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**Hemin rescues adrenodoxin, heme a and cytochrome oxidase activity in  
frataxin-deficient oligodendrogloma cells**

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**Abstract**

Mutations in the frataxin gene cause neurodegeneration and demyelination in Friedreich's ataxia. We previously observed that frataxin deficiency causes iron-sulfur cluster defects, and later causes defects in heme and cytochrome c hemoprotein levels. Iron-sulfur (Fe/S) clusters are required in two enzymes of heme biosynthesis in humans i.e. in ferrochelatase and adrenodoxin. However, decreases in ferrochelatase activity have not been observed in frataxin-deficient Hela cells or patient lymphoblasts. We knocked down frataxin in oligodendrogloma cells using siRNA, which produced significant defects in the activity of the Fe/S cluster enzymes adrenodoxin and aconitase, the adrenodoxin product heme *a*, and cytochrome oxidase, for which heme *a* serves as a prosthetic group. Exogenous hemin produced a significant rescue of adrenodoxin, aconitase, and a trend for increase of heme *a* levels and cytochrome oxidase activity. Thus hemin rescues iron-sulfur cluster defects that are the result of frataxin-deficiency, perhaps as a consequence of increasing the pool of bioavailable iron and thus should be more fully tested for beneficial effects in Friedreich's ataxia models.

## Introduction

Mutations in the frataxin gene result in deficiency of the frataxin protein, expressed in mitochondria, causing the neurodegenerative disease Friedreich's ataxia (FRDA). Although the primary physiological role of frataxin has been debated, a demonstrated function of frataxin is the support of iron-sulfur (Fe/S) clusters [1, 2, 3], which function in iron-sulfur enzymes inside mitochondria.

A microarray study carried out with lymphoblasts and fibroblasts from FRDA patients, human neural cells, and cardiac cells from knockout mice showed that the most consistent transcriptional consequence of frataxin deficiency was an inhibition of the heme pathway transcript, coproporphyrinogen oxidase [4].

Furthermore, we observed in elevated protoporphyrin IX and heme *b* levels, and deficiency of hemes *a* and *c* and cytochrome oxidase in lymphoblasts from Friedreich's patients [4, 5]. Work with an inducible knockdown model showed that Fe/S functions decline rapidly after frataxin deficiency, whereas the heme defect occurs after the decline of Fe/S function in the mitochondria. This is consistent with the idea that heme deficiency is a consequence of a mitochondrial Fe/S cluster defect [6].

There are two known Fe/S enzymes in mammalian heme biosynthesis, ferrochelatase and adrenodoxin. Recently we demonstrated that the activity of ferrochelatase in cells from patients with FRDA is not decreased [5], consistent with work from other groups [7].

The only other Fe/S enzyme beside ferrochelatase known to participate in the heme biosynthetic pathway is adrenodoxin, which carries out the first step of

the conversion of heme O to heme a, that is required for cytochrome oxidase activity. Thus we knocked down frataxin levels using siRNA in a human oligodendroglial cell line (HOG), and observed decreased adrenodoxin activity, decreased heme a levels, decreased cytochrome oxidase activity, and decreased heme c level.

To see if exogenous heme could rescue these frataxin-dependent effects, we supplemented the frataxin-deficient HOGs with hemin, and observed a significant rescue of adrenodoxin, aconitase, and increased cytochrome oxidase activity and heme a levels, suggesting that hemin supplementation may be a rational therapeutic strategy for FRDA patients.

## Results

*There are two iron-sulfur cluster enzymes in the heme biosynthetic pathway*

Frataxin is important for Fe/S cluster biogenesis in the mitochondria [1, 2, 3, 4, 6, 8, 9]; its deficiency in human cells causes a defect in heme *a*, a defect in the activity of the heme *a*-containing enzyme cytochrome oxidase, and also in cytochrome *c* and heme *c* levels [4, 5]. There are only two enzymes of the heme biosynthetic pathway known to have Fe/S clusters, ferrochelatase and adrenodoxin (Fig.1). Frataxin-deficiency does not cause a decrease in the activity of the Fe/S cluster enzyme ferrochelatase in FRDA lymphoblasts or HeLa cells [1, 4]. It has been proposed that the yeast homolog of the human adrenodoxin (ferredoxin) is specifically involved in the first step of conversion of heme O to heme *a* in the heme biosynthetic pathway [10]. Heme *a* is in turn inserted in cytochrome oxidase and acts as the electron transport prosthetic group of the enzyme. Thus, we transfected human HOG cells with frataxin siRNA to see if frataxin level has an effect on the Fe/S enzyme adrenodoxin that participates in the heme pathway.

The human oligodendrogloma cell line (HOG) was transfected with frataxin-specific short interfering RNA (RNAi) [3], which resulted in an overall strong inhibition of the targeted message, and about 40% residual frataxin protein level (Fig. 2) relative to transfection with the 'scrambled' RNAi, i.e. an RNAi of identical nucleotide content but in randomized order.

*Frataxin depletion reduces adrenodoxin and cytochrome oxidase activity in oligodendrogloma cells*

Adrenodoxin and cytochrome oxidase activities were measured in scrambled and si-ftx HOGs; frataxin -deficiency produced a similar and significant reduction, of about 35%, in the activities of these two enzymes, compared with cells transfected with the scrambled oligonucleotide (Fig.3).

*Frataxin-deficiency produces heme a and cytochrome c heme deficiency*

We previously observed that both hemes a [4] and cytochrome c (i.e.'heme c'), were deficient in cells from Friedreich's patients, the latter perhaps as the result of some feedback from the adrenodoxin deficiency [4, 5].

Using HPLC we measured heme a levels in frataxin-deficient HOG cells and observed that it was significantly reduced with respect to the scrambled oligonucleotide (Fig. 4A). Cytochrome c heme amount was evaluated as previously described [6] (Fig. 4C); and was significantly decreased in the frataxin-depleted cells (Fig 4B).

*Hemin stimulates adrenodoxin and aconitase activity*

Because multiple hemes were decreased in frataxin-deficient cells, we supplemented cells with exogenous heme as hemin (10  $\mu$ M). As higher levels of heme are toxic in some cell types [11], the viability of all the cell lines used for our study was evaluated after 22 hours treatment. There was no decrease in viability

of the heme treated cells, as viabilities for treated and untreated were  $\geq 95\%$  (data not shown).

The dose of hemin used for our experiments (10  $\mu\text{M}$ ) stimulated the activities of both the Fe/S enzymes adrenodoxin and aconitase (Fig 5). To exclude that the increased Fe/S enzyme activities were caused by the induction of frataxin expression by hemin, which has been suggested by Sarsero and coworkers in BHK cells [12], we measured the levels of frataxin upon treatment with 10  $\mu\text{M}$  hemin. Western blot and RT-PCR analysis showed no difference in either the protein (Fig. 6) levels or the relative mRNA levels by real-time QRT-PCR (data not shown) of frataxin after treatment for 22 hours.

#### *Hemin increases heme a levels and cytochrome oxidase activity*

Since hemin rescues the activity of adrenodoxin, which is involved in heme a synthesis, we asked if it might rescue heme a levels, and consequently cytochrome oxidase activity. Hemin administration did increase heme a levels and cytochrome oxidase activity in siRNA treated cells (Fig. 7).

## Discussion

### *Frataxin-deficiency causes adrenodoxin and cytochrome oxidase deficiency*

Frataxin's major physiological role appears to be in iron-sulfur (Fe/S) cluster biosynthesis [1, 2, 3, 13]. In the last few years studies conducted in yeast and mammalian cells have supported an involvement of frataxin in heme synthesis [4, 5, 14, 15]. There are two known Fe/S cluster enzymes in the heme synthesis pathway, ferrochelatase and adrenodoxin, and there have been reports that frataxin binds ferrochelatase [16]. We first set out to assay ferrochelatase from FRDA patient's cells, but observed, like another study, no decrease in ferrochelatase activity [1, 4].

We have gone on to show that frataxin-deficiency reliably produces the induction of the heme pathway transcript ALAS1 in another mammalian model system, and that this always follows a defect in mitochondrial Fe/S cluster status [5]. Therefore, we attempted to understand how frataxin, which affects Fe/S clusters and produces a defect in mitochondrial heme *a* and cytochrome oxidase activity, could affect heme synthesis, by assaying adrenodoxin in frataxin-deficient cells. We chose to study this in oligodendrogloma cells, as we have recently observed that mitochondrial defects specifically affect oligodendrocyte-specific transcripts [17].

Heme *a* is the product of the activity of another matrix mitochondrial Fe/S enzyme, adrenodoxin (Adx) (See Fig.1). In yeast, adrenodoxin (Yah1) and adrenodoxin reductase (Arh1) are well-characterized and are involved in heme *a*

biosynthesis [18, 19] and in Fe/S cluster assembly [20]. Adrenodoxin and adrenodoxin reductase provide electrons for the first step of transformation of heme O into heme *a*.

We demonstrated significant heme *a* and *c* deficiency, and a significant defect also in heme *a*-containing enzyme cytochrome oxidase, confirming the previous results we obtained in lymphoblasts from FRDA patients [4].

A heme-*a* deficiency, leading to cytochrome oxidase deficiency, may be relevant to the pathogenetic mechanism of FRDA, which causes degeneration of dorsal root ganglion neurons, for several reasons. Firstly, it has been shown that inhibition of heme metabolism impairs cytochrome oxidase activity, triggers mitochondrial damage, oxidative stress, and iron accumulation, all of which are hallmarks of FRDA [21].

Secondly, previous studies have shown that cultures of myelinated mouse dorsal root ganglia exhibit intense heme fluorescence localized in myelin sheaths but not in axons. Also, treatment with a heme-pathway inhibitor caused a specific demyelination of the dorsal root ganglia [22].

Thirdly, inhibitors of cytochrome oxidase activity (carbon monoxide, cyanide) and chelators of the copper cofactor required for cytochrome oxidase activity (cuprizone), are known to cause a selective demyelination [23, 24, 25].

Fourthly, cytochrome oxidase transcripts are normally up-regulated in the process of oligodendrocyte differentiation (Schoenfeld, in preparation).

Taken together, these data suggest that the frataxin-dependent defect in heme *a* and cytochrome oxidase deficiency may be relevant to the demyelinating phenotype observed in FRDA [26, 27].

*Exogenous hemin rescues frataxin-dependent defects in heme a, adrenodoxin, aconitase and cytochrome oxidase*

Because frataxin-deficiency produced heme deficiency, we tried to rescue these effects with exogenous heme. We observed significant rescue for the activities of adrenodoxin and aconitase, both Fe/S enzymes, for the activity of the heme *a*-containing cytochrome oxidase, and for the heme *a* levels themselves.

The rescue of the Fe/S enzymes adrenodoxin and aconitase confirms what was previously shown in one publication for aconitase [28], i.e. that exogenous hemin stimulates the activity of Fe/S enzymes. Since Friedreich's ataxia seems to be a disease of Fe/S biogenesis, this could be relevant for therapy.

Since we demonstrated that hemin produced no increase in frataxin protein or mRNA relative expression, one possible explanation for the stimulation of Fe/S enzyme activities by heme is that heme and frataxin work together to serve as a source of bioavailable iron in the biosynthesis of iron-sulfur clusters. Therefore a deficiency of frataxin, which decreases availability of iron for Fe/S synthesis, may be overcome by increasing the bioavailability of iron, as heme-iron.

The administration of hemin restores the activity of enzymes that have been shown to be defective in FRDA, suggesting that heme-based strategies should be considered therapeutically. In some ways these results seem consistent with

recent reports that erythropoietin, which is known to stimulate heme synthesis, can rescue defects observed in frataxin-deficient cells [29]. However, given the iron overload observed in some hearts of FRDA patients, also in the context of EPO administration, further testing in cell and animal models to work out this mechanism should be done.

#### *Possible therapeutic use of hemin in FRDA*

Up to now a number of therapeutic approaches have been proposed for Friedreich's ataxia [30, 31, 32, 33, 34], and none appears to alleviate the neurological signs. Idebenone appears to relieve some of the cardiac hypertrophy in small studies [30, 31, 32]; but questions about the use of idebenone as an antioxidant have been raised when it was proven that it actually induces an increased superoxide production in submitochondrial fractions [35].

On the basis of our results demonstrating a heme imbalance in frataxin deficient cells [4] and on recent findings which relate frataxin function to heme synthesis/homeostasis, we propose the consideration of hemin as possible therapeutic approach for the treatment of Friedreich's ataxia. Hemin therapy has already been demonstrated to be effective in the treatment of heme-deficiency related disorders such as porphyria [36]. However, given the possibility of iron toxicity problems, work should first be carried out in cellular and animal models.

*Summary and prospects*

In summary, the data presented here demonstrate that frataxin deficiency produces adrenodoxin, aconitase, and cytochrome oxidase activity impairment and heme a, heme c deficiency in a myelin-producing cell.

This provides the first direct connection between the iron-sulfur cluster defects observed in FRDA, and the heme defects observed by ours and other groups. The cytochrome oxidase deficiency that is a result of heme deficiency could be relevant to the demyelinating phenotype of FRDA. In addition, we observe that exogenous heme administration increases activity of the iron-sulfur cluster enzymes adrenodoxin and aconitase. Thus heme-based stimulation of iron-sulfur cluster biogenesis is a rational strategy for FRDA that should be further tested.

## Materials and Methods

### *Cell culture.*

Oligodendrocytes (HOG) were grown in DMEM containing 500mg/L glutamine and supplemented with 10% (v/v) FBS and maintained at 37° in a humidified atmosphere containing 5% CO<sub>2</sub>.

### *RNA interference (RNAi) for inhibiting frataxin expression in oligodendroglial cells*

RNA interference technique was carried out as previously described [3]. HOG cells were incubated in regular DMEM for 24 hours, washed twice in PBS and then replaced with serum free OPTM 1 medium. A mixture of 200 nM si-RNA (frataxin, si-ftx, AAC GUG GCC UCA ACC AGA UUU; or scrambled, CAG UCG CGU UUG CGA CUG GdTdT) and Oligofectamine (Invitrogen, Gaithersburg, MD, USA) was added to each T75 flask for 4 h, after which OPTIM 1 medium containing 30% serum was added. The day after, the medium was replaced with regular DMEM. Cells were harvested 72 h later and mitochondria were isolated. Western blot was used to confirm the frataxin protein level.

### *Hemin treatment*

For the studies of rescue with hemin one fraction of the cells was added of 10 µM hemin (Frontier Scientific, Inc., UT) and stored in incubator for 22 hours; the untreated fraction was added with the same amount of 100mM of KOH, in which

hemin was dissolved.

#### *Mitochondria isolation*

Mitochondria were isolated from HOG cells transfected with scrambled and si-ftx RNA, treated or untreated with 10  $\mu$ M hemin. Approximately  $1 \times 10^8$  cells were enzymatically released using 2 ml of a 0.25% trypsin-EDTA solution/T75 flask for 3' at 37 °C, following inactivation by addition of 8 ml culture medium and harvested by centrifugation. The pellet was re-suspended with 4 ml of isolation buffer (210 mM mannitol, 70 mM sucrose, 1mM EGTA and 5mM HEPES, pH 7.2) for each gram of packed cells, treated with a final concentration of 0.3 mg/ml digitonin for 2 min and centrifuged at 2800 g for 5'. The pellet was then resuspended with 5 ml for each gram of initial packed cells and homogenized with a chilled glass homogenator (20 passes). After several centrifugation steps at 540 g the final spin was centrifuged and 10000 g and the final pellet was suspended with 0.1 ml of isolation buffer per gram of starting cells, giving a protein concentration of approximately 15-20 mg/ml. For determination of mitochondrial protein concentration, 2  $\mu$ l of the mitochondrial suspension were diluted 1:30 in double distilled water and the protein concentration was estimated using the Bradford assay (Bio-Rad). The lysates were prepared resuspending the mitochondrial pellet in about 100  $\mu$ l per initial gram of packed cell of the following lysis buffer: 50mM Tris, pH 7.8, 100 mM NaCl, 1 mM PMSF and 1% detergent (IGEPAL-CA630) for 30' at 0°C and insoluble material removed by centrifugation at 16000 g. The spin

was collected, the protein amount evaluated using the Bradford assay (Bio-Rad) and the mitochondrial fraction stored at  $-80^{\circ}\text{C}$ .

#### *HPLC analysis of heme a*

A Luna C-18, 4.6  $\times$  100 mm, 5 mm particle size column was used. Standards were prepared from heme a (purified by Dr Eric Hegg, University of Utah and provided as a gift by Dr Brian Gibney, Columbia University). All data were analyzed using Millennium software (Waters, Milford, MA, USA).

Mitochondrial proteins (200  $\mu\text{g}$ ) were lysed with 5% HCl in acetone for 20 min on ice, centrifuged at 18 000g for 5 min and supernatants collected for analysis. The gradient used to measure heme a was from 100% buffer A (35% acetonitrile, 0.08% trifluoroacetic acid) and 0% buffer B (0.08% trifluoroacetic acid in acetonitrile) to 0% A and 100% B over 45 min with a flow rate of 1.0 ml/min. Peaks were monitored at 405 nm.

#### *Heme staining of cytochrome c*

Untreated and treated mitochondrial extracts were separated on a 15% SDS/PAGE gel. Heme staining was performed as previously described [37]. 40  $\mu\text{g}$  of mitochondrial protein from 3 different controls and 5 different FRDA lymphoblast lines were loaded onto a 15% polyacrylamide gel and the electrophoresis was performed at 130mV for 60'. After the run the gel was fixed in 10% TCA for 10', washed 4 times for 5' in double distilled water. The heme staining is based on the oxidation of *o*-dianisidine, a probe which, in presence of hydrogen peroxide

(H<sub>2</sub>O<sub>2</sub>), can be oxidized by the peroxidase activity of some heme-proteins (such as cytochrome c) changing color. The gel was soaked in a solution of 50mM trisodium citrate, 0.7% H<sub>2</sub>O<sub>2</sub> and 1 mg/ml o-dianisidine for 40-60' at 45°C. 0.5 µg of horse heart purified cytochrome c was used as positive controls. The band in correspondence of cytochrome c became evident after 20'.

#### *Western blot analysis of frataxin levels*

Equal amounts of lysates (40µg) untreated or treated with hemin were resolved on a 15% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by electroblotting. After blocking with 4% non-fat dry milk, the blot was incubated with anti-frataxin (kind gift of Dr. Franco Taroni, Istituto Nazionale Neurologico Carlo Besta, Milan, Italy) (1:2000) and developed with AP-conjugated secondary antibodies using a chemiluminescent substrate.

#### *Quantitative RT–PCR analysis*

Total RNA was prepared using Qiagen Mini Kit (Qiagen, CA, USA), and RNA concentration was determined by UV spectrophotometry. Reverse transcription of 1 mg RNA was performed using an RT–PCR kit (Invitrogen, Gaithersburg, MD, USA), and reactions were performed in a 20 ml volume. One microliter cDNA of RT reaction were used for PCR. Quantitative PCR standard curves were set up for frataxin and GAPDH according to a method previously described [38]. The PCR primers for GAPDH are: forward, CCCCTGGCCAAGGTCATCCAT G; reverse,

CAGTGAGCTTCCCGTTCAGTCC. The primers of PCR for frataxin are: forward, AAATCTGGAACTTTGGGCCAC; reverse, ACCTCAGCTGCATAATGAAGC. The PCR reaction was previously described in detail [38]. Results were expressed as frataxin m-RNA levels normalized by GAPDH.

#### *COX, adrenodoxin and aconitase activity assays*

Cytochrome oxidase (COX) activity was evaluated by the single wavelength spectrophotometric assay previously described [39] with some modifications. Intact mitochondria (2  $\mu$ g) were premealed with 2.5 mM of dodecylmaltoside in 0.17 M  $\text{KH}_2\text{PO}_4$ , pH 7.0 for 3' at 30°C, and, after recording the baseline, the reaction was started with 100 mM of reduced cytochrome c. COX activity was calculated from the pseudo-linear rate of cytochrome c oxidation at 550 nm.

Adrenodoxin activity was evaluated in mitochondria lysates. To our knowledge, there's no previous data about the direct measurement of Adx activity in human biological samples so we followed the method described for the purified protein [40] with some modification. Cytochrome c reduction was assayed in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% Tween 20 at 23°C. Reaction mixtures contained 0.05  $\mu$ M purified adrenodoxin reductase, 10 mM cytochrome c, and 350  $\mu$ g of mitochondrial protein. The reaction was initiated by the addition of 140  $\mu$ M NADPH. The absorption change at 550 nm was monitored, and the activity was determined using  $\Delta\epsilon_{550} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$ , after subtracting from the  $\Delta$ abs of the samples prepared as described the  $\Delta$ abs of a "blank" w/o AdR.

The same mitochondrial lysates (150  $\mu$ g protein) used for the evaluation of Adx activity were utilized for the assay of aconitase activity by the BIOXYTECH Aconitase-340 kit (OxisResearch, CA); NADPH formation was recorded at 340 nm.

#### *Statistics*

Statistical analysis was carried out with Prism 3.03 software, the paired t test.

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## Figure Legends

**Figure 1.** Heme *a* and *c* biosynthetic pathways. Adapted from Schoenfeld et al. [4].

**Figure 2.** Mitochondrial frataxin protein expression in oligodendroglial cells transfected with scrambledRNAi or frataxinRNAi (si-ftx). (left) oligodendrogloma cells were transfected with frataxinRNAi or scrambled RNAi, mitochondria extracted and lysed and analyzed by Western Blot. Reported are the results of the densitometry measurements of the frataxin band normalized by an unspecific band appearing in the same blots. Error bars represent S.E.M. of 7 experiments. (Right) Example of Western Blot detection of frataxin protein, using anti-frataxin antibody, 96 h post-transfection with scrambled RNAi or frataxin RNAi. A.U.= Arbitrary densitometry Units. \*\*\*  $P < 0.0005$  with paired t test.

**Figure 3.** Adrenodoxin (left) and cytochrome oxidase (right) activities in HOG cells transfected with scrambled and si-ftx RNAi. Cells were collected 4 days after transfection and mitochondria isolated. For COX activity (here reported as % of scrambled average) “fresh” permealized mitochondria were used; the Adx activity was obtained from the lysate as described in Methods. The results are reported as mean  $\pm$  S.E.M. of 6 (Adx) and 9 (COX) different transfections. Statistics: t test for paired values. \*\* $P < 0.005$ .

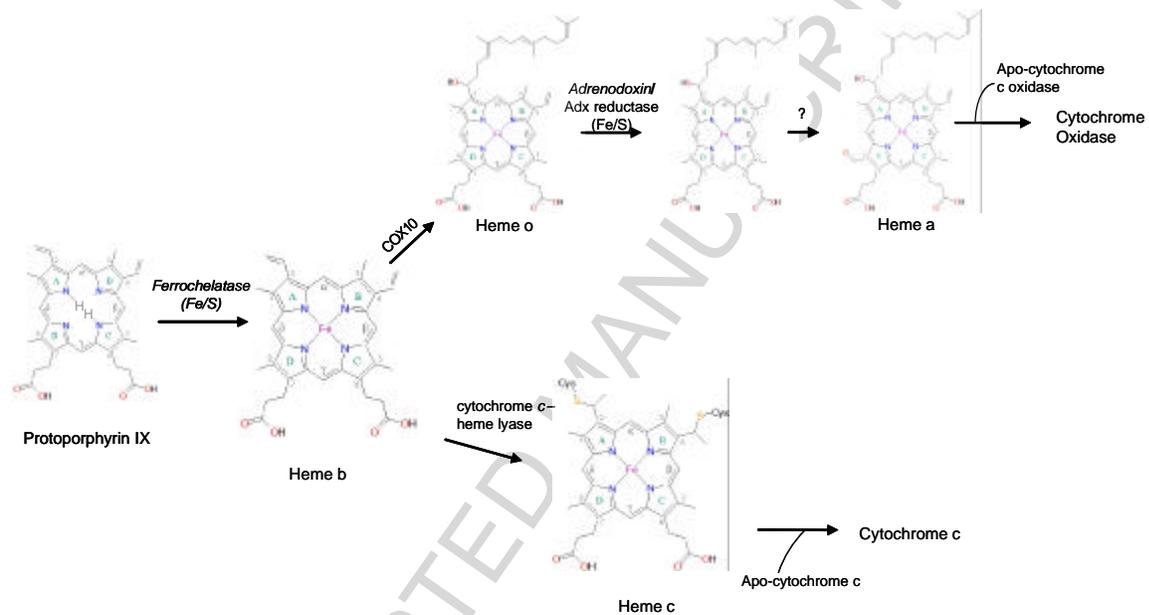
**Figure 4.** (A) Heme a and (B) cytochrome c heme levels in scrambled and si-ftx transfected HOGs. Heme c staining (C, example) was carried out as described in the Methods section. Results are average of 8 different experiments. Mean $\pm$ S.E.M. is reported. A.U.= Arbitrary Units. \*P<0.05 calculated by paired t test.

**Figure 5.** Adrenodoxin (left) and aconitase (right) activities in HOG scrambled and si-ftx mitochondria untreated and treated with 10  $\mu$ M hemin for 22 hours. Reported are the results of 3 (for adrenodoxin activity) and 6 (for aconitase activity) different transfections expressed as mean $\pm$ S.E.M. Statistics was carried out by paired two-tailed t test.

**Figure 6.** Mitochondrial frataxin protein expression in oligodendroglial cells transfected with scrambledRNAi or frataxinRNAi (si-ftx), upon treatment of 10  $\mu$ M hemin for 22 hours. Oligodendrogloma cells were transfected with frataxinRNAi or scrambled RNAi, mitochondria extracted and lysed and analyzed by Western Blot. Every mitochondrial extract was normalized for total mitochondrial protein and the densitometry results reported as ratio between units of frataxin and units of one unspecific band appearing in the same blots; results are expressed as % of the average of the scrambled samples and error bars represent S.E.M. of 7 experiments.

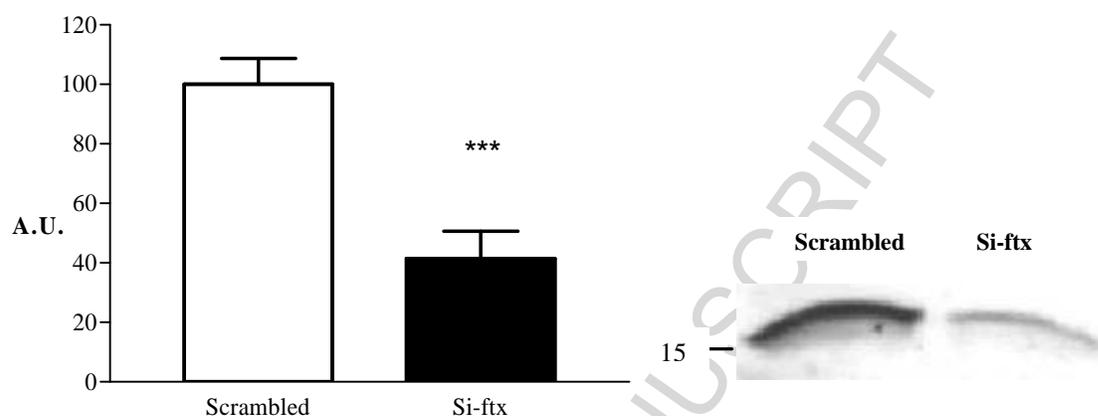
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Figures (legends are included on the same page for convenience to the reviewers)

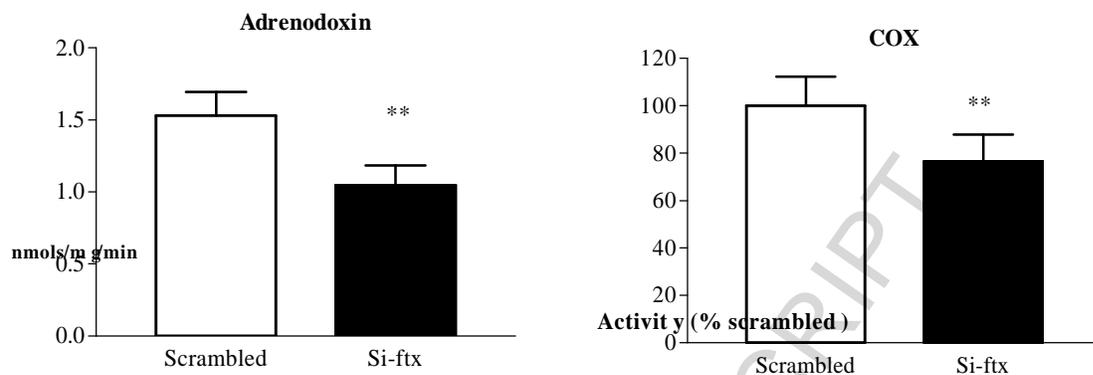


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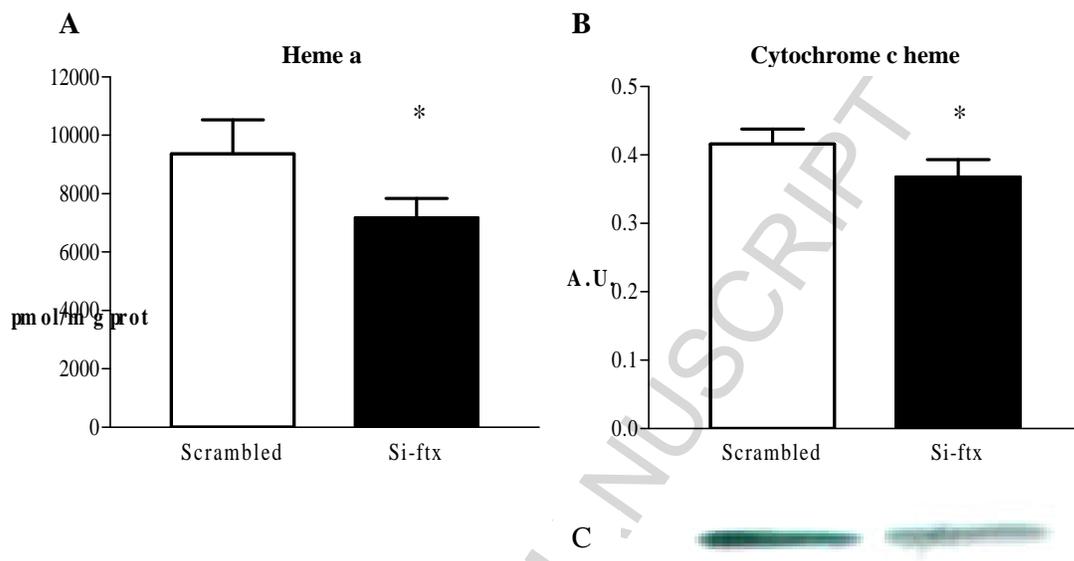
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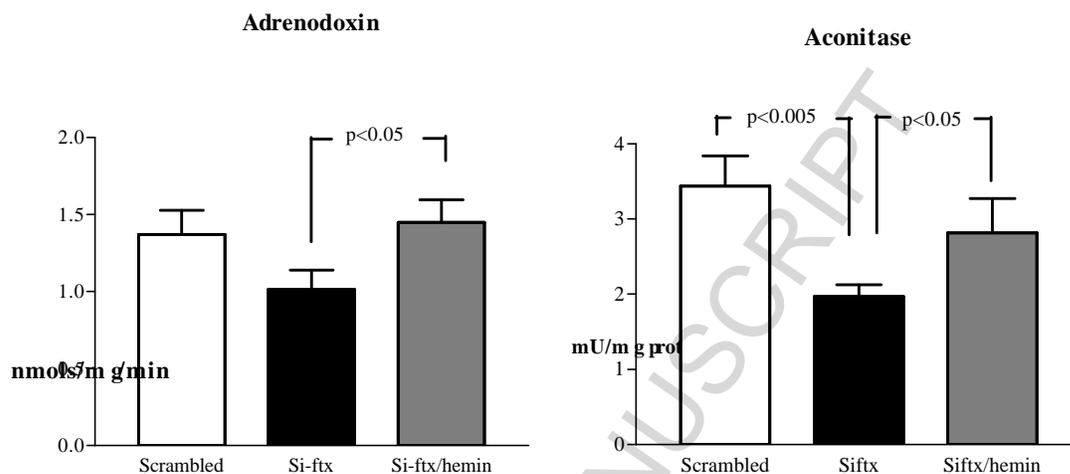
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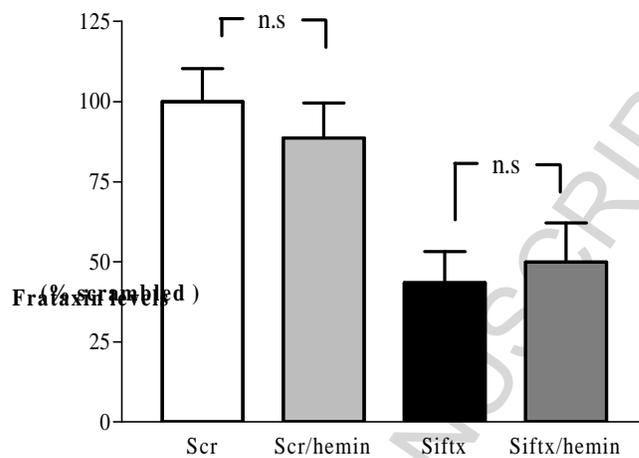
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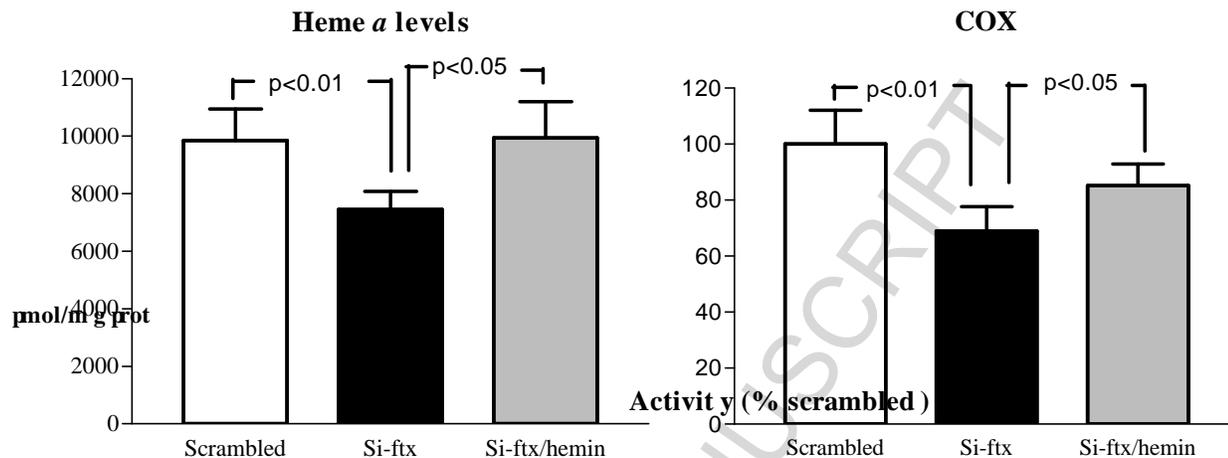
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