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www.elsevier.com

PII: S0891-5849(15)00590-0
DOI: <http://dx.doi.org/10.1016/j.freeradbiomed.2015.07.161>
Reference: FRB12575

To appear in: *Free Radical Biology and Medicine*

Received date: 9 June 2015
Revised date: 30 July 2015
Accepted date: 31 July 2015

Cite this article as: Fabiana Zolea, Flavia Biamonte, Patrizio Candeloro, Maddalena Di Sanzo, Anna Cozzi, Anna Di Vito, Barbara Quaresima, Nadia Lobello, Francesca Trecroci, Enzo Di Fabrizio, Sonia Levi, Giovanni Cuda and Francesco Costanzo, H ferritin silencing induces protein misfolding in K562 cells: A Raman analysis, *Free Radical Biology and Medicine*, <http://dx.doi.org/10.1016/j.freeradbiomed.2015.07.161>

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H ferritin silencing induces protein misfolding in K562 cells: a Raman analysis

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¹Abbreviations

2'-7'-DCF = 2'-7'-dichlorodihydrofluorescein

AβP = β-amiloid protein

BCA = bicinchoninic acid

CM-H₂CFDA = 5-(and-6)-carboxy-2', 7'dichlorofluorescein

DCDR = drop coating deposition Raman

EMSC = extended multiplicative signal correction

FHC = ferritin heavy chain

Fth = ferritin H gene

Ftl = ferritin L gene

FtMt = ferritin mitochondrial

H-bonds = hydrogen bonds

HBSS = Hanks balanced saline solution

H-π = π hydrogen

PD = Parkinson's disease

PFA = paraformaldehyde

PrP^c = prion protein

PrP^{sc} = PrP-scrapie

shFHC = short hairpin FHC

shRNA = short hairpin RNA

SiCaF₂ = calcium floride

Abstract

The redox state of the cell is involved in the regulation of many physiological functions as well as in the pathogenesis of several diseases, and is strictly dependent on the amount of iron in its catalytically active state. Alterations of iron homeostasis determine increased steady-state concentrations of Reactive Oxygen Species (ROS) that cause lipid peroxidation, DNA damage and altered protein folding. Ferritin keeps the intracellular iron in a non-toxic and readily available form and consequently plays a central role in iron and redox homeostasis. The protein is composed by 24 subunits of the H- and L-type, coded by two different genes, with structural and functional differences. The aim of this study was to shed light on the role of the single H ferritin subunit (FHC) in keeping the native correct protein three-dimensional structure. To this, we performed Raman spectroscopy on protein extracts from K562 cells subjected to FHC silencing. The results show a significant increase in the percentage of disordered structures content at a level comparable to that induced by H_2O_2 treatment in control cells. ROS inhibitor and iron chelator were able to revert protein misfolding. This integrated approach, involving Raman spectroscopy and targeted-gene silencing, indicates that an imbalance of the heavy-to-light chain ratio in the ferritin composition is able to induce severe but still reversible modifications in protein folding and uncovers new potential pathogenetic mechanisms associated to intracellular iron perturbation.

Keywords: ferritin heavy chain, oxidative stress, protein misfolding, Raman spectroscopy, K562.

Introduction

Iron is a fundamental cofactor for many biochemical activities in living cells, since it is involved, among others, in energy metabolism, DNA synthesis and oxygen transport. On the other hand, free iron is potentially toxic for the cells since it may catalyse, through Fenton chemistry, the generation of Reactive Oxygen Species (ROS) that damage the structure of DNA, lipids and proteins; all cells have therefore developed a coordinated system of iron-uptake and iron-storage molecules. Ferritin, a globular protein of 450 kDa, localized in eukaryotic cells in cytoplasm, nucleus and mitochondria, fulfils the task of preserving iron in a non-toxic and readily available form.

The cytoplasmic molecule is a heteropolymer of 24 subunits of heavy- and light-type arranged in a hollow spherical shell with a central cavity of about 80 Å of diameter that may store up to 4500 atoms of iron (1), while in the nucleus the shell is only composed by H-type subunits (2). The mitochondrial ferritin (FtMt) is a homopolymer of a single subunit with a 75% sequence identity to the H ferritin, encoded by an intronless gene, located on chromosome 5q23 in human (3).

The ferritin light chain (L; FLC) is coded by a gene (4) located on chromosome 19, and the ferritin heavy chain (H; FHC) is coded by a gene (5) that maps on chromosome 11. FHC has an enzymatic activity that specifically oxidizes ferrous iron Fe^{2+} to ferric iron Fe^{3+} , while FLC is mainly associated with iron nucleation and stabilization of assembled ferritin proteins (1).

An intriguing aspect of the cytoplasmic ferritin is represented by the fact that the ratio of the H to L subunits is not fixed, therefore resulting in a wide combination of H-L chains. It has been largely demonstrated that the structural differences in H/L ratio may depend on: (i) the type of tissue; (ii) the differentiation/proliferation state of the cell; (iii) the cell response to environmental signals; (iv) neoplastic transformation (6). Until now, the molecular basis underlying the existence of such a large variety of isoforms has not been completely clarified.

As already reported, the two ferritin subunits play different roles in relation to iron storage and detoxification within the cell. As a consequence, an imbalance in their ratio has the potential to alter the intracellular iron content and availability: ferritin molecules in cells containing high levels of iron, such as liver and spleen, tend to be L-rich, and may have a long-term storage function, whereas H-rich ferritins are more active in rapid iron uptake and release and lead to a cell phenotype more responsive to acute environmental changes (7).

In the present work, we combined the gene silencing strategy with Raman spectroscopy, a technique developed to analyze vibrational, rotational and other low-frequency modes, to detect the effects of H ferritin knock-down, via ROS production, on the overall proteins structure in K562 cells. The analysis was achieved by mathematical decomposition of Amide I Raman band, which is well known to be sensitive to protein secondary

structure. The data collected indicate that the H-ferritin silencing is accompanied by significant modifications of native protein folding, reversible when ROS are removed. The coupling of gene-specific modifications with a Raman analysis broadens the possibilities to study the biochemical pathways in which a given gene is involved.

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Materials and Methods

Cell culture, lentiviral transduction and protein extracts

K562 cells, a human cell line established from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises (ATCC number: CCL 243), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (Sigma Aldrich, St. Louis, MO) at 37°C in an atmosphere of humidified air containing 5% CO₂. Lentiviral preparations and transductions were performed as previously described (8) using a shRNA as control or a shFHC that targets the 196–210 region of the FHC mRNA. All the experiments were performed using a puromycin-selected pool of clones (1 µg/ml) (Sigma Aldrich, St. Louis, MO). Protein extractions were performed on K562 shRNA and shFHC treated or not with N-acetyl-cysteine 5mM for 30 min or with deferoxamine (desferal) 200 µM for 1 or 5 hrs (Sigma Aldrich, St. Louis, MO). Briefly, for total protein extractions, K562 cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (20 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, and 10 µmol/L leupeptin) (Sigma Aldrich, St. Louis, MO) and after removal of the cell debris by centrifugation (12,000×g, 30 min), the protein content was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

RNA extraction and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted with the TRizol RNA isolation system (Invitrogen, Carlsbad, CA). All the RNA samples were DNase-1 treated (Ambion, Austin, TX), and purity and integrity of the RNA was checked spectroscopically and by gel electrophoresis before use. One microgram of RNA from each sample was used for RT-PCR with a reverse transcriptase system kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using SYBR Green universal PCR master mix (Bio-Rad Laboratories, Hercules, CA) using FHC-specific primers: forward, 5'-cat caa ccg cca gat caa c-3'; reverse, 5'-gat ggc ttt cac ctg ctc at-3'. Each sample was normalized to its glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content. Relative gene expression for FHC mRNA was normalized to a calibrator that was chosen to be the basal condition (K562 shRNA). Results were calculated with the $\Delta\Delta C_t$ method and expressed as n-fold differences in FHC gene expression relative to GAPDH and calibrator and were determined as follows:

$$n\text{-fold} = 2^{-\Delta\Delta C_t} \quad (\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})$$

where the parameter C_t (threshold cycle) is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold. ΔC_t values of the sample and calibrator are determined by subtracting the

average Ct value of the transcript under investigation from the average Ct value of the GAPDH gene, for each sample.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) for 10 min at room temperature (RT) and permeabilized with 0,5% saponin (Sigma Aldrich, St. Louis, MO) in PBS for 30 min at room temperature. Samples were blocked for 30 min in blocking buffer (10% BSA in PBS) (Sigma Aldrich, St. Louis, MO). Primary antibody (ferritin heavy chain H-53, 1:100 Santa Cruz Biotechnology) was diluted in blocking buffer and incubated for 1hr at 37°C. To visualize binding, an appropriate secondary antibody (Alexa Fluor 488, 1:400, Invitrogen, Carlsbad, CA) diluted in blocking buffer was applied for 30 min at 37°C. Nuclear Dapi (1:1000, Invitrogen, Carlsbad, CA) was added for 20 min after secondary antibody incubation, prior to washing and mounting (ProLong Gold antifade Reagent, Molecular Probes, Eugene, OR). Images were collected using a Leica TCS SP2 confocal microscopy system (63X objective).

ROS detection

ROS were determined by incubating K562 shRNA and shFHC cells with the redox-sensitive probe 2'-7'-DCF (CM-H₂CFDA; Molecular Probes, Eugene, OR). Briefly, 1×10^6 K562 cells were plated in 96-well plates and incubated with Hanks balanced saline solution (HBSS), 10 mM glucose and 20 μ M DCF for 15 min at 37°C. After two cycle washes, cells were maintained in HBSS supplemented with 10 mM glucose. Fluorescence was revealed using the Victor3 Multilabel Counter (Perkin Elmer, Turku, FI) at 485 nm and 535 nm for excitation and emission, respectively. Results were normalized on protein concentration evaluated by the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Waltham, MA).

Raman measurements

Raman spectra on proteins extractions from the different cell lines have been recorded by means of an InVia Raman microscope from Renishaw (Renishaw Apply Innovation, New Mills, UK), equipped with an 832nm laser source and in backscattering configuration. All Raman measurements are recorded with a total laser power of about 10mW at the sample level and an accumulation time of 20 sec, in the spectral range from 800 up to 1800 cm^{-1} . Before of each measurement session, the Raman shift has been calibrated acquiring spectra on a Silicon sample and using as a reference the 520 cm^{-1} typical peak of Si. CaF₂ slides have been used as substrates for their negligible Raman signal. For the measurements the drop coating deposition Raman (DCDR) technique (9, 10) has been used. In this technique one drop (5 μ l) of protein solution is deposited on the CaF₂ substrate and measured after that part of the solvent is evaporated, thus increasing the initial concentration of the

protein solute, but before that the dried state occurs. For each proteins extraction, a minimum of 15 spectra are recorded randomly in the drop area, but not too far from the drop ring where the solute concentration is expected to be higher.

Raman data processing and fitting

All the data pre-processing of spectra is carried out by means of a LabView home-developed code for spectra analysis, available at the following web-address (11, 12). First the spectra are normalized to the total spectrum area to account for possible intensity variations due to laser power fluctuations and/or different optical path lengths through the drop. Subsequently a 1st order straight line has been subtracted to correct the slope of the spectra, and each group of spectra recorded on the same proteins extraction has undergone a physical EMSC (extended multiplicative signal correction) procedure (13, 11). Finally, all spectra of the same group are averaged and only the spectral region between 1515 and 1775 cm⁻¹ has been selected for further analysis of the proteins secondary structure. The other spectral regions are excluded due to the fact that some solvents used for proteins extraction have predominant signals below 1450 cm⁻¹, and their background subtraction could cause spectral artifacts. Instead all the solvents exhibit negligible signals above 1450 cm⁻¹, thus allowing for a clean analysis of the Amide I band (1640-1690 cm⁻¹).

As well known (14, 15), the Amide I band mainly arises from the C=O stretching vibration of proteins backbone, and it is very sensitive to secondary structure. This Raman band is composed of three main peaks, centered at 1650-1655 cm⁻¹ for alpha helix structures, at 1665-1670 cm⁻¹ for the beta sheet, and 1675-1685 cm⁻¹ for more disordered structures (15-17). Consequently, decomposition of the Amide I band through fitting procedures permits to separate the contribution from these three different peaks, thus providing clear information about the relative content of alpha, beta and disordered structures. Lorentzian-Gaussian curves are used to model Raman peaks in the fitting procedure, using a Levenberg-Marquardt nonlinear least-squares method. As widely reported in literature (14-17), the mixed Lorentzian-Gaussian curve is the best choice for fitting Raman peaks recorded on liquid samples, even with the DCDR technique where the solvent is partly evaporated. The starting parameters have been chosen so that three peaks are in the Amide I area, while two or three peaks are outside, corresponding to ring modes from aromatic side chains (in the range 1550-1615 cm⁻¹) (18). However no boundary conditions have been imposed on the final position of the modeling curves and center, width and intensity of each curve are free parameters during the calculation. The χ^2 value determined the choice of five- or six-components fit for the whole 1515-1775 cm⁻¹ spectral area, using six curves only if the χ^2 value significantly improved. In all the analyzed proteins extracts, we have found peaks positions for alpha helix, beta sheet and disordered structures lying in the same spectral ranges as reported in the literature (14-16). All the fitting procedures were performed with home-developed LabView codes.

Data analysis and statistical methods

All experiments were conducted at least three times, and the results were from representative experiments. Data were expressed as mean values $\pm$ SD. The Student's t-test was used to compare the groups. $P < 0.05$ was considered statistically significant.

Results

FHC silencing of K562 cells

The aim of this study was to investigate whether the silencing of the ferritin heavy subunit (FHC) might affect, *in vitro*, proper protein folding. The experiments were performed using a pool of K562 cell clones in which the expression of FHC has been stably knocked down by shRNA interference (K562 shFHC) and whose gene repertoire (8) and miRNome profile (19) have already been established. Before Raman analysis, the steady-state amounts of FHC mRNA and protein have been re-evaluated by Real Time RT-PCR, quantitative immunofluorescence and western blot. All together, the results shown in Fig.1, confirm that FHC transcript and protein levels are consistently reduced in the shFHC cells compared with the control K562 shRNA cells.

Raman analysis identifies protein misfolding in FHC-silenced K562 cells

Total protein extracts of K562 cells transfected with scrambled shRNA (K562 shRNA) or with a shRNA for H-ferritin (K562 shFHC) have been measured by Raman spectroscopy as detailed in Materials and Methods section. Amide I band fitting has been performed for evaluating the relative content of alpha helix, beta sheet and disordered structures (Fig. 2), plainly observable respectively at about 1655, 1670 and 1685 cm^{-1} (15). The fitting curves behavior (Panel A of Figure 2) shows that K562 shRNA has the alpha component as the most intense one, followed, in descending order, by the beta and the disordered components. Instead, we have a different appearance for the K562 shFHC cells: the most prominent curve is still the alpha helix, but the beta sheet contribution is smaller than the disordered one, with a reversed behavior compared to K562 shRNA. This observation is not too evident from the fitting curves of Panel A of Fig. 2, because the intensities of beta and disordered components are similar in the last cell line. But it is worthy to say that in Raman spectroscopy, the concentration of a biomolecule and/or of a particular biochemical structure is linearly related to the overall area below one peak, and not to the maximum intensity of the curve. We have then extracted from the fitting parameters the total area below each component. Normalizing the total area below the Amide I band to 100, we have reported the alpha, beta and disordered contributions in one histogram (Fig. 2 Panel B). By comparing the beta sheet and disordered structures percentages, it becomes unambiguous the behavior mentioned above and the difference between K562 shRNA and K562 shFHC cells.

FHC silencing induces oxidative stress

Next, we performed Raman measurements and fitting calculations on protein extracts from K562 shRNA cells treated with increasing H_2O_2 concentrations, i.e. with 1mM, 2mM and 4mM for 2hrs. The results are shown in Panel A of Figure 3 and summarized in one histogram where the percentage of each component of the Amide I band is reported (Panel B). For a better comparison, we also reported in panel B the histograms

relative to untreated K562 shRNA and K562 shFHC cells. It appears that the H_2O_2 treatment of control cells slightly affects the alpha helix content, while the most significant variations regard the beta sheet and the disordered structures. Furthermore, it appears that 2 and 4 mM H_2O_2 are able to induce a fully inverted ratio of beta sheet to disordered structures content to an extent comparable to that observed in the cells after FHC silencing.

We can better appreciate this behavior if we plot the percentage amount of beta sheet and disordered structures as a function of H_2O_2 concentration (Fig. 3 Panel C). It is now evident that the beta to disordered structure ratio reverses its value as the H_2O_2 concentration increases, with decreasing beta sheet content and increasing disordered structures. Again, to compare the effects of H_2O_2 treatment with those induced only by FHC-silencing, the peak area percentage of K562 shFHC cells is also reported.

These results strongly suggest that the altered folding observed after FHC silencing might be related to a state of increased oxidative stress. To confirm this hypothesis, we compared the ROS levels in K562 shRNA with those present in K562 shFHC cells. Quantitative analysis was performed using 2'-7'-DCF as redox-sensitive probe. The results, shown in Figure 4, confirm that FHC silencing is accompanied by an increased intracellular ROS level, since the fluorescence emission in FHC-silenced cells is at least two fold higher than that of the control cells either at 0, 30 and 60 min of incubation.

Protein misfolding in FHC-silenced cells is a reversible phenomenon

Raman analysis was also performed in K562 shFHC cells in which the H ferritin expression was restored by transient transfection of an expression vector containing the full length human FHC cDNA (K562 shFHC^{pc3FHC}) (Fig. 5 Panels A and B). The peak area percentage histograms relative to Amide I band fitting, reported in panel C of Figure 5, highlights the reversion of the beta sheet to disordered structures ratio in reconstituted FHC K562 cells.

We also performed Raman analysis in the silenced cells after treatment with N-acetylcysteine (NAC), an inhibitor of ROS production, or with the Fe chelating agent deferoxamine mesylate (desferal). To this, K562 shFHC cells were treated with 5 mM NAC for 30 min or with 200 μ M desferal for 1hr and 5 hrs. The peak area percentage histograms, relative to Amide I curve fitting, are reported in Figures 6 and 7. Looking at the beta sheet to disordered structure ratio, we can notice that, in control cells, the treatment with NAC does not reverse the behavior of the ratio, with the beta sheet always larger than the disordered contribution. Instead, in K562 shFHC cells, NAC treatment completely reversed the beta sheet/ disordered structure ratio, leading to relative contents of alpha helix, beta sheet and disordered structures fully comparable with those found in the control cells (Fig. 6).

Similar results are found with the Fe chelating agent desferal (Fig. 7). As observed with NAC, also desferal treatment alters the beta to random ratio in K562 shFHC cells, leading, after 5 hrs, to a condition totally comparable to that of the untreated shRNA cells. Notably, the alpha, beta and disordered contributions in the control cells are unaffected by this treatment.

Discussion

In the recent years, several reports showed that mammalian ferritin functions are not uniquely associated to the iron storage–detoxification processes (20). However, the main role of ferritin is to buffer the excess of iron in order to avoid iron-catalyzed Fenton reaction, which is detrimental for biological molecules. Thus, ferritin is recognized as an antioxidant protein, essential to maintain the cellular redox status (21). Its antioxidant role is important in various different conditions like inflammation (6), neurodegeneration (22), vascular protection (23) and apoptosis (24, 25). In cytosolic ferritin, this ability is due to the synergic action of the two ferritin chains, H and L, that cooperate to promote iron oxidation and nucleation inside the ferritin cavity (26, 27). Several previous studies using siRNAs to transiently repress H-ferritin (28), have confirmed the data obtained by up-regulating H-ferritin (29). They essentially showed that H-ferritin levels, by its ferroxidase activity, regulate primarily cell iron availability, and this has secondary effects on the resistance to the oxidative damage. In this work, we confirmed this ferritin primary role and uncovered an unexpected and interesting effect on protein folding in K562 cells caused by the silencing of the H-ferritin chain. We used Raman technique, in combination with different mathematical tools, that have been extensively applied for biochemical studies, such as lipidomics (30, 31), proteomics (32, 33), cellomics (34, 35) and tissue analysis (36, 37). Since it is sensitive to molecular vibrations, a typical Raman spectrum is very rich of information about the biochemical species of the sample. Utilizing this tool, upon silencing of the H chain, we were able to detect profound structural modifications of proteins that are not detectable in cells transfected with scrambled shRNA. The data show an imbalance of the relative amount of alpha/beta/disordered structure in the protein extracts, suggesting an increase in misfolded proteins content. In particular, the most evident effect is the decrease of beta secondary structure content in favor to an enhancement of disordered structure formation. Furthermore, these modifications, evident in the spectral region between 1515 and 1775 cm^{-1} , are statistically significant and reproducible over a series of experiments.

This phenomenon is comparable to cells treatment with strong oxidant molecules, such as H_2O_2 , and is reversible by antioxidant agent treatment. The oxidative effect is iron-dependent, as demonstrated by the partial recover of phenotype obtained by the addition of an iron chelator. This result indicates that the ferroxidase activity of the H-ferritin chain is essential not only to regulate cellular iron homeostasis but also to avoid partial protein misfolding.

The silencing of the H-ferritin is accompanied, in K562 cells, by a consistent increase of ROS levels, as determined by DCF assay. Even though we are aware that this method might present some intrinsic limitations due to artifactual amplification of fluorescence intensity, as reviewed by Kalyanaraman et al. (38), we believe that the magnitude of the phenomenon we observed overcomes any potential methodological caveat.

The production of ROS plays a physiological role within the cell; on the other hand, an increased ROS steady-

state concentration might be dangerous since it induces oxidation of the protein backbone with consequent fragmentation, protein-protein cross-linked derivative formation, oxidation of amino acid side chains. The vast majority of these ROS induced modifications are of a covalent-type. Our results indicate that the alteration in alpha/beta/disordered structure contribution in FHC-silenced cells is a reversible phenomenon, in the presence of appropriate iron or ROS chelating agents. Consequently, we hypothesize that the silencing of H ferritin might induce, along with formation/disruption of S-S bonds, preferentially the formation of not-bonded interactions such as H-bonds, H- π and stacking forces.

The alteration of beta strand conformation is suggestive of protein aggregate formation, which is the principal pathological event in several neurodegenerative conditions. The formation of β -sheets containing amyloid fibrils from disease-associated proteins, such as the β -amyloid protein (A β P), the prion protein, α -synuclein, and polyglutamine, are implicated in the pathogenesis of Alzheimer's disease, prion diseases, Parkinson's disease (PD) and Huntington disease, respectively. One of the best described examples of protein conformational changes induced by iron is the prion protein (PrP^c) that undergoes a conformational change to PrP^{sc} (PrP^{sc}) (39, 40). Furthermore, the loss of normal function of PrP^c due to aggregation to the PrP^{sc} form induces iron dyshomeostasis in prion disease affected brains, resulting in disease associated neurotoxicity (34). Thus, the iron imbalance caused by the reduced H-ferroxidase activity might have a profound effect on the protein structure that may triggers fatal events in the cell. This clearly demonstrates that maintenance of a specific H/L ratio of ferritin is an element of a basic cellular homeostatic mechanism. Indeed, the inactivation of ferritin H gene (*Fth*) in mice is lethal during embryonic development (41), whereas knock-out of the L gene (*Ftl*) is compatible with life, even if the *Ftl*^{-/-} showed a significant decrease in the percentage of newborn mice (42). The *Ftl*^{-/-} mice are characterized by systemic and brain iron dyshomeostasis (42). This L-deficient phenotype was previously described also in human, where homozygous loss of function mutation in the L-ferritin gene is associated with generalized seizure and atypical restless leg syndrome (43). From these overall data, it appears that the existence of H ferritin homopolymers is compatible with life, it can rescue the loss of the L subunit and maintains its capacity to sequester iron in vivo. On the contrary, the L ferritin homopolymers, due to the lacking of ferroxidase ability, is not functional in vivo. In fact, no mutation of *FTH* gene has been described in human, while different human *FTL* gene mutations have been identified (1) and, depending on where the mutations are located, they cause two types of dominant disorders: the hereditary hyperferritinemia with cataract (44) and the neuroferritinopathy (45).

In conclusion, our work confirms that an optimal H/L ratio is essential for ferritin functionality and highlights a previously unrecognized consequence of the presence of intracellular free iron that might be useful to clarify pathogenetic mechanisms associated to iron imbalance in related disorders. Moreover, the innovative use of Raman spectroscopy to analyze protein misfolding in FHC-silenced K562 cells appears as a promising

straightforward and reliable approach to detect biochemical and structural intracellular changes.

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Conclusions

Previous studies have demonstrated that the two ferritin subunits act in a different way in relation to iron storage and detoxification within the cell. A puzzling aspect of cytoplasmic ferritin is represented by the high variability in the H-to-L subunits ratio, resulting in a wide combination of H-L chains. The innovation of the present study relies on the use of an integrated approach involving Raman spectroscopy and targeted-gene silencing, to uncover the role of FHC in controlling the redox state in cells subjected to oxidative injury.

Acknowledgements

This study was supported by the Italian Ministry of Education, University and Research (PON01_02834 – Prometeo grant to GC).

Author Disclosure Statement

No competing financial interests exist.

Accepted manuscript

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Figure Captions

Figure 1. **FHC silencing of K562 cells.** **A.** Real-time PCR analysis of FHC mRNA expression performed on total RNA from K562 shRNA and K562 shFHC. Results are representative of three different experiments (*, $p < 0,001$ compared with K562 shRNA). **B.** Western Blot analysis for FHC expression was performed on 50 μg of total protein extract from K562 shRNA and K562 shFHC cells. γ -Tubulin was used as loading control. Results are representative of three different experiments (*, $p < 0,001$ compared with K562 shRNA). **C.** K562 shRNA and shFHC were fixed and incubated with polyclonal anti-FHC antibody (1:100) followed by incubation with an appropriate secondary antibody. Nuclei were visualized by DAPI staining. Images were collected using a Leica TCS SP2 confocal microscopy system (63X).

Figure 2. **Raman analysis: Amide I band fitting in K562 FHC-silenced and control cells.** Total proteins were extracted from K562 shRNA and K562 shFHC and Raman spectroscopy was used to evaluate protein misfolding. **A.** Curve fitting of Amide I band in K562 shRNA and K562 shFHC cells. Amide I band is composed of three main peaks, centered at $1650\text{--}1655\text{ cm}^{-1}$ for alpha-helix structures, at $1665\text{--}1670\text{ cm}^{-1}$ for the beta-sheet, and $1680\text{--}1685\text{ cm}^{-1}$ for disordered structures. In the graphs, the gray line is the fitting of the Raman experimental curve (black solid line); the three solid lines indicated as alpha, beta and disordered are the Amide I components corresponding to these kind of secondary structures. The other fitting components (dotted lines) outside the Amide I band correspond to ring modes from aromatic side chains and are introduced for numerical convergence of the fitting. In order to avoid overfitting, the number of these components have been kept as low as possible. **B.** The total area below alpha, beta or disordered-structure peak was extracted from the fitting parameters. Normalizing the total area below the Amide I band to 100, we reported the alpha, beta and disordered contributions in one histogram (*, $p < 0,001$ compared with K562 shRNA β -sheet peak area, **, $p < 0,001$ compared with K562 shRNA disordered structure peak area).

Figure 3. **H_2O_2 treatment induces protein misfolding in K562 shRNA cells.** Total proteins were extracted from K562 shRNA untreated or treated with H_2O_2 1, 2 and 4 mM and Raman spectroscopy was used to evaluate protein misfolding. **A.** Using the drop coating deposition Raman (DCDR) technique, spectroscopic measurements (black solid line) were reported in the Amide I range along with the best fitting curves (gray lines), highlighting alpha, beta and disordered components (see also caption of Figure 2). **B.** The total area below alpha, beta or disordered structure-related peaks was extracted from the fitting parameters and reported in histograms (*, $p < 0,001$ compared with K562 shRNA β -sheet peak area, **, $p < 0,001$ compared with K562 shRNA disordered structures peak area). **C.** Percentage amount of beta sheet and disordered structures plotted as

a function of H_2O_2 concentration (beta and disordered contributions of FHC-silenced cells were reported for comparison).

Figure 4. FHC silencing induces ROS production in K562 cells. K562 shRNA and shFHC cells (10^6) were incubated for 15 min with 20 μ M of 2'-7'-DCF and washed with HBSS solution. Fluorescence was measured at 485 nm and 535 nm after 0, 30 and 60 min (**A, B, C**). Results are the mean of three independent experiments in octuplicate (*, $p \leq 0.05$ compared with K562 shRNA, **, $p \leq 0.001$ compared with K562 shRNA).

Figure 5. Raman analysis of K562 silenced cells after FHC reconstitution. **A.** Real-time PCR analysis of FHC mRNA expression performed on total RNA from K562 silenced (shFHC) and reconstituted (K562 shFHC^{pc3FHC}). Results are representative of two different experiments (*, $p < 0.05$ compared with K562 shFHC). **B.** Western Blot analysis for FHC was performed on 50 μ g of total protein extract from K562 shFHC and K562 shFHC^{pc3FHC} cells. γ -Tubulin was used as loading control. Results are representative of two different experiments (*, $p < 0.05$ compared with K562 shFHC). **C.** K562 shFHC and shFHC^{pc3FHC} were fixed and incubated with polyclonal anti-FHC antibody (1:100) followed by incubation with an appropriate secondary antibody. Nuclei were visualized by DAPI staining. Images were collected using a Leica TCS SP2 confocal microscopy system (63X). **D.** Protein extracts from K562 shFHC and K562 shFHC^{pc3FHC} cells were analyzed via Raman spectroscopy (see also caption of Figure 2); the fitting parameters were used to produce the histogram reporting the beta-sheet and disordered-structure contributions ($^\circ$, $p < 0.001$ compared with K562 shFHC β -sheet peak area, $^{\circ\circ}$, $p < 0.001$ compared with K562 shFHC disordered structure peak area).

Figure 6. Raman analysis: Amide I band fitting in NAC treated cells. Total proteins were extracted from K562 shRNA or shFHC not treated or treated with NAC 5mM for 30 minutes. Raman spectroscopy and fitting procedures were used to evaluate the alpha-helix, beta-sheets and disordered-structures contributions to Amide I band. (*, $p < 0.001$ compared with K562 shRNA β -sheet peak area, $^\circ$, $p < 0.001$ compared with K562 shFHC β -sheet peak area, $^{\circ\circ}$, $p < 0.001$ compared with K562 disordered structure peak area).

Figure 7. Raman analysis: Amide I band fitting in desferal treated cells. Total proteins were extracted from K562 shRNA or shFHC not treated or treated with desferal 200 μ M for 1 or 5 hours. The total area below alpha, beta or disordered structures-related peak was extracted from the fitting parameters. Normalizing the total area below the Amide I band to 100, we have reported the alpha, beta and disordered structures contributions in one histogram (*, $p < 0.001$ compared with K562 shRNA β -sheet peak area, $^\circ$, $p < 0.001$ compared with K562 shFHC β -sheet peak area, $^{\circ\circ}$, $p < 0.001$ compared with K562 shFHC disordered structure peak area).

Highlights

1. Raman analysis has been used to study protein conformation in FHC-silenced cells.
2. In K562 cells FHC silencing induces via ROS formation a severe protein misfolding.
3. The observed protein misfolding is a reversible phenomenon.
4. This integrated approach allows the study of specific biochemical pathways.

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