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Review

Insights into the roles of the Sideroflexins / SLC56 family in iron homeostasis and iron-sulfur biogenesis

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Abstract: Sideroflexins (SLC56 family) are highly conserved multi-spanning transmembrane proteins inserted in the inner mitochondrial membrane in eukaryotes. Few data are available on their molecular function but, since their first description, they were thought to be metabolite transporters probably required for iron utilization inside the mitochondrion. Such as numerous mitochondrial transporters, sideroflexins remain poorly characterized. The prototypic member SFXN1 has been recently identified as the previously unknown mitochondrial transporter of serine. Nevertheless, pending questions on the molecular function of sideroflexins remain unsolved, especially their link with iron metabolism. Here, we review the current knowledge on sideroflexins, their presumed mitochondrial functions and the sparse - but growing - evidence linking sideroflexins to iron homeostasis and iron-sulfur cluster biogenesis. Since an imbalance in iron homeostasis can be detrimental at the cellular and organismal levels, we also investigate the relationship between sideroflexins, iron and physiological disorders. Investigating Sideroflexins' functions constitutes an emerging research field of great interest and will certainly lead to main discoveries on mitochondrial physiopathology.

Keywords: sideroflexin; mitochondria; mitochondrial transporters; iron homeostasis; iron-sulfur cluster; heme biosynthesis; one-carbon metabolism; ferroptosis; ferritinophagy.

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1. Sideroflexins: from structure to function

1.1. Sideroflexins from an historical point of view

The mitochondrion is at the crossroad of key metabolic pathways (energy metabolism, central carbon metabolism, one carbon metabolism, lipid, nucleotides and amino acids synthesis, etc.) and is a key player in cell fate and response to stress or infection. In order to ensure its essential functions within the cell, the mitochondrion requires a wide variety of enzymes and transporters. Among these proteins, sideroflexins (SFXN) form a family of recently discovered mitochondrial proteins whose cell functions are progressively being specified. The first mention of the name "sideroflexin" appeared in 2001 [1]. Since then, a few studies have been dedicated to SFXN proteins and, at the

time we are writing this review, only 24 articles are retrieved in Pubmed using the keyword "sideroflexin". Pioneers in the SFXN field, Fleming *et al.* identified a mutation affecting the *Sfxn1* gene in the *flexed-tail* mouse and emitted the hypothesis that the loss of Sfxn1 was responsible for the sideroblastic anemia phenotype. However, it should be noticed that the causal link between the mutation in the *Sfxn1* gene and the phenotype of *flexed-tail* mice has not been clearly established yet. It was even questioned following a study showing that *flexed-tail* mice also had a mutation of the *Madh5/Smad5* gene, involved in the BMP pathway, which could explain the anemia and *flexed-tail* phenotype [2,3]. Anyway, SFXN own their name to the mice in which they were discovered (SIDEROblastic anemia and FLEXed-tail mouse) [1].

1.2. The Sideroflexin family: from genes to proteins

Sideroflexins (forming the SFXN/SLC56 family of mitochondrial transporters [4]) are highly conserved throughout eukaryotes. Only one sideroflexin is found in yeast (Fsf1 for **F**ungal **s**idero**f**lexin 1), whereas there are two SFXNs in *Drosophila* (dSfxn1/3 and dSfxn2) and five SFXN (SFXN1-5) in vertebrates [1,5–7]. Our purpose is not to give an extensive overview of SFXN tissue distribution in this review, but some data are available in the literature. For example, SFXN1 mRNA levels in normal tissues and human cancers, as well as tissue distribution of the five human SFXN, are available in [8].

SFXNs homologues display a high amino acid identity rate in mouse [1], xenopus [5] and human [8]. In humans, SFXN1 and SFXN3 share 76.56% identical amino acids whereas there is 56.05% identity between SFXN1 and SFXN2 and only 22.04% between SFXN1 and SFXN4. An alignment of human SFXNs is shown in **Figure 1**. Identity rates between the different human, *Drosophila* and yeast sideroflexins proteins are described elsewhere [8,9]. The high degree of homology between SFXNs, especially between SFXN1 and SFXN3 in humans, suggest that sideroflexins may ensure redundant functions, as it was proposed for the mitochondrial import of serine that seems to be mediated by SFXN1 [8]. This function will be evoked in more details below (see the section dedicated to the role of SFXN in regulating mitochondrial metabolism). Among the five mammalian SFXNs, SFXN4 is the most divergent member suggesting that this member do not share the same functions (**Figure 1**). Indeed SFXN4 was not able to suppress defects caused by the concomitant loss of SFXN1 and SFXN3 in mammalian cells [8]. Interestingly, up to date no study has been done to specifically uncover Fsf1 function. Because of the high degree of similarity between fungal sideroflexin and SFXN proteins from higher eukaryotes, we think that studies on the functions of Fsf1 will certainly lead to huge advances in the SFXN field and maybe reveal a general function for this family of proteins.

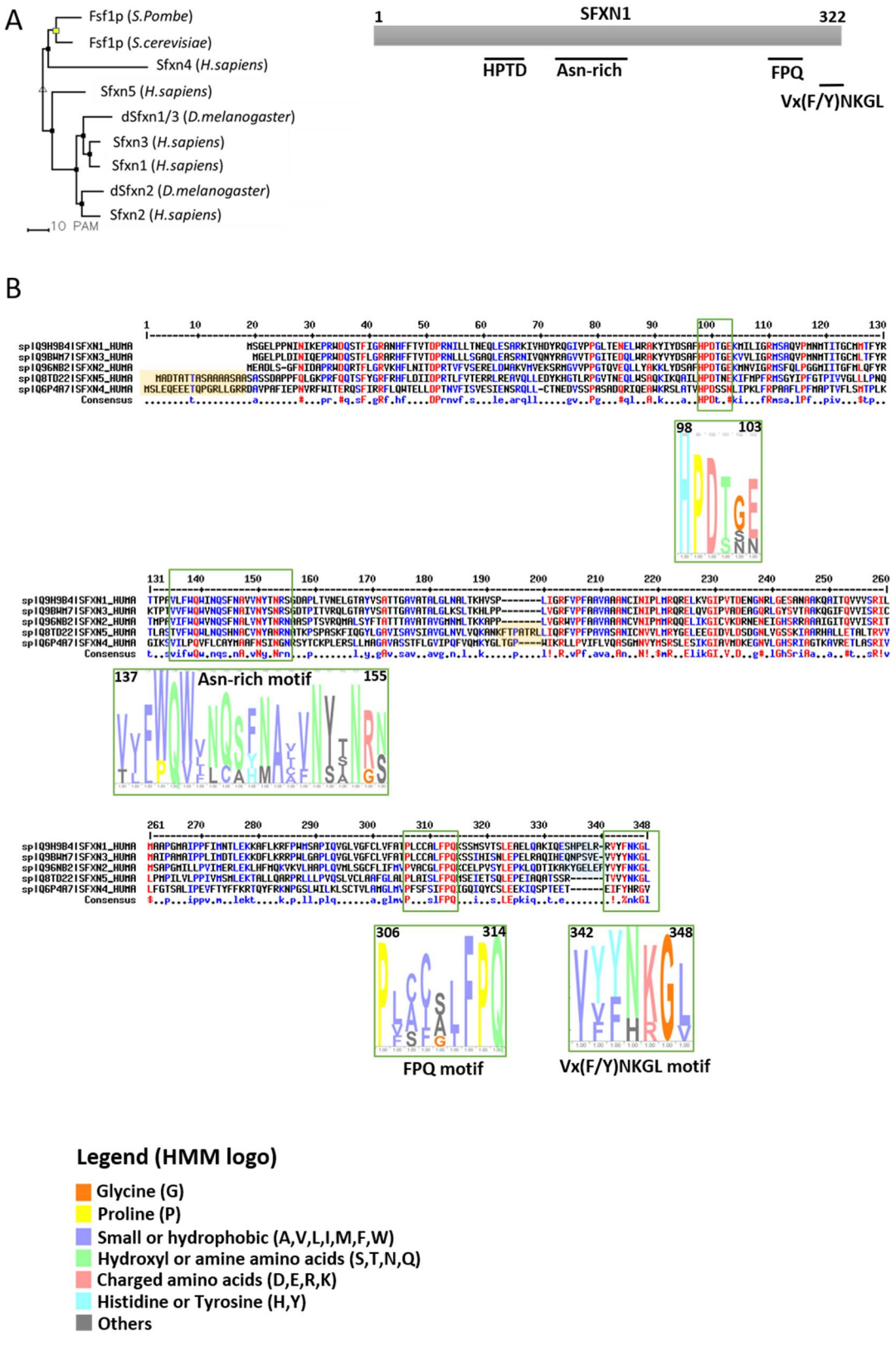

Figure 1. SFXNs form a family of conserved proteins in *Eukarya*. *A.* Left panel: Phylogenetic tree obtained using the MultiAlin software (http://multalin.toulouse.inra.fr/multalin/)[12]. Right panel: scheme of the SFXN1 protein and its conserved motifs. *B.* Alignment of human SFXNs protein sequences. Red amino acids are for high consensus levels (90%), the blue ones are for low consensus levels (50%). Meaning of symbols found in the consensus line: "!" is for Ile or Val, "$" is for Leu or Met, "%" is for Phe or Tyr," #" is anyone of Asn, Asp, Glu, Gln. Conserved motifs are shown and highlighted using an HMM logo created using Skyline (http://skylign.org/) with consensus colors for amino acids according to the ClustalX coloring scheme.

1.3. Sideroflexins are mitochondrial transporters implicated in one-carbon metabolism

SFXNs possess four to six predicted transmembrane domains composed by α-helices revealed by *in silico* modeling [1,6,7]. These proteins share several highly conserved motifs including a HPDT motif and an asparagine-rich sequence (**Figure 1**) [1,6]. The functions of those conserved motifs have not been uncovered yet. Recently, Gyimesi and Hediger performed an *in silico* analysis of human SFXN1-5 sequences and described six well-conserved regions that could be important for SFXNs activity [10]. Whether these conserved regions are essential for metabolite transport need to be further confirmed at the bench.

To date, no crystal structure has been released for SFXNs. We thus tried to model SFXN tridimensional structure using the trRosetta software [11] . SFXN1 predicted structure is shown in **Figure 2**. Interestingly, this structure reveals six internal alpha helices that may correspond to the transmembrane domain of SFXN1.

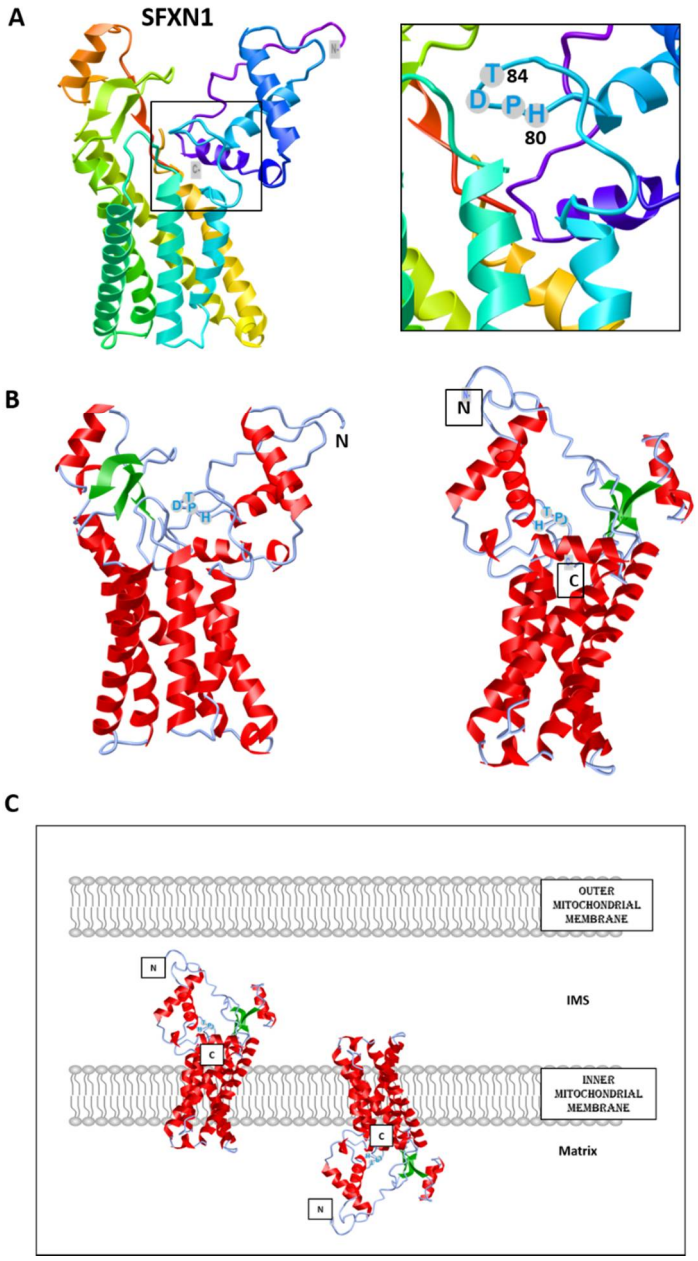

Figure 2. Predicted structure of human SFXN1. Structure prediction was obtained using trRosetta. The confidence of the predicted model shown here is very high (with estimated TM-score=0.806). The model was built by trRosetta based on *de novo* folding, guided by deep learning restraints. iCn3D was used for the visualization of 3D structure [26]. *A.* SFXN1 predicted structure reveals several alpha helices and beta strands. N and C termini are labelled. The inlet shows the position of the HPDT motif (aa 80-83), located just after the fourth helix. *B.* Two views highlighting secondary structures (helices in red, beta sheets in green). C. Models for SFXN1 insertion in the inner mitochondrial membrane.

SFXN1 topology was recently investigated by APEX and classical biochemical experiments [13–15]. Acoba *et al.* [15] performed detergent extraction and protease-protection assays on HEK human cells and confirmed that endogenous SFXN1 is a mitochondrial protein inserted in the inner mitochondrial membrane (IMM). Furthermore, evidence was given for the presence of N-terminus in the intermembrane space (IMS) but not in the matrix contrarily to what is predicted by a *in silico* analysis using Protter. According to biochemical data, the C-terminus seems to protrude in the matrix, in agreement with the previously proposed 5 transmembrane domains. However, our model is rather in agreement with a TM domain composed of six alpha helices and, if this predicted structure is correct, N and C termini could be in the same mitochondrial compartment (**Figure 2**). CryoEM structure of SFXN1 is thus needed to precise the three-dimensional structure of this carrier. Moreover, two recent studies investigated the mechanisms of SFXN1 mitochondrial import and shed light on the role of TIM22 and AGK2 in this process [16,15]. Evidence for a mitochondrial localization of SFXN are listed in the **Table 1.**

144 **Table 1.** Evidence for a mitochondrial localization of Sideroflexins.

SFXN	Model	Localization	Experiment	Reference
SFXN1	Mouse	IMM	Co-fractionation	Fleming *et al*., 2001 [1]
	Human cells (Jurkat, K562)		Immunoblot on affinity-purified mitochondria STED (co-localization of Flag-SFXN1 and COX4)	Kory *et al*., 2018 [8]
	Human cells (MCF7, HT1080), *Drosophila*		Immunoblot on mitochondrial extracts (fractionation) Confocal microscopy, Proteomics (LC-MS/MS on SFXN1 IP)	Our unpublished data
	Human cells (HEK)		SILAC-based proteomics coupled LC-MS/MS, carbonate extraction, digitonin fractionation	Acoba *et al*., 2020 [15]
SFXN2	Human cells (HeLa)	OMM	Confocal microscopy (Tom20 co-localization)	Mon *et al*., 2018 [9]
	Human cells (Jurkat, K562)		Immunoblot on affinity-purified mitochondria	Kory *et al*., 2018 [8]
	Human cells (HEK)		SILAC-based proteomics coupled LC-MS/MS	Acoba *et al*., 2020 [15]
SFXN3	Rat embryonic brain cells	IMM	Fractionation, Confocal microscopy (co-localization with COX4), TEM	Rivell *et al*., 2019 [27]
	Human cells (Jurkat, K562)		Immunoblot on affinity-purified mitochondria	Kory *et al*., 2018 [8]
	Human cells (HEK)		SILAC-based proteomics coupled LC-MS/MS	Acoba *et al*., 2020 [15]
SFXN4	Human cells (HeLa)	IMM	Fractionation and protease protection assay	Hildick-Smith *et al*., 2013 [28]
	Human cells (Jurkat, K562)		Immunoblot on affinity-purified mitochondria	Kory *et al*., 2018 [8]
	Human cells (HEK)		SILAC-based proteomics coupled LC-MS/MS	Acoba *et al*., 2020 [15]
SFXN5	Human cells (HEK)		SILAC-based proteomics coupled LC-MS/MS	Acoba *et al*., 2020 [15]
	Mouse astrocytes, human cortex and spinal cord		Immunocapture of GFP-OMM-tagged mitochondria (MitoTag mice), immunostaining	Fecher *et al*., 2019 [29]

145 [1]IMM: inner mitochondrial membrane, IP: immunoprecipitation, OMM: outer mitochondrial membrane, STED: stimulated emission depletion, TEM: Transmission Electron
146 Microscopy, SILAC: Stable isotope labelling of amino acids, LC-MS/MS: Liquid chromatography and tandem mass spectrometry.
147

Because of their predicted structure, showing several hydrophobic alpha helices, and their mitochondrial location, sideroflexins were proposed to be mitochondrial metabolite transporters. Rat Sfxn3 was presumed to be a tricarboxylate carrier (TCC) and, later, Sfxn5 (also known as BBG-TCC) was reported to transport citrate *in vitro* [17,18]. However, it is only recently that a function of mitochondrial serine transporter was reported for SFXN1 [8].

By a bioinformatic analysis, the *S. cerevisiae* Fsf1 (YOR271cp) was proposed to be a candidate alpha-isopropylmalate transporter but no experimental data ascertained this function [19]. Similarly, the predicted Fsf1 protein from *Schizosaccharomyces pombe*, Spac17g6.15c, is annotated as a serine transporter in the database Pombase (https://www.pombase.org/) based on its homology with human SFXN1 [20,21], although it has not been extensively studied.

Since mice lacking Sfxn1 present similar features to that observed in human syndromes caused by a lack of pyridoxine or ALAS2 mutation (X-linked sideroblastic anemia), it was also proposed that Sfxn1 transports pyridoxine (B6 vitamin) inside the mitochondria [1,22]. Since pyridoxine is the precursor of pyridoxal phosphate that serves as a cofactor for ALAS2 (the erythroid specific enzyme catalyzing the first step of heme biosynthesis), SFXN1 could thus directly regulate heme biosynthesis. However, it has been recently reported that human SFXN1 is not able to transport pyridoxine *in vitro* [8]. Even if we cannot exclude that SFXN1 functions in a complex that is not fully reconstituted in *in vitro* assays, SFXN1 may not be the carrier for pyridoxine. Mtm1p, SLC25A39 yeast homologue, was suggested to import pyridoxal 5'-phosphate inside the mitochondria [23,24]. However, the substrate specificity of the SLC25A39 carrier remains unknown [25].

Thus, the main role of Sfxn1 seems to be the mitochondrial serine import. Inside the mitochondrion, Serine can be catabolized by the serine hydroxymethyl transferase (SHMT2) into glycine, an amino acid necessary for ALA synthesis (see below). So, the lack of Sfxn1 would lead to decreased mitochondrial levels of serine and glycine leading to ALA synthesis impairment (see section 4).

1.4. Sideroflexins in disease

Hildick-Smith *et al.* described for the first time a human syndrome (combined oxidative phosphorylation deficiency-18, OMIM entry # 615578), that was directly associated with the lack of a member of the Sfxn family, namely SFXN4 [28]. Patients showed macrocytic anemia and mitochondriopathy non-explainable by other causes but the lack of SFXN4. Recently, a third patient with SFXN4 mutations was described by Sofou *et al* [30]. The three patients with SFXN4 mutations presented with intrauterine growth retardation, mild to severe intellectual disabilities, microcephaly, , neonatal lactic acidosis, macrocytic anemia and severe visual impairment. Sofou *et al* reported optic nerve hypoplasia in the third case. More recently, some of the mechanisms that could explain those effects in humans were reported in the K562 erythroleukemic cell line [31]. Interestingly SFXN4 loss-of-function leads to a general decrease in the levels of the respiratory chain complexes I-IV, which could be explained by an impaired Fe-S cluster synthesis, as evidenced by a Fe-S fluorescence assay (FeSFA). Nevertheless, Sofou *et al.* showed that the effect of SFXN4 decrease would be exclusively in Complex I but not in the rest of the respiratory chain complexes after muscle bi-

opsy [30]. Despite these discrepancies, which could be due to the different nature of the mutations analyzed in each case, it seems clear that Complex I activity is affected in both studies, which reinforces the hypothesis that SFXN4 could have a role, either direct or indirect, on Fe-S biosynthesis.

Besides the description of mutations in the *SFXN4* human gene causing the COXPD18 syndrome, *SFXN4* was also reported to be a predisposition gene for familial colorectal cancer (CRC). Hence, rare *SFXN4* truncating variants were identified in 3/96 CRC familial cases [32]. An aberrant expression of *SFXN1* and *SFXN5* was also reported in patients with breast cancer or gliomas [33,34].

2. Sideroflexins and mitochondrial respiration

2.1. Overview of the mitochondrial respiratory complexes and the place of iron in RC

Oxidative Phosphorylation (OXPHOS) couples the transport of electrons (through a series of mitochondrial respiratory complexes containing redox-active prosthetic groups) to the production of ATP by the mitochondrial ATP synthase, commonly referred to as the complex V of respiratory chain (**Figure 3**). Respiratory complexes (RC) are arranged in supercomplexes (SC) and megacomplexes in the inner mitochondrial membrane [35,36]. The Electron Transport Chain (ETC) comprises four RC (Complex I-IV) containing more than 70 nuclear DNA encoded subunits and 13 mitochondrial DNA (mtDNA) encoded subunits, some of which including iron-sulfur clusters (ISCs) or heme; those iron-containing groups are essential cofactors for electron transport from one complex to another [37,38]. The purpose of this review is not to give an extensive overview of the abundant literature on RC, so we invite the reader to refer to recent reviews for details on the composition, structure and biogenesis of RC [35,38,39].

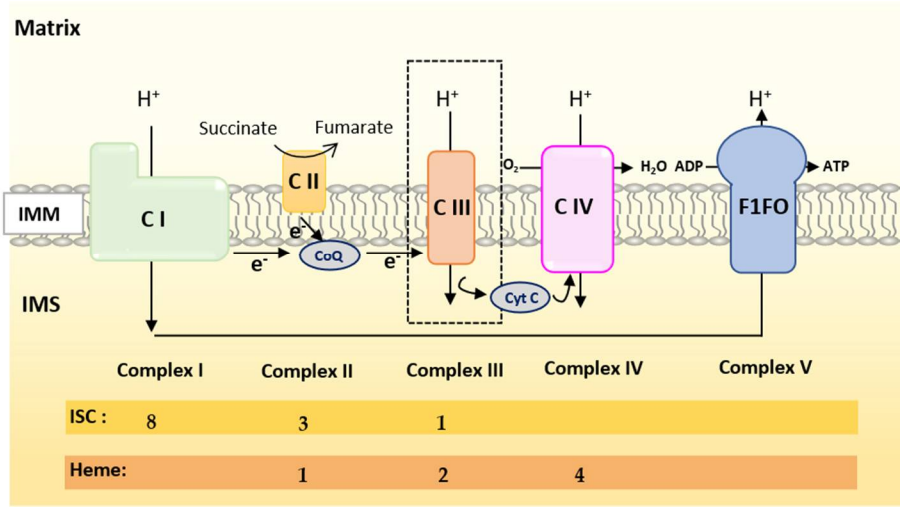

Figure 3. Scheme of the mitochondrial respiratory chain. ISC and heme indicate the respective number of ISC-containing subunits and heme in each complex.

Mammalian Complex I (NADH: Ubiquinone Oxireductase) is a L-shaped megastructure of about 1 MDa comprising 14 core subunits and up to 45 subunits. Among them, five essential subunits (NDUFV1, NUDFV2, NDUFS1, NDUFS7 and NDUFS8) bare the eight ISCs of CI (two [2Fe-2S] and six [4Fe-4S] clusters).

Mammalian Complex II, the smallest of the RC, is composed of only four subunits: succinate dehydrogenase [ubiquinone] flavoprotein (also known as Flavoprotein subunit of complex II, Fp, SDHA), succinate dehydrogenase [ubiquinone] iron-sulfur subunit (a Fe-S protein also named Ip or SDHB), the membrane-anchoring succinate dehydrogenase cytochrome b560 subunit (CybL, SDHC), and finally the succinate dehydrogenase [ubiquinone] cytochrome b small subunit (CybS, SDHD). These subunits are respectively encoded by the *SDHA*, *SDHB*, *SDHC* and *SDHD* nuclear genes. Fp/SDHA and Ip/SDHB are anchored to the IMM thanks to CybL/SDHC and CyBS/SDHD that are the membrane-anchoring subunits of CII. Complex II contains three ICSs ([2Fe-2S], [4Fe-4S] and [3Fe-4S] in SDHB) and a heme shared by SDHC and SDHD.

Mammalian Complex III (also known as bc1 complex) is a dimer made of monomers containing 11 subunits among which three are essential redox subunits: cytochrome b, cytochrome c1 and the Fe-S protein Cytochrome b-c1 complex subunit Rieske (Rieske, ISP, RISP, Rip1 are alternative names that can be found in the literature for this protein). Altogether, these catalytic subunits possess two heme b (Cyt b), a c-type heme (Cyt c1) and a [2Fe-2S] cluster (Rieske) [40]. Heme b is synthesized by Ferrochelatase (FECH) but the mechanism of its insertion into cytochrome b has not been fully elucidated [40].

Mammalian Complex IV contains three mitochondrially-encoded subunits (Cytochrome c oxidase subunit 1, 2 and 3) plus eleven subunits encoded by the nuclear genome. CIV possesses four redox-active metal centers including heme a and heme a3 but no ISCs.

To summarize, Complex I is made of numerous subunits including 8 ISC-containing subunits but none containing heme. Complex IV presents 4 redox-active centers containing heme but no ISC. Both Complexes II and III have ISC and heme containing subunits.

2.2. Current knowledge on the regulation of mitochondrial respiration by SFXN proteins

Kory *et al*. reported decreased basal respiration in SFXN1/SFXN3 double knockout Jurkat cells [8]. Whereas SFXN1 loss alone is not detrimental for respiration of intact cells [8,15], Acoba *et al.* reported a significant decrease in Oxygen Consumption Rates (OCR) of isolated mitochondria from HEK *SFXN1* KO cells with CI, CII and CIII substrates (pyruvate, Glu, Gln, dimethyl- α-ketoglutarate, succinate and glycerol-3-phosphate) [15]. In human embryonic cells, the loss of SFXN1 leads to a marked decrease in the protein levels of three subunits of the Complex III and to a lesser extent in Complex II subunit SDHB (**Table 2**) [15]. SFXN4 KO leukemic cells also showed reduced levels of several RC subunits containing ISCs [31].

Whereas no significant change in the activity of the CI, CII and CIV ETC complexes was observed upon SFXN1 gene knockout in HEK cells, CIII activity was dramatically decreased and partially restored upon SFXN1 overexpression [15]. In agreement with the observed decrease in the levels of cytochrome b (MT-CYB), cytochrome b-c1 complex subunit 2 (UQCRC2) and cytochrome b-c1 complex subunit Rieske (UQCRFS1) subunits, Acoba *et al.* also reported a reduction in CIII2 and in CIII2-CIV subcomplex whereas the assembly of respiratory supercomplexes was unaffected. Mitochondrial translation is not dramati-

289 cally impaired in the absence of a functional SFXN1 protein, neverthe-
290 less a slight decrease in cytochrome b translation was reported in this
291 study.
292 No decrease neither in the quantity of mtDNA nor in the mito-
293 chondrial mass was seen in SFXN1 KO cells, thus a general defect in
294 mitochondrial biogenesis can be excluded [8,15]. Current knowledge
295 on Complex III biogenesis is well-described in [40]. Seven assembly fac-
296 tors are implicated in CIII biogenesis in humans (UQCC1-3, CCHL,
297 BCSL1, LYRM7 and TTC19). The Rieske subunit is first translocated
298 from the cytosol to the matrix where it acquires its ISC and is further
299 incorporated in CIII. In the matrix, Rieske is stabilized by the chaperone
300 LYRM7 [41]. BCS1L is required for the translocation of the folded
301 Rieske iron-sulfur protein in the IMM by a mechanism that remains
302 largely unknown [42]. No regulation of the levels of BCSL1 and LYRM7
303 assembly factors was observed when SFXN1 is absent in mammalian
304 cells [15].
305 Interestingly, HEK *SFXN1*^{KO} cells were reported to have markedly
306 reduced levels of Coenzyme Q (CoQ, ubiquinone), a lipid of the IMM
307 which accepts electron from CI and CII and then donates one electron
308 to the ISC of the Rieske subunit and another one to the heme of the
309 cytochrome b of CIII (see [40] and [43] for more details on the transfer
310 of electrons from CoQ to the IMS soluble electron carrier cytochrome
311 c).
312 Deficiencies of mitochondrial respiration and/or RC activity were
313 also reported for other SFXN, as summarized in **Table 2**. For example,
314 *SFXN2* knockout led to a decreased activity of CII-CIII and CIV [9]. As
315 no specific impairment in complex III activity has been described nor
316 in SFXN2 nor in SFXN4 KO cells, there is presumably no interaction
317 between those SFXN isoforms and the BCS1L protein (responsible of
318 the GRACILE Syndrome), a mitochondrial chaperone which is an-
319 chored to the inner mitochondrial membrane and required for proper
320 Complex III activity [44]. Nevertheless, this possibility cannot be totally
321 discarded, as the patients with S78G point mutation in the BCS1L gene
322 have no decreased Complex III activity when compared with other mu-
323 tations of the same gene.
324

325 **Table 2.** Consequences of SFXN deficiency on the ETC complexes.

SFXN	Model	Complex	Data	Reference
SFXN1	HEK *SFXN1*^{KO} cells HeLa *SFXN1*^{KO} cells	CI	No significant loss of activity SDHB ↓	Acoba *et al*, 2020 [15]
		CII	No significant loss of activity UQCRC2 ↓↓ UQCRFS1 ↓↓	
		CIII	Cytochrome b ↓↓↓ Significant loss of activity Reduced levels of $CIII_2$ and $CIII_2$-CIV respiratory complexes	
SFXN2	HEK *SFXN2*^{KO} cells	CI	No significant loss of activity	Mon *et al*, 2019 [9]
		CII- CIII	Significant loss of activity	
		CIV	Significant loss of activity	
SFXN3	*SFXN3* KO mouse	CI, CIV	No significant loss of activity	Amorim *et al*, 2017 [45]
SFXN4	Primary fibroblasts from two individuals with *SFXN4* mutations	CI+CIII	Decreased activity	Hildick-Smith *et al*, 2013 [28]
	SFXN4 KD zebrafish	CI CI+CIII	Decreased activity	Sofou *et al*, 2019 [30]
	K562 *SFXN4*^{KO} cells	CI CII CIII CIV	NDUFB8 ↓ SDHB ↓ UQCRC2 ↓ COX2 ↓	Paul *et al*, 2019 [31]
SFXN5			N.A.[1]	

326 [1] NA: Not addressed.

327

3. Which place for Sideroflexins in the regulation of mitochondrial metabolism?

3.1. Sideroflexins and one-carbon metabolism (OCM)

Using CRISPR/Cas9 based-screening, Kory *et al.* uncovered a function of mitochondrial serine transporter for SFXN1 [8]. The import of serine inside mitochondria is a key step of the OCM, a major metabolic pathway coupled to the synthesis of methyl donors necessary for purine synthesis, epigenetic methylation processes and synthesis of neurotransmitters [46]. Moreover, glycine - arising from serine catabolism by the SHMT2 enzyme [47] - is a key amino acid for the synthesis of heme, a cofactor present in cytochromes of the respiratory chain and other essential proteins, such as CYP450 proteins. Finally, OCM is known as a central pathway ensuring hyperproliferation of cancer cells. Hence OCM, through the folate cycle, links serine catabolism to purine and nucleotides biosynthesis. Liver, kidney and blood are tissues with high OCM activity, however OCM role is not restricted to these organs but present in all human tissues including brain [46]. Actually, defective one-carbon metabolism during embryonic development is responsible for neural tube defects.

Whereas Jurkat cells lacking SFXN1 proliferate as wild-type cells do, their proliferation rate is markedly reduced in a medium lacking serine but is normal in the absence of glycine that can be provided by the catabolism of serine [8]. A lower proliferative rate compared to that of wild-type cells was also reported for HEK *SFXN1* KO cells in the absence of serine. Interestingly, proliferation of SFXN1 deficient cells was enhanced when formate (OCM metabolite), but not hemin (heme derivative), was added [15]. Additionally, Kory *et al.* showed that the double knockout of SFXN1 and SFXN3 greatly impaired proliferation in a glycine-deficient medium. Apart from human *SFXN4*, overexpression of virtually any *SFXN* family member including *S. cerevisiae* FSF1/YOR271C and the two *Drosophila* orthologues *dSfxn1* and *dSfxn2* can rescue the glycine auxotrophy due to the OCM defect induced by the concomitant loss of SFXN1 and SFXN3 in human leukemic cells. However, the defect in purine synthesis is rescued only by SFXN2, SFXN3, *dSfxn1* and *S. cerevisiae* FSF1 [8]. Thus, most SFXN appear functionally redundant in serine import although probably with different kinetic properties. Moreover, they might also ensure the mitochondrial import of other metabolites.

3.2. Sideroflexins in central carbon metabolism

Disturbance of central carbon metabolism was reported in SFXN1-null cells. A LC-MS analysis of tricarbolylic acid (TCA) cycle metabolites contained in HEK *SFXN1* KO cells showed significantly reduced levels of citrate and isocitrate while α-ketoglutarate (α-KG) was decreased and succinate cellular levels were unchanged [15]. Isotopic labelling experiments helped understanding the role of SFXN1 in mitochondrial metabolism. ^{13}C metabolic flux analysis (^{13}C MFA) is a useful tool to assess intracellular fluxes and get clues on the metabolic pathways that are differentially activated in mammalian cells depending of the genetic context or environmental conditions [48]. Using ^{13}C MFA to investigate metabolic fluxes in HEK *SFXN1* KO cells, Acoba *et al* provided evidence for a reduced activity of the glutamate dehydrogenase (GDH) that converts Glu in α-KG using NAD(P)+ as a coenzyme [49,50]. The lower activity of GDH is unlikely due to a lowering in

NAD(P)+ since NAD(P)+/NADPH ratio was unchanged in SFXN1-deficient cells [15]. In animals, GDH is regulated by a wide variety of ligands (NADH, GTP, ATP, palmitoyl-coA, steroid hormones, leucine) and the mitochondrial enzymes SIRT4 and SCHAD. Alanine aminotransferase (ALT) activity is also markedly reduced in SFXN1-null cells [15]. This deficiency in alanine catabolism is probably due to the lower availability of α-KG in SFXN1-null cells. Alanine aminotransferase (also known as GPT) is implicated in L-alanine degradation via transaminase pathway and uses pyridoxal 5'-phosphate as a cofactor. A comprehensive review of nitrogen utilization and amino acid metabolism can be found in [51]. Mitochondrial levels of GDH and ALT2 (mitochondrial alanine aminotransferase) were not investigated in SFXN1-deficient cells and we wonder if the absence of SFXN1 could trigger a decrease in the mitochondrial import or stability of some mitochondrial enzymes intervening in the catabolism of amino acids that fuel the TCA cycle. Acoba *et al* also performed ^{13}C MFA with [U-^{13}C]-glucose to fuel the TCA cycle with [U-^{13}C]-labelled acetyl-coA and provided evidence for an increase in the incorporation of glucose in the TCA cycle [15].

NAD^+/NADH ratio was also increased in SFXN1 KO cells and, altogether, the results obtained by Acoba *et al* shed light on a disturbance of central carbon metabolism upon the loss of SFXN1. Whether the deficiency in SFXN1 orthologues and the other human sideroflexins also affects central carbon metabolism is an open question that is not fully elucidated.

4. Sideroflexins, iron homeostasis and heme biosynthesis

4.1. A brief overview of iron homeostasis, ISCs and heme biosynthesis

Iron is an essential cofactor for several enzymes involved in redox reactions due to its ability to exist in two ionic forms: ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}). Iron is thus easily oxidized and reduced which makes it suitable for redox reactions. Thus, iron is a key player in many important cellular processes, including energy metabolism, respiration and DNA synthesis. The implication of iron in all these processes is done through the incorporation of this atom in complex structures synthesized mainly in the mitochondria: iron-sulfur clusters and heme. Iron homeostasis is a tightly controlled process in which numerous proteins intervene [52–55]. **Figure 4** depicts the main actors of iron trafficking and metabolism at the cellular level.

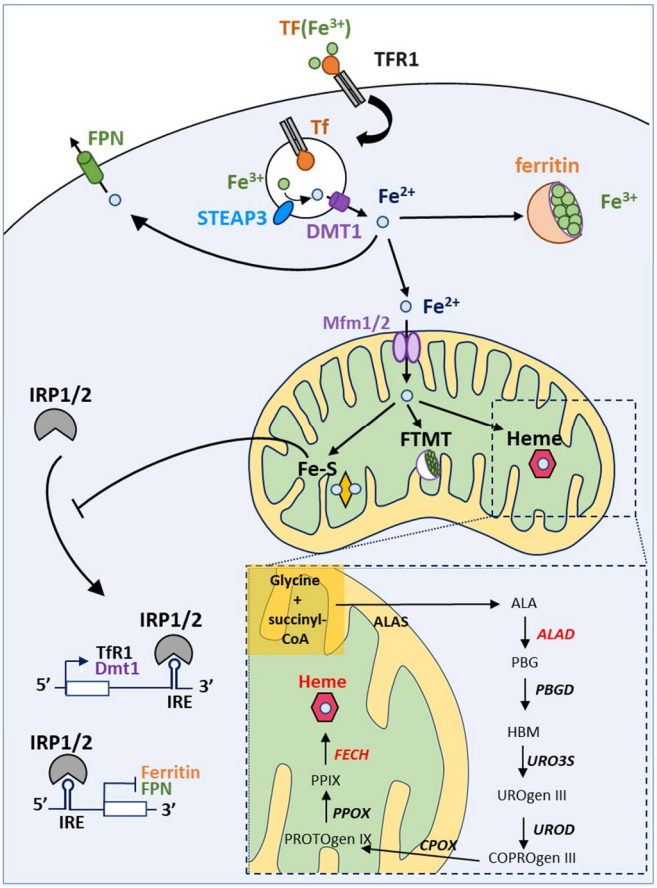

Figure 4. Iron homeostasis and utilization at the cell level. Iron cellular uptake is controlled by transferrin and its receptor (TF and TFR1, respectively). Afterwards, in the endosome, iron is reduced thanks to the action of STEAP3 (which converts the insoluble Fe^{3+} to soluble Fe^{2+}) and released from the endosome into the cytoplasm by the DMT1 channel. Free iron can be stored by ferritin in the cytoplasm or can be transported into the mitochondria, thanks to Mitoferrin 1 and 2 transporters (Mfrn1/2). Excess of iron is released out of the cell by Ferroportin (FPN). Inside the mitochondrion, iron can be stored in FTMT (mitochondrial ferritin) or incorporated in heme or Fe-S clusters. IRP1 and 2 (Iron Related Protein 1 and 2) are the major regulators of iron metabolism. In iron-depleted cells, IRP1 can bind IRE (Iron Response Elements) motifs to promote or repress mRNA translation. If IREs are located in the 5'UTR, IRP1 binding represses mRNA translation under low iron levels. On the contrary, transcripts with IREs at the 3'UTR are stabilized and translated upon IRP binding. Hence, low iron levels lead to decreased Ferritin and FPN levels but promote TFR1 and DMT1 synthesis. High levels of iron prevent IRP1 binding to IREs (see main text for details).

Maintaining iron homeostasis is essential for cell viability and iron intracellular levels are thus tightly controlled by Iron Regulatory Proteins (IRP1 / 2). IRP1/2 regulate the levels of key proteins intervening in iron homeostasis by binding to Iron Responsive Element (IRE) sequences either located in the 5'UTR or in the 3'UTR of mRNA encoding actors of iron metabolism. For example, when cellular iron levels are low, IRP proteins bind to IRE in the 5' UTR of ferritin and ferroportin mRNAs (among others) and thereby inhibit their translation. The IRPs proteins can also bind to IRE in the 3'UTR of iron-regulated mRNAs, such as TfR1 and DMT1 mRNAs encoding two proteins involved in iron uptake, thereby preventing endonuclease-mediated degradation of these mRNAs (see [56] for a review). Thus, this regulation by IRP

proteins under low iron concentration leads to an increase in iron uptake as well as a decrease in iron storage and export. On the opposite, under high iron levels, the synthesis of iron-sulfur clusters is enhanced. The binding of an iron-sulfur cluster to the IRP1 protein leads a conformational change inhibiting its IRE binding activity but promoting its aconitase activity. The ACO1 enzyme (*e.g.,* Fe-S bound IRP1) catalyzes the conversion of citrate and isocitrate in the cytosol enhancing, probably, NADPH generation and lipid synthesis [57]. Our aim is not to give an extensive review of the IRE-IRP signaling pathway and numerous comprehensive reviews can be found elsewhere, such as in [58].

Iron-sulfur clusters are made up of iron and sulfur ions which come together to form [1Fe-0S], [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters [59]. Fe-S clusters (ISCs) are found in numerous metalloproteins such as aconitase 1 [54,60–62]. Thus, ISCs are involved in a wide variety of cellular processes among which we can cite the Krebs cycle, mitochondrial respiration, DNA replication / repair. Assembly of the Fe-S center is carried out by the ISC machinery. Inorganic sulfur is first produced from the cysteine by the cysteine desulfurase NFS1. Then, the Fe-S cluster is formed on the ISC assembly enzyme (ISCU) with the help of frataxin (FXN) [63].

Heme is a complex of ferrous iron and protoporphyrin IX (PPIX). It is an important prosthetic group for many vital proteins, such as hemoglobin, myoglobin, cytochromes and CYP450 proteins [64,65]. Heme is involved in the transport and storage of oxygen, the transfer of electrons for enzymatic redox reactions, signal transduction, ligand binding and control of gene expression [66]. Heme biosynthesis (**Figures 4, 6**) is a pathway comprising eight steps, among which four arise inside the mitochondrion (*e.g.* the first and the last three steps). The rate limiting enzyme of this process is the ALA-synthase (ALAS) responsible for the synthesis of δ-aminolevulinic acid (ALA) from the condensation of glycine and succinyl-CoA, in the presence of pyridoxal-5'-phosphate [67,68]. Two genes encode ALA-synthases: *ALAS1* is the ubiquitously expressed one while *ALAS2* expression is restricted to erythroid cells. Negative feedback regulation of ALAS1 by heme has been reported and will be discussed later. Ferrochelatase (FECH) catalyses the last step of heme biosynthesis, namely the insertion of iron into PPIX. Heme biosynthesis has been extensively reviewed elsewhere [52,55,69].

4.2. Can sideroflexins regulate iron homeostasis ?

The first evidence for a link between sideroflexins and iron metabolism came from a study of the *flexed-tail* mouse, which harbors a mutation in a locus containing the *Sfxn1* gene [1]. Mice mutant for Sfxn1 displayed sideroblastic anemia, microcytic anemia and hypochromic erythrocytes. Furthermore, *flexed-tail* mice were also displaying iron deposits in the mitochondria from erythrocyte precursors. Nevertheless, no mechanisms regarding the iron accumulation in the mitochondria were proposed; but since then, sideroflexins were annotated as proteins implicated in iron metabolism.

Based on the annotation of SFXN as transporters of metabolites required for iron metabolism, we and others have tried to monitor the consequences of the loss of SFXN on iron cellular levels. **Table 3** summarizes the experimental evidence for an iron imbalance in the absence of SFXN. Whereas Mon *et al.* reported increased mitochondrial iron levels in HEK SFXN2 KO cells [9], an ICP-MS analysis did not show significantly modified cellular or mitochondrial iron levels in HEK *SFXN1*

KO cells compared to parental cells but an increase in cellular Mn^{2+} [15]. Of note, albeit not significant, it seems that the loss of SFXN1 also slightly enhanced mitochondrial iron levels measured by ICP-MS, with a more pronounced effect in one of the two SFXN1-KO clones [15]. Maybe, a significant increase could have been seen with more replicates or by quantifying mitochondrial iron by a TEM-EDX analysis as done for SFXN4 KO cells [31]. Despite an appropriate methodology, caution must also be taken when analyzing the results obtained by Mon *et al* because this study was done with only one cellular clone obtained after CRISPR/Cas9 invalidation of the *SFXN2* gene. However, expression of a SFXN2-mCherry fusion protein restored basal mitochondrial Fe^{2+} levels in these SFXN2 KO cells, as measured with a specific fluorescent probe. Loss of SFXN4 was also proven to alter iron levels in the K562 leukemic human cell line. Whereas labile cytosolic iron pool was decreased, Paul *et al*. have provided evidence for a redistribution of cellular iron from the cytosol to the mitochondria in K562 *SFXN4* KO cells [31].

In our lab, we are interested in the early events triggered by the depletion of SFXN1 in mammalian cells. To investigate the effect of a decrease in SFXN1 protein levels, we chose to transiently deplete SFXN1 in HT1080 human cells using siRNA and then, we quantified mitochondrial labile Fe(II) levels using the MitoFerro-Green probe [70]. Depleting SFXN1 in HT1080 cells induced a slight but reproducible increase in mitochondrial iron levels as shown in **Figure 5**. This increase in mitochondrial Fe(II) when SFXN1 levels are lowered, could be either a consequence of a defective heme biosynthesis, since Fe(II) is the substrate of FECH that inserts it into protoporphyrin IX, or a consequence of the catabolism of heme by HO-1 (heme oxygenase-1). Additionally, our data enlighten an erastin-dependent increase in labile Fe(II) mitochondrial levels in HT1080 cells. A similar increase was also reported in erastin-treated MEF cells [71]. In HT1080 and MEF cells, erastin was previously shown to induce HO-1 expression [72,71], which may explain the increase in mitochondrial Fe(II) that we observed in erastin-treated H1080 cells. Whether reducing SFXN1 levels inhibits FECH activity or promotes heme catabolism must be further investigated.

Altogether, the evidence enounced above point towards a role for SFXN in the maintenance of appropriate iron levels since the depletion or loss of SFXN1, SFXN2 and SFXN4 may increase mitochondrial iron by mechanisms that remain unknown. Mitoferrin1 and Mitoferrin2 are known as iron importers into the mitochondria and ABCB8 as an iron exporter [73]. Thus, due to the fact that iron mitochondrial transporters have been already described, and that the lack of either SFXN 1, 2 or 4 leads to intramitochondrial iron accumulation, we do not favor the possibility that SFXN are iron transporters. So, other intriguing possibilities should be explored.

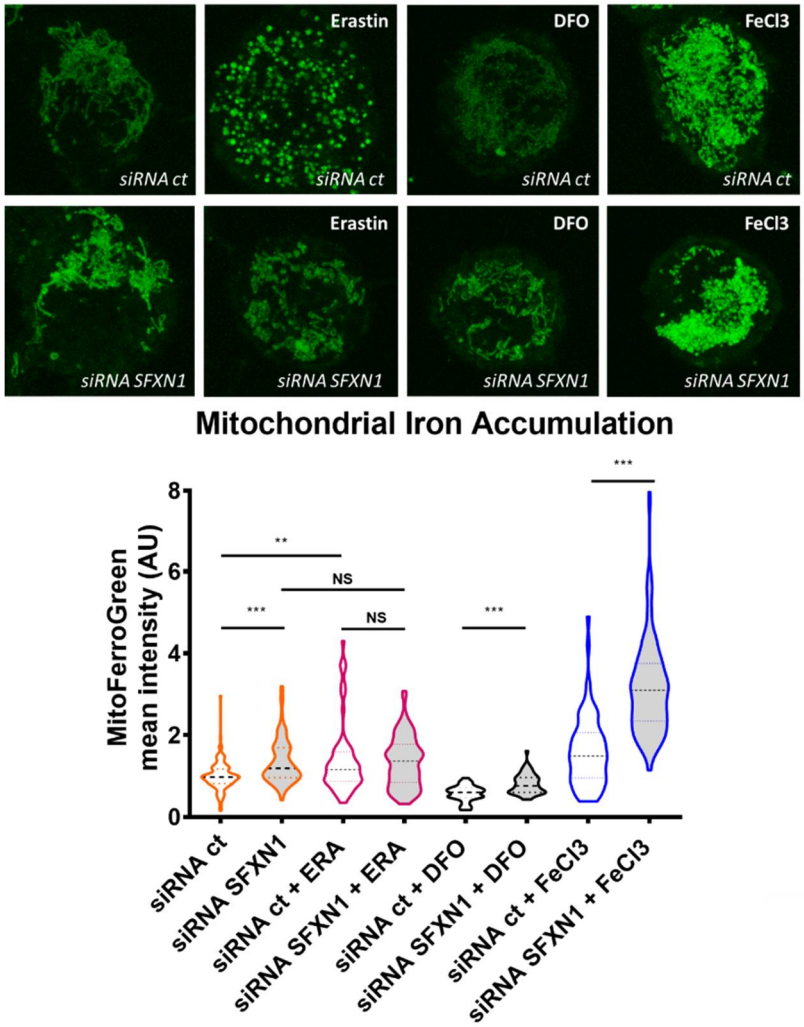

Figure 5. Depleting SFXN1 in HT1080 human cells leads to an intramitochondrial iron accumulation. *Top panel*: mitochondrial labile Fe(II) staining using the MitoFerro-Green probe [70] after transient transfection with a control siRNA (siRNA ct) or a pool of SFXN1-targeting siRNA (siRNA SFXN1). Cells were further treated with DMSO (vehicle), erastin, DFO or $FeCl_3$. Erastin is a drug that is widely used to trigger ferroptosis, DFO (deferoxamine) is an iron chelator that lowers mitochondrial iron levels and is used as a negative control. $FeCl_3$ increases intracellular iron levels and served as a positive control. SFXN1 depleted cells show higher mitochondrial iron levels than control cells (siRNA scramble transfected cells). Erastin promotes iron accumulation. In control cells (siRNA ct), erastin increases mitochondrial iron levels and a punctuate staining is seen, maybe revealing mitochondrial network fission. In SFXN1 depleted cells, erastin does not seem to further increase mitochondrial iron levels. In all conditions except with erastin, iron levels are increased after SFXN1 depletion, compared to control, suggesting that erastin and SFXN1 could use the same mechanisms to lead to an increase in mitochondrial iron. Same magnification is used for all images. *Bottom panel*: quantification of three independent assays (n>50 cells per condition) in which fluorescent signal is measured and values are normalized to siRNA ct mean levels (mean =1). After Mann-Whittney tests, significant differences are shown (** $p<0.01$, *** $p>0.001$, NS Not Significant). *See Appendix A.2 for experimental details.*

Proper iron homeostasis requires a fluid transport of iron and its derivatives through the mitochondrial membranes and the cytosol. In this regard, the ALA (Aminolevunilic acid) synthesis requires Gly import through SCL25A38 on the one hand, and ALA export on the other

hand, presumably through the same transporter (**Figure 6**) [74]. SFXN1 was shown to be a Serine transporter *in vivo* [8]. Intramitochondrial Ser would be catabolized by SHMT2 into Gly and 5,10-meTHF (5,10 methyl tetrahydrofolate) to enter in the OCM pathway, necessary for purine synthesis, pointing out that SFXN1 could be linked to the first and limiting step of heme synthesis. Moreover, once protoporphyrin is generated in the intermembrane space, it must enter the mitochondrial matrix for the last heme synthesis step using both ABCB6 and ABCB10 transporters [75,76]. Anyway, it cannot be excluded that other sideroflexins could be involved in this event. Whether SFXN1 could bind to heme and help in its trafficking is another hypothesis that merits our attention. We thus seek for heme binding motifs (HBMs) in SFXN1 with the SeqD-HBM tool dedicated to the prediction of heme-coordination sites in protein sequences [77,78] and we found four HBMs that are solvent-accessible (**Figure S1, Appendix A**). These predicted HBM may permit transient interactions between heme and SFXN1. Biochemical studies are needed to confirmed these interactions and further investigate their significance regarding SFXN1 activity.

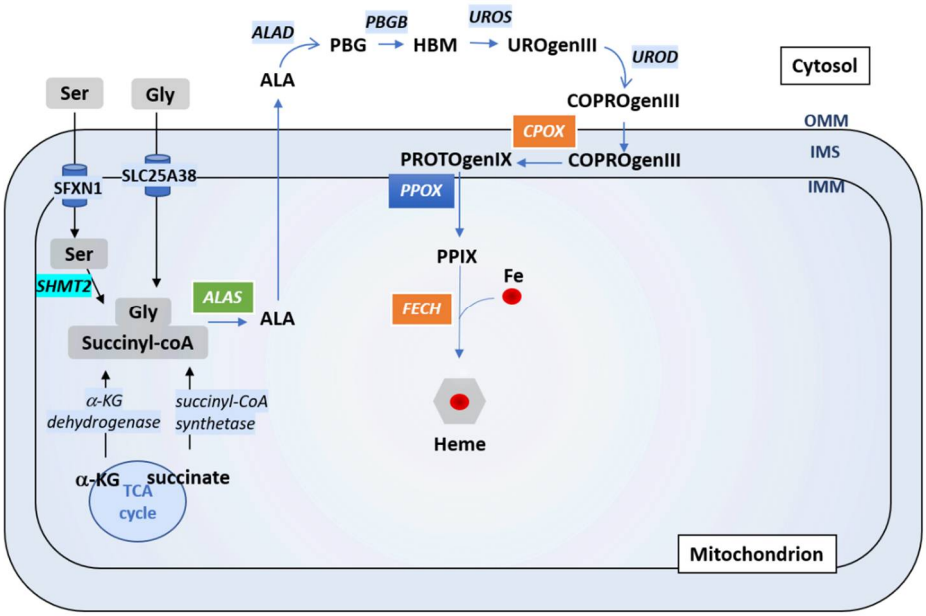

Figure 6. Regulation of heme biosynthesis by SFXN1. Gly and succinyl CoA are the substrates to generate ALA, the first heme precursor, thanks to ALAS enzyme. Gly can enter directly into the mitochondria by SLC25A38, or can be the result of Ser transformation (previously imported by SFXN1) by SHMT2. ALA is further exported to the cytosol where the next steps of heme biosynthesis are catalysed by ALAD, PBGB, UROS and UROD. CPOX, PPOX and FECH are the three mitochondrial enzymes that catalyze the three last steps of heme synthesis (see main text). The last step corresponds to the incorporation of iron into the protoporphyrin PPIX to complete the heme synthesis. Cells lacking SFXN1 show decreased CPOX and FECH mRNA and protein levels (orange box), but higher amount of ALAS protein (green box), according to Acoba *et al*. [15].

To conclude, several open questions are remaining about the role of SFXNs in iron homeostasis. For example, are SFXN3 and SFXN5, like SFXN1, 2 and 4, able to regulate iron levels? No studies have been performed in this regard yet. Do sideroflexins alter iron levels by regulating the activity of other regulators implicated in iron homeostasis?

How can we explain that low SFXN1 levels (as well as low levels of SFXN2 or 4) lead to an increase of mitochondrial iron, and that an increase in SFXN1 may also trigger an increase mitochondrial iron level (see section 5.2)? What are the relationships between iron homeostasis disturbance and one carbon metabolism? To answer those questions, further work in mammalian cells is needed, and later confirmed using *in vivo* models.

4.3. Which role for sideroflexins in heme biosynthesis and ISC biosynthesis ?

Whether SFXN1 and its homologues can regulate heme biosynthesis has not been thoroughly investigated so far, but recent studies gave evidence for an impairment of heme biosynthesis when certain members of the SFXN family are lacking [9,15,31]. Interestingly, SFXN1 loss in human kidney embryonic cells was recently reported to impair heme biosynthesis [15]. Indeed, cells lacking SFXN1 showed reduced heme levels, decreased CPOX and FECH transcripts and protein levels but increased ALAS1 protein levels. It is well-known that heme can induce ALAS1 degradation by a mechanism involving, at least, ALAS1 binding to the mitochondrial protease CplXP [79]. It is thus likely that low heme levels found in SFXN1 cells limits heme binding to ALAS1 and consequently inhibits its degradation by CplXP. These defects in heme biosynthesis may explain the less efficient mitochondrial respiration and, especially, Complex III loss of activity. Accordingly, whereas formate had no effect, hemin supplementation increased CIII activity in wild-type and *SFXN1* KO cells but only partially restored the assembly of CIII in *SFXN1* KO cells [15]. However, hemin was unable to restore basal levels of Complex III subunits in HEK *SFXN1* KO cells suggesting that other defects are present in these cells. Interestingly, DMK (dimethyl- α-KG, a cell permeant analogue of α-KG) rescued almost totally CIII subunits levels and CIII activity in HEK *SFXN1* KO. Succinyl-coA that serves in the first step of heme biosynthesis can originate from α-KG or succinate. Hence, in the mitochondrial matrix, α-KG can be converted in succinyl-coA by α-KG dehydrogenase, a highly regulated enzyme of the TCA cycle [80]. It would thus be interesting to determine if, additionally to the decreased GDH and ALT activity observed in SFXN1 null cells [15], α-KGDH activity is also impaired upon the loss of SFXN1.

646 **Table 3.** Regulation of systemic or cellular iron levels by SFXN.

Protein	*Model*	*Evidence*	*Methodology*	*Reference*
SFXN1	Mouse	Iron overload in mitochondria of erythrocytes in the *flexed-tail* mouse	Iron mitochondrial staining	Fleming *et al*, 2001 Acoba *et al*, 2020
	HEK SFXN1 ^{KO} cells	Increased mitochondrial iron	ICP-MS	
SFXN2	HEK SFXN2 ^{KO} cells	Increased mitochondrial iron levels	ICP-MS MitoFerro-Green staining and confocal microscopy	Mon *et al*, 2019
SFXN3	Mouse Sfnx3 KO	Decreased circulating iron levels in male transgenic mice homozygous for the Sfxn3$^{tm1b(KOMP)Wtsi}$ allele	Biochemical assay	The IMPC database [2]
SFXN4	K562 SFXN4 ^{KO} cells	Decreased labile iron pool Increased mitochondrial iron levels	Indirect biochemical measure based on the dequenching of calcein upon release of iron TEM-EDX	Paul *et al*, 2019
SFXN5	-	-	-	-

647 [1] ICP-MS is for inductively coupled plasma atomic emission - mass spectrometry, TEM-EDX is for Transmission electron microscopy-Energy dispersive X-Ray analysis. [2]
648 website page for SFXN3 : https://www.mousephenotype.org/data/genes/MGI:2137679#phenotypesTab.
649

Impairment of heme biosynthesis upon SFXN loss could be explained by the function of serine transporter attributed to SFXN. Following its import into the mitochondrion, Ser can be converted in Gly and 5,10-me-THF involved in folate cycle and OCM. An imbalance in the cellular Ser/Gly ratio may impair heme biosynthesis since Gly is (with succinyl-coA) the precursor for the synthesis of protoporphyrins into which iron is incorporated in the final step of heme synthesis catalyzed by FECH (**Figure 6**). As SFXN1 is presumed to be the mitochondrial transporter of Ser, its loss could increase cellular Ser and lower Gly levels. Indeed, in Jurkat and K562 *SFXN1* KO cells, the cellular Ser/Gly ratio was increased and associated to increased cellular Ser levels but decreased Gly levels [8]. In agreement with an imbalance in serine levels upon SFXN1 loss, HEK SFXN1 KO cells also have increased cellular Ser levels and Ser/Gly ratio but no decrease in Gly cellular levels were reported [15]. Whether this discrepancy can be explained by a cell type specificity or other reason remains to be elucidated. Of note, mitochondrial levels of those two amino acids have not been assessed and it will be interesting to more specifically address the presence of Ser and Gly inside the mitochondrion by a metabolomics study on this organelle.

SFXN2 has been recently described in HEK293 cells to have a key role in iron metabolism, mainly in heme synthesis [9]. High levels of iron have been shown in mitochondria in SFXN2 knockout HEK293 cells. Also, a decreased activity of Complexes II-IV but not of the Complex I was noticed. Complex I subunits contain Fe-S clusters, in contrast to Complex IV, which is mainly composed by heme groups. Complexes II and III contain both Fe-S clusters and heme groups (**Figure 2**). Thus, as no effect in Complex I was detected, and no decrease in Frataxin (FXN), a mitochondrial enzyme required for the Fe-S cluster formation, nor in ALAS2, the enzyme that catalyzes the first step of the heme biosynthetic pathway, was reported, it was concluded that SFXN2 mutants affected heme synthesis after the first step of heme biosynthesis, but not the Fe-S cluster formation. However, neither the levels of ISC-containing proteins nor those of ALAS1 have been assessed in this study. It is surprising because ALAS2 is the erythroid specific form and ALAS1 the housekeeping one.

We propose few possibilities to explain *SFXN2* knockout cells phenotype. The lack of SFXN2 could either lead to an impaired ALA export or no mitochondrial import of protoporphyrin (PPIX) for the last step of the heme pathway. A defective mitochondrial export of the heme groups is another plausible explanation. Finally, other options could be possible as an interaction of SFXN2 with BCS1L, a chaperone anchored to the inner mitochondrial membrane that is required for proper assembly of the Complex III (see section 2.2 for more details). In all those cases, an intramitochondrial iron accumulation is presumed. All those possibilities, and others, must be studied to be able to clarify the possible role of Sfxn2 in heme biosynthesis.

4.4. Which role for sideroflexins in ISC biosynthesis ?

Loss of SFXN also seems to impair ISC biogenesis. Indeed, in the absence of SFXN4 there is a decrease in Fe-S cluster levels which is consistent with the decrease of Complex I activity seen in SFXN4 KO cells, pointing out that this SLC56 carrier could play a role in Fe-S biosynthesis [30,31]. As a consequence of the low Fe-S levels, IRP1 aconitase activity, as well as labile iron cytosolic levels, also decreases, whereas mitochondrial iron increases, suggesting that iron import in the mitochondria is not impaired, and instead possibly enhanced. Those features are very similar to the lack of mitochondrial frataxin, which leads to Friedreich's Ataxia, also known as X-linked sideroblastic anemia. Frataxin (FXN) is a mitochondrial chaperone that interacts with aconitase in a citrate-dependent manner to convert (3Fe-4S)1+ inactive enzyme into [4Fe-4S]2+ active one within the Krebs cycle. It also interacts with the ISCU-NFS1 (Iron-Sulfur Cluster Scaffold-Cysteine desulfurase) in the final steps of Fe-S formation [81,82]. The reduction of mitochondrial aconitase (ACO2) in SFXN4 KO cells [31] suggests that SFXN4 could participate in the Fe-S biosynthesis maybe through an interaction with Frataxin (FXN). It has been previously reported that FECH, an important enzyme for heme biosynthesis, Mfrn1, an iron transporter into the mitochondria, and ABCB10, a protoporphyrin IX transporter, could form a complex in

mouse erythroleukemia (MEL) cells to direct iron incorporation into protoporphyrin to form heme [54,83]. Taken together, those results open the possibility that SFXN4 and FXN interact with other proteins such as aconitase or the ISCU-NFS1 multimeric complex to maturate the Fe-S clusters. We have recently performed a screen with the aim to identify the direct partners of SFXN1 protein in MCF7 cells (Tifoun et al., in preparation) and, even though Sfxn1 does not interact directly with FXN, it is still possible that Sfxn4 could do so. In Sfxn4 mutants Fe-S synthesis is reduced, pointing out that Sfxn4 may play a role in the first steps of Fe-S cluster formation, maybe through FXN interaction. A recent study shows that the ISC (Iron Sulfur Cluster, composed by NFS1, ISCU and FXN) function requires L-Cysteine to generate de disulfide groups necessary to form the Fe-S clusters [84]. Moreover, it has been postulated that SFXN1 could transport not only serine, but alanine and possibly also glycine and cysteine *in vitro* [8]. Actually, in SFXN1 depleted cells have a proliferative advantage in media containing low cystine (dimer of cysteine formed under oxidant conditions), this could be due to the fact that the amino acid cysteine is necessary for cytosolic glutathione synthesis and that a loss of mitochondrial import would increase its availability for those purposes [8]. The lack of SFXN1 activity can be overcome by SFXN2 and SFXN3 but not by SFXN4 [8]. SFXN4 cannot substitute SFXN1 for Ser import into the mitochondria, but it could maybe have a higher affinity for Cys. This may explain why SFXN1 and SFXN2 mutants present mainly problems in heme synthesis whereas SFXN4 KO cells have deficiencies in Fe-S cluster formation, as Ser and Gly are essential for the ALA synthesis and Cys is required for proper Fe-S maturation.

How could SFXN regulate iron levels and heme biosynthesis remains unanswered and whether SFXN impair mitochondrial iron and heme homeostasis by direct or indirect actions is unknown. We have recently documented the interaction between SFXN1 and ATAD-3 (Tifoun *et al*, in preparation). Because *Caenorhabditis elegans* ATAD-3 was shown to modulate mitochondrial iron and heme homeostasis, heme biosynthesis regulation by SFXN1 may depend on its interaction with ATAD-3. Interestingly in *atad-3* (RNAi) worms, mitochondrial but not cytosolic iron levels were increased and an altered expression of iron homeostasis genes was reported [85]. Indeed *atad-3* knockdown (KD) led to an increase in *ftn-1* but a decrease in *ftn-2* mRNA (respectively encoding the intestinal ferritin heavy chain and a more ubiquitous one). a*co-1 (encoding* the homologue of the mammalian IRP responsible of the post-translational regulation of ferritin), *fpn-1.1 (encoding a C. elegans ferroportin* homologue) *and smf-3 mRNA (involved in the cellular uptake of non-heme iron) were reduced. Expression of mfn-1 (the sole Mitoferrin encoding gene in C. elegans) was unchanged upon atad-3 knockdown. In agreement with a mitochondrial iron overload, atad-3 KD in worms also led to an accumulation of Hemin (a heme-containing protein involved in erythroid differentiation) and a fluorescent analogue of heme.*

Interestingly, a new mutation of ATAD3A (Arg528Trp), which has been described in 7 families [86], is responsible of developmental delay, hypotonia, optic atrophy, axonal neuropathy and hypertrophic cardiomyopathy. In some of those individuals, a deficiency of complex III and citrate synthase was detected. Those results look similar to the consequences of the lack of SFXN1 or SFXN4 proteins. ATAD3A, being a transmembrane protein that binds both external and internal mitochondrial membranes, could interact with SFXN1 and/or SFXN4 to control iron metabolism. Moreover, the use of *Drosophila* in this study, allowed to see that either lack of *bor* (*belphegor*, ATAD3A homologue), either the expression of a R534W form, a variant of Arg528Trp human ATAD3A, in the larval neuromuscular junctions (NMJ) promoted a decreased of mitochondrial content, aberrant mitochondrial morphology and increased autophagy. Complementary, *bor* overexpression promoted larger and elongated mitochondria in the NMJ. Whether SFXN family has a role in autophagy remains completely unexplored and merits attention.

5. Sideroflexins, ferroptosis and ferritinophagy

5.1. SFXN, cell death and ferroptosis

Growing evidence support the key role of iron metabolism in ferroptosis, even if the exact mechanisms are not fully elucidated [87]. Ferroptosis is a physiological cell death contributing to tissue homeostasis and implicated in pathology (cancer, neurodegenerative disease and cardiac injury). Mechanistically, ferroptosis is an iron-dependent but caspase-independent regulated cell death (RCD) triggered by uncontrolled lipid peroxidation leading to dramatic morphological changes in mitochondria. For recent reviews on the place of mitochondria in ferroptosis regulation, the reader is invited to refer to [88,89]. Ferroptosis can be triggered by diverse drugs such as erastin, RSL3 or FIN56, among many others, and this type of RCD is prevented by iron chelators and antioxidants [90]. The mitochondrion appears as a main contributor to ferroptosis because of its central place in iron metabolism and the fact that several mitochondrial metabolic pathways – including TCA cycle, and ETC - contribute to PL-PUFA (polyunsaturated fatty acid containing membrane phospholipids) peroxidation.

Few data are available on the role of SFXN in cell death and ferroptosis regulation. *SFXN4* gene knockout was reported to promote cell death of K562 human cells in galactose-containing medium, together with an increase in caspase 3/7 activity [31]. Whether the loss of SFXN4 triggers ferroptosis was not investigated to our knowledge. Interestingly, in HEK kidney embryonic cells, *SFXN2* gene knockout seems to sensitize cells to erastin-induced cell death however, the underlying mechanisms were not deeply investigated [9].

Recently, SFXN1 was showed to participate in LPS-induced ferroptosis in H9c2 cardiomyocytes, a process depending of NCO4A-mediated ferritinophagy [91]. Li *et al* showed an LPS- and NCOA4-dependent upregulation of SFXN1 and documented the role of SFXN1 in LPS-induced ferroptosis. Briefly, in LPS-treated H9c2 cardiomyocytes cells, knockdown of SFXN1 increased cell viability, restored intramitochondrial basal levels, inhibited mitochondrial ROS production, decreased lipid peroxidation and levels of PTGS2 (also known as cyclooxygenase-2) and MDA. Collectively, these data suggest that SFXN1 promotes LPS-induced ferroptosis, however the molecular mechanisms are far from being clear. Li *et al* explained this role by SFXN implication in the iron mitochondrial import, which has not been proven yet. Further work is thus needed to investigate the relationships between SFXN1 and ferroptosis, and the precise mechanisms whereby SFXN1 could regulate iron levels and cell death. It will be also interesting to determine if SFXN1 mediates LPS-induced ferroptosis in other cell types, as well as its implication in ferroptosis mediated by different inducers (such as erastin, RSL3, FIN56 or other drugs). Because Acoba *et al.* reported lowered CoQ levels in SFXN1 KO cells and CoQ is an antioxidant and a cofactor for the ferroptosis suppressor FSP1 [92], we expect that an imbalance in SFXN1 levels may favor ferroptosis through a direct or indirect regulation of CoQ levels. It would thus be interesting to study FSP1 activity in SFXN1 KO cells.

5.2. SFXN1 and ferritinophagy

To limit the toxicity of free Fe^{2+}, molecular traps – *e.g* Ferritin and FtMt (mitochondrial ferritin) - exist in the cytosol and the mitochondrion respectively, as stated earlier. Ferritinophagy, the lysosome-dependent mechanism whereby iron is mobilized from ferritin, can also contribute to ferroptosis induction. In this process, the selective cargo receptor NCOA4 (nuclear receptor coactivator 4A) binds to ferritin and targets this iron storage protein to the lysosomes, thus promoting ferritin degradation and the subsequent release of iron [93]. In apelin-13 induced cardiomyocytes hypertrophy, Tang *et al* recently reported a decrease in FTH (ferritin heavy chain) together with an upregulation of NCOA4 and SFXN1 [94] . Immunohistochemical analysis of hypertrophic heart tissue also highlighted an upregulation of NCOA4 and SFXN1. The siRNA-mediated depletion of NCOA4 restored basal levels of SFXN1 in cardiomyocytes, suggesting that apelin-13 mediated upregulation of SFXN1 could depend on NCOA4. In the presence of apelin-13, the knockdown of SFXN1 decreased iron overload and mitochondrial ROS production in ferric ammonium citrate – treated cardiomyocytes. How NCOA4 could upregulate SFXN1

remains unanswered, as well as the role of SFXN1 and the other SFXN/SLC56 transporters in cardiac hypertrophy. In this study, SFXN1 is proposed to be an iron importer, together with mitoferrin 1 and 2, which are upregulated. The increase of mitochondrial iron in the induced cardiomyocytes hypertrophy model responds to the elevated SFXN1 levels, and higher amounts of iron would promote ROS production thanks to the Fenton reaction, an increase of lipids peroxidation and finally, an induction of ferroptosis. Nevertheless, the mechanisms that allow SFXN1 to control iron levels are not addressed nor whether SFXN1 is the most important player in regulating mitochondrial iron, aside of mitoferrins and ferritin, is discussed.

NCOA4 mediated regulation of SFXN1 was also reported in a recent study addressing the role of ferritinophagy in sepsis-induced cardiac injury [91]. In this study, SFXN1 was shown to be upregulated at the mRNA level in LPS-treated cardiomyocytes, but whether this upregulation results from a transcriptional activation or an enhanced stability of mRNA was not studied. To date, the regulation of SFXN expression has not been deeply investigated and further work is needed to document this point. However, intracellular iron may be important for NCOA4-mediated SFXN1 regulation since the iron chelator deferoxamine (DFO) was shown to decrease LPS-induced SFXN1 accumulation [91]. Li *et al* used immunofluorescence to show this DFO-mediated downregulation of SFXN1 and this must be confirmed using western blot.

The iron-mediated regulation of SFXN1 levels is intriguing and we wondered if iron could regulate translation or mRNA stability by IRP-dependent molecular mechanisms. We hypothesize that IRP proteins, that are major regulators of iron homeostasis acting at the post-transcriptional levels, could modulate SFXN levels through binding to cis-regulatory IRE response elements in SFXN1 transcripts. We thus searched for IRP-binding sites in SFXN transcripts. IRE found in some of the iron-regulated transcripts are shown in **Figure 7**. Canonical IRE are motifs composed of a six-nucleotide apical loop (5′-CAG-WGH-3′) [95]. Using an IRE prediction tool ("SIREs Web Server 2.0" (http://ccbg.imppc.org/sires/) [96], we retrieved putative IRE in all human SFXN1 variant transcripts except for one (**Table 4**). One IRE of high quality and a second one of low quality are found respectively at the end of the SFXN1 coding sequence and in the 3′ UTR (**Figure 8**). Additionally, human SFXN2 transcripts possess one putative medium-quality IRE and SFXN5 transcripts contain a putative high-quality IRE, at their 3′ UTR. Interestingly, no IREs are predicted neither in SFXN3 nor in SFXN4 mRNAs. As SFXN1 and SFXN3 are closely related and seem to have highly similar three-dimensional structure, it is tempting to hypothesize that they can be differentially regulated depending on iron levels. In *Drosophila*, no putative IREs are predicted in any of the two mRNAs encoding dSfxn1/3 and dSfxn2, the SFXN orthologues found in flies. The presence of putative IREs at the 3′UTR of some of SFXN transcripts is suggestive of their IRP-mediated stabilization. We thus expect an increase of SFXN1 levels under low iron levels, when IRP1 lacks its Fe-S cluster and IRP2 is degraded. This latter is not in agreement with the DFO-mediated downregulation of SFXN1 levels reported by Li *et al* [91]. IRE motifs found in SFXN transcripts are non-canonical IRE motifs derived from IRE sequences identified in IRP-interacting mRNAs uncovered in the genome-wide SELEX experiments [97–99]. Having found IRE in SFXN1 transcripts is in favor of an iron-mediated regulation of SFXN levels, however, whether the IREs found in SFXN1, SFXN2 and SFXN5 transcripts are functional, is a point that needs to be further investigated.

To conclude, despite two works started to shed light on SFXN1 role in iron homeostasis and ferroptosis [94,91], how this metabolite transporter exerts its function is far from being clear and more work is required to properly elucidate mechanistically how SFXN1 is implicated in iron homeostasis.

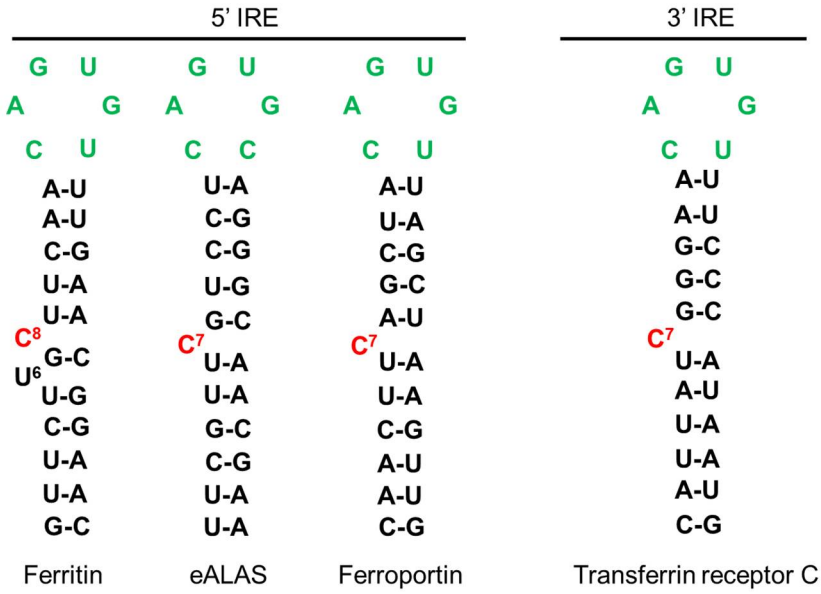

Figure 7. IRE sequences from known proteins involved in iron metabolism. IRE sequences can be localized at 5′ or 3′. In the absence of iron, IRP1 binds the sequences located at 5′ of blocking the translation of the RNA. Ferritin, ALAS and Ferroportin are proteins involved in iron storage, heme synthesis and iron export, respectively. In the same situation, IRP binding to 3′ sequences, stabilizes the RNA promoting the translation of, for example, Transferrin receptor, involved in iron import. In the opposite situation, with high iron levels, IRP binds to iron, which unbinds the IREs, thus promoting translation of Ferritin, ALAS and Ferroportin and leading to Transferrin receptor RNA decay, which is no more protected by IRP1.

Table 4. Location of predicted IRE in SFXN1 splicing variants.

Sequence ID	mRNA length	CDS position	Product	IRE position	
NM_022754.7 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 1, mRNA	4066	90-1058	sideroflexin-1 isoform 1	1000-1031	2698-2729
NM_001322977.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 2, mRNA	4094	118-1086	sideroflexin-1 isoform 1	1028-1059	2726-2757
NM_001322978.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 3, mRNA	4037	244-1029	sideroflexin-1 isoform 2	971-1002	2669-2700
NM_001322980.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 4, mRNA	3938	90-875	sideroflexin-1 isoform 4	872-903	2570-2601
NM_001322981.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 5, mRNA	3966	118-903	sideroflexin-1 isoform 4	900-931	2598-2629
NM_001322982.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 6, mRNA	4065	272-1057	sideroflexin-1 isoform 2	999-1030	2697-2728
NM_001322983.2 Homo sapiens sideroflexin 1 (SFXN1), transcript variant 7, mRNA	959	90-818	sideroflexin-1 isoform 3	No IRE	

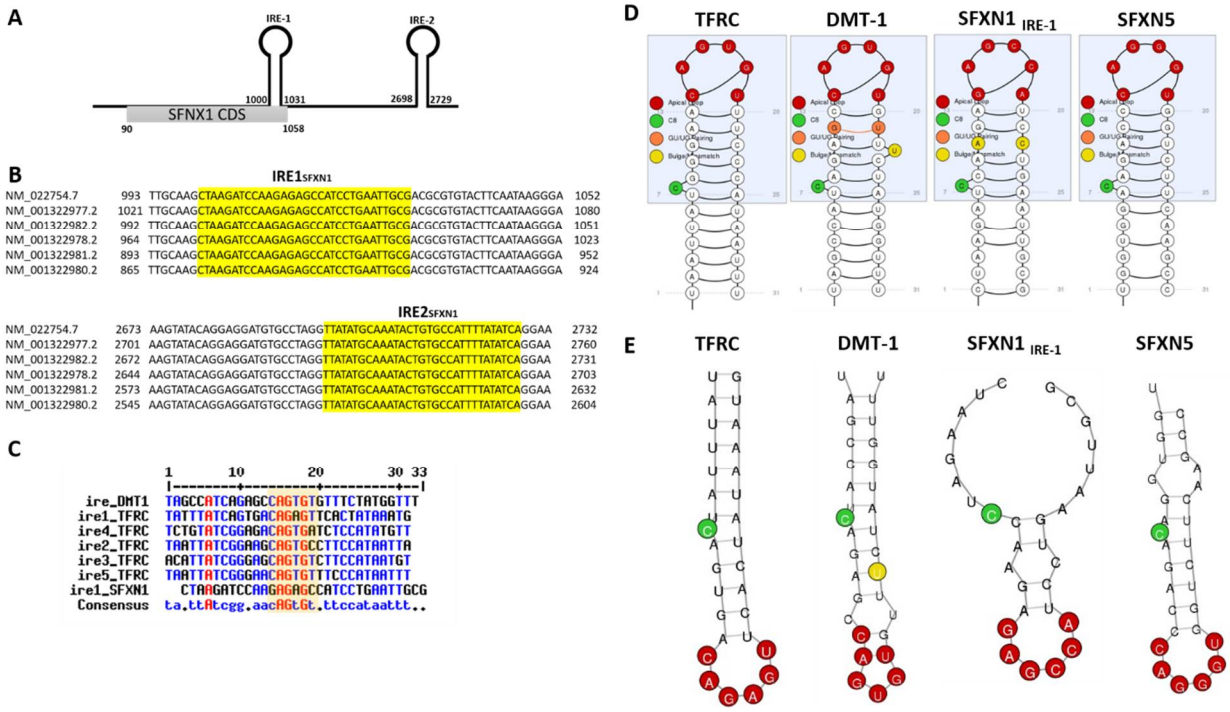

Figure 8. Predicted IRE in SFXN transcripts. *A*. Two IREs were found in the 3'UTR of SFXN1 transcripts using the SIREs Web Server 2.0. The first one is located at the end of the coding sequence. *B*. Alignment showing the position of the two IREs in SFXN1 transcripts. All except one shorter SFXN1 transcript variant possess putative IREs. *C*. Alignment of the IREs of DMT-1, transferrin receptor (TFRC) and SFXN1 transcripts using MultiAlin. The consensus highlights the position of the six-nucleotide apical loop (5'-CAGWGH-3') as shown in the yellow box. *D, E*. Schemes (*D*) and RNA fold prediction (*E*) for the IREs from TFRC, DMT-1, SFXN1 and SFXN5 transcripts generated by the SIREs Web Server 2.0.

6. Sideroflexins in aging: may SFXN regulate neuronal physiology and retinal function?

In this part, we discuss the potential role of SFXN in neuronal pathophysiology, aging and retinal function.

6.1. Sideroflexins and biometals in neuronal physiopathology

Brain accumulation of biometals - including iron and manganese - has been observed in neurodegenerative diseases and associated with a decline in cognitive functions [100–102]. Accumulation of biometals can be detrimental and may promote protein aggregation. Hence, Amyloid beta peptide (Aβ), which forms toxic aggregates in the brain of patients who suffered from Alzheimer's disease, is known to interact with iron [103–106]. Aβ toxicity was reported to be suppressed by the iron storage protein Ferritin in *Drosophila* [107].

We postulate that some SFXN may share a neuroprotective role because SFXN are present in brain neurons (Human Protein Atlas, [27] and our unpublished data) and a decreased expression of SFXN1 and SFXN3 was linked to Alzheimer's and Parkinson's diseases (AD and PD). Indeed, SFXN1 is decreased in brains of AD patients [108] and SFXN3 downregulated in late stage PD dopamine neurons from *substantia nigra* [109]. Additionally, downregulation of the *Drosophila* orthologue dSfxn1/3 enhanced tau toxicity in a *Drosophila* model commonly used to study neurodegeneration [108]. Under physiological conditions, SFXN3 and alpha-synuclein (α-Syn, a PD marker protein) levels were inversely correlated in a murine model, whereas overexpressing dSfxn1/3 impaired synapse morphology at the *Drosophila* neuromuscular junction [45]. It is tempting to link a putative iron-dependent regulation of SFXN, as discussed above, and the known regulation of α-Syn by IRPs. Hence, an IRE is found in the 5'UTR of α-Syn mRNAs and IRP-mediated translational inhibition is relieved upon high iron levels [58,110]. This could explain the

opposite regulation of α-Syn and SFXN levels. However, we did not find putative IRE in SFXN3 transcripts, as stated above.

Decreased levels of SFXN1 in the hippocampus were also observed in a rat model with bilateral ovariectomy displaying depressive behaviors and cognitive impairment [111]. Recent evidence point towards a regulatory role for SFXN in iron homeostasis / utilization at the cell level [9,112,15]. However, iron homeostasis and heme biosynthesis have not been investigated specifically in SFXN-deficient neurons yet, and it would be interesting to question this point. Besides the mitochondrial accumulation of iron reported when some SFXN are lacking, Acoba *et al* also reported a decrease in manganese levels in SFXN1-null cells [15]. Manganese is an essential metal element required for the activity of certain enzymes (such as MnSOD) and both insufficiency and overexposure can affect neuronal physiology and cognitive functions [113]. Thus, SFXN might regulate neuronal physiology in participating in biometals homeostasis.

Whether SFXN are able to regulate ferroptosis is also an important concern, because ferroptosis is one of the most important regulated cell death in brain [114]. Ferroptosis was reported in Parkinson's disease, Alzheimer 's disease and Huntington's disease and other neurologic disorders. Using *in vitro*, *ex vivo* and *in vivo* (mouse) PD models, Do Van *et al.* [115] reported ferroptosis in PD dopaminergic neurons, a process that was reversed by Ferrostatin-1, a selective inhibitor of erastin-induced ferroptosis which inhibits lipid ROS. Growing evidence also highlight the implication of ferroptosis in Alzheimer's disease [116], a neurodegenerative disease characterized by cognitive functions and memory impairment, synaptic loss and neuronal cell death. In mouse, conditional deletion in forebrain neurons of glutathione peroxidase 4 (Gpx4) gene altered cognitive functions (spatial learning and memory) and triggered hippocampal neurodegeneration with hallmarks of ferroptosis [117].

To conclude, further investigations must be undertaken to precisely specify the role of SFXN1 and its homologues in brain biometals homeostasis and neurodegeneration.

6.2. Sfxn and retinal degeneration

Iron levels vary during retina development, with gender and it accumulates during aging. When supply does not equal demand (*e.g.* if retinal blood flow is impaired), retinal neurons are at risk of excitotoxic cell death and vision is impaired or lost [118,119].

Many proteins are involved in iron homeostasis in the retina, and most of the rodent models studied, are related to human pathologies, like human atransferrinemia (lack of transferrin), hemochromatosis type IV (lack of ferroportin) or microcytic hypochromic anemia with iron overload (decrease in DMT1), among others (see [119] for a review). Human transferrin electrotransfection in rodents was shown to protect retinal structure and function, reducing microglial infiltration and preserving the integrity of the outer retinal barrier in a photo-oxidative model. Transferrin, a natural iron chelator, delayed also the retina degeneration and decreased oxidative stress [120]. This work validates iron overload as a therapeutic target for pathologies as retinitis pigmentosa or age-related macular degeneration. Taking into account the relationships between SFXN and iron metabolism, we expect that the loss of SFXN could impair retinal function. Accordingly, in mice, Sfxn3 mutations lead to retinal degeneration [121]. Using forward genetics and screening by optical coherence tomography, Chen et al. identified the *pew* and *basilica* mutations in the Sfxn3 gene leading to a significant decrease in the outer retina thickness. Mice with CRISPR-Cas9-induced Sfxn3 loss-of-function mutations were further generated to investigate the consequences on retinal structure and function. Mice with predicted dramatically shortened Sfxn3 proteins showed retinal impaired morphology (decreased retinal thickness, especially that of the outer retina, and loss of the hexagonal shape of retinal pigmentary epithelium cells) and abnormal fundus and vasculature compared to controls. Retinal thickness even decreased with age in favor of a retinal degeneration due to the lack of functional Sfxn3. Whether those defects are associated to impairment in iron ho-

meostasis is not explored nor discussed. Anyway, we favor the idea that SFNX3 contributes to regulate intracellular iron levels, thus protecting the retina from oxidative stress. Moreover, in humans, SFXN4 loss-of-function is associated with optic atrophy [19,21], pointing to SFXNs as a central family of proteins required for proper retina development and homeostasis.

7. Conclusion and open questions

SFXN/SLC56 is a new family of mitochondrial proteins that have important roles in amino acid transport and in iron homeostasis. Several studies associate SFXN depletion with an increase in mitochondrial iron, deficiencies in carbon metabolism and RC activity and ferroptosis, in cell culture, in animal models and in human pathology, making the SFXN an interesting target for tissue degeneration therapy. But even though those links seem clear and reproducible, nothing is known about the mechanism of action of SFXN. Do all the isoforms have the same functions (different members are expressed in different tissues)? As there are several transcripts for each isoform, do those different transcripts generate different proteins with different kinetic properties? If SFXN are not iron transporters, how can they control iron levels in the mitochondria? How can they control mRNA and/or protein levels of some key heme regulators (CPOX, FECH and ALAS)? Some SFXN present putative IRE but other don't; are all SFXN sensible to iron content and to IRP1/2 regulation?

We think that a better knowledge on SFXN biochemistry is needed to properly decipher the functions of each SFXN member, to know whether they all have redundant functions, their interaction with other proteins or with other SFXN, and how they are regulated.

Abbreviations

ABCB6: ATP Binding Cassette Subfamily B Member, ACO1: Aconitase1, AD: Alzheimer's disease, α-KG: α-ketoglutarate, α-KGDH: α-ketoglutarate deshydrogenase, AGK2 AcylGlycerol Kinase, ALA: Aminolevulinic acid, ALAD: Aminolevulinic acid deshydratase, ALAS: Aminolevulinic acid synthase, ALT2: Alanine aminotransferase 2, ICP-MS: Inductively coupled mass spectrometry, a-Syn: Alpha-synuclein, ATAD-3: ATPase family 3A domain containing protein, Aβ: Amyloid beta peptide, BBG-TCC: Brain Bergmann Glial cell-Tricarboxylate carrier, BCS1L: Ubiquinol-Cytochrome C Reductase Complex Chaperone, BMP: Bone Morphogenetic Protein, CCHL: Cytochrome c heme lyase, COPROgenIII: Coproporphyrinogen III, CoQ: Coenzyme Q, COX4: Cytochrome c oxidase subunit 4, COXPD18: Combined oxidative phosphorylation deficiency 18, CPOX: Coproporphyrinogen oxidase, CYP450: Cytochrome P450, Cyt b: Cytochrome b, Cyt c1: Cytochrome c1, DFO: Deferoxamine, DMK: dimethyl-α-ketoglutarate, DMSO: Dimethyl sulfoxide, DMT1: Divalent metal transporter 1, dSfxn: Drosophila sideroflexin, ETC: Electron transport chain, Fe^{2+}: Iron ferrous, Fe^{3+}: Iron ferric, Fe-S: Iron–sulfur, FECH: Ferrochelatase, FeSFA: Fluorescence assay, FIN56: ferroptosis inducing 56, FLVCR1b: Feline leukemia virus subgroup C receptor 1, Fp: Flavoprotein, fpn-1.1: Ferroportin 1.1, Fsf1: Fungal sideroflexin 1, FtMt: Ferritin Mitochondrial, FXN: Frataxin, GDH: Glutamate dehydrogenase, Gpx4: Glutathione peroxidase 4, GTP: Guanosine Triphosphate, HBM: Heme Binding Motif, HEK: Human embryonic kidney, HO-1: Heme oxygenase-1, ISCs: Iron-sulfur clusters, IMM: Inner mitochondrial membrane, IMPC: International Mouse Phenotyping Consortium, IMS: Intermembrane space, IRE: Iron Response Elements, IRP1/2: Iron Related Protein 1 and 2, ISCU: Iron-sulfur cluster assembly enzyme, ISP: Iron-sulfur protein, LC-MS/MS: Liquid chromatography-coupled to tandem mass spectrometry ,LPS: Lipopolysaccharide, LYRM7: LYR motif-containing protein 7, Madh5: Mothers against decapentaplegic homolog, MDA: Malondialdehyde, MEF: Mouse Embryonic Fibroblasts, MEL: Mouse erythroleukemia, Mfrn1/2: Mitoferrin 1/2, MnSOD: Manganese superoxide dismutase, NAD(P)+ : Nicotinamide adenine dinucleotide phosphate, NADH: Nicotinamide adenine dinucleotide hydrogen, NCO4A: nuclear receptor coactivator 4, NDUFB8: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, NDUFS1: NADH dehydrogenase (ubiquinone) Fe-S protein 1, NDUFS7: NADH dehydrogenase (ubiquinone) Fe-S protein 7, NDUFS8: NADH dehydrogenase (ubiquinone) Fe-S protein 8, NDUFV1: NADH dehydrogenase [ubiquinone] flavoprotein 1, NFS1: nitrogen fixation 1 homolog (S. cerevisiae), NMJ : Neuromuscular

junctions, NUDFV2: NADH dehydrogenase [ubiquinone] flavoprotein 2, OCM: One-carbon metabolism, OCR: Oxygen Consumption Rates, OXPHOS: Oxidative Phosphorylation, PD: Parkinson's disease, PGB: Porphobilinogen, PL-PUFA: phospholipid-bound polyunsaturated fatty acids, PPIX: Protoporphirin IX, PPOX: Protoporphyrinogen oxidase, PTGS2: Prostaglandin-Endoperoxide Synthase 2, RC: Respiratory complexes , RCD : Regulated Cell death, RISP: Rieske iron-sulfur protein, ROS: Reactive oxygen species, RSL3: Ras-selective lethality protein 3, SCHAD: Short-Chain 3-Hydroxyacyl-Coenzyme A, SDHA: Succinate dehydrogenase complex, subunit A, SDHB: Succinate dehydrogenase complex, subunit B, SDHC: Succinate dehydrogenase complex, subunit C, Ser: Serine, SFXN: Sideroflexins, SHMT2: Serine Hydroxymethyltransferase 2, SILAC: Stable isotope labeling by amino acids, SIRT4: Sirtuin 4 , SLC25A38 Solute carrier Family 25 Member 38, SLC25A39: Solute Carrier Family 25 Member 39, SLC56: Solute carrier family , STEAP3: Six-Transmembrane Epithelial Antigen of Prostate 3, STED: Stimulation Emission Depletion, TCA: Tricarboxylic acid, TCC: Tricarboxylate carrier, TEM-EDX: Transmission electron microscopy linked with energy-dispersive X-ray spectroscopy , TF: Transferrin, TFR1: Transferrin receptor protein 1, TIM22: Translocase of Inner Mitochondrial Membrane 22, TTC19: Tetratricopeptide Repeat Domain 19, UQCC1-3: ubiquinol-cytochrome c reductase complex assembly factor 1, UQCRC2: Cytochrome b-c1 complex subunit 2, UQCRFS1: Cytochrome b-c1 complex subunit Rieske, UROD: Uroporphynogen decarboxylase, UROgenIII: Uroporphyrinogen III, UROS: Uroporphyrinogen Synthase

Author Contributions: methodology, A.G.; data curation, N.T., J.M.H; writing—original draft preparation, N.T, J.M.H, N.L.; writing—review and editing, N.L, S.B and B.M.; supervision, project administration, funding acquisition, N.L. All authors have read and agreed to the published version of the manuscript."

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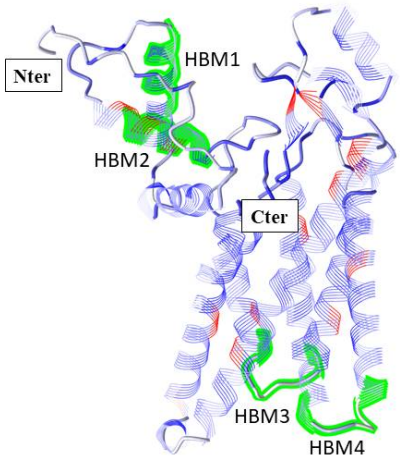
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

A.1. Prediction of Heme Binding Motifs in SFXN1

The computational tool *SeqD-HBM* (131.220.139.55/SeqDHBM/) was used for the determination of heme binding motifs in human SFXN1 (Uniprot entry Q96NB2). The default mode released 13 possible heme-coordination sites and the WESA mode, which passes the sequence through a sequence-based solvent accessibility meta-predictor, gave 4 putative HBMs. These putative HBMs were further located on SFXN1 predicted structure, highlighting 4 sites that may transiently interact with heme (**Figure S1**). If our predictions are correct, two sites would be located in the matrix and the others would be in the intermembrane space.



Motif	Sequence
HBM1	RKIV**H**DYRQ (52)
HBM2	WRAK**Y**IYDS (73)
HBM3	MMTF**Y**RTTP (111)
HBM4	ALTK**H**VSPL (171)

Figure S1. Predicted HBMs in human SFXN1. *Left panel:* The putative HBMs on SFXN1 predicted structure are highlighted (in green). *Right panel*: Sequences of the 9mer motifs in SFXN1 corresponding to predicted HBMs. The position of the potential heme-coordination site (Cys, His or Tyr) is shown (bold).

A.2. Mitochondrial labile iron staining with the MitoFerro-Green fluorescent probe

Prior to the staining, HT1080 cells were seeded in a 6-well plate and transiently transfected with a validated scrambled control siRNA (Control siRNA-A sc-37007, Santa Cruz Biotechnology, INC) or a pool of specific siRNA for SFXN1 (sc-91814, Santa Cruz Biotechnology, INC) using Interferin™ transfection reagent (Polyplus-transfection Inc., New York, NY) following manufacturer instructions. Briefly, a mix of siRNA and Interferin™ transfection reagent was prepared and incubated for 10 min at room temperature, and then, added to each well at a final concentration of 10 nM. Cells were incubated at 37°C under standard culture conditions and amplified. 24h post-transfection, cells were seeded in µ-Slide 2 Well (Ibidi) and further incubated at 37°C under standard culture conditions for 24 h. The following day, cells were eventually treated with deferoxamine (DFO) with or without FeCl3 for 1h30 or erastin for 6 h before adding the MitoFerro-Green probe (Dojindo, TEBU, France). MitoFerro-Green staining was done according to the manufacturer's recommandations. Live imaging images were acquired on a Leica TCS SPE confocal microscope with a 63X oil immersion objective (CYMAGES imaging facility, UVSQ). Image analysis of three independent experiments was done using ImageJ software with a macro developed in the lab.

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