Atp23p and Atp10p coordinate to regulate the assembly of yeast mitochondrial ATP synthase

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Abstract
Two chaperones, Atp23p and Atp10p, were previously shown to regulate the assembly of yeast mitochondrial ATP synthase, and extra expression of ATP23 was found to partially rescue an atp10 deletion mutant, by an unknown mechanism. Here, we identified that the residues 112-115 (LRDK) of Atp23p were required for its function in assisting assembly of the synthase, and demonstrated both functions of Atp23p, processing subunit 6 precursor and assisting assembly of the synthase, were required for the partial rescue of atp10 deletion mutant. By chasing labeling with isotope 35S-methionine, we found the stability of subunit 6 of the synthase increased in atp10 null strain upon overexpression of ATP23. Further co-immunoprecipitation (Co-IP) and blue native PAGE experiments showed that Atp23p and Atp10p were physically associated with each other in wild type. Moreover, we revealed the expression level of Atp23p increased in atp10 null mutant compared with the wild type. Furthermore, we found that, after 72 hours growth, atp10 null mutant showed leaky growth on respiratory substrates, presence of low level of subunit 6 and partial recovery of oligomycin sensitivity of mitochondrial ATPase activity. Further characterization revealed the expression of Atp23p increased after 24 hours growth in the mutant. These results indicated, in atp10 null mutant, ATP10 deficiency could be partially complemented with increased expression of Atp23p by stabilizing some subunit 6 of the synthase. Taken together, this study revealed the two chaperones Atp23p and Atp10p coordinated to regulate the assembly of mitochondrial ATP synthase, which advanced our understanding of mechanism of assembly of yeast mitochondrial ATP synthase.

Keywords
assembly, Atp10p, Atp23p, coordinate, mitochondrial ATP synthase

Abbreviations:
Atp6p, subunit 6 of mitochondrial ATP synthase; Atp9p, subunit 9 of mitochondrial ATP synthase; BN-PAGE, blue native PAGE; Co-IP, co-immunoprecipitation; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time polymerase chain reaction; TBST, Tris-buffered saline with 0.05% Tween 20; TCA, tricarboxylic acid.

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1 | INTRODUCTION

Mitochondrial ATP synthase (F$_1$-Fo) catalyzes the synthesis or hydrolysis of ATP by promoting a transfer of protons across the lipid layer.$^{1,2}$ Proton transfer occurs at an interface between subunit 6 (Atp6p) and subunit 9 ring (Atp9p ring) of Fo part of the enzyme.$^{3,4}$ Biogenesis of yeast mitochondrial ATP synthase is assisted by a dozen nuclear gene products.$^5$ Three nuclear-encoded proteins, Atp11p, Atp12p, and Fmc1p, have been shown to be necessary for oligomerization of the F$_1$ component of the Saccharomyces cerevisiae (S. cerevisiae) ATP synthase.$^6$ Some nuclear-encoded proteins, such as Nca2p, Nca3p, Atp22p, Smt1p, Atp10p, and Atp23p, have been implicated in the expression of subunit 6 and in its interaction with subunit 9 ring of Fo.$^9,10$

Nuclear-encoded Atp23p has been shown to have dual functions of processing the yeast Atp6p precursor and of assisting assembly of Fo of ATP synthase, and the metalloprotease motif (HEXXH) of Atp23p is responsible for its function of processing Atp6p precursor.$^{11,12}$ Atp10p has also been shown to regulate the assembly of Fo, acting at a post-translational stage. Atp10p interacts physically with newly translated Atp6p to promote the assembly of Atp6p with the Atp9p ring.$^{5,13,14}$ It has been reported that mitochondrial ATP synthase assembly in the $atp10$ or $atp23$ mutants is arrested at the same or closely related stage,$^{11}$ which is consistent with the finding of the partial rescue of the $atp10$ deletion mutant by an extra single copy or multi-copy expression of Atp23.$^{12}$ However, the mechanism of rescue of $atp10$ null mutant by extra expression of ATP23 and whether Atp23p and Atp10p coordinate to regulate the assembly of yeast mitochondrial ATP synthase in wild type remain unclear.

In the present study, we elucidated that both functions of Atp23p were required for the partial rescue of the $atp10$ null mutant and the rescue was due to increased stability of Atp6p of the synthase. Furthermore, we revealed that Atp23p and Atp10p coordinated to regulate the assembly of yeast mitochondrial ATP synthase through their physical and functional interactions. This was evidenced by detection of physical association of Atp23p and Atp10p in wild type, and detection of increased expression of Atp23p and partial recovery of mitochondrial ATP synthase assembly in $atp10$ null mutant which results in leaky growth of the mutant on respiratory substrates after 3-day growth. These advanced our understanding of assembly of yeast mitochondrial ATP synthase.

2 | MATERIALS AND METHODS

2.1 | Yeast strains and growth media

Yeast strains of the $S$ cerevisiae used in this study were listed in Table 1. The compositions of the media for growth of yeast were: YPD (2% glucose, 1% yeast extract, and 2% peptone), YPGal (2% galactose, 1% yeast extract, and 2% peptone), and YPEG (2% Ethanol, 3% Glycerol, 2% peptone, and 1% yeast extract).$^{15}$

2.2 | Construction of hybrid genes expressing Atp23p or Atp10p with a C-terminal hemmagglutinin tag and construction of $atp23$ mutants

To express Atp23p tagged with hemagglutinin (HA) at the C-terminus (Atp23p-HA), the wild-type ATP23 locus from nucleotide position 180 upstream of the initiator codon to the stop codon was PCR-amplified using plasmid pG200/T1.$^{12}$ The primers used were 5$′$-cgcggatccgtgggccaaatattgcaatacgagctctttttgat-3$′$ and 5$′$-cccaagctttcaagcgtagtctgggacgtcgtatgggttatctgtaatctcataaac-3$′$. The PCR fragment was digested with BamH1 and HindIII and cloned into YEp352 and Ylp352.$^{18}$ The plasmid constructs were confirmed by sequencing. ATP23 site-directed mutagenesis was performed using the ClonExpress One Step Cloning Kit (Vazyme Biotech, Nanjing, Jiangsu, China) according to the supplier's instructions.

Similarly, to express Atp10p tagged with HA at the C-terminus (Atp10p-HA), the wild-type ATP10 locus from nucleotide position 286 upstream of the initiator codon to the stop codon was PCR-amplified using plasmid pG165/T1.$^{19}$ The primers used were 5$′$-ccacggatccgcaaggcagaggtctgggacgtcgtatggtgtagtacagaagctttggcaaaacttcc-3$′$ and 5$′$-acacggatcctacaagctttgacagcttgctagcctgtggtgtagtgtagtacagagctttggcaaaacttcc-3$′$. The PCR fragment was digested with BamH1 and PstI and cloned into YEp352 and Ylp352.$^{18}$ The resultant plasmids were confirmed by sequencing.

2.3 | Serial dilution growth

The OD$_{600}$ of cells grown overnight in liquid YPD was adjusted to 1.0. Four serial dilutions were made, and 3 $\mu$L each were plated on YPD and YPEG plates and incubated at 30°C for 2-3 days.

2.4 | Preparation of yeast mitochondria and ATPase activity assays

Mitochondria were prepared by the method of Faye et al.$^{20}$ except that Zymolyase 20,000 instead of Glusulase was used to convert cells to spheroplasts. Mitochondria concentration was determined by the procedure of Folin-phenol.$^{21}$ ATPase activity was assayed by measuring release of Pi from ATP at 37°C in the presence or absence of oligomycin.$^{22}$
2.5 | In vivo labeling of mitochondrial gene products

Cells grown overnight in YPGal media were inoculated into 10 mL of minimal galactose medium (containing 0.67% yeast nitrogen base without amino acids, 2% galactose) and the appropriate auxotrophic requirements for about 2 hours. Cells equivalent to an OD$_{600}$ of 0.5 were harvested at a growth density of OD$_{600}$ of 1-2. After centrifugation and washing with minimal galactose medium, the cells were suspended in 500 μL of the same buffer and 10 μL of a freshly prepared aqueous solution of cycloheximide (7.5 mg/mL) and incubated at 24°C for 5 minutes. Then, 5 μL of $^{35}$S-methionine (10 mCi/mL, PerkinElmer, Boston, MA, USA) was added to the cells and incubated at 24°C for 30 minutes. After centrifugation, the cells were resuspended with 500 μL of 20 mmol/L methionine and 75 μL of 1.8 mmol/L NaOH, 1 mmol/L β-mercaptoethanol and 0.01 mmol/L phenylmethylsulfonyl fluoride (PMSF). Then, 575 μL of 50% tricarboxylic acid (TCA) was added and the mixture was centrifuged. The precipitated proteins were washed once with 0.5 mmol/L Tris (free base) and two times with water, and were suspended in 45 μL of sample buffer. Total cellular proteins were separated by SDS-PAGE on 12% polyacrylamide gel with 4 mol/L urea and 25% glycerol. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics GmbH, Mannheim, Germany) and the radiolabeled mitochondrial gene products were visualized by autoradiography.

2.6 | Quantitative real-time PCR

Yeast strains were grown overnight in YPGal medium, and the cultures were diluted to an OD600 of 0.2 in the same medium. The diluted cultures were grown until OD600 reached 0.6-0.8, and the cells were harvested. RNA was isolated using an EZNA Yeast RNA Kit (OMEGA). Isolated RNA was treated with RNase-free DNase I (Fermentas) to remove contaminating genomic DNA. RNAs were reverse-transcribed using the HiScript II Reverse Transcriptase Kit (Vazyme Biotech), quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green Master Mix-High ROX Premixed (Vazyme Biotech) according to the manufacturer's instructions in a Stepone Plus system (Applied Biosystems). The relative fold changes were determined using the 2$^{-\Delta\Delta C_t}$ method, in which GAPDH was used for normalization. All reaction was performed in triplicate. The primer sequences for qRT-PCR were listed in Table S1.

2.7 | Western blot

Proteins were separated by SDS-PAGE, and then transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with primary antibodies for 2 hours. The primary antibodies used were rabbit polyclonal antibodies, including anti F$_{1}$-β, anti-Atp9p, anti-Atp10p, anti Atp22p (gifts from Dr Alexander Tzagoloff), anti-Atp6p (gift from Dr Jean-Paul di Rago and Dr Marie-France Giraud), anti-Atp23p (prepared in my lab), anti-VDAC1 (#PA1-954A, Invitrogen), or mouse monoclonal antibody against HA (#901513, Biolegend). Then, the membranes were washed with TBST three times (15 minutes each time) and blotted with peroxidase-coupled anti-rabbit IgG (#70010200, Biosharp) or anti-mouse IgG (#69041060, Biosharp) for 2 hours. The membranes were washed with TBST three times, and the antibody complexes were visualized with the Super Signal chemiluminescent substrate kit (Pierce Chemical).

2.8 | Co-immunoprecipitation

For Co-IP, mitochondria were lysed at a concentration of 10 mg/mL in buffer containing 1.5% of digitonin, 150 mmol/L of KAc, 4 mmol/L of MgAc, 30 mmol/L of HEPES, pH 7.4, 1 mmol/L of PMSF, and 1 mmol/L of ATP for 30 minutes at 4°C. After centrifuged at 70,000 g for 15 minutes at 4°C, the supernatant was subjected to immunoprecipitation by subsequent incubation with polyclonal antibody against HA (#51064-2-AP, Proteintechn) or Atp23p (prepared in our lab) and protein A-Sepharose (Millipore). Beads were washed three times with 1 mL of 20 mmol/L Tris/HCl, pH 7.4 and resuspended in SDS loading buffer. The samples were analyzed by western blot with anti-HA, anti-Atp23p, or anti-Atp10p antibodies.

2.9 | Blue native PAGE (BN-PAGE)

Protein complexes were analyzed by BN-PAGE following published procedures. In brief, mitochondria were solubilized in buffer containing 50 mmol/L of sodium chloride, 50 mmol/L of imidazole, 2 mmol/L of 6-aminohexanoic acid, and 1 mmol/L of EDTA and digitonin with final concentration of 3 mg digitonin per mg mitochondrial proteins, and incubated on ice for 15 minutes. Unsolubilized material was removed by centrifugation at 100,000g at 4°C for 10 minutes and the supernatant was mixed with BN-PAGE loading buffer (0.5% Coomassie Brilliant Blue G-250, 50 mmol/L 6-aminocaproic acid, and 10 mmol/L Bis-Tris/ HCl pH 7.0), and loaded on a 4%-13% polyacrylamide gel.

2.10 | Miscellaneous procedures

Standard methods were used for the preparation and ligation of DNA fragments and for transformation and extraction of plasmid DNA from Escherichia coli. Yeast was
transformed following the LiAc procedure of Schiestl and Gietz. Polycolonal antiserum against Atp23p was raised in rabbits against synthetic peptide corresponding to residues 30-46 of Atp23p. Protein concentrations were determined by the method of Lowry et al.

3 | RESULTS

3.1 | Residues 112-115 (LRDK) of Atp23p are required for its function in assisting the assembly of mitochondrial ATP synthase

Atp23p has dual functions of processing yeast Atp6p precursor and of assisting assembly of mitochondrial ATP synthase. To determine which domain(s) of Atp23p is required for its function in assisting the assembly of mitochondrial ATP synthase, we selected four segments (LRDK, HELIH, LSGE, and VDEV) that are relative conserved in fungal Atp23p sequences and constructed four deletion mutants, respectively (Figure 1A). Mutants harboring each of the deletions failed to show growth on respiratory substrates (YPEG medium), as did an atp23 null mutant (Figure 1B). None of the mutations affected the expression level of the β subunit of F₁ component of mitochondrial ATP synthase (Figure 1C). This is consistent with previous findings that mutations in genes for biogenesis of Fo subunits do not affect the synthesis and assembly of F₁ subunits. In contrast, the Atp6p of Fo was absent in the atp23 null mutant and in the four deletion mutants (Figure 1C), suggesting that Atp6p failed to assemble into the ATP synthase in the mutants, which was confirmed by the insensitivity of the mitochondrial ATPase activity to oligomycin in all mutants (Table 2). The lower ATPase activity in the atp23 mutants was due to the absence of Fo assembly, which was consistent with previous findings of lower ATPase activity in strains unable to assemble the Fo. Furthermore, no stable Atp23p was detected by Western blot in all the mutants except for the strain expressing mutant Atp23p with the LRDK deletion (Figure 1C). The absence of Atp23p indicates that the deletions affect the tertiary structure of the mutant protein making it protease susceptible. However, the strain with the LRDK deletion in Atp23p contained certain amount of Atp23p, but failed to assemble the synthase, suggesting the LRDK segment may not be crucial for Atp23p stability but should be in a domain enabling Atp23p to get a functional structure for assembly of the ATP synthase.
Residues 112-115 (LRDK) of Atp23p are required for its function in assisting assembly of mitochondrial ATP synthase. A, Alignment of yeast Atp23p among fungi. The sequences of *Saccharomyces cerevisiae* (Sc), *Komagataella pastoris* (Kp), *Candida albicans* (Ca), and *Monascus purpureus* (Mp) were aligned with the ClustalX 2.1 program. Identical and conserved residues were marked by asterisks. The domains selected for construction of deletion mutants in this study were boxed in red. B, Growth phenotype of the *atp23* mutants. The indicated strains were serially diluted and spotted on YPD (rich glucose) and YPEG (rich ethanol/glycerol) plates. The images were taken after growth for 2 days at 30°C. C, Western blot analysis of mitochondrial β subunit of F1, Atp6p of Fo and Atp23p. Mitochondria (40 μg) prepared from the same strains shown in (B) were analyzed by SDS-PAGE and immunoblotted with antibodies against the β subunit, Atp6p, Atp23p, and VDAC1. VDAC1 was used as a loading control. D, Growth phenotype of the *atp23* mutants. The indicated strains were serially diluted and spotted on YPD and YPEG plates. The photograph was taken after growth for 2 days at 30°C. E, Western blot analysis of β subunit of F1, Atp6p, Atp23p, and VDAC1. F, Mitochondrial translation products in the same strains shown in (D) were labeled with 35S-methionine in the presence of cycloheximide as detailed in Experimental Procedures. The labeled mitochondrial translation products marked on the left are subunit 3 of cytochrome oxidase (Cox3) and subunit 6 of the ATPase (mAtp6). The precursor form of subunit 6 (pAtp6) seen in the null mutant and deletion mutants migrated slightly higher than the mature protein (mAtp6).
We next investigated whether all the four residues in LRDK segment of Atp23p were required for its function in assembly of ATP synthase. We constructed mutants with double or triple residues of LRDK segment depleted from wild-type Atp23p. All the mutants failed to grow on respiratory substrates medium (Figure 1D). This growth phenotype, together with the lack of oligomycin sensitivity of mitochondrial ATPase activity (Table 2), the presence of Atp23p, Atp6p translated but absence of stable Atp6p (Figure 1E,F) in the mutants indicated a requirement of the entire LRDK segment enabling Atp23p to get a functional structure for assembly of the ATP synthase.

### 3.2 Both functions of Atp23p are required for the partial rescue of atp10 null mutant

Previous work showed that extra one or more copies of ATP23 could suppress atp10 deletion mutant, but ATP23 mutant lack of processing function could not. To determine whether only assisting assembly function of Atp23p or both functions of Atp23p are required for the suppression of atp10 null mutant, ATP23 with deletion of LRDK segment (lack of assisting assembly function) or with the E168Q mutation (lack of processing function) or were applied to rescue of the atp10 null mutant. The result showed that unlike wild-type ATP23 which could rescue atp10 null mutant, overexpression of ATP23 lack assisting assembly function was not able to rescue the atp10 null mutant (Figure 2A), similar with the result of the E168Q mutation reported before. This result indicated that both functions of Atp23p were necessary for the rescue, which was further confirmed by the partial recovery of expression of Atp6p and partial recovery of sensitivity of the mitochondrial ATPase activity to oligomycin in the strain overexpressing wild-type ATP23 but no recovery in the strains overexpressing ATP23 mutants (Figure 2B, Table 3).

### 3.3 Stability of Atp6p increased in the atp10 mutant upon overexpressing ATP23

To elucidate why overexpression of ATP23 could rescue atp10 null mutant, the stability of newly synthesized Atp6p of mitochondrial ATP synthase was investigated by chasing labeling. As Cox1 is relative stable, the ratio of Atp6/Cox1 was used to reflect the stability of Atp6p. Newly synthesized mitochondrial translation products were labeled with 35S-methionine for 30 minutes. After stopping the reaction and

| Strains        | % p+ | ATPase activity (μmol/mg/min) | Inhibition (%) | Oligomycin | +Oligomycin |
|----------------|------|-------------------------------|----------------|------------|-------------|
| W303           | >99  | 4.42                          | 0.71           | 83.9       |
| ΔATP23         | 27   | 1.20                          | 1.08           | 10.0       |
| ΔATP23 + ATP23 | >99  | 4.31                          | 1.10           | 74.5       |
| ΔATP23 + ΔLRDK | 62   | 1.98                          | 1.54           | 16.3       |
| ΔATP23 + ΔHELIH| 57   | 1.79                          | 1.50           | 16.2       |
| ΔATP23 + ΔVDEV | 60   | 1.69                          | 1.46           | 13.6       |
| ΔATP23 + ΔLSGE | 50   | 1.18                          | 1.07           | 9.3        |
| W303           | >99  | 4.57                          | 0.93           | 79.6       |
| ΔATP23         | 32   | 1.21                          | 1.09           | 9.9        |
| ΔATP23 + ATP23 | >99  | 4.14                          | 0.96           | 76.8       |
| ΔATP23 + ΔLRDK | 58   | 1.34                          | 1.14           | 14.9       |
| ΔATP23 + ΔLRD  | 59   | 1.09                          | 0.96           | 11.9       |
| ΔATP23 + ΔRD   | 62   | 1.17                          | 1.02           | 12.8       |
| ΔATP23 + ΔR    | 57   | 1.17                          | 1.05           | 10.3       |
| ΔATP23 + ΔD    | 60   | 1.05                          | 0.92           | 12.4       |
| ΔATP23 + ΔDK   | 60   | 1.08                          | 0.93           | 13.9       |

**Note:** Mitochondria were prepared from wild-type, the null mutant, and mutants with the indicated chromosomally integrated alleles grown in YPGal. The ATPase activity was measured at 37°C.
chasing for 120 minutes, compared with chasing for 0 minute (normalized as 100%), the percentage of starting radioactivity of Atp6/Cox1 in \(\text{atp10}^{\text{null}}\) strain overexpressing \(\text{ATP23}\) was higher than in \(\text{atp10}^{\text{null}}\) mutant (Figure 3A,B) and showed significant difference compared with \(\text{atp10}^{\text{null}}\) mutant (Figure 3C), indicating the stability of Atp6p increased upon overexpression of Atp23p in \(\text{atp10}^{\text{null}}\) strain.

### 3.4 Atp23p and Atp10p are physically associated with each other

The above results indicated overexpression of \(\text{ATP23}\) could partially rescue \(\text{atp10}^{\text{null}}\) mutant by increasing the stability of Atp6p. We next wondered whether Atp23p and Atp10p coordinate to regulate the assembly of mitochondrial ATP

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**Figure 2** Both functions of Atp23p are required for the rescue of \(\text{atp10}^{\text{null}}\) mutant. A, Growth phenotype of the \(\text{atp10}^{\text{null}}\) mutant and the transformants. The indicated strains were serially diluted and spotted on YPD and YPEG plates. The photograph was taken after growth for 2 days at 30°C. B, Western blot analysis of β subunit of F₁ and Atp6p of Fo of mitochondria prepared from the same strain shown in (A). C, Growth phenotype of the \(\text{atp23}^{\text{null}}\) mutant and the transformants. D, Western blot analysis of β subunit of F₁ and Atp6p of Fo of mitochondria prepared from the same strain shown in (C).

**Table 3** ATPase activities of mitochondria from wild-type and cross-transformation strains

| Strains                        | % ρ+ | ATPase activity (μmol/mg/min) | Inhibition (%) |
|--------------------------------|------|------------------------------|---------------|
|                                |      | −Oligomycin                  | +Oligomycin   |
| W303                           | >99  | 4.27                         | 0.81          | 81.0          |
| \(\Delta\text{ATP10}\)         | 39   | 2.11                         | 1.92          | 9.0           |
| \(\Delta\text{ATP10} + \text{ATP10(e)}\) | 85   | 3.08                         | 1.47          | 52.3          |
| \(\Delta\text{ATP10} + \text{ATP23(e)}\) | 81   | 2.81                         | 1.68          | 40.2          |
| \(\Delta\text{ATP10} + \text{ATP23E168Q (e)}\) | 62   | 2.38                         | 2.20          | 7.5           |
| \(\Delta\text{ATP10} + \text{ATP23∆LRDK(e)}\) | 50   | 2.94                         | 2.62          | 10.9          |
| W303                           | >99  | 4.52                         | 0.87          | 80.8          |
| \(\Delta\text{ATP23}\)         | 25   | 1.58                         | 1.48          | 6.3           |
| \(\Delta\text{ATP23} + \text{ATP23(e)}\) | 80   | 3.07                         | 1.52          | 50.4          |
| \(\Delta\text{ATP23} + \text{ATP10(e)}\) | 56   | 1.61                         | 1.51          | 6.2           |
| \(\text{ATP23∆LRDK} + \text{ATP10(e)}\) | 50   | 1.73                         | 1.61          | 7.4           |
| \(\text{ATP23∆LRDK,E168Q} + \text{ATP10(e)}\) | 55   | 1.78                         | 1.69          | 5.1           |

Note: Mitochondria were prepared from the indicated strains grown in YPGal. The ATPase activity was measured at 37°C.

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synthase in wild type. To investigate whether Atp23p and Atp10p are physically associated with each other, strain expressing Atp10p-HA was constructed and mitochondrial proteins were immunoprecipitated with an anti-HA antibody. Both Atp10-HA and Atp23p were co-immunoprecipitated with antibody against HA (Figure 4A). Reciprocally, both Atp23p and Atp10p were co-immunoprecipitated with antibody against Atp23p in W303 (wild type) strain (Figure 4B). These data indicated that Atp23p and Atp10p were physically associated with each other in the mitochondria of wild-type strain. The native state of Atp23p-Atp10p-containing complex was further investigated by BN-PAGE. A protein complex containing Atp23p, Atp10p, Atp6p, Atp9p, and β subunit was detected (Figure 4C,D), which further confirmed the coordinately regulation of assembly of mitochondrial ATP synthase by Atp23p and Atp10p.

It was previously reported that both Atp23p and Atp10p could bind Atp6p, respectively. 12,14 To elucidate whether the interaction between Atp23p and Atp10p was dependent on the presence of Atp6p, mitochondrial proteins from atp6 null mutant was immunoprecipitated with antibody against Atp23p. The result showed the interaction between Atp23p and Atp10p still could be detected in the atp6 deletion mutant (Figure 4B), suggesting the association of the two proteins was independent of the presence of Atp6p. This was confirmed by the still detection of Atp23p-Atp10p containing complex in the atp6 null mutant by BN-PAGE (Figure 4E,F).

3.5 | Expression of Atp23p increases in atp10 null mutant

Next, we investigated the expression level of Atp23p in the atp10 null mutant and the expression of Atp10p in the atp23 null mutant. The results showed that the mRNA level and protein level of Atp23p in atp10 null mutant increased

![Figure 3](https://example.com/f3.png)
FIGURE 4  Atp23p is physically associated with Atp10p. A, Co-IP of Atp10p-HA and Atp23p with antibody against HA. Mitochondria were prepared from yeast strain expressing Atp10p-HA and extracted with 1.5% digitonin. The extracts were purified with ployclonal rabbit antibody against HA or rabbit antibody against IgG (as control) by protein A beads. The extraction super (Input), IP supernatant (IP sup), and IP beads were separated by SDS-PAGE and immunoblotted with monoclonal antibody against HA or polyclonal antibody against Atp23p. B, Co-IP of Atp23p and Atp10p with antibody against Atp23p. Mitochondria were prepared from W303 strain or atp6 deletion mutant and extracted with 1.5% digitonin. The extracts were purified with polyclonal rabbit antibody against Atp23p or rabbit antibody against IgG (as control) by protein A beads. The extraction super (Input), IP supernatant (IP sup), and IP beads were separated by SDS-PAGE and immunoblotted with polyclonal antibodies against Atp23p or Atp10p. C, Analysis of Atp23p-Atp10p-containing complex by BN-PAGE. Mitochondria from wild-type W303 was solubilized with 3 mg digitonin/mg mitochondrial protein, and was analyzed by BN-PAGE on a 4%-13% polyacrylamide gel. The proteins were transferred to a PVDF membrane and probed with polyclonal antibodies against Atp23p, Atp10p, Atp6p, Atp9p, β subunit of F1, or Atp22p. Arrow indicates the Atp23p-Atp10p-containing protein complex. D, Second dimension SDS-PAGE analysis of the protein complex. The gel slice of BN-PAGE was analyzed by 12% SDS-PAGE and transferred to a PVDF membrane and probed with the indicated antibodies. Stars indicate Atp23p or Atp10p. E, Analysis of Atp23p-Atp10p-containing complex from MR6ΔATP6 mitochondria by BN-PAGE. Mitochondrial proteins from MR6ΔATP6 was analyzed by BN-PAGE and probed with the indicated antibodies as panel C. Arrow indicates the Atp23p-Atp10p-containing protein complex. F, Second dimension SDS-PAGE analysis of the protein complex. The gel slice of MR6ΔATP6 mitochondria separated by BN-PAGE as panel D was analyzed by 12% SDS-PAGE and transferred to a PVDF membrane and probed with the indicated antibodies. Stars indicate Atp23p, Atp10p, Atp9p, or β subunit of F1.
compared to that of wild type (Figure 5A-C). However, no obvious change in the mRNA level or protein level of Atp10p in atp23 deletion mutant were observed compared to that of wild type (Figure 5D-F).

3.6 Increased expression of Atp23p in atp10 null mutant results in slow growth of the mutant after 3 days growth

Above results showed that overexpression of ATP23 could partially rescue atp10 null mutant, and the expression level of Atp23p increased in atp10 null mutant compared with wild type. We wondered whether the increased expression of Atp23p in atp10 null mutant could be helpful for the assembly of mitochondrial ATP synthase in the mutant and result in partial rescue of the mutant. We observed that the atp10 null mutant showed leaky growth after 3 days (72h) growth on respiratory substrates (YPEG) medium (Figure 6A). This is consistent with the previous observation that atp10 null mutant displays slow growth on respiratory substrates.13 Meanwhile, we detected a low level of Atp6p and certain recovery of sensitivity of ATPase activity to oligomycin in the atp10 null mutant after 72 hours growth (Figure 6B, Table 4), indicating partial recovery of assembly of mitochondrial ATP synthase in the mutant. Further analysis showed that, after 24 hours growth, the mRNA level and protein level of Atp23p increased in atp10 deletion mutant compared with wild type (Figure 6B,C). Combining with our finding of overexpression of ATP23 could partially suppress atp10 deletion

**FIGURE 5** Expression of Atp23p in atp10 null mutant increases. A, Western blot analysis of protein level of Atp23p in atp10 null mutant. B, Quantification of the Atp23p protein level. The band intensity was quantified and normalized against W303 (WT) cells. The graph shows the average of Atp23p protein level of three independent experiments. C, qRT-PCR analysis of the steady-state levels of ATP23 mRNA. Level of ATP23 mRNA in W303 cells was normalized to the level of VDAC1 mRNA. The graph showed the average of ATP23 mRNA level of three independent experiments. D, Western blot analysis of protein level of Atp10p in atp23 null mutant. E, Quantification of the Atp10p protein level. The band intensity was quantified and normalized against W303 cells. The graph showed the average of Atp10p protein level of three independent experiments. F, qRT-PCR analysis of the steady-state level of ATP10 mRNA. Level of ATP10 mRNA in W303 cells was normalized to the level of VDAC1 mRNA. The graph showed the average of ATP10 mRNA level of three independent experiments. Statistically significance was determined by the t test (*P < .05, **P < .01, ***P < .001).
mutant and the stability of Atp6p increased upon overexpression of ATP23 in atp10 null mutant, these results suggest the slow growth on respiratory substrates of atp10 null mutant after 72 hours might be due to the increased expression of Atp23p in the mutant. It was previously reported that a single mutation Ala249Val of Atp6p could suppress atp10 null mutation.\(^\text{13}\) To exclude the possibility that the partial assembly of mitochondrial ATP synthase of atp10 null mutant is due to Atp6p point mutation, ATP6 gene in the atp10 null mutant after 72 hours growth on respiratory substrates was sequenced and no mutation was found in the ATP6 sequence (Data not shown), indicating the slow growth of the atp10 null mutant on respiratory substrates was not due to ATP6 mutation but might be due to increased expression of Atp23p.

4 | DISCUSSION

Assembly of mitochondrial ATP synthase complex depends on the assistance of specific chaperones that interact with their cognate subunits. Three nuclear gene-encoded proteins (Atp23p, Atp10p, and Oxa1p) have been shown to be required for assisting subunit 6 assembly into S. cerevisiae mitochondrial ATP synthase.\(^\text{27}\) Both Atp23p and Atp10p are involved in assisting assembly of Atp6p and Atp9p ring, which was proposed to be the last assembly step of the ATP synthase. The present study indicated that both functions of Atp23p (function in processing of Atp6p precursor and function in assisting assembly of the ATP synthase) were required for the rescue of atp10 deletion mutant. We also revealed that overexpression of ATP10 could rescue neither atp23 null mutant nor atp23 mutant deficiency of assembly function, and meanwhile, overexpression of ATP23E168Q with only assisting assembly function could not rescue atp10 null mutant, indicating that the function of assisting assembly of the two proteins Atp23p and Atp10p are not complementary to each other. Moreover, we revealed that Atp23p was physically associated with Atp10p in wild type by co-IP and BN-PAGE, indicating the two proteins could physically interact with each other to co-regulate assembly of mitochondrial ATP synthase. Furthermore, we found increased expression of Atp23p in atp10 mutant and partial recovery of mitochondrial ATP synthase assembly in atp10 null mutant after 72 hours growth. Combining with our finding that overexpressing ATP23 could increase the stability of Atp6p and partially rescue atp10 null mutant, we suggest that Atp23p could partially complement the function of Atp10p in ATP10 deletion mutant. These data revealed a coordinately regulating
### TABLE 4 ATPase activities of mitochondria from wild-type and null mutants

| Strains         | ATPase activity (μmol/mg/min) | Inhibition (%) |
|-----------------|-------------------------------|----------------|
|                 | − Oligomycin | +Oligomycin |                 |
| W303            | >99           | 4.47         | 0.64            | 85.6  |
| ΔATP23 (48 h)   | 24            | 1.42         | 1.28            | 9.8   |
| ΔATP10 (48 h)   | 38            | 2.29         | 2.08            | 9.2   |
| ΔATP23 (72 h)   | 18            | 1.03         | 0.92            | 10.7  |
| ΔATP10 (72 h)   | 25            | 2.35         | 1.46            | 37.9  |

Note: Mitochondria were prepared from wild-type, the atp23 null mutant, and atp10 null mutant grown on YPGal medium for different time. The ATPase activity was measured at 37°C.

**FIGURE 7** Model of coordinately regulating assembly of yeast mitochondrial ATP synthase by Atp23p and Atp10p. The top panel shows in the presence of Atp23p and Atp10p, Atp6p precursor is processed and mature Atp6p is stable. With assistance of the two proteins, mitochondrial ATP synthase is assembled. Moreover, Atp23p and Atp10p form protein complex co-regulating assembly of the ATP synthase. The middle panel shows the subunit 6 precursor is degraded as a result of the absence of Atp23p. The bottom panel shows, in the absence of Atp10p, expression of Atp23p increases, which processes pre-Atp6p and stabilizes some Atp6p resulting in partial assembly of the ATP synthase. Meanwhile, most of Atp6p is degraded.
assembly of \textit{S. cerevisiae} mitochondrial ATP synthase by Atp23p and Atp10p through their physical and functional interactions. The increased expression of Atp23p in \textit{atp10} deletion mutant might be due to abnormal functional status of mitochondria caused by \textit{ATP10} deficiency, as increased expression of Atp23p was also detected in \textit{atp6}, \textit{atp7}, \textit{cox1}, or \textit{rip1} deletion mutant (data not shown). However, the partial complementation of \textit{atp10} deletion by increased Atp23p was specific, as overexpression of Atp23p could rescue \textit{atp10} deletion mutant but not \textit{atp6}, \textit{atp7}, \textit{cox1}, or \textit{rip1} deletion mutant. Interestingly, we revealed Atp23p could be associated with Atp10p independent from presence of Atp6p, and detected a subcomplex containing Atp23p, Atp10p, Atp9p, and of β of F1 in the absence of Atp6p by BN-PAGE, suggesting that Atp23p and Atp10p might possibly interact with other assembly factor(s) for the complex assembly independent of Atp6p.

\textit{S. cerevisiae} mitochondrial ATP synthase consists of three parts: F1, Fo, and stator. Recent studies indicate that mitochondrial ATP synthases are built up from several pre-assembled subcomplexes. The soluble F1 module is assembled independently of both Fo and the stator module.\textsuperscript{28,29} Formation of the subunit 9 ring is probably the first step in Fo assembly, which occurs independently of F1 and forms the second assembly module. Another assembly module is composed of Atp6p, Atp8p, at least two stator subunits, Atp10p and Atp23p chaperones.\textsuperscript{30,31} The assembly of yeast subunit 6 and subunit 9 ring is proposed to be the last step in order to avoid a futile proton efflux concomitant with the dissipation of the mitochondrial membrane potential.\textsuperscript{9} Combining with the findings in this study, we integrated the coordinate regulation of yeast mitochondrial ATP synthase by Atp23p and Atp10p in the model shown in Figure 7. During the assembly of \textit{S. cerevisiae} mitochondrial ATP synthase, Atp23p and Atp10p form protein complex co-regulating assembly of ATP synthase in wild type. Atp23p cleaves N-terminal leader peptide of Atp6p and assists assembly of Atp6p into Atp9p ring to form the ATP synthase complex. Meanwhile, Atp10p and Atp6p form complex and assists assembly of Atp6p with Atp9p ring (Top panel in Figure 7). In the absence of Atp23p, even in the presence of Atp10p, the Atp6p precursor is unstable and degrades, which results in no assembly of the ATP synthase (Middle panel in Figure 7), suggesting that Atp23p plays essential roles for assembly of Atp6p into the synthase.

This might explain why overexpression of \textit{ATP10} could not rescue \textit{atp23} mutant deficiency of assisting assembly of ATP synthase function in this study. In the absence of Atp10p, the cells express more Atp23p which stabilizes some Atp6p and promotes the partial assembly of mitochondrial ATP synthase (Bottom panel in Figure 7). It was previously showed that proteolytically inactive Atp23p could promote assembly of a functional ATPase complex indicating that the Atp6p precursor is functional.\textsuperscript{12} However, in the present study, the proteolytically inactive Atp23p could not rescue \textit{atp10} null mutant, which might because Atp6p precursor became more unstable in the absence of ATP10.

\textit{S. cerevisiae} Atp23p is associated with the mitochondrial inner membrane and is conserved from yeast to human.\textsuperscript{12} \textit{S. cerevisiae} Atp10p is an integral inner membrane protein facing the matrix side of the inner membrane.\textsuperscript{13} Alignment of Atp10p sequences from different species revealed that the sequences in fungus, plant, lower animals (such as \textit{Stylophora pistillata}) were relatively conserved (Figure S1), but homolog of Atp10p were not found in higher animals or human. We propose that because Atp23p is able to partially compensate for the absence of \textit{ATP10}, with biological evolution and functional improvement of Atp23p, the functions of Atp23p might replace that of Atp10p and Atp10p disappeared in higher animals. This broadens our knowledge about the biological evolution of some yeast proteins. Subunit 6 is not synthesized as a precursor in many eukaryotes, including mammals that have \textit{ATP23} homologs. The Atp23p in mammals also contains metalloprotease motif (HEXXH) which is conserved from yeast to human. This raises the possibility Atp23p may also act on other substrates or has other roles in mammals, which is worthy of further study.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflict of interest in connection with this article.

AUTHORS CONTRIBUTIONS

X. Zeng and G. Yang designed the research. G. Yang, Y. Ding, X. Shang, T. Zhao, S. Lu, and J. Tian performed the research. G. Yang, J. Weng, and X. Zeng analyzed the data. X. Zeng, J. Weng, and G. Yang wrote the paper.

REFERENCES

1. Boyer PD. The ATP synthase—a splendid molecular machine. \textit{Annu Rev Biochem}. 1997;66:717-749.
2. Senior AE, Nadanaciva S, Weber J. The molecular mechanism of ATP synthesis by F1-Fo-ATP synthase. \textit{Biochim Biophys Acta}. 2002;1553:188-211.
3. Nakamoto RK, Ketchum CJ, al-Shawi MK. Rotational coupling in the FoF1 ATP synthase. \textit{Annu Rev Biophys Biomol Struct}. 1999;28:205-234.
