Iron regulatory protein deficiency compromises mitochondrial function in murine embryonic fibroblasts

Huihui Li¹, Hongting Zhao¹, Shuangying Hao¹, Longcheng Shang¹, Jing Wu¹, Chuanhui Song¹, Esther G. Meyron-Holtz², Tong Qiao³ & Kuanyu Li¹

Iron is essential for growth and proliferation of mammalian cells. The maintenance of cellular iron homeostasis is regulated by iron regulatory proteins (IRPs) through binding to the cognate iron-responsive elements in target mRNAs and thereby regulating the expression of target genes. Irp1 or Irp2-null mutation is known to reduce the cellular iron level by decreasing transferrin receptor 1 and increasing ferritin. Here, we report that Irp1 or Irp2-null mutation also causes downregulation of frataxin and IscU, two of the core components in the iron-sulfur cluster biogenesis machinery. Interestingly, while the activities of some of iron-sulfur cluster-containing enzymes including mitochondrial aconitase and cytosolic xanthine oxidase were not affected by the mutations, the activities of respiratory chain complexes were drastically diminished resulting in mitochondrial dysfunction. Overexpression of human ISCU and frataxin in Irp1 or Irp2-null cells was able to rescue the defects in iron-sulfur cluster biogenesis and mitochondrial quality. Our results strongly suggest that iron regulatory proteins regulate the part of iron sulfur cluster biogenesis tailored specifically for mitochondrial electron transport chain complexes.

Iron is essential for growth and proliferation of mammalian cells mainly because iron is a crucial component of heme and iron-sulfur clusters (Fe-S) therefore indispensable for DNA synthesis, oxygen transport and ATP production. Iron deficiency is known to cause anaemia and permanent neurocognitive and motor impairments1,2. Yet, excess cellular iron could lead to the generation of reactive oxygen species that damage macromolecules such as DNA, lipid, and proteins. Excess iron in brain has been found to be associated with common neurodegenerative disorders, including Alzheimer’s, Parkinson’s, and Friedreich’s ataxia1,3. Therefore, organisms and cells must precisely regulate iron metabolism.

In mammals, cellular iron metabolism is regulated by iron regulatory protein (IRP) 1 and 24,5. IRPs post-transcriptionally regulate the expression of target protein by binding to iron responsive elements (IREs) located within the 5′- or 3′-untranslated region (UTR) of the target gene transcripts. These transcripts mostly encode iron metabolism proteins, including the iron storage protein, ferritin, and iron import protein, transferrin receptor 1 (TfR1)⁶. When cells are iron-deficient, IRPs bind IREs located in the 5′-UTR of ferritin to inhibit the translation and in the 3′-UTR of TfR1 to stabilize the mRNA to facilitate iron import. When cells are iron-sufficient, IRP1 is converted to a [4Fe-4S]-containing aconitase and IRP2 is degraded through iron mediated proteasomal degradation⁷-⁹, which increases ferritin translation and promotes TfR1 mRNA degradation to prevent more iron absorption and avoid the excess iron-induced injury. Thus, IRPs play critical roles in cellular iron homeostasis.

The mouse models of Irp1 and Irp2 deficiency have been generated¹⁰-¹². Irp1⁻/⁻ mice display polycythemia due to derepression of the Irp1-specific target mRNA of hypoxia-inducible factor 2α¹³-¹⁵. Two mouse models of global Irp2 deficiency display dysregulation of ferritin and TfR1 and abnormal iron content in several tissues, and
develop microcytic anaemia and erythropoietic protoporphyria\(^{16,17}\). Mice lacking \(\text{Irp2}\) also show symptoms of neurological disorders with motor neuron death\(^{1,18,19}\). The motor neuron death observed in the \(\text{Irp2}\)-null mice is likely caused in part by the diminished size of the ‘functional’ iron pool (compared to ‘total’) due to the reduced \(\text{TIR1}\) expression and increased ferritin expression\(^{20}\). Importantly, the role of IRPs in mitochondrial iron homeostasis has also been uncovered\(^{21}\). Irp1 activation is thought to be critical for sustaining the mitochondrial iron supply therefore maintaining normal mitochondrial function\(^{22}\). \(\text{Irp2}\)-null mice showed tissue-specific iron insufficiency and compromised motor neurons and their mitochondrial function\(^{20}\). However, the exact mechanism by which IRPs sustain normal mitochondrial function has yet to be determined.

Both Fxn and IscU are essential proteins, with Fxn acting as an iron chaperone for iron delivery or an allosteric factor that modulates the cysteine desulfurase activity (see review\(^{23}\)), and IscU as a scaffold protein for Fe-S cluster biogenesis. Deficiency of human Fxn causes Friedreich ataxia, a neurodegenerative mitochondrial disease\(^{24}\). A muscle-specific alternative mis-splicing of human ISCU renders ISCU myopathy, also known as “myopathy with deficiency of succinate dehydrogenase and aconitase”\(^{25}\). It is widely believed that deficiency of Fe-S cluster biogenesis is causative of mitochondrial dysfunction in both above mentioned diseases and that iron subcellular mislocalization is a common phenomenon in the disorders of Fe-S biogenesis\(^{4}\). Thus, we wonder whether the biological manifestation of IRPs deficiency shares the same or part of the mechanism of Fxn or IscU deficiency.

In this study, we confirmed that murine embryonic fibroblasts (MEFs), derived from global \(\text{Irp1} \text{ or } \text{Irp2}\) deficient mice, showed cellular iron starvation due to the low expression of \(\text{TIR1}\) and the increased expression of ferritin. More importantly, we found that depletion of IRPs led to deficiencies of Fxn and IscU. Furthermore, the IRP depletion-induced deficiencies in Fxn and IscU impaired specifically Fe-S cluster-dependent mitochondrial respiratory chain activities, but not the activity of either mitochondrial aconitase or cytosolic xanthine oxidase (Xod). Our results suggest that Irp affects the Fe-S cluster biogenesis tailored specifically for mitochondrial electron transport chain (ETC) complexes.

**Results**

**Irp1 or Irp2 ablation induces downregulation of the components of Fe-S cluster biogenesis machinery.** It has been reported that mice lacking \(\text{Irp1}\) show systemic iron deficiency in the later age\(^{13}\) and lacking \(\text{Irp2}\) show neurodegenerative symptoms\(^{11,26}\), which is thought due to the reduction of “functional iron pool”\(^{28}\). To gain further understanding of this phenotype, we used MEFs derived from global \(\text{Irp1} \text{ or } \text{Irp2}\) deficient mice and measured iron content in mitochondrial and extra-mitochondrial (mito and extra-mito) fractions. First, fractionation was performed to separate mito from extra-mito fraction. Xod and cytochrome C (CytC) were used as markers to define the purity of extra-mito and mito fractions, respectively (Fig. 1a, upper panel). We quantified the purity of extra-mito and mito fractions by calculating the relative percentage of Xod or CytC in extra-mito and mito fractions after normalisation with total proteins as shown in Fig. 1a (bottom panel). Using ferrozone assays, we verified that in comparison with wild type (WT), MEFs from systemic knockout of \(\text{Irp1} \text{ or } \text{Irp2}\) were iron deficient in both extra-mitochondrial and mitochondrial compartments (Fig. 1b). No significant difference between \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells was observed. To further detect the cytosolic and mito labile iron pool (IIP, chelatable iron), we used both Calcein-AM and RPA, respectively, two well accepted iron probes, by monitoring the decay of these two fluorescent dyes. As shown in Fig. 1c, the cytosolic iron decreased significantly in both \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells comparing with WT cells, while the mito available iron content exhibited little if any changes (Fig. 1c).

Since cellular iron homeostasis is mainly controlled by the IRP/IRE machinery in mammalian cells, we verified Irps-posttranscriptionally regulated target proteins ferritin and TIR1. In comparison with WT cells, the protein level of \(\text{TIR1}\) drastically decreased and of ferritin increased in both \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells (Fig. 2a), which is consistent with the previous studies\(^{13,20}\). These results confirm that \(\text{Irp1}\) or \(\text{Irp2}\) deficiency reduces iron uptake and increases intracellular iron chelation, which both cause cellular iron deprivation.

Iron is revealed as a key regulator of mitochondrial biogenesis\(^{37}\) and expression of Fxn and IscU is regulated by iron\(^{28,29}\), we therefore examined whether Fxn and IscU levels were altered in \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells. We first confirmed our self-made anti-Fxn antibody\(^{30,31}\), then compared Fxn and IscU protein levels among WT, \(\text{Irp1}^{-/-}\), and \(\text{Irp2}^{-/-}\) cells by immunoblotting. As shown in Fig. 2b, the antibody against Fxn was able to specifically detect the endogenous Fxn in both human and mouse samples as well as the overexpressed human Fxn. When over-expressed, Fxn (precursor, intermediate, and mature forms\(^{32,36}\) were clearly detected as marked with asterisks (Fig. 2b lane 2). Importantly, the levels of Fxn and IscU were markedly lower in both \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells than in WT cells (Fig. 2a), with quantification showed a more than 50% reduction (Fig. 2c). The data revealed that Irp deficiency resulted in a significant reduction in both Fxn and IscU levels.

**Downregulation of the components of Fe-S biogenesis machinery in Irp1 and 2 deficient fibroblasts is specifically associated with impaired mitochondrial respiratory chain.** Both Fxn and IscU are known as two of core components for Fe-S cluster biogenesis. Reduction of both Fxn and IscU suggests that Fe-S cluster dependent pathways were likely defective in \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells. To ascertain whether this is the case, we first measured the activities of Fe-S cluster-dependent enzyme aconitase of WT, \(\text{Irp1}^{-/-}\), and \(\text{Irp2}^{-/-}\) cells using an in-gel assay. As shown in Fig. 3a, the cytosolic aconitase activity (c-Aco) in \(\text{Irp2}^{-/-}\) cells was indeed decreased compared with WT (Fig. 3a), and its protein level was lower in this mutant than in WT cells. Surprisingly, no reduction was detected for mitochondrial aconitase (m-Aco) in both \(\text{Irp1}^{-/-}\) and \(\text{Irp2}^{-/-}\) cells. Instead, both activities and protein levels were clearly higher in the mutants than in WT cells. Additionally, we examined the cytosolic Fe-S cluster-containing enzyme Xod in the \(\text{Irp1}^{-/-}\) or \(\text{Irp2}^{-/-}\) cells. Similar to m-Aco described above, the protein level and activity of Xod were also higher in \(\text{Irp1}^{-/-}\) or \(\text{Irp2}^{-/-}\) cells than in WT (Fig. 3a and b). Based on these data, it appears that \(\text{Irp1}\) deficiency has similar effects to \(\text{Irp2}\) deficiency on some of Fe-S enzymes with increased both protein level and activity.
However, iron deficiency or downregulation of Fe-S cluster biogenesis generally brings about the low activities of Fe-S cluster dependent enzymes. We wonder if the phenotype we observed above is universal for Irp deficiency. Next, Fe-S cluster richest and dependent ETC were analysed in the Irp1−/− and Irp2−/− cells. We measured the activities of complex I, II, III and IV. As shown in Fig. 3c, the activities of both complex I and II in the mutant
cells were only about 40% of the WT level. The activity of complex III was reduced only in Irp2−/− cells, not in Irp1−/− cells. Complex IV activity was at the WT level in Irp1−/− cells and 30% higher in the Irp2−/− cells than in WT (Fig. 3c). The negative effects of Irp deficiency are specific on ETC complexes, which are known to be functionally dependent on Fe-S clusters except complex IV.

To further analyse the effects of Irp deficiency on complex I, II, and III, we also detected the levels of complex subunits by immunoblotting. As shown in Fig. 3d, the levels of Fe-S containing subunit Ndufs3 (Complex I) and SdhB (Complex II) were lower in both Irp1−/− and Irp2−/− cells than in the WT cells. The level of SdhA, a non-Fe-S subunit that complexes with SdhB, was higher in Irp1−/− cells and lower in Irp2−/− cells than WT cells. The expression of two subunits of complex III, Uqcrc1 (not containing Fe-S cluster) and Uqcrfs1 (Fe-S cluster-containing), was markedly decreased in Irp2−/− cells and mildly increased in Irp1−/− cells. To see a broader effect of Irp deficiency on mitochondrial biogenesis, we detected more other mitochondrial proteins, such as ferrochelatase (Fech, a matrix protein), CytC (an intermembrane protein), and Vdac (an outer membrane protein). The protein levels of CytC and Vdac were significantly decreased in both Irp1−/− and Irp2−/− cells compared with WT (Fig. 3d). However, the level of Fech was not changed in Irp1−/− cells and decreased in Irp2−/− cells (Fig. 3d). For comparison, we measured the enzymatic activity and protein level for citrate synthase, a critical and non-Fe-S cluster dependent enzyme in Krebs cycle. As shown in Fig. 3d and e, the activity and protein level in both Irp1−/− and Irp2−/− cells were similar to those in the WT. Taken together, these results reveal that Irp deficiency, particularly Irp2−/−, mainly affects Fe-S cluster dependent protein levels and activities of respiratory chain complexes.

**Figure 3.** Downregulation of the components of Fe-S cluster biogenesis machinery in Irp1 and 2 deficient fibroblasts is specifically associated with impaired electron transport chain (ETC). (a) A representative graph of in-gel assays of mitochondrial (m-Aco, encoded by Aco2) and cytosolic (c-Aco, encoded by Irp1) aconitases in Irp deficient MEF cells (upper panel). The protein levels of Aco2, Irp1, and cytosolic Fe-S containing enzyme xanthine oxidative (Xod) were detected with western blotting. (b) The activities of Xod, m-Aco, and c-Aco were quantified. (c) Activities of ETC complexes were determined in Irp deficient MEFs. (d) Western blot analysis of mitochondrial proteins including Ndufs3 (a subunit of CI), SdhA and SdhB (subunits of CII), Uqcrcl and Uqcrfs1 (subunits of CIII), Fech (matrix protein), CytC (intermembrane space protein), Vdac (outer membrane protein), and citrate synthase (Cs, matrix non-Fe-S protein). A representative image set is presented. (e) Activities of citrate synthase, which is a mitochondrial non-Fe-S enzyme, were determined. CI/CII/CIII/CIV: Complex I/II/III/IV. Values represent mean ± SEM (n = 10 for (e), n = 3, each duplicates for (b) and (c)). A one-way ANOVA was performed. *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT.
The increased expression of FXN and ISCU in Irp1 and 2 deficient fibroblasts reverses the cellular iron content and restores the activities of complexes. Because most of mitochondrial Fe-S cluster dependent ETC proteins are affected, we examined the role of IscU and Fxn downregulation in Irp depletion-induced malfunction of mitochondria. Human ISCU and FXN were expressed in Irp depleted cells to assess the effect on ETC basing on the conserved function of these proteins in human and rodents. Western blot analysis confirmed the successful expression of exogenous ISCU and FXN (Fig. 4a). Then, we measured the relative levels of cytosolic and mitochondrial LIP were measured with Calcein-AM and RPA, respectively (details see Materials and Methods). Values represent mean ± SEM (n = 3, each duplicates for (a), n = 5 for (b)). A one-way ANOVA was performed. ***p < 0.001 compared with WT. ###p < 0.001 compared with expression-plasmid transfected cells (+FXN + ISCU vs. -FXN-ISCU).

The co-expression of FXN and ISCU in Irp1 or Irp2-ablation cells improves mitochondrial integrity. To further confirm the effect of co-expression of FXN and ISCU in Irp depletion cells on mitochondria, we examined the integrity and quantity of mitochondria by measuring the intensity of mito-tracker staining and the copy number of mitochondrial DNA (mtDNA), respectively. Mito-tracker stains mitochondria in living cells and its accumulation is dependent upon mitochondrial membrane potential (MMP), a subcellular marker for mitochondrial integrity. The intensity of mito-tracker staining represents the level of MMP, revealed by Flow cytometry. The data repeatedly showed a clear right shift of the peak representing increased MMP in Irp1−/− and Irp2−/− cells. Although the cytosolic and mitochondrial labile iron pool increased after overexpression of TIR1 (Fig. 5c), the protein levels of Fxn and IscU were not changed and of mitochondrial complex subunits also kept constant (Fig. 5d). These results indicate that IRPs regulate respiratory chain function likely through modulating the expression of IscU and Fxn for Fe-S biogenesis and its targeted delivery.
Figure 5. Co-expression of FXN and ISCU in Irp1 or 2 deficient MEFs rescues the mitochondrial function.

(a) Activities of ETC CI, CII, and CIII were determined in Irp1 or Irp2 deficient MEFs after co-transfection with pcDNA3.1-FXN-myc and pXS-ISCU-myc (same plasmids as used in Fig. 4). Values represent mean ± SEM, n = 3, each duplicates. *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT. #p < 0.05, ##p < 0.01 compared +FXN+ISCU with −FXN−ISCU. (b) Western blot analysis of endogenous and exogenous FXN and ISCU, and mitochondrial respiratory chain proteins including Ndufs3 (a subunit of CI), SdhB (a subunit of CII), Uqcrcl (a subunit of CIII) in MEFs after co-transfection with pcDNA3.1-FXN-myc and pXS-ISCU-myc. The arrows indicate the precursor and mature forms of exogenous ISCU. A representative dataset is presented. (c) The relative levels of cytosolic and mitochondrial LIP were measured with Calcein-AM and RPA, respectively. Values represent mean ± SEM (n = 3, each duplicates). ****p < 0.0001 vs. WT, ***p < 0.001, **p < 0.01, *p < 0.05, +TfR1 vs. −TfR1. (d) Western blot analysis of iron related proteins including Tfr1, Fxn, IscU, and mitochondrial respiratory chain proteins including Ndufs1 (a subunit of CI), SdhB (a subunit of CII), Uqcrfs1 (a subunit of CIII) in MEFs after transfection with plasmid pcMV3-TFR1. A representative dataset is presented.
the ratio in mutant cells between before and after FXN and ISCU co-expression, but without significance (Fig. 6c).

Our data indicate that enhanced mitochondrial Fe-S cluster biogenesis by increasing expression of the core components improves the mitochondrial quality and quantity in Irp depletion cells.

Discussion

In this study, we report that Irp1 or Irp2-null mutation causes the decreased expression of Fxn and IscU, two important components of Fe-S cluster biogenesis machinery. Though Irp ablation down-regulates the expression of Fxn and IscU, the enzymatic activities of Fe-S cluster-containing aconitase and Xod were not diminished. Surprisingly, mitochondrial respiratory chain is severely impaired. Moreover, increasing the expression of FXN and ISCU reversed the Irp depletion-induced deficits including cellular iron content and activities of complex I, II, and III. Our results indicate that compromised function of the respiratory chain in response to Irp-depletion could be due to the downregulation of Fxn and IscU, which specifically reduces the acquisition of Fe-S cluster by respiratory complexes in MEFs.

Irp1 and Irp2 regulate the expression of target proteins posttranscriptionally by binding to IREs in transcripts that mostly encode iron metabolism proteins for iron uptake, storage, or export. Their targeted deletion causes changes in the levels of their direct targets including reduced levels of TIR and increased levels of ferritin in
Iron deprivation could strongly impair the mitochondrial respiratory chain and mitochondrial biogenesis in both IRP deficient cells, not in IRP−/− MEFs. Different from IRP−/− cells, IRP1−/− cells only showed defects of complex I and II activities. Expression of Uqcrcl and Uqcrfs1, two subunits of complex III (non-Fe-S cluster and Fe-S cluster-containing proteins, respectively), dramatically decreased in IRP2−/− cells, while IRP1−/− cells even expressed slightly more Uqcrcl and Uqcrfs1 than WT cells to maintain the complex III activity. Given the instability of proteins devoid of their Fe-S domain as for Sdhb and Ndufs3 in this study due to the iron starvation, a compensatory mechanism would raise the expression of non-Fe-S cluster-containing proteins as for SdhA and Uqcrcl. This was only observed in IRP1−/− cells, not in IRP2−/− cells. We did not check the expression of SDHAF1, a LYR complex-II specific assembly factor, important for SDH activity by interaction with SDHB. The pathogenic mutations of SDHAF1 abrogate binding to SDHB, which impairs biogenesis of holo-SDH and results in LONP1-mediated degradation of SDHB. The decreased expression of SdhA in IRP2−/− cells might also induce downregulation of SdhB protein expression. Alternatively, IRP ablation-induced iron deprivation might induce a decrease at mRNA levels of the components of complexes through dynamic alterations of histone acetylation and methylation. IRP ablation not only affects cellular iron content, but also affects the expression of components of Fe-S biogenesis machinery and subunits of ETC complexes to further trigger the deficiency of complex activities. This selective effect of IRP2 depletion on mitochondrial iron supply might partially explain the symptoms of neurological disorders in IRP2−/− mice. However, overexpression of human FXN and ISCU in IRP1−/− and IRP2−/− cells significantly improves the mitochondrial function and recovers the deficits of ETC complex subunits. Curiously, genetic and hypoxic alterations of the microRNA-210-ISCU1/2 axis promote iron-sulfur deficiency and pulmonary hypertension, the same preclinical phenotype raised from IRP1 depletion. IRP1 activation sustains mitochondrial iron supply and function rather than driving detrimental iron overload in Fxn deficient mice. These results support that the reduced expression of Fxn and Iscu is involved in the effects of IRP1 and IRP2 deficiency on impaired mitochondrial function to further cause the phenotypes in IRP−/−/− and IRP2−/− mice.

Collectively, our data provide in vitro evidence that depletion of IRP1 or IRP2 downregulates the expression of Fxn and Iscu and specifically compromises the activities of Fe-S cluster-containing ETC in MEFs. The current results reveal the role of IRP in securing mitochondrial function through regulating the expression of the core components, Fxn and Iscu, of Fe-S cluster biogenesis machinery. Our data also imply that IRPs specifically tailor Fe-S delivery for mitochondrial ETC complexes.

Materials and Methods

Cell culture and transfection. All media and reagents for cell culture were purchased from Invitrogen (Shanghai, China). MEFs (generously given by Dr. Tracey Rouault) derived from wild type (WT) and global IRP1 and IRP2 deficient mice were cultured in DMEM medium with 10% heat inactivated fetal bovine serum, 4 mM glutamine, penicillin, and streptomycin. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. For transfection, HG-Trans293 TM transfection reagent (Genomeditech, Shanghai, China) was used according to the supplier’s instructions. The transfected plasmids included pXS-ISCU-myc, pcDNA3.1-FXN-myc, and pcMV3-TRI1 for overexpression of ISCU, FNX, and TRI, respectively. Cells were harvested 24 h post-transfection for further analysis.
Subcellular fractionation. Mitochondria were isolated from cultured WT, Irp1−/−, and Irp2−/− cells, respectively, with the specialized cell mitochondrial isolation kit (Beyotime, Jiangsu, China) following the manufacturer’s instructions. Briefly, cells were harvested in mitochondrial isolation buffer with PMSF and transferred to a grinder for crushing until the isolation became homogeneous. The mitochondria were isolated by differential centrifugation at 600 g and 3500 g for 10 min at 4 °C, respectively. The latter pellet was washed with PBS one time, then stored in mitochondrial lysis buffer as mitochondrial fraction. The supernatant was transferred into new EP tubes and centrifuged at 12000 g for 10 min at 4 °C. This supernatant was considered as extra-mitochondrial fraction.

Ferrozine iron assays and labile iron pool (LIP) measurement. Iron content was measured using a colorimetric ferrozine-based assay with some modifications56. Briefly, 22 μl concentrated HCl (11.6 mol/L) was added to 100 μl cell lysate (~500 μg total protein). The mixed sample was heated at 95 °C for 20 min, then centrifuged at 12,000 g for 10 min. Supernatant was transferred very gently into fresh tubes. Ascorbate was added to reduce the Fe (III) into Fe (II). After 2 min of incubation at room temperature, ferrozine and saturate ammonium acetate (NH4Ac) were sequentially added to each tube and the absorbance was measured at 570 nm (BioTek EL x 800, Shanghai, China) within 30 min.

Labile iron was measured using the iron-sensitive probes Calcein-AM (Aladdin, Shanghai, China) and Rhodamine B-[(1, 10-phenanthroline-5-yl)-aminocarbonyl] benzyl ester (RPA, Squarix GmbH, Elbestr, Germany). Briefly, 2 × 105 WT, Irp1−/−, and Irp2−/− cells were incubated with 10 μM Calcein-AM for 10 min at 37 °C in PBS and then washed one time with PBS buffer. Cells were obtained in 100 μl PBS. Cytosolic LIP was measured using fluorescent microplate reader at 495 nm (excitation) and 530 nm (emission). For mitochondrial LIP, cells were incubated with 10 μM RPA for 15 min at 37 °C in HBSS, then incubated with 100 μl HBSS buffer for 15 min following washing once with HBSS. Mitochondrial iron was measured at 543 nm (excitation) and 601 nm (emission) using fluorescent microplate reader.

Western blot analysis. Proteins from lysates were prepared and resolved by 12% SDS-PAGE at 100 V, transferred for 1.5 h at 250 mA onto Nitrocellulose membranes, and analysed by immunoblotting as described previously56. The information for primary antibodies is as follows: anti-ferritin (cat# 69090), SdhA (cat# 137040), and SdhB (cat# 178423) from Abcam (Cambridge, MA, USA), anti-Xod (cat# 55156-1-AP), citrate synthase (cat# 16131-1-AP), aconitate 2 (Aco2) (cat# 11134-1-AP), Ndufs1 (cat# 12444-1-AP), Ndufs3 (cat# 15066-1-AP), Uqcr1 (cat# 21705-1-AP), and Uqcrfs1 (cat# 1843-1-AP) from Proteintech Group Inc. (Chicago, IN, USA), anti-Actin (cat# BM0627) from Boster (Wuhan, China), anti-Tubulin (cat# T0198) from Sigma-Alrdich (St. Louis, MO, USA), anti-TIR1 antibody (cat# 136800) from Zymed (San Francisco, CA, USA), anti-IsuU, Fech, Irp1, and Irp2, anti-Fxn (self-made)51, anti-CytC (cat# 1896-1) from Epitomics (Burlingame, CA, USA), anti-Vdac1 (cat# 4661S) from Cell Signaling Technology Inc (Shanghai, China).

Enzymatic activities. In-gel aconitase activity assays were performed as described previously28. Related chemicals used were purchased from Sigma-Alrdich. The activities of complex I, II, III, and IV, Xod, and citrate synthase were measured following the manufacturer’s protocols, respectively. Purchase information is as follows: Complex I from Abcam, Complex II, Complex IV, and Citrate synthase from Comin Biotechnology Co. (Suzhou, Jiangsu, China), Complex III from Biovision Inc. (Milpitas, CA, USA), Xod from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Flow cytometric analysis. WT, Irp1−/−, and Irp2−/− cells were incubated with Mito-tracker Red for 5 min at room temperature in PBS and then washed twice with PBS. Cells were harvested, pelleted by centrifugation and resuspended in PBS. The fluorescence intensity was measured by flow cytometry (BD, Franklin, NJ) and data were analysed using Flowjo software.

Quantitative real-time PCR (qRT-PCR). Total DNA was prepared with QiaAmp DNA mini kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. qRT-PCR experiments were performed with SYBR Green PCR master mixture (Thermo Fisher Scientific). The relative copy number of mtDNA was determined by comparing the copy number of mtDNA-locating CytB to that of nDNA-locating Act. The used primer sequences (5′−3′) were: TTCATGTCGGACGAGGCTTA and CTGTGGCACCTCAGAATGAT for mouse CytB; ACACTGGGCCTTGAGTGTA and GTACGACCAGGGAGCATACAG for mouse Act.

Statistical analysis. The values were expressed as mean ± SEM from, at least, three independent experiments. A one-way analysis of variance (ANOVA) was carried out using SPSS ver. 22.0 software (IBM Corporation, Armonk, NY, USA). Significance was considered at p < 0.05.

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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H.L. and K.L. conceived and designed experiments. H.L., H.Z., S.H., L.S., C.S., and J.W. performed experiments. K.L., Q.T., and E.G.M. contributed to data analysis. H.L. and K.L. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

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