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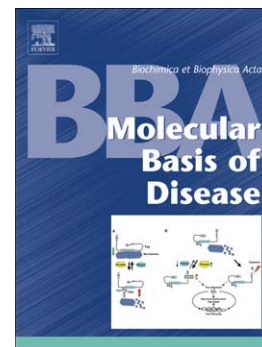
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**The effects of frataxin silencing in HeLa cells are rescued by the expression of human mitochondrial ferritin.**

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**Short Title:** Frataxin silencing and mitochondrial ferritin in HeLa cells.

**Keywords:** Iron, frataxin, ferritin, mitochondrial iron, aconitase

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Abbreviations: ISC, iron sulfur cluster; FRDA: Friedreich's ataxia; Dox: doxycycline; MtF, mitochondrial ferritin; Mt53: HeLa cell clone expressing the MtF; FAC, ferric ammonium citrate; GAPDH, glyceradehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; SDH, succinate dehydrogenase; SOD2, superoxide dismutase 2. IRE: Iron Regulatory Element; IRP1: iron responsive protein-1

**SUMMARY.**

Frataxin is a ubiquitous mitochondrial iron-binding protein involved in the biosynthesis of Fe/S clusters and heme. Its deficiency causes Friedreich's ataxia, a severe neurodegenerative disease.

Mitochondrial ferritin is another major iron binding protein, abundant in the testis and in sideroblasts from patients with sideroblastic anaemia. We previously showed that its expression rescued the defects caused by frataxin deficiency in the yeast. To verify if this occurs also in mammals, we silenced frataxin in HeLa cells. This caused a reduction of growth, inhibition of the activity of aconitase and superoxide dismutase-2 and reduction of cytosolic ferritins without alteration of mitochondrial iron content. None of these effects were evident when silencing was done in cells expressing mitochondrial ferritin. These data indicate that frataxin has some roles in controlling the balance between different mitochondrial iron pools that are partially in common with those of mitochondrial ferritin. (143 words)

ACCEPTED MANUSCRIPT

## INTRODUCTION

Iron is essential for many functions in the cell, but is also potentially toxic for its capacity to catalyse free radical production. Therefore iron homeostasis must be tightly regulated, particularly in the mitochondria, where the synthesis of heme and Fe/S clusters (ISC) occurs. The mechanisms of this regulation in the mitochondrion are unclear, and they must involve proteins for iron transport and storage. Mitochondrial ferritin (MtF) is probably one of them, since it has ferroxidase activity and can sequester mitochondrial iron and reduce its toxicity [1-4]. Another candidate is frataxin, a mitochondrial iron binding protein with homologues in eukaryotes and prokaryotes.

In yeast, the deficiency of the frataxin homologue Yfh1p causes mitochondrial iron accumulation, increased sensitivity to oxidative damage, depletion of mitochondrial DNA, decreased activities of ISC enzymes and impaired respiration [5]. These may possibly be related to an inhibition of Mn-superoxide dismutase 2 (SOD2) activity [6]. In the yeast Yfh1p seems to act as a mitochondrial iron chaperone, which interacts with members of ISC biosynthesis like Isu and Nfs1 [7], with ferrochelatase [8], with succinate dehydrogenase (SDH) and the putative electron transfer flavoprotein complex homologue Etf [9]. Alternatively it may act as an iron storage protein [10]. Frataxin has been extensively studied because its deficiency is the cause of Friedreich's ataxia (FRDA), the most common genetic ataxia [11]. However, the role of frataxin in mammalian cells is more complex and controversial than in yeast. Mitochondrial iron accumulation is not a common finding in frataxin deficient mammalian cells [12], and it occurs in the heart of mouse models only in the late stage of the disorder [13]. High sensitivity to oxidative stress has been observed in frataxin deficient mammalian cells [14], but not in FRDA mice models [15]. Frataxin deficiency reduces the activity of cytosolic ISC proteins [16,17], inducing a phenotype similar to that caused by the silencing of other proteins of ISC biosynthesis, such as the cysteine desulfurase Nfs1 [18], and the Fe/S scaffold protein Isc-u [19]. A direct role of frataxin in ISC biosynthesis was indicated by its physical interaction with members of the ISC machinery, including ISD11 [20]. A reduction of ISC biosynthesis is expected to activate the RNA-binding activity of the bifunctional protein cytosolic aconitase/Iron Regulatory protein-1 and thus repress ferritins and other iron binding proteins. In addition, cells from FRDA patients showed a downregulation of genes involved in sulphur amino acid metabolism [21] and frataxin deficiency alters heme pathway transcripts and decreases mitochondrial heme metabolites in mammalian cells [22-24]. Conversely, frataxin is downregulated during erythroid differentiation [25].

MtF is highly expressed in a limited number of cell types with high metabolic activity [26]. Its overexpression causes modifications of cytosolic and mitochondrial iron homeostasis [1-4] and may modify heme biosynthesis. In fact it is abnormally upregulated in the bone marrow cells of

sideroblastic anemia, characterized by mitochondrial iron overload and defects in heme synthesis [27]. We previously demonstrated the capacity of human MtF to rescue most of the defects due to the Yfh1p deficiency in yeast [28]. In the present work we report that human MtF is likewise able to recover the most evident effects of frataxin deficiency in human cells. Our data further support a role of frataxin in the cellular iron homeostasis and suggest some common functions between MtF and frataxin.

## MATERIALS AND METHODS

**siRNAs and transient transfections.** The ds-siRNAs specific for frataxin were first produced by the method described in ref [29]. The most effective and specific (F3: 5'-GAAAGACUAGCAGAGGAAAC-3', nt 286-304) was also prepared by chemical synthesis (Qiagen-Xeragon, Germantown, MD). HeLa Tet-off cells (Clontech Laboratories, Mountain View, CA) were cultured in Dulbecco's Modified Eagle Medium (D-MEM, Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% tetracycline-free fetal bovine serum (FBS, Clontech Laboratories), 1 mM L-glutamine, 40 µg/ml gentamicin and 150 µg/ml hygromycin. The HeLa cell clone expressing MtF, named Mt53 clone [1], was cultured in DMEM supplemented with 10% tetracycline-free FBS, 1 mM L-glutamine, 40 µg/ml gentamicin, 150 µg/ml hygromycin, 100 µg/ml geneticin without doxycycline (Dox) to induce the expression of human MtF. Transient transfections were performed with 100 pmol of siRNAs derived by *in vitro* transcription or 50 pmol of synthetic ones in 12 well plates with 2 µl Oligofectamine (Invitrogen). To extend frataxin silencing up to 5 days, we performed two sequential transfections at day 0 and again at day 2 and cells were harvested at day 5. Transfection efficiency was monitored by the use of a fluorescently labelled unrelated siRNA (Qiagen-Xeragon) and found to be about 90%. All experiments used Oligofectamine alone (mock) and unrelated siRNA as negative controls.

**Stable cell lines.** The vector for inducible frataxin silencing was constructed in our laboratory. It was derived from pEGFP vector (Clontech Laboratories) that constitutively expresses EGFP under the control of the CMV promoter, and it contained a short hairpin RNA (sh-RNA) under the control of a human U6 promoter mutagenized for inducible tetracycline-dependent expression [30]. The F3 sequence was cloned as sh-RNA downstream of this promoter. T-Rex<sup>TM</sup> HeLa cell line stably expressing the Tet repressor from the pcDNA6/TR plasmid (Invitrogen,) were maintained in Minimum Essential Medium with Earle's Salts (E-MEM, Gibco, Invitrogen) containing 1 mM L-glutamine, 40 µg/ml gentamicin, 5 µg/ml blasticidin and 10% tetracyclin-free FBS, accordingly to the manufacturer's instructions. Vector transfection was performed with 5 µl Lipofectamine

(Invitrogen) per  $\mu\text{g}$  plasmid DNA. Stable transfectants cells were selected in the medium containing  $400\mu\text{g/ml}$  geneticin 48 h after transfection. Resistant clones were subcultured and analysed for EGFP expression by FACS analysis. The clones with the strongest expression of EGFP were analysed for frataxin inducible silencing. Dox induction of the shRNA in these cells was performed at different times (0-15 days) and concentrations ( $0.5\text{-}10\ \mu\text{g/ml}$ ) to optimise silencing. Following optimisation studies, the cells were induced with  $7.5\ \mu\text{g/ml}$  Dox and harvested at day 5.

**Biochemical and immunological methods.** L-ferritin levels were determined with a commercial serum ferritin assay (Abbott Laboratories, Abbott Park, IL). H-ferritin levels were evaluated in total cellular extracts by an ELISA assay using the monoclonal antibody rH02 calibrated on the recombinant homopolymer [31]. In immunoblot experiments, equal amounts of proteins were separated on SDS-PAGE and transferred to a PVDF membrane. The PVDF filters were incubated at  $4^\circ\text{C}$  for 16 h with specific antibodies: rabbit anti-frataxin polyclonal antibody (1:2000), rabbit anti-SOD2 polyclonal antibody (1:4000, Upstate, Bilerica, NA), a mouse anti-MtF polyclonal antibody produced in our laboratory, a rabbit anti-poly (adenosine diphosphate-ribose) polymerase (anti-PARP) p85 fragment (1:700, Promega, Madison, WI), a rabbit anti- $\beta$ -actin monoclonal antibody (1:1000, Sigma) and anti-voltage-dependent anion channel (VDAC)1/porin antibody (Sigma). The filters were then washed and further incubated for 1 hour with secondary peroxidase-labelled antibodies, anti-rabbit Ig (Pierce) or anti anti-mouse Ig (Dako, Denmark).

Immunocomplexes were detected by a chemiluminescence detection kit (ECL Advance Western Blotting Detection kit, Amersham Biosciences, Uppsala, Sweden) with Kodak Image Station 440 CF (Kodak, Rochester, New York). The densitometric analysis was performed with the Kodak 1D 3.6 program. For the determination of the activity of aconitase, citrate synthase, SDH and SOD2 we followed previously described methods [32-35]. When mitochondrial fractionation was required, cells were washed with ice-cold PBS and resuspended with mitochondrial isolation buffer HMS ( $220\ \text{mM}$  D-mannitol,  $70\ \text{mM}$  sucrose,  $2\ \text{mM}$  HEPES pH 7.4);  $1.75\ \text{mg/ml}$  digitonin was added and the cell suspension was incubated for 5 min on ice. The lysed cells were centrifuged at  $11,000\ \text{rpm}$  for 6 min to collect the crude mitochondrial pellet and the post-mitochondrial fraction. The mitochondrial-enriched pellet was then washed with cold HMS and resuspended in lysis buffer. Absolute enzyme activities were expressed as  $\text{mU/mg}$  of protein. For all assays, protein concentration was determined by the BCA method (Pierce, Rockford, IL), calibrated on bovine serum albumin (BSA). Free protoporphyrin content was evaluated by flow cytometry as in [35].

**Real Time quantitative RT-PCR.** Total RNA was extracted with RNAzolB (Tel-Test Inc., Friendswood, Texas, U.S.A.) according to the manufacturer's instructions and quantified

spectrophotometrically. cDNA synthesis was performed with 1 µg of DNase-treated RNA using ImProm-II reverse transcriptase (Promega) and oligodT as primer. Real-time PCR was carried out with PCR primers specific for frataxin mRNA (sense, 5'-GTGGAGATCTAGGAACCTATG-3', and antisense 5'-TTAAGGCTTTAGTGAGCTCTG-3') and a specific fluorogenic probe (5'-TCCAGTCATAACGCTTAGGTCCAC-3'). The sense and antisense primers were designed to be complementary to regions on two different exons and the probe to span intron-exon boundaries to avoid amplification and recognition of genomic DNA. The mRNA encoding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The PCR reaction was carried out using the GeneAmp™ 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Results were normalized to GAPDH levels using the  $2^{-\Delta\Delta Ct}$  method. A value of 1 was given to control samples chosen as calibrators and the sample values expressed the *n*-fold reduction of frataxin mRNA with respect to the calibrator (normalized dose = nd).

**Cell proliferation assays.** Cell proliferation was assessed both by trypan blue exclusion assay and by vital dye reduction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma). Briefly, parental and stably transfected cell lines were seeded in T25 flasks ( $10^5$  cells per flask) with Dox and cultured for 5 days. At day 5, cells were trypsinised and viable cells were counted with trypan blue. For MTT assay, cells were seeded in 24-well plates ( $5 \times 10^4$  cells per well) with Dox and cultured for 5 days. At day 5, the medium was removed and replaced with 0.5 ml fresh medium without FBS and with 50 µl MTT solution (5 mg/ml in PBS) for 2-3 h. The solution was removed and the formazan blue crystals were dissolved with 1 ml DMSO. The absorbance was read at 570 nm for the quantification of living metabolically active cells. For transient transfection experiments, after two rounds of transfection, cells were trypsinised at day 5 and counted with trypan blue or alternatively were treated for MTT assay as above. MTT assay was also used to assess sensitivity to oxidative stress: at day 5 post-induction for stable line and post-transfection for transient silencing, the cells were washed and incubated in serum-free medium for 1h. Then cells were treated with H<sub>2</sub>O<sub>2</sub> (0-1200 µM) for 2 h in serum-free medium, plates were washed twice with PBS and analysed by incubation with MTT as above. Cells were also cultured with 0.25 g/L glucose for 3 days or with 1.0 mM ferric ammonium citrate (FAC) for 18 h prior to assess sensitivity to H<sub>2</sub>O<sub>2</sub> treatment. For the determination of cell apoptosis,  $1 \times 10^4$  cells were plated in 96-well culture black plates (Corning, Corning, NY) and lysed with the same volume of Caspase-Glo 3 Assay (Promega) and the luminescence was analysed. The genomic DNA was extracted according to the protocol for Wizard Genomic DNA Purification Kit (Promega) and samples were analysed on a 1.5% agarose gel with ethidium bromide staining.



**[<sup>55</sup>Fe] uptake.** Stable cell lines were grown for 4 days with Dox and then trypsinised, counted and seeded ( $2.5 \times 10^4$ /well) in 12 well plates with the antibiotic. The day after, cells were washed twice with PBS and grown for an additional day with 0.5% tetracycline-free FBS, 0.5% BSA, with or without Dox and in the presence of 74 kBq/ml [<sup>55</sup>Fe] FAC and 200  $\mu$ M ascorbic acid. Cells were then washed 3 times with PBS and harvested. After preparation of extracts, 10  $\mu$ l of each sample were mixed with 1 ml scintillation liquid (Ready Safe, Beckman Coulter, Fullerton, CA) and counted in a liquid scintillation counter. Data were normalized with protein content. Equal amounts of protein extracts (10  $\mu$ g) were separated on 7% non-denaturing PAGE and the dried gels were exposed to autoradiography. The intensity of ferritin bands was quantified by densitometry in the linear range.

**Statistical analysis.** Statistical analysis was performed using Student's *t* test for unpaired data. Values were considered significant when  $p < 0.05$ .

## RESULTS

### **Frataxin silencing in HeLa cells.**

To determine the most evident effects of frataxin deficiency, we initially produced by *in vitro* transcription 4 different ds-siRNAs. All of them induced significant silencing of frataxin mRNA (down to 25%) and protein (down to 10%) in HeLa cells at 72 h post-transfection (not shown). One of them, coded F3, was produced by chemical synthesis and used at 25-100 pmol per well for maximal inhibition of frataxin (Fig. 1). Frataxin protein level remained below 25% at 72 h post-transfection, a level below that found in FRDA patients. This caused reduction of cell proliferation, which became more evident after a second sequential transfection to extend silencing up to 5 days (see below). For a better characterization of the phenotype we chose to establish HeLa clones with inducible expression of sh-RNA. To this aim we constructed a vector in which the human U6 promoter was mutagenised for being controlled by tetracycline and located upstream of a sh-RNA for the F3 sequence. This vector, named sh-RNA F3, expressed also EGFP under the control of CMV promoter to facilitate cell selection. It was used to transfect T-Rex™ HeLa cells expressing the Tet repressor, and the clones were selected initially with geneticin and for EGFP expression and then analysed for frataxin level after incubation with Dox. In different clones Dox caused a dose dependent suppression of frataxin at concentrations between 1 and 10 µg/ml (not shown). One clone named F3-clone was chosen and further characterized. Its frataxin mRNA was down to about 20% after 5 days of growth in 5 µg/ml Dox, and below 5% in 7.5 µg/ml Dox (Fig. 2A). Frataxin protein was essentially undetectable in 7.5 µg/ml Dox at day 5 and remained so until day 11 (Fig. 2 B). The following experiments were done in 7.5 µg/ml Dox and the cells analysed at day 5.

### **Frataxin-deficient HeLa cells show a decrease of cell proliferation rate.**

In the experiments we used as a control the parental T-Rex™ HeLa cells treated with the same Dox concentration. We initially observed that F3-clone always took a longer time to reach confluence, then to quantify proliferation we analysed the number of viable cells after 5 days of growth. Unviable, trypan blue positive cells were a minor proportion (<5%) in the F3 and parental clone. The total viable cells in the F3-clone were about 40% of the control (Fig. 3A). Similar results were obtained when cell proliferation was evaluated by MTT assay or by protein content (not shown). We observed that the F3-clone had an altered morphology, with roundish and grainy cells that easily detached from the plate, suggesting a general sufferance not seen in the parental cells in the same conditions, but analysis of PARP fragmentation, caspase-3 activation and DNA laddering did not reveal evident signs of apoptosis (not shown).

**Frataxin deficiency reduces the activity of mitochondrial enzymes**

We analysed two ISC containing enzymes known to be inhibited by frataxin deficiency [13, 16]. In the F3-clone the activity of succinate dehydrogenase (SDH) decreased to about 60% of the control (Fig. 3B), that of total aconitase to about 45% (Fig. 3C), with a similar decrease in the mitochondrial (~51%, Fig. 3D) and cytosolic fractions (~56%, not shown). Also citrate synthase (CS) activity slightly decreased in F3 clone without a parallel decrease in porin protein level, as detected by Western Blot (Fig. 3E). Since porin is an index of mitochondrial content, the result suggests that frataxin depletion has a negative effect also on non-ISC mitochondrial enzymes. Treatments for 18 h with H<sub>2</sub>O<sub>2</sub> concentrations up to 1.2 mM caused a similar reduction of cell viability in the F3 and the parental clone, even after iron supplementation (1 mM ferric ammonium sulphate, FAC, for 18 h), but the reduction of glucose content of the medium from 1 g/L to 0.25 g/L caused a minor increase of H<sub>2</sub>O<sub>2</sub> toxicity in the F3-clone compared to the control (not shown), possibly related to enhanced oxidative phosphorylation. A decrease of the activity of the mitochondrial superoxide dismutase-2 (SOD2) has been reported in cellular [14] and animal FRDA models [15]. Total homogenates of the F3 clone were separated on non-denaturing PAGE and SOD2 activity was developed. This was largely decreased (Fig. 3F), but SOD2 protein level detected by immunoblot was unchanged (Fig. 3F), indicating enzyme inactivation.

**Modification of indices of iron status.**

To study intracellular iron distribution we incubated the cells with [<sup>55</sup>Fe] FAC, following the recent observation that also non-transferrin bound iron is readily delivered to mitochondria in cultured cells [1,36]. The mitochondrial fraction was separated, its specific radioactivity and that of the total homogenates were counted and expressed as their ratio. They were analogous in the F3 clone and parental cells (Fig. 4A) indicating that frataxin deficiency did not cause mitochondrial iron accumulation. Autoradiography of total cell homogenates run on non-denaturing PAGE showed a single band corresponding to cytosolic ferritin iron, whose intensity was lower in F3-clone than in control cells (Fig. 4B). ELISA assays confirmed that the level of cytosolic ferritin protein was lower in F3 clone, both the L-ferritin (Fig. 4C) and the H-ferritin (not shown). Thus, ferritin and iron homeostasis was altered in frataxin deficient cells, possibly as a consequence of the increased RNA-binding activity of c-Aconitase/Irp1.

**Mitochondrial ferritin rescues defects of frataxin depleted HeLa cells.**

Mitochondrial ferritin (MtF) has a cytoprotective role in mammalian cells [2] and its expression rescued the defects associated with frataxin deficiency in yeast [28]. Therefore it was of interest to evaluate if the same occurs also in mammalian cells. Initial experiments showed that transfection of the F3-clone with MtF cDNA was difficult, probably because Dox interferes with transfection or reduces the resistance to the stress of transfection. Thus, we chose to transiently transfect with F3-siRNA the HeLa cell line that expresses human MtF in the absence of Dox (clone Mt53) [1]. We performed two sequential transfections with F3-siRNA to silence frataxin for 5 days and have a more evident phenotype. Frataxin protein level was comparable in the parental and Mt53 clone and similarly decreased after silencing (fig 5B). The silencing in control HeLa cells caused a significant decrease in cell proliferation (35%, Fig. 5A), in cytosolic L ferritin level (20%, Fig. 5B), in total (20%, Fig. 5C) and mitochondrial aconitase activity (40%, Fig. 5D). The effects were similar although less evident than those detected with stable silencing described above. In contrast, neither of these parameters was modified by the transient frataxin silencing in the Mt53 clone overexpressing MtF (Fig. 5A-D). Also the activity of citrate synthase was slightly reduced by F3 transfection in HeLa cells but not in the Mt53 clone, while the porin protein levels were not affected (not shown). The activity of SOD2 was significantly reduced in the silenced control cells in conditions of low glucose content (0.25 g/L) to force aerobic metabolism, but not in the Mt53 clone grown in the same conditions (Fig. 5E). An alteration of heme synthesis in HeLa cells may result in a modification of free protoporphyrin level, which can be evaluated by its intrinsic fluorescence. The basal level of fluorescence in the control HeLa cells was low, but increased of about 40% after frataxin silencing. Interestingly this did not occur in Mt53 clone, where frataxin silencing did not have any evident effect on protoporphyrin fluorescence (fig 5F). Altogether our results strongly suggest that MtF expression in HeLa cells inhibits all the major effects associated with frataxin silencing.

**DISCUSSION.****Characterization of frataxin deficient clone.**

Present work shows that transient and stable frataxin silencing in HeLa cells causes a significant reduction of SDH and cytosolic and mitochondrial aconitase activity, of cytosolic ferritin and of cell proliferation, in agreement with previous data [16]. Similar effects in mammalian cells were shown to be caused by the silencing of other mitochondrial proteins involved in the ISC biogenesis, like the ISC transporter ABCB7 [35], the cysteine desulphurase m-Nfs1 [18] and the protein scaffold Isc-U [19]. This further supports that frataxin is involved in ISC biosynthesis in mammals, and consequently has a role in cellular iron homeostasis. For example, the inhibition of cytosolic ISC biosynthesis reduces the cytosolic aconitase/Irp1 activity and at the same time increases its RNA binding activity, resulting in the suppression of some IRE containing transcripts, including the ferritin ones. Although the downregulation of ferritin has not been observed before, the activation of IRPs RNA binding activity was described in frataxin deficient mammalian cells [16], in *Drosophila* cells [37] and also in Nfs1 and IscU silenced cells [19,38].

To evaluate mitochondrial iron content, the cells were incubated with radioactive FAC, which was readily incorporated in mitochondria of cells overexpressing MtF [1] or deficient in the transporter ABCB7 [35]. In addition, non transferrin-bound iron was readily taken up by mitochondria in cultured myocytic cells [36], confirming that this form of iron supplementation is regulated by mitochondria similarly to the more physiological transferrin iron. Using this approach we could not detect an increase of mitochondrial iron in the silenced cells. The finding is in agreement with other studies [12,16] and with the finding that in mouse models mitochondrial iron accumulation occurs late in the disease [13]. This stresses the differences in iron homeostasis between mammalian and yeast cells, where defects in frataxin invariably cause mitochondrial iron deposition [5,8]. The lack of evident mitochondrial iron deposition supports the hypothesis that the decrease in cytosolic ferritin iron and protein level is more likely due to the defect in cytosolic ISC proteins such as cytosolic aconitase/IRP1, as mentioned above, than to iron sequestration in the mitochondria, as found to occur after MtF expression [1].

The reduction of growth is an effect of frataxin deficiency shared by mammalian and yeast cells. This suggests that critical ISC enzymes like yeast Rli1p [39] are involved in the proliferation also of mammalian cells [17]. Alternatively this effect could be attributed to an iron detoxification function of frataxin that in yeast preserved cell longevity [40].

The role of oxidative stress in frataxin deficiency is still controversial and the FRDA animal model showed a decrease of SOD2 activity without other evident signs of oxidative damage [15]. We also

observed a decrease in SOD2 activity in the silenced cells that was expected to reduce antioxidant protection. However, the cellular model showed a slightly higher sensitivity to H<sub>2</sub>O<sub>2</sub> toxicity only after forcing aerobic metabolism by limiting glucose availability, probably because HeLa cells use only marginally the oxidative metabolism under normal condition of culture. Similarly, the phenotypic effect of frataxin deficiency in yeast becomes more evident when cells are grown in aerobic conditions [41].

### **Frataxin deficiency and MtF expression.**

From the characterization of the F3-clone we concluded that robust indices of frataxin silencing consisted in the reduction of cell proliferation, of ferritin levels, of aconitase and SOD2 activity. They were significantly modified after two rounds of F3-siRNA transfection in HeLa cells although not as strongly as in the F3-clone, probably because of the less continuous level of silencing and the absence of Dox. This allowed us to evaluate if MtF expression can protect HeLa cells from frataxin deficiency, as it was shown to occur in yeast cells [28]. We found that frataxin silencing in a clone expressing MtF did not cause inhibition of cell growth, reduction of ferritin level, of aconitase and SOD2 activities. Also free protoporphyrin level, which was increased in the silenced parental HeLa cells, was not affected by the silencing in the ones expressing MtF. Thus, MtF inhibited all the detectable effects associated with frataxin silencing in HeLa cells.

The only known function of MtF is to interact with Fe(II) and sequester it, thus reducing iron excess. However, MtF has never been detected in cultured mammalian cells, and when expressed it reduced cytosolic iron availability [1,3], cell proliferation in cancer cells [4], and protected the cells from oxidative damage [2]. Frataxin too has ferroxidase activity and binds Fe(II) in vitro [42] and, unlike MtF, is ubiquitously expressed in cultured cells. Thus frataxin deficiency may affect the iron redox status and reduce iron-sequestering and detoxification activity inside the mitochondrion [40]. This may possibly cause a deregulation of the mitochondrial iron pools without alteration of total mitochondrial iron. For example, frataxin deficient mammalian cells showed an increase of filterable, but not of total iron in the mitochondria [43]. Possibly more intriguing was the decrease of SOD2 activity [14,15], which we confirmed. It was proposed that mitochondria contain a distinct iron pool that competes with Mn for SOD2 binding and inactivates the enzyme [35,44]. Thus, SOD2 activity is an index of this “SOD2 reactive” iron pool, which in yeast increased in the absence of Mtm1p, of Ssq1, and of Grx5, proteins required for ISC assembly [44]. The pool increased also after ABCB7 silencing in HeLa cells [35]. In all these models its expansion was accompanied by increased mitochondrial iron deposition, unlike the present frataxin deficient model. The increase of PPIX was another index of abnormal mitochondrial iron homeostasis in the

frataxin-silenced cells. Individuals with FRDA have increased free protoporphyrin levels, but without evident signs of erythropoietic defects [45]. Moreover deficient heme synthesis was described in frataxin deficient mammalian cells [23] and frataxin was described to act as an iron chaperone for ferrochelatase [46]. Altogether our data suggest that in HeLa cells frataxin deficiency may alter the balance between various iron pools, including the one available to heme synthesis. MtF may restore the balance, since it reduced the “SOD2 reactive” pool and increased that available for heme synthesis. Similar effects were observed in ABCB7 deficient cells [35]. These findings confirm that frataxin is involved in mitochondrial iron handling and possibly also in oxidative damage via the regulation of SOD2 activity, and indicate that it can be partially substituted by MtF. In conclusion, the finding that MtF expression inhibits the effects of frataxin suppression in HeLa cells confirms the hypothesis that they have some common functions, such as iron binding and ferrooxidation. This may help to interpret the unexpected finding that MtF overexpression in tumor cells strongly suppressed the expression of frataxin protein [4], although we show that this does not occur in HeLa cells. If the tight connection between frataxin and the regulation of cellular iron homeostasis observed in the HeLa cell model occurs also in the neuronal and cardiac cell types affected in FRDA, the findings may be of importance for disease treatment.

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## LEGENDS TO FIGURES

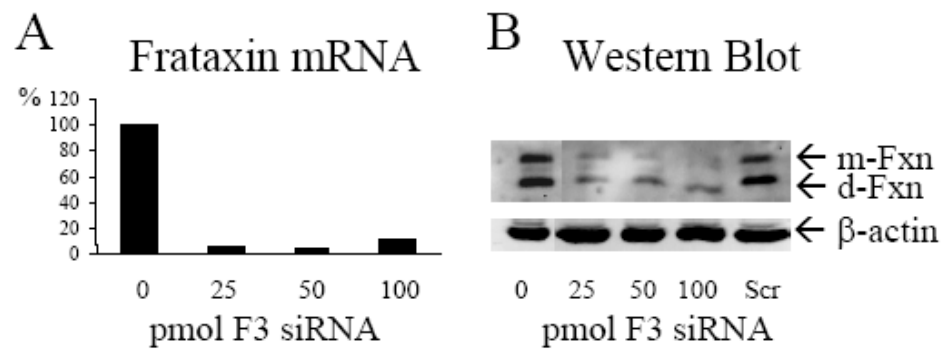
**Figure 1. Efficiency of the F3 siRNA.** **A.** HeLa cells were transfected with 25-100 pmol of synthetic F3 siRNA and the level of frataxin mRNA was quantified by real-time RT-PCR 24 hrs after transfection. The results were expressed as percentage of the control mock-transfected cells. **B.** The same transfected cells were analyzed for frataxin levels by western blot 72 hrs after transfection. Cell lysates (20  $\mu$ g total proteins) were loaded on 12% SDS-PAGE and immunoblotted with specific antibodies for frataxin and actin. Scr.: scrambled siRNA; m-fxn: mature form of frataxin (17 kDa); d-fxn: frataxin cleavage product (about 14 kDa) also identified in mouse heart extracts [10].

**Figure 2. F3 shRNA induction in the F3 clone.** **A.** Stable F3 clone (empty bars) was induced with 1-10  $\mu$ g/ml of Dox for 5 days and compared to parental line (solid bars). Frataxin mRNA levels were analysed by real-time RT-PCR and expressed as percentage of the control cells. **B.** The same clone was analysed for frataxin levels by immunoblot after 5 and 11 days in the presence or absence of 7.5  $\mu$ g/ml Doxycycline (Dox). Cell lysates (20  $\mu$ g total proteins) were loaded on 12% SDS-PAGE and immunoblotted with the specific antibodies.

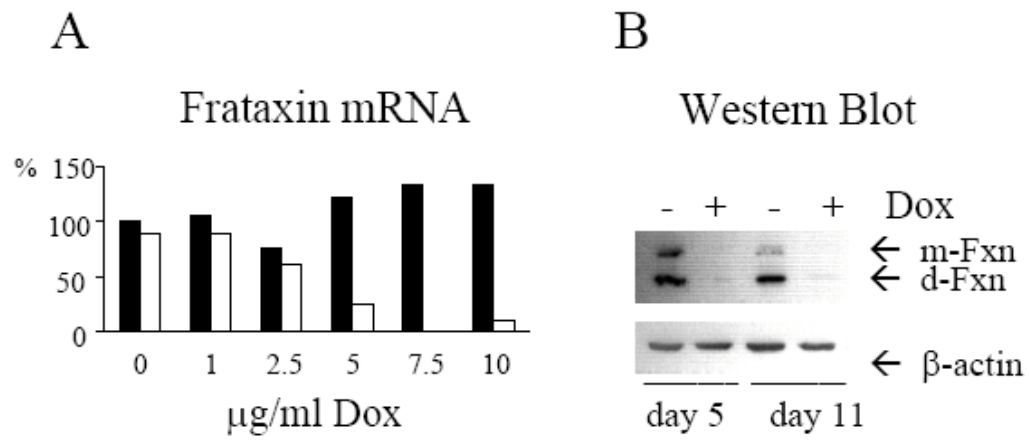
**Figure 3. Phenotype of the stably silenced F3 clone.** After 5 days of growth in 7.5  $\mu$ g/ml Dox, HeLa T-Rex (solid bars) and F3 clone (empty bars) were analysed. **A:** Viable cells were counted by trypan blue exclusion assay. Trypan blue stained only <5% of cells, and the data were expressed as percentage of cells that excluded the dye in comparison with the control cells. Mean of 10 experiments. **B:** The level of SDH activity in total cellular homogenates was analysed and expressed as mU/mg of proteins. Mean and SD of 4 experiments. **C:** The level of aconitase activity in the total cellular homogenates was evaluated and expressed as mU/mg of proteins. Mean and SD of 5 experiments. **D:** The mitochondrial fraction was separated and mitochondrial aconitase activity was measured. Results were expressed as percentage of the control. Mean and SD of 5 experiments. **E:** the level of citrate synthase activity (CS) in the total cellular homogenates was analysed and expressed as mU/mg of proteins. Mean and SD of 5 experiments. The asterisks indicate a significant difference (\*\*  $p < 0.01$ , \*  $p < 0.05$ ). Porin protein levels in the total cellular lysates were analyzed by immunoblot. B-actin was used for normalization. Representative of 3 independent experiments with similar results. **F:** The SOD2 activity in the total cellular homogenates was analysed by nitro blue tetrazolium staining of non-denaturing PAGE. SOD2 protein levels were analysed by immunoblot. Coomassie blue stain was used for normalization of enzyme activity and  $\beta$ -actin was used for normalization of protein blotting.

**Figure 4. Iron indices.** **A:** After 5 days of growth in 7.5  $\mu\text{g/ml}$  Dox, parental cells and F3-clone were incubated for 18 h with 74 kBq/ml [ $^{55}\text{Fe}$ ] FAC, and the specific radioactivity of the mitochondrial fraction and of the total cellular extracts were counted (cpm/ $\mu\text{g}$  of protein). Results were then expressed as the ratio between the two values. Mean and SD of 5 experiments. **B:** The total extracts were separated on non-denaturing PAGE (10  $\mu\text{g}$  of protein per lane) and exposed to autoradiography to analyse protein-bound [ $^{55}\text{Fe}$ ]. The single evident band corresponds to cytosolic [ $^{55}\text{Fe}$ ] ferritin. **C:** After Dox induction the L-ferritin content (FTL) was evaluated in total homogenates of T-Rex (solid bars) and F3 clone (empty bars) and the data expressed as ng of ferritin per mg of total proteins. Mean and SD of 4 experiments. The asterisks indicate significant differences (\* $p < 0.05$ ).

**Figure 5. Frataxin silencing in the presence of mitochondrial ferritin.** Two sequential transient transfections were performed with F3-siRNA to silence frataxin in the HeLa cell clone expressing mitochondrial ferritin (Mt53) and in parental control cells. **A:** Cell proliferation was monitored by trypan blue exclusion assay. Trypan blue stained only  $< 5\%$  of cells in all clones. The data are expressed as percentage of total viable cells in comparison with mock-transfected cells. Mean of 5 experiments. **B:** Western Blot with anti-frataxin and with anti-MtF polyclonal antibody (which cross-reacts with cytosolic H-ferritin) of the total cellular extracts (20  $\mu\text{g}$  per lane). B-actin was used for normalization. Representative of 3 independent experiments with similar results. **C:** Levels of aconitase activity in the total cellular homogenates. Data were expressed as mU/mg of proteins. Mean and SD of 5 experiments. **D:** The mitochondrial fraction was separated and aconitase activity was evaluated and expressed as percentage of the control. Mean and SD of 5 experiments. **E:** SOD2 activity was analysed by nitro blue tetrazolium staining of non-denaturing PAGE. Densitometric analyses were normalized with Blue Coomassie stain and expressed as percentage of the control. Mean and SD of 4 experiments. **F:** The levels of protoporphyrin were analysed for its intrinsic fluorescence by flow cytometry. Data were expressed as percentage of the mock-transfected cells. Mean and SD of 4 experiments. The asterisks indicate significant differences (\* $p < 0.05$ ).

**Fig. 1**

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**Fig. 2**

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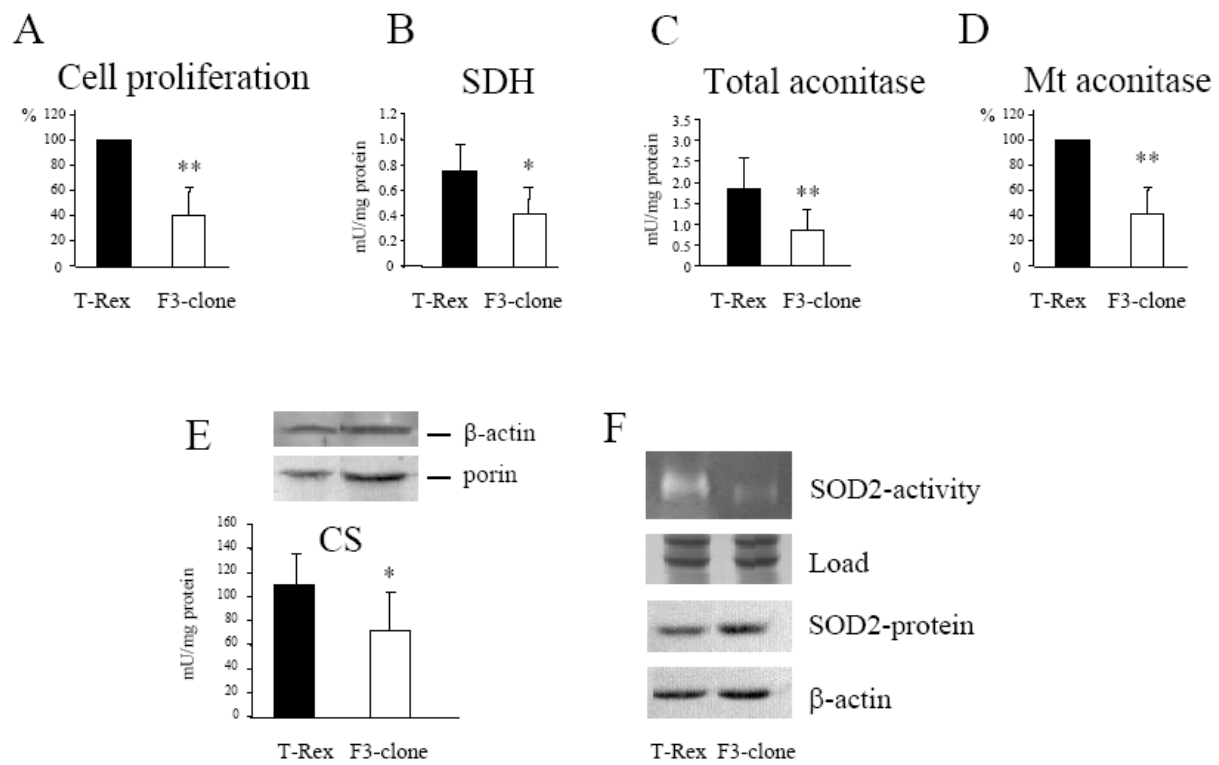


Fig. 3

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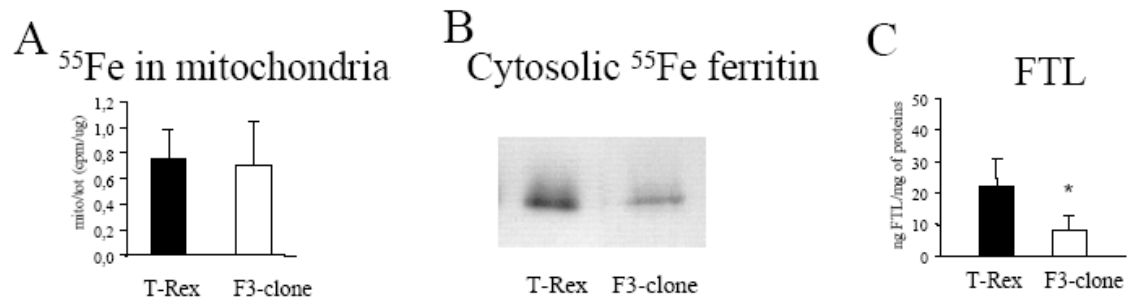
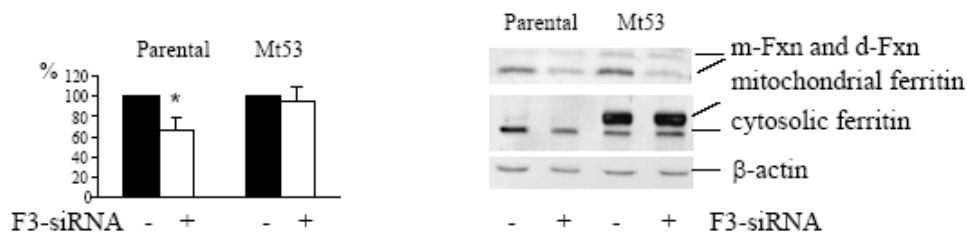


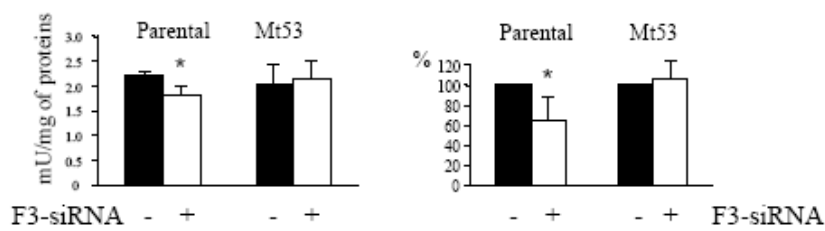
Fig. 4

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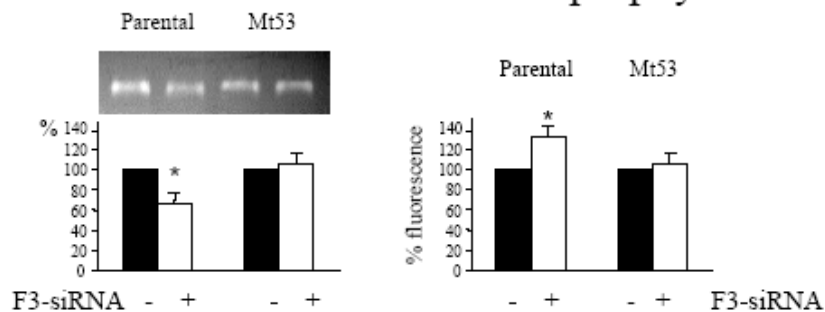
**A** Cell proliferation **B** Frataxin and ferritin level



**C** Total aconitase **D** Mt aconitase



**E** SOD2 activity **F** Protoporphyrin



**Fig. 5**