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Iron supplementation delays aging and extends cellular lifespan through potentiation of mitochondrial function

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Abstract

Aging is the greatest challenge of humankind worldwide. Aging is associated with a progressive loss of physiological integrity due to a decline in cellular metabolism and functions. Such metabolic changes lead to age-related diseases, thereby compromising human health for the remaining life. Thus, there is an urgent need to identify geroprotectors that regulate metabolic functions to target the aging biological processes. Nutrients are the major regulator of metabolic activities to coordinate cell growth and development. Iron is an important nutrient involved in several biological functions, including metabolism. In this study, using yeast as an aging model organism, we show that iron supplementation delays aging and increases the cellular lifespan. To determine how iron supplementation increases the lifespan, we performed the gene expression analysis of mitochondria, the main cellular hub of iron utilization. Quantitative analysis of gene expression data reveals that iron supplementation upregulates the expression of mitochondrial tricarboxylic acid (TCA) cycle and electron transport chain (ETC) genes. Furthermore, in agreement with expression profiles of mitochondrial genes, ATP level is elevated by iron supplementation, which is required for increasing the cellular lifespan. To confirm, we tested the role of iron supplementation in the AMPK knockout mutant. AMPK is a highly conserved controller of mitochondrial metabolism and energy homeostasis. Remarkably, iron supplementation rescued the short lifespan of AMPK knockout mutant confirmed the anti-aging role through enhancement of mitochondrial functions. Thus our results suggest a potential therapeutic use of iron supplementation to delay aging and prolong healthspan.

Introduction

The aging population is growing dramatically worldwide [1–3]. Aging is the biggest risk factor for several chronic diseases, including cancer, neurodegeneration, heart disease, diabetes, cognitive impairment, and immune system decline [4–8]. A gradual decrease in metabolic functions increases the vulnerability to age-related pathologies in the older population that affect health for the remaining life. Recent research in different organisms from single-celled yeast to animal model systems has shown that aging mechanisms are conserved and controlled by a highly interconnected and functionally redundant gene and protein interactions network of cellular metabolism [9–11].

The budding yeast *Saccharomyces cerevisiae* is a powerful model organism for studying biology of aging as many of its cellular processes are conserved up to humans. Yeast has a tractable short lifespan and is amenable for high-throughput screening under various environmental conditions [9–12]. Yeast has been extensively used to study human replicative and chronological aging by analyzing its replicative lifespan (RLS) and chronological lifespan (CLS) [13–15]. The RLS analyzes the number of times a mother cell divides to form daughter cells, a replicative human aging model for mitotic cells such as stem cells. The CLS is the duration of time that a non-dividing cell is viable in the stationary phase, a chronological human aging model for post-mitotic cells such as neurons.

Ongoing efforts in aging biology research have demonstrated that delaying aging is feasible by modulating the biological processes of aging [11, 16]. Currently, the most promising anti-aging interventions are rapamycin and metformin drugs [11, 16]. Rapamycin inhibits a highly conserved protein kinase metabolic regulator target of rapamycin complex 1 (TORC1). Metformin increases the adenosine monophosphate-activated protein kinase (AMPK) activity. TORC1 promotes cellular anabolic processes such as the synthesis of protein, nucleotide, and lipid [17, 18]. On the other hand, TORC1 inhibits the catabolic process, including oxidative phosphorylation and autophagy. However, AMPK has opposite metabolic functions as it potentiates catabolic processes and inhibits anabolic processes [19]. Under the nutrients limited conditions, AMPK is activated and inhibits TORC1. The resulting metabolic response increases the cellular energy production by inducing catabolic processes, which coordinate with the decrease in ATP utilization in anabolic processes [19–21].

Rapamycin and metformin are in clinical trials for their use as anti-aging therapeutics [16, 22].

Iron is one of the important nutrients and essential for almost all organisms, including humans [23–26]. Iron plays a crucial role in cellular metabolism, and functions as a cofactor for several enzymatic reactions. Since cellular metabolism is the major determinant of aging and iron is crucial for several diverse metabolic functions, we investigated the effect of iron supplementation on aging. We examined the effect of iron supplementation on the CLS of yeast. We found that iron supplementation delays aging and increases the cellular lifespan. We further revealed the anti-aging mechanism of iron supplementation by enhancing mitochondrial functions. We found that iron supplementation improves mitochondrial functions and rescues the short lifespan of the mutant of AMPK.

Materials and Methods

Yeast strains and gene deletion

The prototrophic *Saccharomyces cerevisiae* CEN.PK wildtype strain was used in all experiments to avoid the effects of amino acid auxotrophy on cell growth and survival [27, 28]. Gene knockout strain was generated by PCR-mediated homologous recombination whereby the entire locus was replaced by an amplified selection marker containing the upstream and downstream flanking sequences of the target gene [29]. PCR confirmed stable integration of the amplified selection marker.

Medium composition and chemicals

The rich medium YPD contains 1% yeast extract, 2% peptone, and 2% glucose, YPD agar (2.5% Bacto agar), and SD medium contains 6.7 g/L yeast nitrogen base with ammonium sulfate without amino acids (Difco) and 2% glucose. $FeSO_4.7H_2O$, $FeCl_3$, $CaSO_4.2H_2O$, $MgSO_4.7H2O$, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$ and BPS were purchased from Sigma, and their stocks solution was prepared in water. Antimycin A (Sigma) stock solution was prepared in dimethyl sulfoxide (Sigma). The final concentration of DMSO did not exceed 1% in any assay.

Yeast growth conditions

The wild type and deletion strains were recovered from frozen glycerol stock on YPD agar medium at 30°C. Yeast cells were grown in SD medium overnight at 30°C with shaking at 220 rpm. The cell culture grown overnight were diluted to OD600nm ~ 0.2 in fresh SD medium to initiate growth assay in 96-well plate or flask with or without chemicals and incubated at 30°C. The cell growth (OD600nm) was measured at different time points using a microplate reader or spectrophotometer.

Chronological aging assay

Chronological aging was determined by measuring the chronological lifespan (CLS) of yeast cells, as previously reported with slight modifications [30, 31]. The CLS experiment was carried out on a 96-well plate. Overnight cell culture was diluted to OD600nm ~ 0.2 in fresh SD medium and transferred into the 96-well plate containing different concentrations of chemicals and incubated at 30°C. Cells were grown to stationary phase was considered as Day1 (100% cell survival) for the CLS analysis. Yeast cells survival was quantified at various age time points by outgrowth assay. Chronological aging cells (3µL) at different age time points were transferred to a second 96-well plate. YPD medium (200µL) was added to a 96-well plate and incubated for 24 hours at 30°C. Cell outgrowth was measured by the absorbance (OD600nm) using the microplate reader. Cell survival for different age points was quantified relative to Day1 (considered 100% cells survival).

Oxidative resistance assay

Yeast cells were grown to stationary phase stage (72h) in SD medium. After that, cells were washed and diluted to OD600nm ~0.2 in YPD medium with different concentrations of H_2O_2 and grew at 30°C. Oxidative stress resistance was analyzed by comparing the cell growth of H_2O_2 treated cells with non-treated control.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen), following the manufacturer's mechanical disruption protocol. RNA concentration and quality were determined by spectrophotometer (NanoDrop 2000 Thermo Scientific). Typically, 1 µg total RNA was reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen). During the synthesis of cDNA, two negative controls including without

reverse transcriptase and RNA template were also performed. All RT-PCR was performed in a final volume of 20 µl containing 20ng of cDNA using SYBR Fast Universal qPCR Kit (Kapa Biosystems) and analyzed using the Quant Studio 6 Flex system (Applied Biosystems). The RT-PCR condition is one hold at [95 °C, 180 s], followed by 40 cycles of [95 °C, 1 s] and [60 °C, 20 s] steps. After amplification, a melting-curve was performed to verify PCR specificity and the absence of primer dimers. The quantitative abundance of each gene was determined relative to the house-keeping transcript ACT1. The relative gene expression between the control and treated conditions was calculated using the $2^{-\Delta\Delta Ct}$ method [32]. A list of primers used for RT-PCR is provided in the supplementary table 1.

ATP analysis

Yeast cultures were mixed with tricholoroacetic acid to a final concentration of 5% and kept on ice for at least 5 min. Next, the cells were spun down, resuspended in 150µl 5% trichloroacetic acid, and lysed with glass beads on Precellys® 24 homogenizer (Bertin Technologies). ATP level was measured using an Enliten luciferin/luciferase reagent kit (Promega) in a luminometer (Biotek). Protein concentration was estimated with Bradford reagent (Biorad). The ATP level in the samples was normalized to total protein concentration.

Statistical analysis

All experiments were performed at least in triplicate on different days. Statistical analysis such as mean value and standard deviations were calculated using GraphPad Prism 9 software.

Results

Iron supplementation extends the cellular lifespan of yeast

We investigated the effect of Iron(II) sulfate ($FeSO_4$) supplementation on the chronological lifespan (CLS) of yeast in a 96-well plate. First, cells were incubated with different concentrations of $FeSO_4$ in a synthetic defined (SD) medium and growth was analyzed at different time points (16h, 24h, and 48h). Cell growth reached saturation approximately 24h after incubating with $FeSO_4$ concentrations (Supplementary fig. 1a). Next, we determined the CLS of cells incubated with different concentrations of

FeSO4. We considered 48h stationary phase cell culture as Day1 for CLS analysis. The viability of aged cells at different points was normalized with Day1 (100% viable) and plotted the survival graph. We found that different concentrations of $FeSO_4$ supplementation into medium extend the CLS of yeast (Fig. 1a). We also tested the Iron(III) chloride ($FeCl_3$) and found similar result to $FeSO_4$ (Fig. 1b and supplementary fig. 1b).

To clarify whether $FeSO_4$ and $FeCl_3$ salts or its components Iron(II) and Iron(III) or sulfate and chloride extend CLS, we examined the other sulfate and chloride-containing salts. Yeast cells were incubated with $CaSO_4$, $MgSO_4$, $CaCl_2$, $MgCl_2$ including $FeSO_4$ and $FeCl_3$ in the SD medium. Growth of cells incubated with different salts reached saturation after 24h (Supplementary fig. 1c). We next measured the survival and found that except $FeSO_4$ and $FeCl_3$ other sulfate or chloride-containing salts did not extend the lifespan of yeast (Fig. 1c). These results revealed that iron but not sulfate and chloride extend CLS of yeast.

For further validation, we depleted the iron and analyzed the CLS of yeast. Bathophenanthrolinedisulfonic acid (BPS) is a specific iron chelator compound that sequesters the iron and leads to iron deficiency in the cells [33]. Yeast cells were incubated with iron and different concentrations of BPS in the SD medium. Cell growth of different concentrations reached saturation after 24h (Supplementary fig. 1d). We further measured the cell survival and found that BPS addition reduced the lifespan of iron-supplemented cells (Fig. 1d). Altogether these results confirmed that iron supplementation increases the lifespan of yeast.

Iron supplementation increases oxidative stress resistance

Cellular aging is associated with increased oxidative stress that damages the biological systems and mitigates age-related diseases [34]. Since iron supplementation delayed aging, we investigated their effect on oxidative stress. We used an oxidative stress inducer compound, hydrogen peroxide (H_2O_2), to test the oxidative resistance of cells. Cells were first grown in the presence of iron to stationary phase stage (72h) in SD medium. After that, cells were washed and incubated with different concentrations of H_2O_2 in the YPD medium and grew for 24h. Oxidative stress resistance was analyzed by comparing the cell growth of H_2O_2 treated cells with non-

treated control. We found that cells supplemented with iron were resistant to oxidative stress compared to control (Fig. 2a). To confirm that iron supplementation provides resistance to oxidative stress, BPS was added to iron and cell growth with H_2O_2 was analyzed. The addition of BPS reduced the oxidative stress resistance of iron-supplemented cells (Fig. 2b). These results correlate with the role of iron supplementation in delaying aging and extending of cellular lifespan.

Iron supplementation potentiates mitochondrial functions

Mitochondria are the major cellular hub for iron utilization and metabolism [35–38]. A decline in mitochondrial functions is associated with aging [39–43]. Iron serves as a cofactor of several mitochondrial proteins, including iron-sulfur cluster and heme-containing proteins [44–46]. These iron-containing proteins are involved in the mitochondrial tricarboxylic acid (TCA) cycle and electron transport chain (ETC) (Fig. 3a). We investigated whether the increase in cellular lifespan by iron supplementation required mitochondrial functions. To test this, we analyzed the expression of mitochondrial TCA cycle genes. We found that iron supplementation significantly induces the expression of several TCA cycle genes (Fig. 3b and Supplementary fig. 2). TCA-cycle metabolites α-ketoglutarate and oxaloacetate can produce glutamate and aspartate by cataplerotic reactions in mitochondria (Fig. 3a). These amino acids are utilized in the biosynthesis of proteins, lipids, and nucleotides [47–49]. Interestingly, expression of glutamate (*GDH1* and *GDH3*) and aspartate (*AAT1* and *AAT2*) biosynthetic genes was decreased in iron-supplemented cells (Fig. 3c). This expression profile suggests that iron supplementation promotes preservation instead of the consumption of TCA cycle intermediates. In agreement with this idea, gene expression of the mitochondrial anaplerotic pathway genes (*PYC1*, *PYC2*, and *GDH2*) was significantly increased in iron supplemented cells (Fig. 3c). *PYC1* and *PYC2* encode pyruvate carboxylase that converts pyruvate to oxaloacetate. *GDH2* encodes glutamate dehydrogenase which synthesize α-ketoglutarate from glutamate. Together, these results suggest that iron supplementation configures the cells in a metabolic state that favors anaplerosis and prevents cataplerosis to boost the TCA cycle metabolites.

TCA cycle reactions generate NADH and $FADH_2$, which are oxidized by ETC complexes I and II and required for the functionality of the TCA cycle (Fig. 3a).

Although *S. cerevisiae* lacks complex I, reducing equivalents are transferred to the respiratory chain through NADH dehydrogenases. Succinate dehydrogenase plays a central role and participates in both the TCA cycle and the ETC complex II (Fig. 3a). Strikingly, the expression of succinate dehydrogenase (*SDH1* and *SDH2*) was highly upregulated among all analyzed TCA cycle genes (Figs. 3b and 3d). These results indicate that the TCA cycle flux continues towards ETC instead of accumulating a particular intermediate. Likewise, the expression of all other genes of ETC complexes was highly upregulated in iron-supplemented cells (Fig. 3d). ETC is associated with generation of reactive oxygen species (ROS), which regulate the expression *SOD2* gene [50]. It encodes a manganese-superoxide dismutase (MnSOD) which is the principal scavenger of mitochondrial superoxide. *SOD2* gene expression was upregulated in iron-supplemented cells (Supplementary fig. 2), which is consistent with the expression of ETC genes. Altogether, expression analysis indicates that iron supplementation potentiates mitochondrial functions of cells.

Iron supplementation increases the ATP level required for extension of cellular lifespan

Mitochondrial TCA cycle reactions produce reducing equivalents NADH and FADH2, which transfer electrons to ETC and generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (Fig. 3a). Since the expression of the TCA cycle and ETC genes were enhanced by iron supplementation, we tested its effect on ATP synthesis. In agreement with the expression profile of mitochondrial TCA cycle, ETC genes, ATP level was high in iron-supplemented cells (Fig. 4a).

Then, we asked whether ATP is required for the extension of the lifespan of iron-supplemented cells. We inhibited the ATP synthesis and measured the CLS of yeast. Antimycin A (AMA) is an inhibitor of ATP synthesis, which binds to complex III and blocks electron transfer in the mitochondrial ETC [51]. We first examined the ATP level and found that AMA treatment inhibits ATP synthesis (Fig. 4b). We further tested the effect of AMA on the cellular lifespan of iron-supplemented cells. Yeast cells were incubated with iron and AMA in the SD medium. Growth of cells reached saturation after 24h (Supplementary fig. 3). Subsequently, we measured the survival and found that AMA treatment inhibits the iron supplementation mediated extension of lifespan (Fig. 4c). Together, these findings reveal that iron supplementation increases the ATP

level which is required for the extension of cellular lifespan. We also observed that the lifespan of AMA-treated cells was lower than the control (Fig. 4c), further confirming that the inability to synthesize ATP compromised lifespan.

Iron supplementation prevents accelerated aging of AMPK knockout mutant

AMPK is the master regulator of cellular energy homeostasis [20, 21]. The highly conserved human analog of AMPK in yeast *S. cerevisiae* is the Snf1 protein [52]. AMPK activates mitochondrial functions to produce ATP under energy-limited conditions. Recent reports have shown that the decline in mitochondrial functions with age occurs in part through the impaired activity of AMPK in different aged organisms [53, 54]. Thus, absence in the AMPK activity affects the mitochondrial functions that compromise numerous cellular functions, including metabolism, resistance to stress, and cell survival, which are the most critical determinants of aging and lifespan. Consistent with the previous studies, we found that the *snf1* knockout mutation compromised ATP level, oxidative stress, and lifespan (Figs. 5a, 5b, and 5c).

Since *snf1* mutant is defective in mitochondrial functions, we tested whether iron supplementation can rescue the accelerated aging phenotypes. Therefore, we supplemented the iron to *snf1* mutant and analyzed the ATP level, oxidative stress lifespan. We found that iron supplementation increased the ATP level, oxidative stress, and lifespan (Figs. 5d, 5e, and 5f). Altogether, these findings confirmed that iron supplementation delays aging and extends cellular lifespan by increasing mitochondria functions.

Discussion

Nutrients determine the functional status of cells, and deficiency of essential nutrients compromises human health [55, 56]. Furthermore, nutrients are the major regulator of cellular metabolism, which controls several biological processes, including aging, a major risk factor of several chronic diseases. A decline in metabolic activity is one of the hallmarks of aging [10]. Recent research in different organisms, including mammals, has demonstrated that delaying aging and increasing healthspan is feasible by anti-aging interventions including rapamycin and metformin drugs administration [11, 16]. These drugs target the nutrient-sensing complexes TORC1 and AMPK, which are the important metabolic regulators of the cells [11, 16].

Iron is an essential nutrient involved in several crucial metabolic reactions in the cells [23–26]. Iron deficiency impairs metabolic activity resulting in compromised cellular functions, leading to many diseases, including anemia, cognitive development, and loss of muscle strength [26, 57–59]. Iron deficiency is widespread in elderly populations aged ≥65 years [60–62].

Since iron regulates metabolic processes, we investigated its role in aging. We utilized yeast as a model organism to examine the role of iron in chronological aging. We investigated the effect of iron supplementation on the CLS of yeast. We found that both $FeSO_4$ and $FeCl_3$ increased the cellular lifespan. Using different salts of sulfate, chloride, and iron chelator, we confirmed that the cellular lifespan is extended by iron. Aging is related to a gradual accumulation of oxidative stress, which is harmful to cellular functions and decreases cell survival [34]. Since we found that iron supplementation delayed aging, we tested whether it can provide resistance to oxidative stress. To examine the oxidative stress phenotype, we treated the cells with oxidative inducer agent H_2O_2 and measured the cell survival. We found that iron supplementation increased oxidative stress resistance compared to control. These findings correlate with the role of iron supplementation in the extension of lifespan.

We further unravel the anti-aging mechanism of iron supplementation. Mitochondria are the main cellular consumer of iron utilization and metabolism [35–38]. We first analyzed the expression of mitochondrial TCA cycle genes. We found that the expression of almost all TCA cycle genes was upregulated by iron supplementation. Importantly, iron supplementation downregulated the expression of mitochondrial anaplerotic and upregulated cataplerotic metabolic genes. These results reveal that iron supplementation enhances the synthesis of TCA cycle intermediates and prevents their cellular utilization. These findings supported the anti-aging activity of iron supplementation, as regular export of TCA cycle intermediates affects the mitochondrial integrity [47]. Moreover, replenishing the mitochondrial carbon pool is essential to maintain the mitochondrial functions required for survival during cellular aging.

TCA cycle intermediate α-ketoglutarate has been shown to extend the lifespan of different organisms [63]. However, we found that iron supplementation increased the expression of α-ketoglutarate dehydrogenase (*KGD1* and *KGD2*), which converts α-ketoglutarate to form succinyl-CoA. Moreover, we observed that the expression of succinate dehydrogenase genes (*SDH1* and *SDH2*) was highly upregulated among other tested genes of the TCA cycle. Importantly, succinate dehydrogenase participates in both the TCA cycle and the ETC complex II. These results suggested that instead of accumulating a particular TCA cycle intermediate, the anti-aging activity of iron supplementation could involve the ETC pathway.

Since the TCA cycle is functionally connected with ETC, we analyzed the expression of ETC genes. We found that iron supplementation highly upregulated the expression of ETC genes. TCA cycle products NADH and $FADH_2$ are oxidized by ETC complexes and generate ATP through OXPHOS. We found that iron supplementation increases the cellular ATP level, which is correlated with the upregulation of the TCA cycle and ETC genes. Next, we elucidated whether the increase in ATP level by iron supplementation is required for the extension of cellular lifespan. We found that lifespan extension by iron supplementation was abolished by inhibiting ATP synthesis. Thus, these findings suggest that iron supplementation increases the level of ATP which is required for the extension of cellular lifespan. Our results are consistent with the previous reports showing the role of ATP in cellular lifespan [51, 64, 65]. Further, we utilized iron supplementation to enhance the mitochondrial functions and rescued the short lifespan and oxidative stress-sensitive phenotype of the AMPK mutant. Altogether, these results revealed that iron supplementation potentiates the mitochondrial functions that delay aging and increase the lifespan of cells.

Recent studies have shown that iron supplementation restores the mitochondrial defect of lysosome-impaired mutants and prevents mitochondrial decline during aging [66, 67]. Thus, our results support the previous findings that iron supplementation improves mitochondrial functions. Interestingly, one of the earlier studies showed that iron supplementation rescued the accelerated replicative aging of lysosome-impaired mutants; however, the effect on the wildtype cells was not included in report [67]. Nevertheless, our results are correlated with previous findings, and despite different aging models (chronological aging), we found that iron supplementation delays aging

and increases cellular lifespan. Thus, collectively our results and previous reports clearly suggest that iron supplementation could be a potential therapeutic to target aging and increase the healthspan.

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Contributions

Jovian Lin Jing: Methodology, Formal analysis, Investigation and Reviewing

Trishia Cheng Yi Ning: Methodology, Formal analysis, Investigation and Reviewing

Federica Natali: Validation, Formal analysis and Reviewing

Frank Eisenhaber: Reviewing, Editing and Supervision

Mohammad Alfatah: Conceptualization, Data curation, Methodology, Writing, Reviewing, Editing and Supervision.

Competing interests

The authors declare no competing financial and non-financial interests.

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Figure legends

Fig. 1. Iron supplementation increases the chronological lifespan of yeast. The prototrophic yeast strain was incubated with different chemical conditions in the synthetic defined (SD) medium and grown in 96-well plates at 30°C. Cells grown to stationary phase were considered Day1 (100% cell survival) for the chronological lifespan (CLS) analysis. Cell survival was quantified at various age time points by an outgrowth assay. (a) CLS of cells supplemented with different concentrations of FeSO4. (b) CLS of cells supplemented with different concentrations $FeSO_4$ and $FeCl_3$. (c) CLS of cells supplemented with 100μM of $FeSO_4$, $FeCl_3$, $CaSO_4$, $MgSO_4$, $CaCl_2$ and $MgCl_2$. (d) CLS of cells supplemented with 100μM of $FeSO_4$ in the presence of different concentrations of BPS.

Fig. 2. Iron supplementation increases oxidative stress resistance. The prototrophic yeast strain was incubated with different conditions and grown to stationary phase stage in SD medium for 72h at 30°C. After that, cells were washed and diluted to OD600nm ~0.2 in YPD medium with different concentrations of H_2O_2 and grew at 30°C. (a) Oxidative stress analysis for cells incubated with different concentrations of $FeSO_4$. (b) Oxidative stress analysis for cells incubated with different concentrations of $FeSO_4$ and 250μM BPS. Oxidative stress phenotype was analyzed by comparing the cell growth of H_2O_2 treated cells with non-treated control.

Fig. 3. Iron supplementation enhances mitochondrial functions. (a) An overview of mitochondrial TCA (tricarboxylic acid) cycle, ETC (electron transport chain) with the major cataplerotic and anaplerotic reactions illustrated. (b) The prototrophic yeast strain was incubated with 100μM $FeSO_4$ and grown in SD medium for 8h at 30°C. RNA was extracted from the cultures, and the expression of the indicated TCA cycle genes was analyzed by quantitative RT-PCR. (c) Expression analysis of cataplerotic genes (*GDH1*, *GDH3*, *AAT1,* and *AAT2*) and anaplerotic genes (*PYC1*, *PYC2,* and *GDH1*) by quantitative RT-PCR. (d) Expression analysis of ETC genes by quantitative RT-PCR.

Fig. 4. Iron supplementation increases cellular lifespan by enhancing the ATP level. (a) The prototrophic yeast strain was incubated with different concentrations of

$FeSO_4$ and grown to stationary phase stage in SD medium for 72h at 30°C. ATP was extracted from the cultures, measured using a luciferin/luciferase reagent in a luminometer, and normalized to total protein concentration. (b) ATP analysis of cells incubated with different concentrations of $FeSO_4$ and 50μM antimycin A (AMA). (c) Chronological lifespan (CLS) analysis of cells incubated with varying concentrations of FeSO4 and 50μM AMA.

Fig. 5 Iron supplementation rescues accelerated aging of AMPK knockout mutant. The yeast prototrophic wildtype and AMPK knockout mutant (*snf1*Δ) strains were grown to stationary phase in SD medium for 72h at 30°C. (a) ATP analysis of wildtype and *snf1*Δ strains. (b) Oxidative stress analysis of wildtype and *snf1*Δ strains with different H_2O_2 concentrations. (c) Chronological lifespan (CLS) analysis of wildtype and *snf1*Δ strains. (d) ATP analysis of wildtype and *snf1*Δ strains incubated with different concentrations of $FeSO_4$. (e) Oxidative stress analysis of wildtype and *snf1*Δ strains incubated with different concentrations of $FeSO_4$. (f) CLS analysis of wildtype and *snf1*Δ strains incubated with different concentrations of $FeSO_4$.

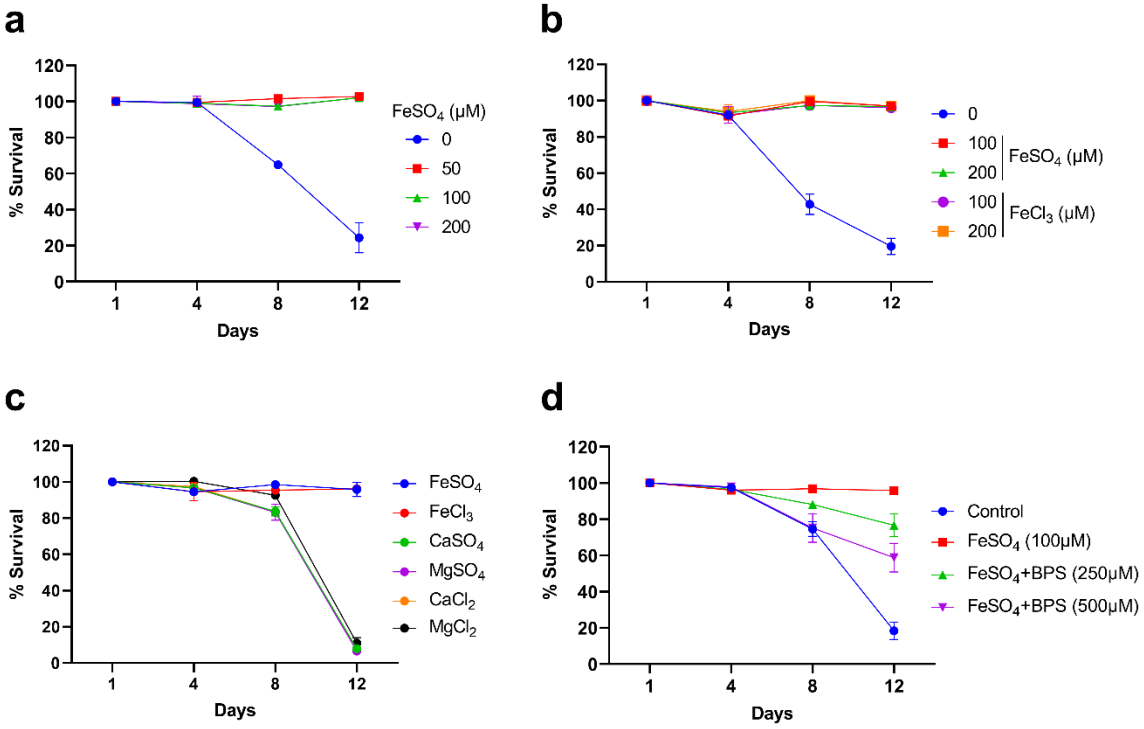
a
b
c
d
% Survival
Days
FeSO4 (μM)
0
50
100
200
FeSO4 (μM)
FeCl3 (μM)
FeSO4
FeCl3
CaSO4
MgSO4
CaCl2
MgCl2
Control
FeSO4 (100μM)
FeSO4+BPS (250μM)
FeSO4+BPS (500μM)

Figure 1

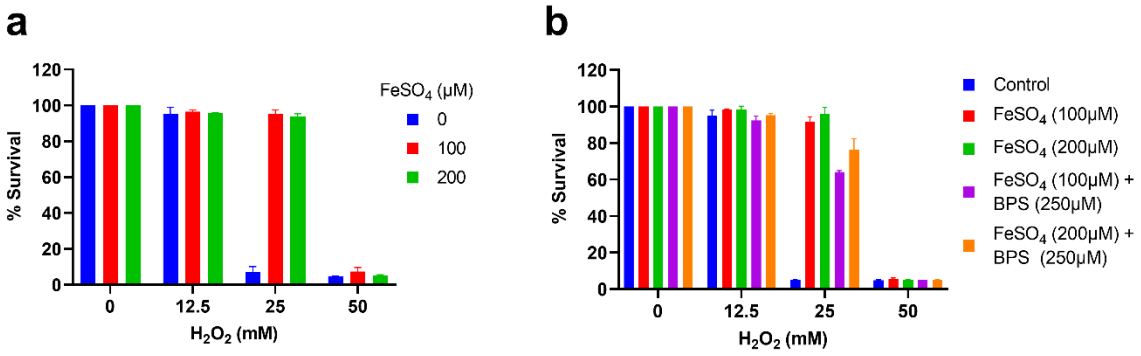
a
% Survival
120
100
80
60
40
20
0
0
12.5
25
50
H2O2 (mM)
FeSO4 (μM)
0
100
200
b
% Survival
120
100
80
60
40
20
0
0
12.5
25
50
H2O2 (mM)
Control
FeSO4 (100μM)
FeSO4 (200μM)
FeSO4 (100μM) + BPS (250μM)
FeSO4 (200μM) + BPS (250μM)

Figure 2

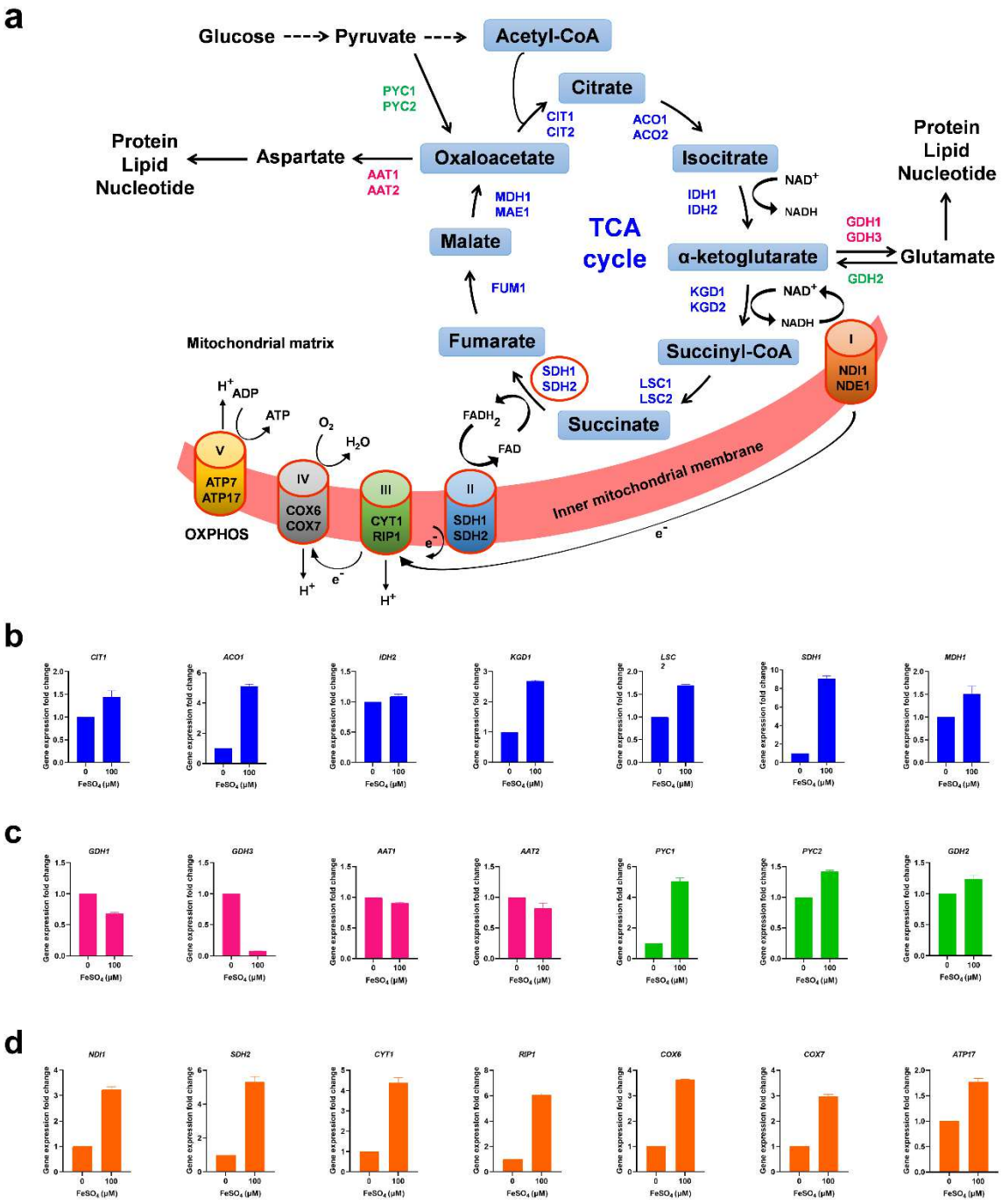
a
Glucose ----> Pyruvate ----> Acetyl-CoA
PYC1
PYC2
Citrate
CIT1
CIT2
ACO1
ACO2
Protein
Lipid
Nucleotide
Aspartate
Oxaloacetate
Isocitrate
AAT1
AAT2
MDH1
MAE1
IDH1
IDH2
NAD+
NADH
TCA
cycle
GDH1
GDH3
Glutamate
GDH2
Malate
α-ketoglutarate
FUM1
KGD1
KGD2
Mitochondrial matrix
Fumarate
Succinyl-CoA
SDH1
SDH2
LSC1
LSC2
Succinate
H+ ADP
ATP
O2
H2O
FADH2
FAD
V
ATP7
ATP17
IV
COX6
COX7
III
CYT1
RIP1
II
SDH1
SDH2
I
NDI1
NDE1
Inner mitochondrial membrane
OXPHOS
e-
H+
b
c
d
CIT1
ACO1
IDH2
KGD1
LSC2
SDH1
MDH1
GDH1
GDH3
AAT1
AAT2
PYC1
PYC2
GDH2
NDI1
SDH2
CYT1
RIP1
COX6
COX7
ATP17
Gene expression fold change
FeSO4 (μM)
0
100

Figure 3

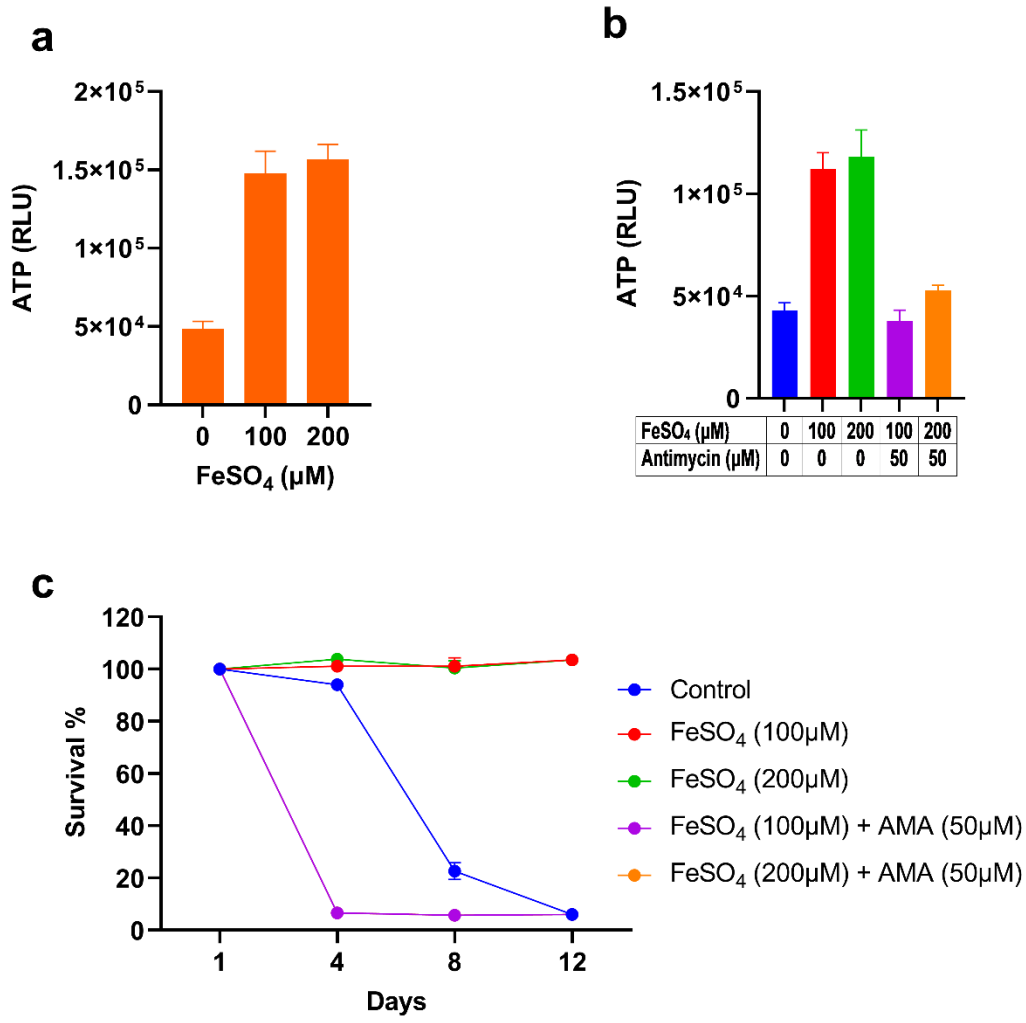
a
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
0
ATP (RLU)
0
100
200
FeSO4 (μM)
b
1.5×10⁵
1×10⁵
5×10⁴
0
ATP (RLU)
FeSO4 (μM) 0 100 200 100 200
Antimycin (μM) 0 0 0 50 50
c
120
100
80
60
40
20
0
Survival %
1
4
8
12
Days
Control
FeSO4 (100μM)
FeSO4 (200μM)
FeSO4 (100μM) + AMA (50μM)
FeSO4 (200μM) + AMA (50μM)

Figure 4

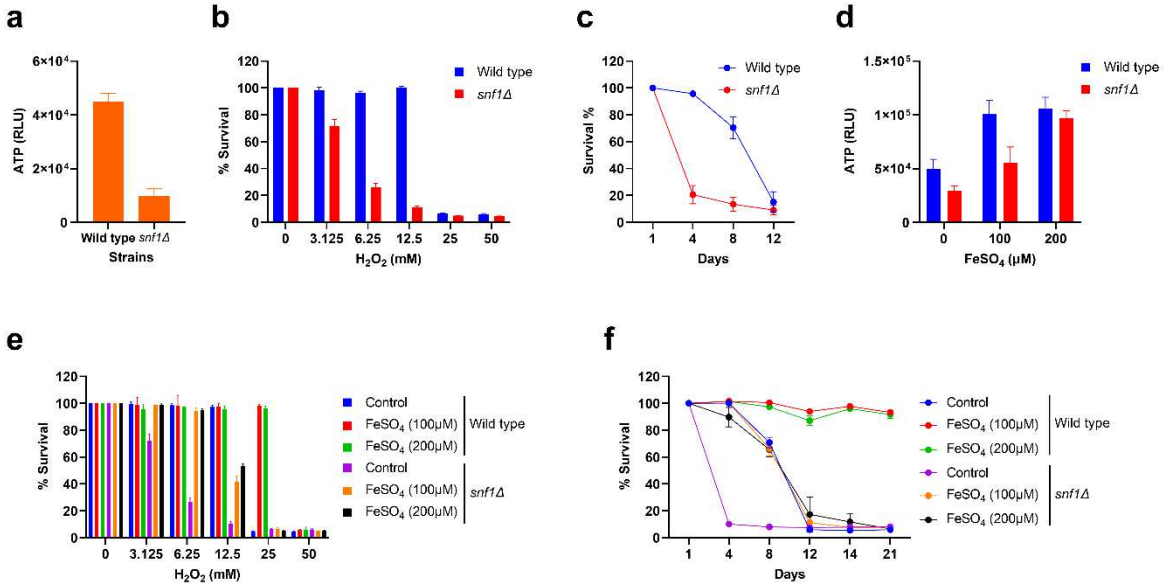
a
b
c
d
ATP (RLU)
Wild type snf1Δ
Strains
% Survival
Wild type
snf1Δ
H2O2 (mM)
Survival %
Days
FeSO4 (μM)
e
f
Control
FeSO4 (100μM)
FeSO4 (200μM)
Wild type
snf1Δ

Figure 5

Supplementary Files

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