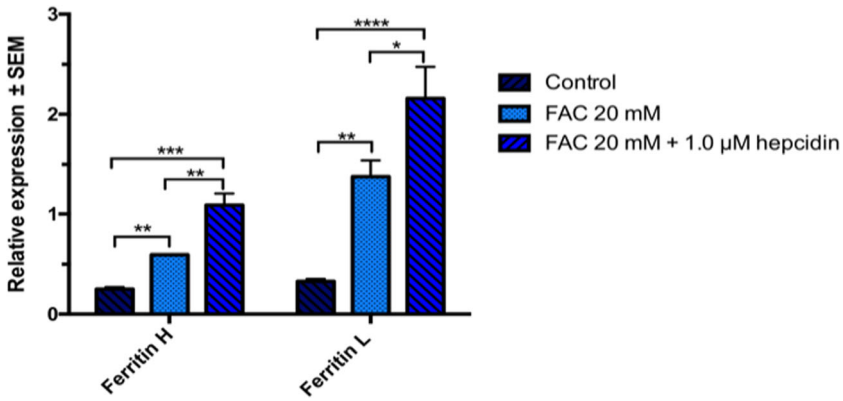

Fig. 5 To verify the affection of iron on the gene expression in the PC12 cells, the relative expression of *Fth* and *Ftl* was investigated after iron challenge with 20 mM FAC and 20 mM FAC + 1.0 μM hepcidin. Both *Fth* and *Ftl* expression increased after iron challenge, and the expression was further increased following addition of 20 mM FAC + 1.0 μM hepcidin

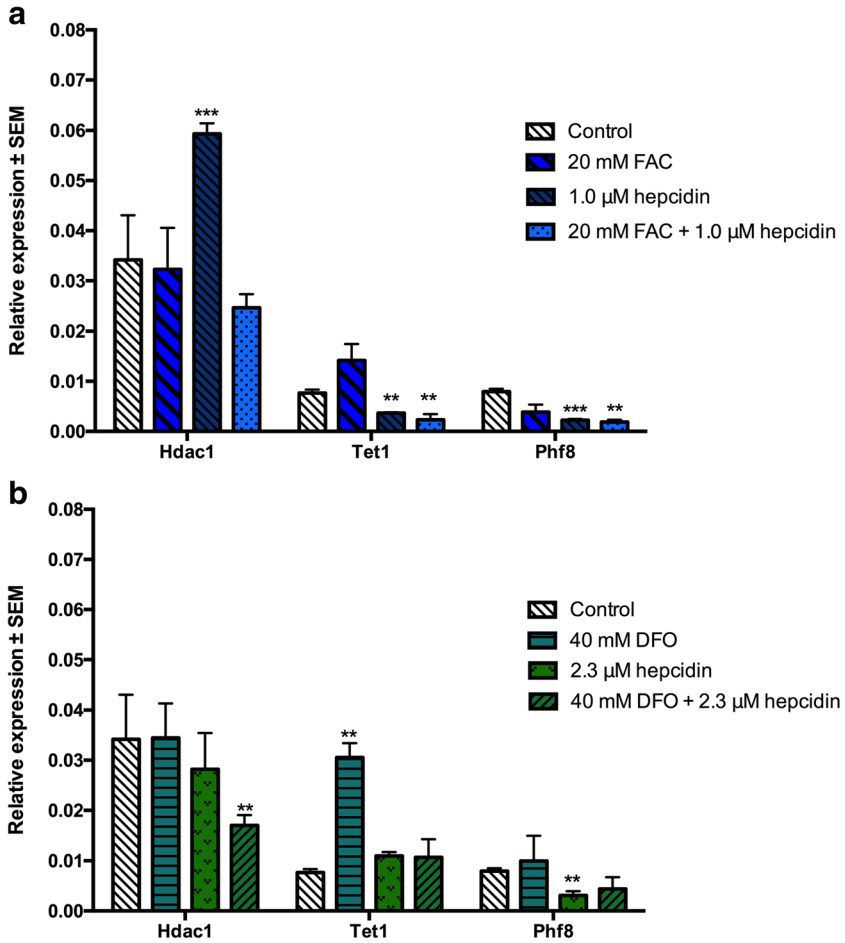

Fig. 6 The effects of FAC, DFO, and hepcidin on the relative gene expression of various epigenetic regulators in the NGF-β differentiated PC12 cells after treatment with 20 mM FAC, 40 mM DFO, or hepcidin in combination with 20 mM FAC (**a**) or 40 mM DFO (**b**). **a** Treatment with 20 mM FAC did not alter expression of *Hdac1*, but treatment with 1.0 μM hepcidin alone increases the expression significantly. FAC blunts the increased expression of *Hdac1* when combined with 1.0 μM hepcidin. There is seen a significant downregulation of Tet1 and Phf8 after treatment with 1.0 μM hepcidin, as well as with 20 mM FAC + 1.0 μM hepcidin. **b** The expression of Hdac1 is significantly lower after treatment with 40 mM DFO in combination with 2.3 μM hepcidin. Tet1 expression is significantly increased after treatment with 40 mM DFO. Phf8 expression is significantly decreased after treatment with 2.3 μM hepcidin. Statistics: $*p = 0.01–0.05$, $**p = 0.001–0.01$, $***p = 0.0001–0.001$, $****p < 0.0001$. Figure created in Prism

dependent cell death that occurs in cancer cells and neurons [29, 30], releasing their intracellular iron to the extracellular environment. This iron then becomes available for the remaining cells. The purpose of the iron challenge was to investigate how the cells would react when reaching their threshold for iron tolerance. The differentiated PC12 cells of the present study proved very resistant to FAC challenges and were only affected when iron exposures ranked in the level of millimolar, which is contrasted by other studies that report on *Fpn* expression in other cell lines exposed in the micrometer range [31, 32]. This dramatic tolerance for iron may be explained by the fact that PC12 cells are derived from a cancer cell line with a major need for iron to satiate their proliferative stage. At 20 mM FAC and upwards, a significant decrease in viability was seen with only 4% still viable at 50 mM. The reason why the viability was so strongly affected between 20 and 30 mM FAC may be explained by a combination of cellular iron saturation within the cells and a decreased capability for iron export. Ferritin is an iron storage protein capable of storing 4500 iron molecules. It consists of 24 subunits of Ferritin Heavy chain (Fth) and Ferritin Light Chain (Ftl) [33]. When looking at the *Fth* and *Ftl* mRNA expression, iron induced a significant increase in both genes. This is to be expected, as ferritin expression is stimulated by high iron levels [34]. The dramatic increase of *Fth* and *Ftl* after simultaneous treatment with hepcidin is in line with Thomsen *et al.* (2015) findings where high hepcidin expression correlated with high *Fth* and *Ftl* expression [35].

Ferroportin mRNA Expression During Cellular Iron Overload Combined with Hepcidin Treatment

Concerning *Fpn* expression, a slight but not significant upregulation was seen at 20 mM. Chen et al. (2005) previously

reported that iron loading inhibits *Fpn* expression in differentiated PC12 cells but the concentrations of FAC applied was 100 μM, which is much lower than the concentrations in the current study [31]. Zhao et al. (2014) conversely reported on an increase in *Fpn* expression with increasing treatment with FAC in the range of 0 to 200 μM [31], which implicates that lower concentrations of FAC may inhibit *Fpn* expression only in some cell types, and further emphasizes a dynamic regulation of *Fpn* dependent on cell type.

Hepcidin, often called the master iron regulator, binds to ferroportin triggering its internalization and lysosomal degradation, thereby directly regulating iron export from cells to plasma [30, 36–38]. The incapability to export iron increases the cellular iron content, gradually lowering the cells viability, and poses an increased risk of ROS-mediated damage. This is seen in the steadily declining viability of the cells after 1.0–2.3 μM hepcidin treatment. The treatment with hepcidin though did not cause a dramatic fall in viability to the extent as that of treatment with FAC, which may be explained by that hepcidin binds and degrades ferroportin, thereby trapping the iron that is already present within the cell, where the extracellular iron concentration remains more stable than following FAC treatment.

To investigate how the PC12 cells react to hepcidin in an iron overload stage, FAC treatment was combined with hepcidin. After treatment with 20 mM FAC and 1.0 μM hepcidin, which both had induced about 20% decrease in viability as a single treatment, the viability remained surprisingly unaffected (Fig. 3c). The *Fpn* expression was significantly upregulated after treatment with FAC and hepcidin, and when comparing the effect of 20 mM FAC treatment with that of 20 mM FAC in combination with 1.0 μM hepcidin, the *Fpn* expression was approximately 100 times greater in the latter. This indicates that the strong upregulation of *Fpn* expression during iron overload is mediated by hepcidin, not FAC. This upregulation of *Fpn* expression, mediated by iron overload in combination with hepcidin has not been reported before in neuronal-like cells. Domenico et al. reported similar transcriptional changes in macrophages, both *in vitro* and *in vivo* [39], and Nairz et al. has demonstrated that salmonella-infected macrophages are able to upregulate *Fpn* expression, by increase in transcriptional factor Nrf2 mediated by nitric oxide [40]. Increase in *Fpn* expression increases the survival of the cell by preventing ROS-mediated damage due to a great amount of unbound intracellular iron (Fig. 7).

Hepcidin is secreted in response to inflammation and has been thought cause iron accumulation in neurons and microglia, promoted by changes in *Dmt1* and *Fpn* expression [41]. On the contrary, hepcidin levels are reduced in human and mouse brains exhibiting severe Alzheimer's pathology, but unaltered in the early course of the disease [42]. This could this indicate that hepcidin is beneficial in situations combining iron overload and inflammation as seen in neurodegenerative diseases. Another study showed that microglial activation and oxidative damage could be prevented by treating the cells with hepcidin prior being subjected to amyloid-beta injection. Hepcidin was additionally able to suppress oxidative damage in amyloid-beta injected mice [43]. These results indicate that hepcidin may have an anti-inflammatory effect on cells within the CNS, which could be mediated by a change in capability to manage cellular iron. The effect of hepcidin within the CNS needs further investigation, as studies disagree on whether it is to responsible for iron accumulation or beneficial in neurodegeneration. It has been suggested that macrophage ferroportin is more sensitive to hepcidin than enterocyte ferroportin, which might be explained by a tissue specific hepcidin-mediated transcription [44].

In the investigation of the potential epigenetic regulation of *Fpn* after treatment with FAC, hepcidin and a combination of these revealed significant increase in expression of *Hdac1*, a histone deacetylase. HDAC1 suppresses hepcidin expression through SMAD4-dependent transcriptional regulation [45]. An upregulation of *Hdac1* after treatment with hepcidin would thereby suppress endogenous hepcidin expression, preventing the level of hepcidin from becoming toxic. HDAC1 promotes either neuroprotection or neurotoxicity, depending on its interacting partners [46]. When examining the viability of the cells after treatment with 1.0 μM hepcidin, the viability was significantly affected, but when comparing the viability of the cells after treatment with 20 mM FAC and 20 mM FAC + 1.0 μM hepcidin, the viability was higher in the latter group. It can, therefore, be assumed the observed neuroprotection might be due to the upregulation of *Hdac1* mediated by hepcidin.

The expression of *Tet1* was slightly increased after treatment with 20 mM FAC but decreased significantly after treatment with 1.0 μM hepcidin and 20 mM FAC + 1.0 μM hepcidin. This decrease in expression seems also to be a resultant of hepcidin treatment. TET1 is a dioxygenase predominantly expressed in the brain and is able to promote DNA demethylation [22, 25, 26]. The decreased expression of *Tet1* correlates well with the upregulation of *Hdac1* after hepcidin treatment. The expression of *Phf8*, a histone demethylase [25], was also significantly decreased after treatment with 1.0 μM hepcidin, 2.3 μM hepcidin, and 20 mM FAC + 1.0 μM hepcidin. *Phf8*, like *Tet1*, works by activating transcription [47]. The decreased expression of *Phf8* seems to be caused by hepcidin as in the case of *Tet1* expression. These results indicate an epigenetic regulation mediated by hepcidin in iron-loaded differentiated PC12 cells, but this should be investigated further, preferably in primary cells.

Ferroportin mRNA Expression in Cellular Iron Deficiency Combined with Hepcidin Treatment

A significant upregulation in *Fpn* expression was seen after treatment with 40 mM DFO, which is in good accordance

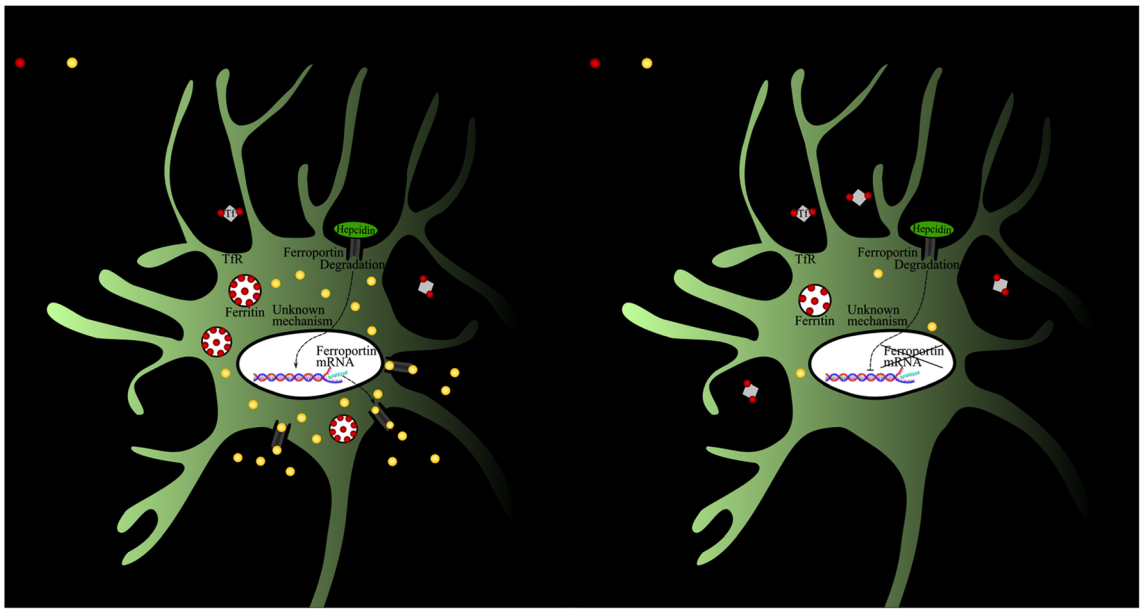

Fig. 7 Overview of our hypothesis regarding the action of hepcidin on *Fpn* mRNA in NGF-β differentiated PC12 cells depending on the iron status. (Left) In iron overload, a strong upregulation of *Fpn* mRNA is mediated by hepcidin. This could lead to increase translation of *Fpn* mRNA and hence more ferroportin protein (yellow dots) in spite hepcidin also provokes a degradation of the ferroportin when present in the cellular membrane. A raised cytosolic appearance of ferroportin may nonetheless aid capture of excess iron and prevent ROS-mediated damage caused by unbound intracellular iron. (Right) In cellular iron deficiency, hepcidin mediates a decrease in *Fpn* mRNA expression, which lowers the translational and presence of ferroportin protein (yellow dots) in the cellular membrane. In turn, this increases the survival of the cell by retaining iron intracellularly to facilitate its availability

with a previous observation by Chen et al. (2005) [32], although upregulation during iron deficiency might seem illogical as neurons devoid the demand for transcellular transport of iron as transporting enterocyte. The expected mechanism of iron deficiency in the differentiated PC12 cell would be for the IRP/IRE system to impair *Fpn* expression to ensure cellular survival. Accordingly, the reason for the upregulation of *Fpn* may be linked to the oxidative phosphorylation as heme production occurs in the mitochondria and gets incorporated in, e.g., oxygen transporting molecules like neuroglobin and myoglobin, both present in neurons. Heme synthesis demands the presence of Fe^{2+} as a cofactor, and cellular iron deficiency can therefore also lead to hypoxia [48]. Hypoxia-dependent mechanisms, e.g., DFO induced hypoxia, limit the IRP1-mediated translational repression of *Fpn* during iron deficiency in some cells such as enterocytes leading to increased *Fpn* expression despite iron deficiency [12, 49]. The results of the present study support this hypothesis. Suppression of IRP1 is mediated by HIF2α [50] and HIF2α increases the expression of *Fpn* in the intestines of mice during iron deficiency [41, 42]. The results of this study imply that the control of *Fpn* expression in differentiated PC12 cells seems to be at the transcriptional level, rather than translational level and seem to resemble the mechanism in enterocytes [41, 42] and macrophages [51].

The cells were also treated with a combination of DFO and hepcidin to investigate how the cells in an iron deficient state would react to hepcidin, which is actively secreted in response to neuroinflammation. These are two conflicting signals that might force the cells to choose between the two signals. The cells were subjected to low DFO concentrations that had not altered the cell viability as a single treatment and 2.3 μM hepcidin, which had resulted in 50% decrease in cell viability. Low concentrations of DFO are thereby able to reverse the toxicity of iron loading mediated by a high concentration of hepcidin in response to inflammation.

When looking at the expression level of *Fpn*, there is not seen any increase in expression in response to the combined treatment of DFO and hepcidin, which may be the reason why the viability of the cells did not decrease. Probably, the cells did not export excessive amounts of iron, as they did in response to DFO. This decrease in *Fpn* expression after treatment with DFO and hepcidin, mediated by hepcidin, will enhance the cell survival, as seen in Fig. 3e. Hepcidin treatment alone resulted in a downregulation of *Fpn* expression. This is in line with suggestions that iron signals dominate over inflammatory cues [52]. These results emphasize the importance of chelator therapy, as DFO was able to upregulate the *Fpn* expression, which should reduce any iron accumulation within the cell.

For the study of the possible epigenetic regulation of *Fpn* after treatment with DFO, hepcidin and a combination of these revealed that expression of *Hdac1* was not altered by 40 mM DFO nor 2.3 μM hepcidin as a single treatment but decreased

significantly after treatment with 40 mM DFO in combination with 2.3 μM hepcidin. The expression of *Tet1* was significantly increased after treatment with 40 mM DFO. TET proteins are belived to serve as stress sensors, modulating their activity in response to changes in, e.g., cellular iron levels and hypoxia [25, 53]. The upregulation which is seen after 40 mM DFO treatment may, therefore, be a direct response to the reduced iron level within the cell. The alterations in expression of multiple epigenetic regulators emphasize the need of better understanding of *Fpn* expression patterns in the CNS. The epigenetic alterations in iron-deficient PC12 cells treated with hepcidin may be an unknown mechanism that promotes cell survival.

Summary

The importance of understanding how iron transporters are regulated during conditions with changes in cellular iron availability is crucial. The results of the present study indicate that the regulation of the *Fpn* gene is more complex than originally comprehended. Hepcidin decreased the cell mortality in both iron-loaded and iron-deficient cells, and we believe that this putative protective effect is due to induction of the ferroportin mRNA expression in iron-loaded cells, and the reverse in iron-deficient cells (Fig. 7). We suggest that the *Fpn* gene could be regulated both transcriptionally and epigenetically.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

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