Iron Supply via NCOA4-Mediated Ferritin Degradation Maintains Mitochondrial Functions

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ABSTRACT Iron is an essential nutrient for mitochondrial metabolic processes, including mitochondrial respiration. Ferritin complexes store excess iron and protect cells from iron toxicity. Therefore, iron stored in the ferritin complex might be utilized under iron-depleted conditions. In this study, we show that the inhibition of lysosome-dependent protein degradation by bafilomycin A1 and the knockdown of NCOA4, an autophagic receptor for ferritin, reduced mitochondrial respiration, respiratory chain complex assembly, and membrane potential under iron-sufficient conditions. However, autophagy did not contribute to degradation of the ferritin complex under iron-sufficient conditions. Knockout of the ferritin light chain, a subunit of the ferritin complex, inhibited ferritin degradation by decreasing interactions with NCOA4. However, ferritin light chain knockout did not affect mitochondrial functions under iron-sufficient conditions, and ferritin light chain knockout cells showed a rapid reduction of mitochondrial functions compared with wild-type cells under iron-depleted conditions. These results indicate that the constitutive degradation of the ferritin complex contributes to the maintenance of mitochondrial functions.

KEYWORDS mitochondria, NCOA4, ferritin complex, ferritin heavy chain, ferritin light chain, iron, lysosome, mitochondrial respiration, mitochondrial respiratory chain complex

Mitochondria require iron to maintain their functions, i.e., numerous biosynthesis, electron transport, and oxidative phosphorylation functions. Iron is utilized in mitochondria as iron-sulfur cluster (ISC) proteins and heme-containing proteins (1, 2), mainly present in the electron transport chain. ISC proteins and heme-containing proteins are present in mitochondrial respiratory chain complexes (3, 4), especially complexes I and II and complex IV, respectively (5, 6).

Intracellular iron transfer to mitochondria is regulated by several pathways: (i) transferrin-containing endosomal iron in erythrocytes is transferred directly to mitochondria (7), the “kiss-and-run” model (8); (ii) non-transferrin-bound iron independently permeates cardiac cells (9, 10); and (iii) cytosolic labile iron generated from the transferrin/transferrin receptor pathway forms a transient pool of ferrous iron, which is delivered to the mitochondria of yeast and rat hepatocytes in a membrane potential-dependent manner (11, 12). Despite the identification of these pathways, especially in erythrocytes, the important and common pathways remain unclear.

The ferritin complex is formed by ferritin heavy chain (FTH1) and ferritin light chain (FTL), which are major iron storage proteins containing ~4,500 iron atoms (13). This protects cells or tissues against free radical generation via Fenton-like reactions (14). Ferritin is degraded by autophagy mediated by NCOA4, an autophagy receptor (15–19). Intracellular iron levels are maintained by ferritin degradation under iron-deficient conditions.

Citation Fujimaki M, Furuya N, Saiki S, Amo T, Imamichi Y, Hattori N. 2019. Iron supply via NCOA4-mediated ferritin degradation maintains mitochondrial functions. *Mol Cell Biol* 39:e00010-19. <https://doi.org/10.1128/MCB.00010-19>.

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Received 3 January 2019

Returned for modification 28 January 2019

Accepted 29 April 2019

Accepted manuscript posted online 6 May 2019

Published 27 June 2019

Here, we report that iron derived from NCOA4-mediated ferritin complex degradation in lysosomes is supplied to mitochondria to maintain their functions.

RESULTS AND DISCUSSION

Iron deficiency reduces mitochondrial respiration, the respiratory chain complex assembly, and membrane potential. To test the effect of treatment with deferoxamine (DFO), an iron chelator, on ferritin degradation, we performed Western blotting of FTH1 and FTL (Fig. 1A). FTH1 and FTL were reduced time dependently with DFO. FTL was more rapidly decreased than FTH1 by DFO. To investigate the effect of DFO treatment on ferritin complex degradation, we performed blue native polyacrylamide gel electrophoresis (BN-PAGE), a method to observe native protein complexes (20, 21), followed by immunoblotting. FTH1 and FTL form a 480-kDa complex comprising 24 ferritin molecules (22). Iron chelation degraded ferritin complexes in a time-dependent manner, and ferritin complexes were significantly reduced after DFO incubation for 8 h and reached baseline levels at 16 h (Fig. 1B).

To examine the effect of DFO treatment on mitochondrial respiration, oxygen consumption rates (OCRs) were measured by using an extracellular flux analyzer (Fig. 1C). OCRs were reduced by DFO in a time-dependent manner. Basal respiration, ATP production, and maximum respiration were significantly reduced by 8, 12, and 6 h of DFO treatment, respectively (Fig. 1D). Large numbers of ferritin complexes were degraded by DFO treatment at 8 h, implying that there is an association between ferritin complex degradation and the reduction of mitochondrial respiration. To assess respiratory chain complex levels, BN-PAGE was performed (Fig. 1E). Although protein levels of respiratory chain complex subunits were slightly lower (complex I) or unchanged (complexes II, III, and IV), mitochondrial respiratory chain complex I, II, and III levels were reduced in mitochondrial fractions prepared from DFO-treated cells (decreases in complexes I, II, and III were 74%, 33%, and 21%, respectively) (Fig. 1E and F). Iron chelation did not affect the assembly of complexes IV and V. Reduction of complexes I and II by DFO treatment was confirmed using another antibody (Fig. 1G). Mitochondrial membrane potential is produced by the activities of complexes I, III, and IV (23). To test mitochondrial membrane potential in DFO-treated cells, we costained cells with Tom20 antibody and MitoTracker Red CMXRos, which is taken up only by mitochondria with preserved membrane potentials. Mitochondria in untreated cells were stained with Tom20 antibody and MitoTracker, whereas valinomycin, a mitochondrial uncoupler, significantly reduced MitoTracker staining. Loss of MitoTracker staining in mitochondria of DFO-treated cells occurred in a time-dependent manner (Fig. 1H and I).

These results showed that iron deprivation induces the reduction of respiratory complexes I to III, which is associated with the reductions of OCRs and mitochondrial membrane potential. Cytoplasmic iron is vital for maintaining the assembly of respiratory chain complexes and respiratory activity in mitochondria.

Lysosomal function is required for the maintenance of mitochondrial respiration, respiratory chain complex assembly, and membrane potential. When ferritin complex levels reached their nadir (or they were faintly detected) (upon DFO treatment for 16 to 24 h) (Fig. 1B), OCR reduction was clearly observed. Therefore, we hypothesized that iron is supplied to mitochondria by the degradation of ferritin complexes during DFO treatment. Ferritin is degraded in lysosomes, and its degradation is inhibited by lysosome inhibitors such as bafilomycin A1 (BAF) or E64d with pepstatin A (15, 16). Indeed, FTL degradation by DFO was blocked by BAF and E64d with pepstatin A (Fig. 2A) but not by epoxomicin, a proteasome inhibitor (see Fig. S1A in the supplemental material). Degradation of ferritin complexes was completely inhibited by BAF but not by epoxomicin (Fig. 2B). Immunofluorescence (IF) staining revealed that ferritin colocalized with LAMP2, a lysosomal membrane protein, in control cells. DFO increased the colocalization of ferritin and LAMP2, which was abrogated by BAF (Fig. S1B and C).

FIG 1 Iron deficiency disrupts mitochondrial respiratory chain complex assembly and reduces mitochondrial respiratory activity and membrane potential. (A) HeLa cells were incubated with 100 μ M DFO for the indicated times and immunoblotted using anti-FTH1 and (Continued on next page)

As was the case for DFO treatment for 12 h, treatment with BAF for 24 h significantly decreased OCRs (Fig. 2D and E). BAF is known to severely disrupt endocytic trafficking and, importantly, the acidification of endosomes. Therefore, we examined the effect of the lysosomal protease inhibitor E64d with pepstatin A on OCRs (Fig. S1D and E). While E64d with pepstatin A also reduced OCRs, the effect of E64d with pepstatin A treatment on OCRs was smaller than that of BAF treatment. These results suggest that not only ferritin degradation in lysosomes but also the endocytic pathway affects mitochondrial functions. Intracellular ferritin levels were increased by the inhibition of ferritin degradation or extracellular supplementation with ferric ammonium iron (FAC) (Fig. 2C). In addition to the reduction of OCRs by 24-h treatment with BAF or 12-h treatment with DFO, FAC supplementation partially rescued the reduction in respiration by BAF, DFO, or E64d with pepstatin A (Fig. 2D and E and Fig. S1D and E). Combined treatment with DFO and E64d with pepstatin A for 6 h significantly decreased OCRs compared with treatment with each of these agents alone, indicating that DFO-mediated ferritin degradation in lysosomes provided iron for mitochondrial usage in the initial phase of iron chelation (Fig. S1F and G). Respiratory chain complex assembly was also reduced by BAF but not by epoxomicin (Fig. 2F). Similarly, complex disassembly was completely rescued by FAC (Fig. 2G). The mitochondrial membrane potential also collapsed due to BAF (Fig. S1H and I). Notably, partial amelioration of respiratory chain complex assembly and membrane potential in BAF-treated cells was similarly achieved by FAC treatment (Fig. 2D, E, and G and Fig. S1H and I). These results suggest that lysosomal functions are required for the supply of iron to mitochondria via ferritin degradation, and the extracellular addition of iron rescued mitochondrial respiration lysosome independently.

NCOA4-mediated ferritin complex degradation contributes to the maintenance of mitochondrial respiration. NCOA4 functions as a selective autophagic receptor for ferritin, and the knockdown (KD) of NCOA4 suppressed the degradation of ferritin and colocalization of ferritin and lysosomes upon treatment with DFO in several cell types (17). To investigate the role of NCOA4-mediated ferritin degradation in mitochondrial respiration, we used NCOA4 KD using small interfering RNA (siRNA) in HeLa cells. The degradation of FTH1, FTL, and ferritin complexes by DFO treatment was inhibited by NCOA4 KD (Fig. 3A and B). Increased ferritin levels (especially FTL) were observed in not only NCOA4 KD HeLa cells but also NCOA4 KD SH-SY5Y cells, even under basal conditions (Fig. S2A), suggesting that ferritin is constantly degraded by an NCOA4-dependent pathway. To confirm that the elevated FTL levels in NCOA4 KD cells were caused by the inhibition of degradation, we treated cells with cycloheximide (CHX), an inhibitor of translation. The protein levels of ferritin and NCOA4 were reduced by CHX in a time-dependent manner, but the reduction was inhibited by BAF treatment or NCOA4 KD (Fig. S2B). The increase of FTL levels in NCOA4 KD was blocked by CHX (Fig. S2C). Surprisingly, the increase in the levels of NCOA4 by BAF treatment was also reduced by CHX (Fig. S2C). Therefore, FTL and NCOA4 were constantly synthesized and subjected to rapid turnover in lysosomes. Immunostaining analysis revealed that NCOA4 KD induced a more diffuse distribution of FTH1 than that in the controls and prevented the colocalization of ferritin with lysosomes under basal and iron-deprived conditions (Fig. 3C and D).

FIG 1 Legend (Continued)

anti-FTL antibodies. (B) The postnuclear supernatant (PNS) fraction obtained from DFO-treated HeLa cells was subjected to BN-PAGE followed by immunoblotting. (C) OCRs in DFO-treated HeLa cells were measured by using an extracellular flux analyzer. Next, 1 μ M oligomycin A, 1 μ M FCCP, and 5 μ M antimycin A with 10 μ M rotenone were injected at the indicated times to determine the proportions of oxygen consumption due to ATP production and the basal and maximum rates of respiration. The results are from three independent experiments. (D) Basal respiration, ATP production, and maximum respiration were quantified. (E and G) Isolated mitochondria were prepared from HeLa cells treated with 100 μ M DFO for 12 and 24 h. BN-PAGE and SDS-PAGE were performed using the indicated antibodies. Data are representative of results from three independent experiments. (F) Results of quantification of data in panel E from three independent experiments. (G) HeLa cells were treated with 100 μ M DFO or 1 μ M valinomycin for the indicated times and with anti-Tom20 and MitoTracker CMXRos. Bar = 10 μ m. (H and I) Results of quantification of the fluorescence intensity ratios of MitoTracker Red CMXRos and Tom20 from 30 cells. Error bars represent mean values $\pm$ standard errors of the means (SEM). ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Dunnett's multiple-comparison test was performed for panel I).

FIG 2 A lysosomal inhibitor reduces respiratory chain complex assembly and induces mitochondrial dysfunction. (A) HeLa cells were incubated with 100 μ M DFO, 10 nM BAF, and 50 μ g/ml E64d with pepstatin A for 24 h. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. Data are representative of results from three independent experiments. (B) The PNS obtained from HeLa cells treated with 100 μ M DFO and 10 nM BAF or 1 μ M epoxomicin was subjected to BN-PAGE prior to Western blotting using the indicated antibodies. Data are representative of results from three independent experiments. (C) Cell lysates from HeLa cells incubated with 100 μ M DFO, 100 μ M FAC, 10 nM BAF, or 100 μ M FAC for 24 h were immunoblotted with the indicated antibodies. Data are representative of results from three independent experiments. (D) OCRs in cells treated with 100 μ M DFO, 10 nM BAF, or 10 nM BAF with 100 μ M FAC for the indicated times were measured. Results are from five independent experiments. (E) Basal respiration, ATP production, and maximum respiration were quantified. (F and G) Mitochondria were isolated from HeLa cells treated under the indicated conditions. SDS-PAGE and BN-PAGE followed by immunoblotting were performed using the indicated antibodies. Error bars represent mean values $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$ (a Tukey-Kramer test was performed for panel E).

Levels of mitochondrial respiratory chain complex assembly and functions were assessed in NCOA4 KD cells. Complex I and II assembly in mitochondrial fractions isolated from NCOA4 KD cells was clearly reduced, even under basal conditions (Fig. 3E). NCOA4 KD reduced OCRs and membrane potential, and these effects were abrogated by FAC (Fig. 3F to H and Fig. S2D and E). Consistent with the findings in HeLa

FIG 3 The importance of NCOA4 in maintaining mitochondrial function via ferritin complex degradation in lysosomes. (A) HeLa cells were transfected with siNCOA4 or scrambled siRNA. Cells were incubated with 100 μ M DFO for 24 h and immunoblotted with the indicated antibodies. (B) The PNS of NCOA4 KD (Continued on next page)

cells, reduced levels of mitochondrial respiratory chain complex assembly and OCRs under basal conditions were observed in NCOA4 KD SH-SY5Y cells (Fig. S3F to H). These reductions were canceled by FAC treatment. Furthermore, the expression of an siRNA-resistant version of green fluorescent protein (GFP)-NCOA4 significantly and partially rescued respiratory chain assembly and OCRs suppressed by NCOA4 KD, respectively (Fig. 3I to L). To assess the effect of NCOA4 KD on mitochondrial iron, the fluorescent dye Mito-FerroGreen was used. Fluorescence intensity reflecting mitochondrial iron was significantly reduced in DFO-treated cells and NCOA4 KD cells compared with the levels in control cells (Fig. 3M and N). These results indicate that NCOA4-mediated ferritin complex degradation is required to maintain mitochondrial iron storage, mitochondrial respiratory activity, respiratory chain complex assembly, and membrane potential. Mitochondrial iron shortage caused by a deficiency in the NCOA4-mediated pathway can be rescued by the supply of extracellular iron.

Autophagy partially contributes to ferritin complex degradation under iron-deprived conditions. NCOA4 mediated the delivery of ferritin to lysosomes via autophagy by iron chelator treatment or starvation (17, 18). However, endosomal sorting complex required for transport (ESCRT) also participated in ferritin and NCOA4 turnover (24, 25). To evaluate the role of autophagy or ESCRT in mitochondrial respiration and NCOA4-mediated ferritin degradation, we knocked down FIP200, an essential component for autophagy, and the ESCRT components Hrs, USP8, and CHMP4B. The OCRs in FIP200 KD cells were significantly lower than those in control cells under basal and iron-deprived conditions, and respiratory activities in UPS8 and CHMP4B KD cells were comparable to those in control cells under basal and iron-deprived conditions (Fig. 4A and Fig. S3A and B). It was difficult to assess the effects of Hrs KD for OCRs, because more than 50% of Hrs KD cells died. Furthermore, the levels of ferritin and mitochondrial respiratory chain complex assembly in UPS8 and CHMP4B KD HeLa cells were comparable to those in control cells under basal and iron-deprived conditions (Fig. S3C to F). Consistent with the findings in FIP200 KD cells, ATG7 knockout (KO) mouse embryo fibroblasts (MEFs), an established model of autophagy deficiency (26), had lower OCRs than wild-type (WT) MEFs under basal and iron-deprived conditions (Fig. S3J). Thus, autophagy but not ESCRT components contributes to mitochondrial respiratory activity.

Although FTH1 and FTL degradation was partially inhibited by FIP200 KD and ATG7 KO upon DFO treatment, FIP200 KD and ATG7 KO did not influence FTH1 and FTL levels under basal conditions, in contrast to NCOA4 KD (Fig. 4B and Fig. S3G). Ferritin complex degradation was slightly inhibited by FIP200 KD and ATG7 KO under iron-deprived conditions (Fig. 4C and Fig. S3H).

The levels of assembly of complexes I and II in FIP200 KD cells and ATG7 KO MEFs were unchanged under basal and iron-deprived conditions (Fig. 4D and Fig. S3I). Moreover, OCRs were not recovered by FAC treatment of FIP200 KD cells and ATG7 KO MEFs (Fig. 4E and Fig. S3J). Thus, autophagy partially contributes to NCOA4-mediated ferritin degradation only under iron-deprived conditions. The reduction in OCRs in

FIG 3 Legend (Continued)

and control cells was subjected to BN-PAGE followed by immunoblotting with the indicated antibodies. (C) NCOA4 KD and control cells incubated with 100 μ M DFO were stained with DAPI (4',6-diamidino-2-phenylindole), anti-LAMP2, and anti-FTH1. Bar = 10 μ m. (D) LAMP2-positive lysosomes that colocalized with FTH1 from 30 cells. (E and F) HeLa cells transfected with siNCOA4 or control siRNA (siControl) were incubated with 100 μ M DFO for 12 h or 100 μ M FAC for 24 h. Mitochondria isolated from these cells were subjected to SDS-PAGE and BN-PAGE followed by immunoblotting using the indicated antibodies. (G) OCRs in NCOA4 KD and control cells treated under the indicated conditions were measured. (H) Results of quantification of basal respiration, ATP production, and maximum respiration. The results are from five independent wells. (I) HeLa cells transfected with control siRNA and siNCOA4 were expressed with siRNA-resistant GFP-NCOA4 or GFP. Western blotting was performed using the indicated antibodies. (J) HeLa cells transfected with control siRNA and siNCOA4 were expressed with siRNA-resistant GFP-NCOA4 or GFP. Isolated mitochondria were prepared from these HeLa cells. BN-PAGE and SDS-PAGE were performed using the indicated antibodies. (K) OCRs in NCOA4 KD and control cells transfected with or without siRNA-resistant GFP-NCOA4 or GFP were measured. (L) Quantification of data in panel K, from three independent experiments. (M) HeLa cells transfected with siNCOA4 or siControl were expressed with pMTS-mCherry, in which the mitochondrial targeting sequence of human PINK1 was ligated into mCherr-N (30). These cells, incubated with or without 100 μ M DFO for 24 h, were stained with 5 μ M Mito-FerroGreen. (N) Results of quantification of the fluorescence intensity ratios of mCherry and Mito-FerroGreen from 30 cells. Error bars represent mean values $\pm$ SEM. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Tukey-Kramer tests and Dunnett's multiple-comparison tests were performed for panels H and L and panels D and N, respectively).

FIG 4 Autophagy contributes to ferritin complex degradation under iron-deprived conditions. (A) HeLa cells transfected with siFIP200 or siControl oligonucleotides were incubated with 100 μ M DFO for the indicated times. OCRs were obtained from five independent wells. (B) Cells transfected with siFIP200 or siControl were treated with 100 μ M DFO for 24 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. (C and D) Mitochondria and the PNS were obtained from FIP200 KD and control cells treated with 100 μ M DFO for 24 h (C) and 12 h (D). BN-PAGE and SDS-PAGE followed by immunoblotting were performed with the indicated antibodies. (E) HeLa cells transfected with siFIP200 or siControl oligonucleotides were incubated with 100 μ M DFO or 100 μ M FAC for the indicated times. OCRs were obtained from five independent wells. Error bars represent mean values $\pm$ SEM.

autophagy-deficient cells was not due to a shortage of mitochondrial iron, indicating that the mitochondrial dysfunctions in autophagy-deficient cells might be caused by defects in other cellular mechanisms.

Ferritin light chain supplies iron to mitochondria via the NCOA4-mediated pathway under iron-deprived conditions. We showed that NCOA4-mediated ferritin complex degradation supplies iron to mitochondria. The formation of ferritin complexes, as an intracellular iron pool, might be important for mitochondrial respiration. To confirm this, we knocked out FTL in HeLa cells using CRISPR/Cas9. Interestingly, similarly sized ferritin complexes (FTH1 homopolymer) were formed in FTL KO cells, but the degradation of FTH1 and FTH1 complexes by DFO was reduced in FTL KO cells compared with that in WT cells (Fig. 5A and B). Immunostaining showed that FTH1 was diffusely distributed and that LAMP2 colocalization was lost in FTL KO cells (Fig. 5C). FTH1 was diffusely localized in NCOA4 KD cells (Fig. 3C); therefore, FTL KO might influence the colocalization of or interaction between NCOA4 and ferritin. While GFP-NCOA4 colocalized with FTH1 in GFP-NCOA4-expressing WT cells, colocalization of GFP-NCOA4 and FTH1 was not observed in FTL KO cells (Fig. 5D). Consistent with this, the interaction of NCOA4 and FTH1 was reduced in FTL KO cells compared with that in WT cells (Fig. 5E). Thus, the lack of FTL expression reduced the interaction between NCOA4 and FTH1 and delayed the degradation of the ferritin complex.

Levels of mitochondrial respiratory activity in FTL KO cells were comparable to those in WT cells under basal conditions (Fig. 5F and G). FTL KO delayed FTH1 degradation by DFO; therefore, mitochondrial respiration of FTL KO cells might be influenced by DFO

FIG 5 The ferritin light chain is required for iron supply to mitochondria under iron-deprived conditions. (A) Wild-type (WT) and FTL knockout (KO) HeLa cells treated with 100 μM DFO for the indicated times were immunoblotted with the indicated antibodies. (B) The PNS was obtained from WT and FTL KO HeLa (Continued on next page)

with a shorter incubation time than for WT cells. We subjected cells to DFO for 6 h, which was the initial time point where differences of OCRs were observed (Fig. 1C and D). As expected, OCRs were significantly reduced in FTL KO cells compared with those in WT cells after 6 h of iron deprivation (Fig. 5F and G). Lower levels of complexes I and II were observed in FTL KO cells than in WT cells after DFO treatment for 6 h (Fig. 5H). Thus, FTL KO cells acquire iron via routes other than ferritin complex degradation to maintain mitochondrial functions under basal conditions. However, shutting off alternative routes of iron supply by DFO reduced mitochondrial respiration more rapidly in FTL KO cells.

The exogenous expression of FLAG-tagged FTL rescued the FTL KO phenotype, including impaired FTH1 degradation, formation of FTH1 punctate structures, and colocalization of LAMP2 and NCOA4 (Fig. 5I to K). Furthermore, the reduction of complexes I and II and OCRs was rescued by the exogenous expression of FLAG-FTL in FTL KO cells (Fig. 5H, L, and M).

In addition to iron binding capacity, FTH1 has ferroxidase activity and interacts with NCOA4 (27), but the functions of FTL are unclear. A loss-of-function mutation in the FTL gene was associated with idiopathic generalized seizures and atypical restless leg syndrome (28). Increased iron incorporation into FTH1 homopolymers reduced cellular iron availability and increased reactive oxygen species in fibroblasts obtained from the patient (28). These phenotypes of FTL-mutated fibroblasts are similar to those of FTL KO HeLa cells. Therefore, FTL might be important for ferritin complex formation, interactions with NCOA4 and the ferritin complex, and the degradation of ferritin complexes.

Taking these findings, NCOA4-mediated ferritin complex degradation in lysosomes is important for supplying iron to mitochondria to maintain mitochondrial respiratory chain complexes, respiratory activity, and membrane potential. Under conditions of iron deprivation, autophagy partially contributes to the degradation of ferritin and its complexes but not to mitochondrial function. FTL is important for the formation of “regular” ferritin complexes and the production of the intracellular iron pool used by mitochondria.

MATERIALS AND METHODS

Antibodies and reagents. Anti-FTH1 (catalog number 4393) and anti-SDHA (catalog number 11998) antibodies were obtained from Cell Signaling Technology (Boston, MA). Anti-FTL (catalog number ab69090), anti-NDUFA9 (catalog number ab14713), anti-MTDC1 (catalog number ab14705), anti-SDHB (catalog number ab14714), anti-UQCRCF51 (catalog number ab14746), and Oxphos cocktail (catalog number ab110413) antibodies were obtained from Abcam (Cambridge, UK). Anti- β -actin (clone C4) and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (catalog number 2271144) were obtained from Millipore (Billerica, MA). Anti-UQCRC1 (catalog number 21705-1-AP) and anti-FIP200 (RB1CC1) (catalog number 17250-1-AP) antibodies were obtained from Proteintech (Rosemont, IL). Anti-Tim23 (catalog number 61222) antibody was obtained from BD Transduction Laboratories (San Jose, CA). Anti-NDUFA9 (catalog number GTX113878) antibody was obtained from Genetex (Irvine, CA). Anti-COX2 (catalog number EP1978Y) and anti-LAMP2 (catalog number NBP2-22217) antibodies were obtained from Novus (Littleton, CO). Anti-Tom20 (catalog number sc-11415) antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Antiferritin (catalog number 29925) (for IF staining) antibody was

FIG 5 Legend (Continued)

cells treated with 100 μ M DFO for 24 h. Shown are results from BN-PAGE followed by immunoblotting with the indicated antibodies. (C) Representative images of WT and FTL KO cells incubated with 100 μ M DFO for 24 h and immunostained with anti-LAMP2 and anti-FTH1 antibodies. Bar = 10 μ m. (D) WT and FTL KO HeLa cells expressing GFP-NCOA4 incubated with 100 μ M DFO for 24 h. Immunostaining with anti-FTH1 antibodies was performed. Arrows indicate GFP-NCOA4 puncta that colocalized with FTH1. Bars = 10 μ m. (E) FTL WT and KO cells expressing GFP-NCOA4 or GFP were incubated for 48 h. Cell lysates were subjected to immunoprecipitation (IP) using anti-GFP magnetic beads. Immunoprecipitates were analyzed by Western blotting. (F) OCRs in WT and FTL KO cells were measured. OCRs were obtained from four independent wells. (G) Results of quantification of basal respiration, ATP production, and maximum respiration. The results are from five independent wells. (H) Mitochondria were isolated from FTL WT and KO cells expressing FLAG-FTL or an empty vector incubated with 100 μ M DFO for 6 h. BN-PAGE and SDS-PAGE followed by immunoblotting were performed with the indicated antibodies. (I) FTL KO cells, FLAG-FTL cells, or empty vector cells were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (J and K) FTL KO cells were transfected with FLAG-FTL or an empty vector with (J) or without (K) GFP-NCOA4. Cells treated with 100 μ M DFO for 24 h were immunostained with anti-LAMP2 and anti-FTH1 (K) or anti-FTH1 (J) antibodies. Bars = 10 μ m. (L) OCRs in FTL KO cells transfected with FLAG-FTL or an empty vector were measured. OCRs were obtained from five independent wells. (M) Results of quantification of basal respiration, ATP production, and maximum respiration from panel L. Error bars represent mean values $\pm$ SEM. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Tukey-Kramer tests were performed for panels G and M).

obtained from Rockland Immunochemicals Inc. (Limerick, PA). Anti-NCOA4 (ARA70) (catalog number A302-272A) antibody was obtained from Bethyl Laboratories Inc. (Montgomery, TX). Anti-DYKDDDDK tag antibody was obtained from Wako Laboratory Chemicals (Osaka, Japan) (catalog number 017-25151). Anti-ATG7 antibody was a kind gift from Takashi Ueno and Isei Tanida (Juntendo University, Japan) (29). Valinomycin (catalog number V0627), bafilomycin A1 (catalog number B1793), E64d (catalog number E8640), pepstatin A (catalog number P5318), epoxomicin (catalog number E3652), ferric ammonium iron (catalog number F5789), and cycloheximide (catalog number C7698) were obtained from Sigma-Aldrich (St. Louis, MO). Deferoxamine mesylate (catalog number 1883-500) was obtained from BioVision (Milpitas, CA). MitoTracker Red CMXRos (catalog number M7512) was obtained from Thermo Fisher Scientific (Waltham, MA). Mito-FerroGreen was obtained from the Dojindo Laboratory (Kumamoto, Japan). The ON-TARGETplus human siRNA oligonucleotides for NCOA4 (catalog number L-010321-00-0005), FIP200 (catalog number L-021117-00-0005), USP8 (catalog number L-005203-00-0005), and CHMP4B (catalog number L-018075-01-0005) were obtained from Dharmacon (Lafayette, CO).

Plasmid construction. Human FTL and NCOA4 were cloned by PCR using HeLa cell cDNA as a template. To construct expression plasmids for FTL and NCOA4, PCR products and a FLAG tag or GFP were ligated into pcDNA3.1-Hyg(+) (Life Technologies, Carlsbad, CA). For the cloning of an siRNA-resistant NCOA4 expression plasmid, we replaced nucleotide sequences for NCOA4 siRNA (siNCOA4) target regions so as not to change the amino acid sequence by PCR.

Cell culture and transfection. HeLa cells and FTL KO HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Nacalai Tesque, Kyoto, Japan), at 37°C in a 5% CO₂–95% air atmosphere. Plasmid DNA was transfected into cells using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were used in the experiments. For RNA interference experiments, siRNA oligonucleotides were transfected into cells using Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's instructions. Ninety-six hours after siRNA transfection, cells were used in the experiments. ATG7 KO MEFs were originally generated by Takashi Ueno and Masaaki Komatsu and were a kind gift from them.

Immunofluorescence staining. Cells grown on glass chamber slides were fixed in 4% paraformaldehyde. For immunostaining, cells were incubated with appropriate primary antibodies or MitoTracker CMXRos followed by Alexa Fluor 488- or 594-conjugated secondary antibodies. Fluorescence staining was visualized using a Zeiss LSM770 confocal microscope (Carl Zeiss, Oberkochen, Germany). FTH1 colocalization with LAMP2 was quantified, and the intensity of MitoTracker CMXRos and Tom20 was determined using ImageJ software.

Western blotting. For SDS-PAGE, cells were lysed in lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a protease inhibitor cocktail). Lysates were centrifuged at 15,000 $\times g$ for 10 min at 4°C to remove debris. For immunoblotting, supernatants were subjected to 10%-to-20% gradient SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a polyvinylidene fluoride membrane and probed with the indicated primary antibodies. This was developed by using a LAS-4000 mini instrument (GE Healthcare UK Ltd., Little Chalfont, UK).

Immunoprecipitation. FTL KO or WT HeLa cells expressing GFP-NCOA4 or GFP were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease and phosphatase inhibitor cocktails (catalog numbers 11873580001 and 04906837001; Roche Diagnostics) for 20 min on ice. Supernatants obtained after centrifugation at 15,000 $\times g$ were incubated with anti-GFP monoclonal antibody (mAb) magnetic beads. Immunoprecipitates were eluted in SDS-PAGE sample buffer.

BN-PAGE. Cells were scraped and soaked in 250 mM isotonic sucrose buffer (10 mM HEPES-KOH [pH 7.4], 1 mM EDTA, and a protease inhibitor cocktail). Needle homogenization was performed, followed by centrifugation at 1,000 $\times g$ for 5 min at 4°C to remove debris. To assess the ferritin complex, 1% *n*-dodecyl- β -D-maltoside was added to the supernatant. To assess mitochondrial respiratory chain complexes, the rest of the supernatant was centrifuged at 8,000 $\times g$ for 10 min at 4°C to isolate mitochondrial fractions. Next, 10 μ g mitochondria obtained from the pellet was subjected to 0.5% *n*-dodecyl- β -D-maltoside treatment. Samples were subjected to BN-PAGE using 3% to 12% Bis-Tris protein gels (Invitrogen), followed by Western blotting.

Extracellular flux analyzer. Cells were seeded in XF24 cell culture plates (catalog number 100867-100; Agilent) at 70,000 cells per well and cultured for 12 h, followed by treatment with the indicated drugs. XF Cell Mito stress test compounds (catalog number 103015-100) [including oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine (FCCP), antimycin A, and rotenone] optimized for each cell type were loaded into the assay cartridge. OCR measurement was performed by using an extracellular flux analyzer (Agilent Technologies, Seahorse Bioscience, Santa Clara, CA). After OCR investigation, cells were lysed in 100 μ l lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a protease inhibitor cocktail) to measure protein concentrations.

Establishment of an FTL knockout cell line. FTL KO HeLa cells (catalog number CL0035195934A) with CRISPR/Cas9 technology were generated by EdiGenes Biotechnology Inc. (Beijing, China). A 1-bp deletion was included in allele 1, and oligonucleotides were inserted into allele 2 in exon 1. Sanger sequencing confirmed 100% KO.

Statistical analysis. Multiple data sets were examined for statistical significance using one-way analysis of variance with a Tukey-Kramer *post hoc* test or Dunnett's multiple-comparison test. Both analyses were performed by using JMP 13.0.0 (SAS Institute Inc.).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MCB.00010-19>.

SUPPLEMENTAL FILE 1, PDF file, 5.7 MB.

SUPPLEMENTAL FILE 2, PDF file, 4.9 MB.

SUPPLEMENTAL FILE 3, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Takashi Ueno (Laboratory of Proteomics and Biomolecular Science, Research Support Centre, Juntendo University) and Masaaki Komatsu (Niigata University) for providing ATG7 KO MEFs. We also thank Yukiko Sasazawa (Juntendo University), Soichiro Kakuta (Juntendo University), and Kazuhiro Iwai (Kyoto University) for helpful comments and discussion. We thank Annie Li, the senior manager of Edigene Biotechnology Inc., and coworkers for establishing FTL KO HeLa cells. We thank the Edanz Group for editing a draft of the manuscript.

This work was supported by grants-in-aid for scientific research (C) (15K09325 to N.F.), grants-in-aid for scientific research on innovative areas (25111007 to S.S.), grants-in-aid for scientific research (B) (15H04842 to N.H., 15H04843 to S.S., 18H02744 to S.S., and 18KT0027 to S.S.), grants-in-aid for scientific research (A) (18H04043 to N.H.), and the MEXT-supported Program for the Scientific Research Foundation at Private Universities, 2014 to 2017 (to N.F.).

We declare no competing interests.

M.F. and N.F. conceptualized the study; M.F. performed formal analysis; N.F., S.S., and N.H. acquired funding; M.F., N.F., Y.L., and T.A. performed the investigation; S.S., T.A., and N.H. supervised the experiments; and M.F. and N.F. wrote the original draft.

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