Regulation of mitochondrial iron homeostasis by sideroflexin 2

Ei Ei Mon1 · Fan-Yan Wei1,4 · Raja Norazireen Raja Ahmad1 · Takahiro Yamamoto1 · Toshiro Moroishi2,3,4 · Kazuhito Tomizawa1,3,5

Received: 25 October 2018 / Accepted: 10 December 2018 / Published online: 20 December 2018 © The Author(s) 2018

Abstract
Mitochondrial iron is indispensable for heme biosynthesis and iron–sulfur cluster assembly. Several mitochondrial transmembrane proteins have been implicated to function in the biosynthesis of heme and iron–sulfur clusters by transporting reaction intermediates. However, several mitochondrial proteins related to iron metabolism remain uncharacterized. Here, we show that human sideroflexin 2 (SFXN2), a member of the SFXN protein family, is involved in mitochondrial iron metabolism. SFXN2 is an evolutionarily conserved protein that localized to mitochondria via its transmembrane domain. SFXN2-knockout (KO) cells had an increased mitochondrial iron content, which was associated with decreases in the heme content and heme-dependent enzyme activities. By contrast, the activities of iron–sulfur cluster-dependent enzymes were unchanged in SFXN2-KO cells. Moreover, abnormal iron metabolism impaired mitochondrial respiration in SFXN2-KO cells and accelerated iron-mediated death of these cells. Our findings demonstrate that SFXN2 functions in mitochondrial iron metabolism by regulating heme biosynthesis.

Keywords Mitochondria · Iron · Respiration · OXPHOS · Heme

Introduction
Iron is an essential element that is involved in the regulation of diverse biological processes, including oxygen transport, metabolism, respiration, and the cell cycle [1–3]. A large portion of cellular iron is bound to heme, which is an essential component of hemoglobin and respiratory complexes [4]. In addition, iron is utilized for the biogenesis of iron–sulfur clusters, which are indispensable for the activities of several enzymes related to RNA modification and redox signaling [5–7]. Given the essential role of iron, its deficiency often induces anemia in women and children, which can lead to severe complications such as inflammation and heart failure [8–11]. On the other hand, excess iron exerts cytotoxic effects because ferrous iron is highly active and can generate reactive oxygen species [12]. Indeed, iron overload has been linked to several diseases, such as osteoporosis, cancer, and neurological disorders [13–17].

Extracellular iron is mainly transported to the cytosol via transferrin receptor-mediated endocytosis [18]. Subsequently, a portion of cytosolic iron is transported to mitochondria for the biosynthesis of heme and iron–sulfur clusters, which are exported back to the cytosol [18]. Biosynthesis of heme and iron–sulfur clusters involves
The present study investigated the physiological role of human SFXN2, an uncharacterized SFXN family protein. We report that SFXN2 is an outer mitochondrial membrane protein that functions in mitochondrial iron homeostasis by regulating heme biosynthesis.

Results

SFXN2 is an evolutionarily conserved transmembrane protein

To examine the conservation of SFXN2 across species, we compared its sequences between eight representative eukaryotic species: Homo sapiens, Mus musculus, Bos taurus, Xenopus tropicalis, Drosophila melanogaster, Caenorhabditis elegans, Danio rerio, and Saccharomyces cerevisiae. Phylogenetic analysis demonstrated that SFXN2 is evolutionarily conversed from yeast to humans (Fig. 1a). SFXN2 proteins in vertebrates and invertebrates exhibit 70–91% and 44–56% similarity with human SFXN2, respectively. A previous in silico study demonstrated that fungal sideroflexin-1 (FSF1) is a fungal homolog of human SFXN proteins [36]. Indeed, human SFXN2 shows 31.8% similarity to yeast FSF1. Alignments of human SFXN2 with its homologs showed that conserved amino acids are evenly distributed from the N-terminus to the C-terminus. All these SFXN2 proteins contain five putative transmembrane domains and a long N-terminal region consisting of ~100 amino acids (Fig. 1b). The C-terminus of these SFXN2 proteins is relatively short (~40 amino acids), and the final eight amino acids are almost identical across the eight species.

SFXN2 is a mitochondrial protein

We conjugated human SFXN2 to the C-terminus or N-terminus of mCherry and examined its localization in HeLa cells to verify whether it is a mitochondrial protein. Both mCherry-SFXN2 and SFXN2-mCherry colocalized with the mitochondria-specific fluorescent dye MitoTracker (Fig. 2a, b). The majority of mCherry-SFXN2 and SFXN2-mCherry colocalized with endogenous Tomm20, an outer mitochondrial membrane protein (Fig. 2c, d). Notably, some mitochondria were surrounded by mCherry-SFXN2 or SFXN2-mCherry, while the interior of mitochondria lacked mCherry fluorescent signals (Fig. 2c, d). This observation led us to speculate that SFXN2-mCherry and mCherry-SFXN2 localizes to the outer mitochondrial membrane. We purified mitochondria from HEK293 cells expressing SFXN2-mCherry and treated them with trypsin in the absence of detergent. Trypsin rapidly digested mitofusion1, which is an outer mitochondrial membrane protein,
and SFXN2-mCherry (Fig. 2e, f). By contrast, Timm50, which is an inner mitochondrial membrane protein, was resistant to trypsin digestion (Fig. 2e, f). These results suggest that SFXN2-mCherry is targeted to the outer mitochondrial membrane.

Mitochondrial targeting signal in SFXN2

Targeting of proteins to mitochondria is very complicated, and the targeting signal might be embedded in various regions of the protein. A mitochondrial targeting signal was previously proposed to be located in the N-terminal region
of SFXN proteins [36]; therefore, we conjugated the N-terminal region of SFXN2 to mCherry and examined its localization. In contrast with full-length SFXN2 shown in Fig. 2, the chimeric SFXN2<sub>N</sub>-mCherry protein failed to localize to mitochondria (Fig. 3a). Likewise, mCherry conjugated to the C-terminal tail of SFXN2 failed to localize to mitochondria (Fig. 3b). These data demonstrate that the mitochondrial targeting signal is not located in the N-terminus or C-terminus of SFXN2; therefore, we speculated that it is present in the transmembrane domains. We truncated SFXN2 at the end of the second transmembrane domain and conjugated it to mCherry. This chimeric protein (SFXN2<sub>N-TM2</sub>-mCherry) colocalized with MitoTracker (Fig. 3c). Furthermore, the combination of the first transmembrane domain (TM1) and the N-terminus of SFXN2 (SFXN2<sub>N-TM1</sub>-mCherry) was sufficient for targeting to mitochondria (Fig. 3d). Mitochondrial targeting of SFXN2<sub>N-TM1</sub>-mCherry was less efficient than that of SFXN2<sub>N-TM2</sub>-mCherry (Pearson’s colocalization efficient = 0.728 for SFXN2<sub>N-TM1</sub>-mCherry and 0.627 for SFXN2<sub>N-TM2</sub>-mCherry, Fig. 3e).

Iron accumulates in mitochondria of SFXN2-knockout (KO) cells

The mitochondrial localization of SFXN2 prompted us to examine its role in mitochondrial iron homeostasis. Sfxn2 was highly expressed in mouse kidney and liver (Fig. S1A). Notably, SFXN2 was also expressed in human embryonic kidney 293 (HEK293) cells, and was the third most highly expressed isoform among the five SFXN isoforms (Fig. S1B). We then generated SFXN2-knockout (KO) HEK293 cells using CRISPR-Cas9-mediated genome editing [37]. We designed a guide RNA (gRNA) that targets exon 4 of SFXN2. After colony selection and genomic PCR, one clone showed a potential deletion near to exon 4 (Fig. 4a). Subsequent DNA sequencing revealed that a part of exon 4 and the entire exon 5 of SFXN2 was deleted (Fig. 4b). To examine whether overall transcription of SFXN2 was impaired by this targeted deletion, we performed quantitative PCR using primers complementary to exon 1 of SFXN2. The mRNA level of SFXN2 in SFXN2-KO cells was 10% of that in control cells stably expressing Cas9 (Fig. 4c). Notably, the levels of other SFXN family members did not differ between SFXN2-KO and control cells (Fig. 4c).

To examine the mitochondrial iron content, we purified mitochondria and extracted total iron using nitric acid. The extract was subjected to inductively coupled plasma-mass spectrometry (ICP-MS) for iron quantification. The iron content was significantly higher in mitochondria purified from SFXN2-KO cells than in mitochondria purified from control cells (Fig. 4d). ICP-MS cannot distinguish between ferric and ferrous iron when measuring the iron content; therefore, we treated control and SFXN2-KO cells with a fluorescent iron probe that localizes to mitochondria and specifically labels ferrous iron [38]. Confocal microscopy revealed that the fluorescence intensity of this iron probe was significantly higher in SFXN2-KO cells than in control cells (Fig. 4e, f). Importantly, expression of SFXN2-mCherry significantly reduced the mitochondrial ferrous iron level in SFXN2-KO cells, while expression of Mito-DsRed, a fluorescent mitochondrial protein, did not (Fig. 4g–i). These results clearly demonstrate that SFXN2 is required for maintenance of mitochondrial iron homeostasis.

The heme level is decreased in SFXN2-KO cells

The majority of mitochondrial iron is utilized to synthesize heme and iron–sulfur clusters [18]. Dysfunctional heme biosynthesis and iron–sulfur cluster assembly prevent efficient iron usage, leading to iron accumulation and defective respiration in mitochondria. The labile heme content is generally proportional to the total heme content [39]. Therefore, we measured the labile heme content in SFXN2-KO cells by monitoring peroxidase activity. The labile heme content was significantly lower in SFXN2-KO cells than in control cells (Fig. 5a). We also measured the total heme content by mass spectrometry. Similar to the labile heme content, the total heme content was significantly lower in SFXN2-KO cells than in control cells (Fig. 5b, c).

Heme and iron–sulfur clusters are essential for the activities of mitochondrial respiratory complexes. Complex I contains iron–sulfur clusters, complex IV contains heme, and complexes II and III contain both heme and iron–sulfur clusters [40]. The activities of complexes II–III and IV were significantly lower in SFXN2-KO cells than in control cells (Fig. 5d, e). On the other hand, the activity of complex I did not differ between SFXN2-KO and control cells (Fig. 5f). Collectively, the defective activities of complexes II–IV were associated with a decrease in the maximum oxygen consumption rate (OCR) in SFXN2-KO cells (Fig. 5g). In addition to respiratory complex activities, we examined the level of the 2-methylthio modification of mitochondrial tRNA by mass spectrometry. This modification is mediated by Cdk5 regulatory subunit-associated
protein 1 (CDK5RAP1), a mitochondrial iron–sulfur cluster-dependent tRNA-modifying enzyme [41, 42]. Similar to complex I activity, the level of the 2-methylthio modification of tRNA did not differ between SFXN2-KO and control cells (Fig. 5h). Thus, SFXN2 might be involved in heme biosynthesis, but not in iron–sulfur cluster assembly.

We further examined expression of genes related to iron transport and heme biosynthesis in SFXN2-KO cells.
Expression of *Mitoferrin1* (*MFRN1*), *Mitoferrin2* (*MFRN2*), and *Frataxin* (*FXN*), which are related to iron import and iron–sulfur cluster assembly [43, 44], did not differ between *SFXN2*-KO and control cells (Fig. 6a–c). The mRNA level of *SFXN2* was significantly lower in *SFXN2*-KO cells than in control cells. Expression of *SFXN1*, *SFXN3*, *SFXN4*, and *SFXN5* did not differ between *SFXN2*-KO and control cells. *n* = 3 each. ***p* < 0.001. 

The mitochondrial iron content was measured using ICP-MS. This content was significantly higher in *SFXN2*-KO cells than in control cells. *n* = 3 each. **p* < 0.01. 

The mitochondrial iron contents of control and *SFXN2*-KO cells were investigated using Mito-FerroGreen. Bar = 10 μm. 

The fluorescence intensity of Mito-FerroGreen was significantly higher in *SFXN2*-KO cells than in control cells. *n* = 100 control cells and 200 *SFXN2*-KO cells. ****p* < 0.0001. 

Control and *SFXN2*-KO cells were transfected with Mito-DsRed (g) or *SFXN2*-mCherry (h), and iron was stained with Mito-FerroGreen. 

Quantification of the fluorescence intensity of Mito-FerroGreen in mock transfected cells and in cells transfected with Mito-DsRed and *SFXN2*-mCherry. *n* = 10–12. ****p* < 0.0001. Expression of *SFXN2*-mCherry, but not of Mito-DsRed, suppressed iron accumulation in *SFXN2*-KO cells.

These results demonstrate that abnormal iron homeostasis in *SFXN2*-KO cells is not due to dysfunction of other proteins related to iron import and iron–sulfur cluster assembly.

**SFXN2-KO cells are sensitive to iron-mediated cytotoxicity**

Given the decrease in mitochondrial respiration and the accumulation of iron in mitochondria, we speculated that *SFXN2*-KO cells might be sensitive to iron-induced
cytotoxicity. SFXN2-KO cells grew significantly slower than control cells (Fig. 7a). Upon exposure to excess iron, the growth rate of SFXN2-KO cells was suppressed more than that of control cells (Fig. 7b). Furthermore, we treated cells with erastin in an attempt to induce cytotoxic ferroptosis, a form of iron-mediated cell death [46]. Erastin treatment significantly decreased the viability of SFXN2-KO cells (Fig. 7c, d). In addition, we assessed cell death by performing trypan blue staining and monitoring release of lactate dehydrogenase (LDH) into the culture medium (Fig. 7e, f). Erastin treatment increased the number of SFXN2-KO cells stained with trypan blue (Fig. 7e) and significantly augmented release of LDH into the culture medium by SFXN2-KO cells (Fig. 7f). Taken together, these results demonstrate that SFXN2-KO cells are sensitive to iron toxicity.

Discussion

The present study demonstrated that human SFXN2 is a mitochondrial protein that regulates mitochondrial iron homeostasis. SFXN2-KO cells exhibited abnormal mitochondrial iron accumulation, which was associated with a decrease in mitochondrial respiration. In addition, SFXN2-KO cells displayed a growth defect upon iron overload and were susceptible to erastin-induced death. These results demonstrate that SFXN2 is important for maintenance of mitochondrial iron homeostasis. Iron homeostasis is also perturbed in Sfxn1- and SFXN4-deficient cells [29, 34]; therefore, it is conceivable that SFXN family proteins are evolutionarily conserved to regulate mitochondrial iron homeostasis.

What is the role of SFXN2 in mitochondrial iron homeostasis? Iron is imported from the cytosol into mitochondria for biosynthesis of heme and assembly of iron–sulfur clusters, which are subsequently exported back to the cytosol. Defects in genes related to heme biosynthesis or iron–sulfur cluster assembly lead to aberrant iron accumulation in mitochondria [18]. The high mitochondrial iron content in SFXN2-KO cells was associated with decreases in the heme content and heme-dependent enzyme activities. By contrast, the activities of iron–sulfur cluster-dependent enzymes were unchanged in SFXN2-KO cells. These results suggest that SFXN2 is involved in heme biosynthesis, but not in iron–sulfur cluster assembly.

Heme biosynthesis involves multiple enzymatic reactions that occur sequentially in the cytosol and mitochondria. Accordingly, the intermediates of these chemical reactions must be imported into and exported from mitochondria. Despite the complexity, only a few transmembrane proteins have been reported to mediate this transport. For example, the inner mitochondrial membrane protein SLC25A38 transports glycine, which is required for the first step of heme synthesis [24], while ABCB6 and ABCB10 are involved in import of protoporphyrin into mitochondria [28, 47]. However, these transporters cannot explain the entire heme transport system. Interestingly, a very recent study revealed that SFXN1 is a mitochondrial serine transporter [48]. Serine hydroxymethyltransferase 2 metabolizes serine into glycine in mitochondria; therefore, loss of SFXN1 reduces de novo glycine synthesis in mitochondria, leading to defects in one-carbon metabolism including purine synthesis. Given the essential role of glycine in biosynthesis of heme, it is conceivable that defective heme synthesis in SFXN2-KO cells is due to impaired serine transport and de novo glycine synthesis. A further study is required to elucidate the role of SFXN2 in serine transport and its relevance to heme biosynthesis.

SFXN2 (and SFXN1) is predominantly expressed in the liver and kidney, and is poorly expressed in other tissues. This differential expression pattern might be due to differences in the requirement for hemoglobin and heme synthesis. The fetal liver is the major organ involved in erythropoiesis during the late phase of embryogenesis [49]. In adults, the liver and cortex of the kidney have a high heme-synthesizing capacity, which sustains the activity of cytochrome P450 to detoxify endogenous and exogenous substances [50]. The high demand for hemoglobin and heme in the liver and kidney might explain why SFXN2 is highly expressed in these organs. On the other hand, despite its low abundance, SFXN2 is also required for synthesis of heme and purine nucleotides to sustain cellular activity in other tissues. SFXN3–SFXN5 may compensate for low expression of SFXN2 in these tissues. A further study using knockout mice is required to elucidate the physiological roles of SFXN2 and other SFXN family members.

Proteins are targeted to mitochondria by specific signals. The targeting signals are usually located in the N-terminal, internal, and C-terminal regions of mitochondrial proteins with a single transmembrane segment [51]. Some outer mitochondrial membrane proteins, such as peripheral
benzodiazepine receptor, contain several transmembrane segments, and the location of the targeting signal is unclear [52]. A previous in silico study predicted that the targeting signal of SFXN2 is located in its N-terminal region [36]. However, neither the N-terminus nor the C-terminus was sufficient for targeting of SFXN2 to mitochondria in the present study. Instead, the transmembrane domains of SFXN2 functioned as a mitochondrial targeting signal and guided SFXN2 to the mitochondria. In contrast to SFXN2, SFXN3, and SFXN4 are targeted to the inner mitochondrial membrane [33, 34]. Sequence homology is 54% between SFXN2 and SFXN3, but only 20% between SFXN2 and SFXN4 (Fig. S2). SFXN3 contains one fewer putative transmembrane domains, which are potentially important for mitochondrial targeting (Fig. 3), than...
SFXN2 (Fig. S2). A further study is needed to elucidate the localizations of endogenous SFXN proteins.

All SFXN family genes are expressed in HEK293 cells, but only SFXN2 was deleted in the present study. Given the moderate decrease in the heme level, the remaining SFXN proteins likely compensated for the loss of SFXN2 function. Consistently, the hematological phenotypes are mild in Sfxn1-deficient f/f mice [29], but severe in transgenic mice with defective heme biosynthesis [28, 45]. Simultaneous KO of multiple SFXN genes is required to elucidate the functions of SFXN proteins.

In conclusion, SFXN2 is a mitochondrial membrane protein that regulates heme biosynthesis and contributes to mitochondrial iron homeostasis.
Materials and methods

Cell culture

HEK293 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 4500 mg/l glucose, 10% fetal bovine serum (Corning), and 1× penicillin/streptomycin (Invitrogen) in an incubator containing 5% CO2 at 37 °C.

Plasmids and cDNA

Human SFXN2 was amplified from human cDNA derived from HEK293 cells and subcloned into the pEF1a-mCherry-C1 and pEF1a-mCherry-N1 vectors (Clontech). pDsRed2-Mito was obtained from Clontech. For the generation of lentiviruses, lentiCRISPRv2 (#52961), psPAX2 (#12260), and pVSVg (#8454) were purchased from Addgene.

Generation of SFXN2-KO cells

A gRNA targeting exon 4 of SFXN2 was selected using the CRISPR DESIGN web tool. DNA oligonucleotides were annealed and ligated into LentiCRISPRv2 that had been digested with BsmBI (New England BioLabs). Lentiviruses were generated in HEK293FT cells according to the manufacturer’s instructions. Culture media containing viruses were filtered and stored at −80 °C until use. HEK293 cells were infected with lentiviruses carrying the Cas9 gene and gRNA. Two days later, cells were seeded in 10-cm culture dishes at a density of 40 cells/dish in the presence of 2 μg/ml puromycin. Colonies were plated in 96-well dishes and subjected to genotyping.

Immunocytochemistry

HeLa and HEK293 cells were transfected with mCherry-SFXN2 or SFXN2-mCherry using Lipofectamine 3000. Cells were stained with 100 nM MitoTracker Green FM (Invitrogen) or 100 nM MitoTracker Deep Red FM (Invitrogen) for 30 min. Thereafter, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Finally, cells were stained with an anti-Tomm20 antibody (Abcam, ab186734) and an anti-mCherry antibody (Abcam, ab125096), and then incubated with secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 546. Fluorescence was observed and analyzed using a confocal microscope (Olympus 3000) and FluoView software.

Iron staining

To label iron in mitochondria, cells were incubated with Mito-FerroGreen (Dojindo) in accordance with the manufacturer’s instructions. Briefly, cells were washed thrice with serum-free DMEM. Mito-FerroGreen was freshly dissolved in dimethyl sulfoxide (Sigma) and added to cells at a final concentration of 5 μM. Cells were incubated with the reagent for 30 min at 37 °C and then washed with phosphate-buffered saline (PBS). Fluorescence was observed using a confocal microscope.

Isolation of mitochondria

Cells were grown in 10-cm dishes until they reached 80% confluency, washed with PBS, and suspended in homogenization buffer (250 mM sucrose, 20 mM Mops, 1 mM EDTA, and a protease inhibitor cocktail (Sigma)). Thereafter, cells were homogenized with a Teflon homogenizer until 70–80% of cells were lysed. The homogenate was centrifuged at 800g for 10 min at 4 °C, and the supernatant was centrifuged at 8000g for 10 min. The resulting pellet was used as the crude mitochondrial fraction. Frataxin was detected using an antibody (Abcam, ab175402).

Trypsin digestion

Isolated mitochondria were incubated with 25 μg/ml trypsin (Promega) on ice in homogenization buffer for the indicated durations. A trypsin inhibitor (Sigma) was added to stop the digestion. Mitochondria were washed with homogenization buffer and subjected to SDS-PAGE. Mitofusin1 and Timm50 were selected as representative outer and inner mitochondrial membrane proteins, respectively. Mitofusin1, Timm50, and mCherry were detected using specific antibodies (ab104274, ab109436, and ab125096, respectively) purchased from Abcam.

Measurement of mitochondrial respiratory complex activities

The activities of mitochondrial complexes I, II–III, and IV were measured using previously described methods [41]. To measure complex I activity, 50 μg of isolated mitochondria was mixed with 50 mM potassium phosphate (pH 7.4), 2 mM KCN, 75 μM NADH (nicotinamide adenine dinucleotide reduced disodium salt), and 50 μM coenzyme Q1. Absorbance at 340 nm was measured for 200 s. To measure complex II–III activity, 50 μg of isolated mitochondria was mixed with 50 mM potassium phosphate (pH 7.4), 20 mM succinate, 0.5 mM EDTA, 2 mM KCN, and 30 μM cytochrome C. Absorbance at 550 nm was measured for 200 s. To measure complex IV activity, 50 μg of isolated
mitochondria was mixed with 10 mM potassium phosphate (pH 7.4) and 10 μM ferrocytochrome C. Absorbance at 550 nm was measured for 200 s.

**Flux analysis**

Oxygen consumption was measured using a Seahorse XF Analyzer (Agilent), according to the manufacturer’s instructions. Briefly, cells were seeded in a 24-well culture plate (Agilent) at a density of 50,000 cells/well. Oligomycin (Sigma), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma), and rotenone (Sigma) were added sequentially and oxygen consumption was measured under each condition.

**Cell viability**

Control and SFXN2-KO cells were seeded in a 96-well plate at a density of 5000 cells/well. The following day, cells were treated with 10 μM erastin (Sigma) and incubated for 24 h. Cell viability was monitored using WST-8 reagent (Dojindo) according to the manufacturer’s instructions. Absorbance was measured at 405 nm. To investigate cell death, control and SFXN2-KO cells were seeded in a 96-well plate at a density of 10,000 cells/well. The following day, cells were treated with 10 μM erastin (Sigma) and incubated for 24 h. Cell death was assessed by the LDH assay and trypan blue staining in the same well. The culture medium in each well was transferred to a fresh 96-well plate. The amount of LDH released into the culture medium was measured using a Cytotoxicity LDH Assay Kit-WST (Dojindo) according to the manufacturer’s instructions. Cells were stained with trypan blue (Sigma) and examined under a microscope (Olympus) equipped with a CCD camera.

**Quantitative PCR**

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was generated using PrimeScript RT Master Mix (Takara), and then quantitative PCR was performed using TB Green Premix Ex Taq (Takara) and a Rotor-Gene Q system (Qiagen). The primer sequences are provided in Supplementary Table 1. The level of the target gene was normalized against that of 18S rRNA for analyses in mouse tissues, while the levels of SFXN1–SFXN5 were normalized against that of Ubiquitin C (UBC) for analyses in HEK293 cells. Relative expression was calculated using the 2ΔΔCt method as described previously [53]. Expression levels of target genes were normalized against that of 18S rRNA.

**Mass spectrometry**

The total mitochondrial iron content was measured using ICP-MS (Agilent 7900). Mitochondria were suspended in 2% nitric acid and heated at 80 °C for 15 min to extract iron. Denatured proteins were pelleted by centrifugation at 10,000g for 15 min, and the supernatant was subjected to ICP-MS analysis according to the manufacturer’s instructions.

The level of the 2-methylthio-N6-isopentenyladenosine modification of mitochondrial tRNA was measured using liquid chromatography-mass spectrometry (Shimadzu LCMS-8050) as previously described [42]. Briefly, total RNA was purified from control and SFXN2-KO cells. Five micrograms of total RNA was digested with nuclease P1 (FujiFilm-Wako). Nucleosides were fractionated using an Inertsil ODS-3 column and analyzed in multiple reaction monitoring mode.

**Measurement of labile heme**

After reaching 80% confluency, cells were washed with PBS, lysed in Tris-buffered saline containing 1% Triton X-100, sonicated, and centrifuged at 10,000g for 10 min. The heme content was measured using a Hemin Assay kit (Abcam, ab65332), which utilizes peroxidase activity to measure the level of heme. Protein concentrations were measured using a Pierce BCA assay kit (Thermo Fisher Scientific). Peroxidase activity was normalized against the total protein concentration.

**Measurement of total heme**

The total heme (hemin) content was measured by mass spectrometry according to a previously described method [54]. Briefly, 2 × 10⁷ cells were suspended in 1 ml of PBS, and an aliquot of the cell suspension was transferred to a fresh tube for measurement of the total protein concentration. The cell suspension was mixed with 3 ml of acetonitrile to extract heme from proteins. Next, 2 ml of acetonitrile/HCl (8:2) was added to the sample, followed by 0.5 ml of saturated MgSO₄ and 0.05 g of NaCl. After brief centrifugation at 2600g for 5 min, the upper organic phase, which contained free hemin, was analyzed by mass spectrometry (Agilent 6470). The peak corresponding to hemin was normalized against the protein concentration.

**Statistical analysis**

At least three independent replicates were performed in all experiments. All data were analyzed using GraphPad Prism 6 software. The unpaired Student’s t test was used to assess the significance of differences between two groups.
A two-way ANOVA followed by Tukey’s multiple comparison test was used to examine the significance of differences between more than two groups. A two-tailed p value of 0.05 was considered significant. Data are presented as the mean ± SEM.

Acknowledgements We thank Nobuko Maeda (Kumamoto University) and Megumi Nagayama (Kumamoto University School of Medicine, Core Laboratory for Medical Research and Education) for providing technical assistance.

Funding This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan [17905074 and 18959602 to KT, 18H02599 and 18K19521 to FYW, and 18K19433 and 18H02438 to TM], the Japan Agency for Medical Research and Development (AMED) [17935694 to KT], and the Takeda Science Foundation [to KT].

Compliance with ethical standards

Conflict of interest All of the authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

1. Zhang C (2014) Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. Protein Cell 5:750–760. https://doi.org/10.1007/s13238-014-0083-7
2. Muckenthaler MU, Rivella S, Hentze MW, Galy B (2017) A red carpet for iron metabolism. Cell 168:344–361. https://doi.org/10.1016/j.cell.2016.12.034
3. Lv H, Shang P (2018) The significance, trafficking and determination of labile iron in cytosol, mitochondria and lysosomes. Metallomics 10:899–916. https://doi.org/10.1039/C8MT00048D
4. Kafina MD, Paw BH (2017) Overview of iron metabolism in health and disease. The Journal of Physiological Sciences 68:221–232. https://doi.org/10.1007/s12576-018-0600-1
5. Apostolakis S, Kypraiou A-M (2017) Iron in neurodegenerative disorders: being in the wrong place at the wrong time? Rev Neurosci 28:893–911. https://doi.org/10.1515/reneuro-2017-0020
6. Winterbourn CC (1995) Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicol Lett 82–83:969–974
7. Lertsuwan K, Wongdee K, Teerapornpuntakit J, Charoenphandhu N (2018) Intestinal calcium transport and its regulation in thalassemia: interaction between calcium and iron metabolism. J Physiol Sci 68:221–232. https://doi.org/10.1016/j.jps.2017.10.006
8. Lopez CA, Skaar EP (2018) The impact of dietary transition metals on host-bacterial interactions. Cell Host Microbe 23:737–748. https://doi.org/10.1016/j.chom.2018.05.008
9. Pope M, Kalra PR (2018) Iron deficiency in heart failure: to treat or not to treat? Curr Treat Options Cardiovasc Med 20:65. https://doi.org/10.1007/s11936-018-0661-8
10. Martins AC, Almeida JJ, Lima IS et al (2017) Iron metabolism and the inflammatory response. IUBMB Life 69:442–450. https://doi.org/10.1002/iub.1635
11. Lopez A, Cacoub P, Macdougall IC, Peyrin-Biroulet L (2016) Iron deficiency anaemia. Lancet 387:907–916. https://doi.org/10.1016/S0140-6736(15)60865-0
12. Manz DH, Blanchette NL, Paul BT et al (2016) Iron and cancer: recent insights. Ann N Y Acad Sci 1368:149–161. https://doi.org/10.1111/nyas.13008
13. Dev S, Babilt J (2017) Overview of iron metabolism in health and disease. Hemodial Int 21:S6–S20. https://doi.org/10.1111/hdi.12542
14. Baevya M, Khchedaduri A, Wu R et al (2013) ATP-binding cassette B10 regulates early steps of heme synthesis. Circ Res 113:279–287. https://doi.org/10.1161/CIRCRESAHA.113.301552
15. Apostolakis S, Kypraiou A-M (2017) Iron in neurodegenerative disorders: being in the wrong place at the wrong time? Rev Neurosci 28:893–911. https://doi.org/10.1515/reneuro-2017-0020
16. Belaïdi AA, Bush AI (2016) Iron neurochemistry in Alzheimer’s disease and Parkinson’s disease: targets for therapeutics. J Neu rochem 139:179–197. https://doi.org/10.1111/jnc.13425
17. Kafina MD, Paw BH (2017) Overview of iron metabolism in health and disease. Metallomics 10:899–916. https://doi.org/10.1039/C8MT00048D
18. Lane DJR, Merlot AM, Huang ML-H et al (2015) Cellular iron uptake, trafficking and metabolism: key molecules and mechanisms and their roles in disease. Biochim Biophys Acta Mol Cell Res 1853:1130–1144. https://doi.org/10.1016/j.bbamcr.2015.01.021
19. Cavazzini C, Lode H, Campana DR et al (2006) The mitochondrial ATP-binding cassette transporter Abcb7 is essential in mice and participates in cytosolic iron–sulfur cluster biogenesis. Hum Mol Genet 15:953–964. https://doi.org/10.1093/hmg/ddi012
20. Bekri S, Kispal G, Lange H et al (2000) Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron–sulfur protein maturation. Blood 96:3256–3264
21. Raskind WH, Wijsman E, Pagon RA et al (1991) X-linked sideroblastic anemia and ataxia: linkage to phosphoglycerate kinase at Xq13. Am J Hum Genet 48:335–341
22. Shimada Y, Okuno S, Kawai A et al (1998) Cloning and chromosomal mapping of a novel ABC transporter gene (hABC7), a candidate for X-linked sideroblastic anemia with spinocerebellar ataxia. J Hum Genet 43:115–122. https://doi.org/10.1007/s10038050051
23. Crsere P, Lill R, Kispal G (1998) Identification of a mammalian mitochondrial ABC transporter, the functional ortholog of yeast Atm1p. FEBS Lett 441:266–270.
24. Lentsuwan K, Wongdee K, Teerapornpuntakit J, Charoenphandhu N (2018) Intestinal calcium transport and its regulation in thalassemia: interaction between calcium and iron metabolism. J Physiol Sci 68:221–232. https://doi.org/10.1016/j.jps.2017.10.006
25. Dhaka S, Babilt J (2017) Overview of iron metabolism in health and disease. Metallomics 10:899–916. https://doi.org/10.1039/C8MT00048D
26. Baevya M, Khchedaduri A, Wu R et al (2013) ATP-binding cassette B10 regulates early steps of heme synthesis. Circ Res 113:279–287. https://doi.org/10.1161/CIRCRESAHA.113.301552
27. Guernsey DL, Jiang H, Campagna DR et al (2009) Mutations in mitochondrial carrier family gene SLC25A38 cause non-syndromic autosomal recessive congenital sideroblastic anemia. Nat Genet 41:651–653. https://doi.org/10.1038/ng.359

28. Yamamoto M, Arimura H, Fukushige T et al (2014) Abcb10 role in heme biosynthesis in vivo: Abcb10 knockout in mice causes anemia with protoporphyrin IX and iron accumulation. Mol Cell Biol 34:1077–1084. https://doi.org/10.1128/MCB.00865-13

29. Fleming MD, Campagna DR, Haslett JN et al (2011) A mutation in a mitochondrial transmembrane protein is responsible for the pleiotropic hematological and skeletal phenotype of flexed-tail (f/f) mice. Genes Dev 15:652–657. https://doi.org/10.1101/gad.78300

30. Gregory CJ, McCulloch EA, Til JE (1975) The cellular basis for the defect in haemopoiesis in flexed-tailed mice. III. Restriction of the defect to erythroid progenitors capable of transient colony formation in vitro. Br J Haematol 30:401–410

31. Chui DH, Sweeney GD, Patterson M, Russell ES (1977) Hemoglobin synthesis in siderocytes of flexed-tailed mutant (f/f) fetal mice. Blood 50:165–177

32. Yoshikumi Y, Mashima H, Ueda N et al (2005) Roles of CTPL/Syntaxin 3 and Syntaxin 4 in heme biosynthesis in vivo: Abcb10 knockout in mice causes anemia with protoporphyrin IX and iron accumulation. Mol Cell Biol 34:1077–1084. https://doi.org/10.1128/MCB.00865-13

33. Sanjana NE, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 13:783–784. https://doi.org/10.1038/nmeth.3047

34. Miotto G, Tessaro S, Rotta GA, Bonatto D (2007) In silico analyses of Fsf1 sequences, a new group of fungal proteins orthologous to the metazoan sideroblastic anemia-related sideroflexin family. Fungal Genet Biol 44:740–753. https://doi.org/10.1016/j.fgb.2006.12.004

35. Sanjana NE, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 11:783–784. https://doi.org/10.1038/nmeth.3047

36. Hirayama T, Kadota S, Niwa M, Nagasawa H (2018) A mitochondrial serine transporter required for one-carbon metabolism. J Cell Sci 130:325–331. https://doi.org/10.1242/jcs.194241

37. Puccio H, Simon D, Cossee M et al (2017) Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe–S enzyme deficiency followed by intramitochondrial iron deposits. Nat Genet 27:181–186. https://doi.org/10.1038/ng.8481

38. Hirayama T, Kadota S, Niwa M, Nagasawa H (2018) A mitochondrial serine transporter required for one-carbon metabolism. Science 362(6416):eaat9528. https://doi.org/10.1126/science.aat9528

39. Baron MH, Isen J, Fraser ST (2012) The embryonic origins of erythropoiesis in mammals. Blood 119:4828–4837. https://doi.org/10.1182/blood-2012-01-153486

40. Otera H, Taira Y, Horie C et al (2007) A novel insertion pathway of mitochondrial proteins with multiple transmembrane segments. J Cell Biol 179:1355–1363. https://doi.org/10.1083/jcb.200702143

41. Livan KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2ΔΔCt method. Methods 25:402–408

42. Fyrestam J, Östman C (2017) Determination of heme in microorganisms using HPLC-MS/MS and cobalt(III) protoporphyrin IX inhibition of heme acquisition in Escherichia coli. Anal Bioanal Chem 409:6999–7010. https://doi.org/10.1007/s00216-016-0610-5

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations