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| Author(s) | Imamura, Yuko; Yu, Feifei; Nakamura, Misaki; Chihara, Yuhki; Okane, Kyo; Sato, Masahiro; Kanai, Muneyoshi; Hamada, Ryoko; Ueno, Masaru; Yukawa, Masashi; Tsuchiya, Eiko |
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| Relation | |
RESEARCH ARTICLE

RSC Chromatin-Remodeling Complex Is Important for Mitochondrial Function in Saccharomyces cerevisiae

Yuko Imamura¹, Feifei Yu¹, Misaki Nakamura¹, Yuhki Chihara¹, Kyo Okane¹, Masahiro Sato¹, Muneyoshi Kanai², Ryoko Hamada², Masaru Ueno¹, Masashi Yukawa¹, Eiko Tsuchiya¹*

¹ Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima, 739–8530, Japan, ² National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima, 739–0046, Japan

* yukoima@hiroshima-u.ac.jp

Abstract

RSC (Remodel the Structure of Chromatin) is an ATP-dependent chromatin remodeling complex essential for the growth of Saccharomyces cerevisiae. RSC exists as two distinct isoforms that share core subunits including the ATPase subunit Nps1/Sth1 but contain either Rsc1 or Rsc2. Using the synthetic genetic array (SGA) of the non-essential null mutation method, we screened for mutations exhibiting synthetic growth defects in combination with the temperature-sensitive mutant, nps1-105, and found connections between mitochondrial function and RSC. rsc mutants, including rsc1Δ, rsc2Δ, and nps1-13, another temperature-sensitive nps1 mutant, exhibited defective respiratory growth; in addition, rsc2Δ and nps1-13 contained aggregated mitochondria. The rsc2Δ phenotypes were relieved by RSC1 overexpression, indicating that the isoforms play a redundant role in respiratory growth. Genome-wide expression analysis in nps1-13 under respiratory conditions suggested that RSC regulates the transcription of some target genes of the HAP complex, a transcriptional activator of respiratory gene expression. Nps1 physically interacted with Hap4, the transcriptional activator moiety of the HAP complex, and overexpression of HAP4 alleviated respiratory defects in nps1-13, suggesting that RSC plays pivotal roles in mitochondrial gene expression and shares a set of target genes with the HAP complex.

Introduction

In eukaryotes, chromatin structure remodeling plays crucial roles in various nuclear processes, including transcription, DNA replication, repair, and recombination. Two general classes of enzymes that regulate chromatin remodeling are as follows: enzymes that covalently modify histone molecules and enzymes that alter nucleosome structures using energy from ATP hydrolysis. These enzymes are highly conserved in eukaryotes. ATP-dependent chromatin remodeling factors can be further divided into four groups, SWI/SNF, Ino80/SWR, ISWI, and
CHD complexes, based on characteristics of the ATPase subunits molecular structures in each complex. Out of these, SWI/SNF complexes are known to be tumor suppressors in mammalian cells (reviewed in [1] and [2]). Therefore, insights into the functions of this SWI/SNF-type complex will facilitate a better understanding of the role of chromatin remodeling in both DNA-metabolism regulation and cancer formation. In mammalian cells, however, several hundred variant SWI/SNF complexes are thought to possibly exist because of the large number of subunits encoded by their gene families, of which variants differ among cells of different lineages; such variations cause difficulty in the analysis of complex function [1].

Saccharomyces cerevisiae possesses the following two SWI/SNF-type complexes: the non-growth-essential SWI/SNF complex [3, 4] and RSC complex, which is essential for both mitotic and meiotic growth [5–7]. The RSC complex is composed of 17 subunits, and at least two distinct types of complex containing either Rsc1 or Rsc2 are present. Previous studies have shown that RSC functions in a pleiotropic manner to regulate transcription, DNA repair, and chromosome segregation (reviewed in [8] and [9]); however, the scope of RSC function is still enigmatic.

To obtain a more global insight into the role of RSC in cell growth, we performed a synthetic genetic array (SGA) analysis, which comprised a genome-wide screening of synthetic lethality/sickness, using nps1-105, a temperature-sensitive mutant allele of the NPS1/STH1 gene that encodes the ATPase subunit of RSC, as a query. Using this screening procedure, we determined that RSC played pivotal roles in mitochondrial function. A part of this RSC function was achieved via the action of HAP complex, a transcription factor composed of Hap2, Hap3, Hap4, and Hap5 that plays an essential role in respiratory gene expression [10].

Materials and Methods

Strains and culture conditions

All strains were isogenic to BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). The yeast strains used in this study are listed in Table 1. Standard procedures were used for mating, sporulation, transformation, and tetrad dissection. All media were prepared as described previously [11]. Because our rsc2Δ haploid strain frequently bore diploids, possibly due to some chromatin defect, we constructed homozygous diploids for the rsc mutation-bearing strains used in this study. The homo-diploid of null mutations for RSC1, RSC2, and RSC7 was constructed by the transformation of the HO endonuclease gene on a plasmid (YEp13 HO), using standard methods [12]. Alleles of nps1-105 and nps1-13 were described previously [13, 14]. To construct HAP4-HA, NPS1-TAP HAP4-HA, and nps1-13 HAP4-HA, we designed primers to introduce the 6 HA sequence in frame with the C-terminus of the HAP4 gene, followed by a CYC terminator and URA3 gene. PCR reactions were performed with each primer pair, using the plasmid pBS6HA–URA3 as the template; appropriate strains were transformed with the resulting DNA fragments. Correct insertion was verified by sequencing. All primer sequences for PCR reactions are listed in Table 2. Cells were grown at 28°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose), YPEG medium (1% yeast extract, 2% peptone, 3% ethanol, 3% glycerol) or YPL medium (1% yeast extract, 2% peptone, 2% lactic acid, pH 5.5 adjusted with NaOH). Spot assays were performed by spotting 5–10 μl of cells at a concentration of 1 10⁷ cells/ml after 5-fold serial dilutions onto YPD or YPEG plates. The plates were incubated at various temperatures from 30°C to 35°C as necessary.

Plasmids

The plasmids used in this study are listed in Table 3. YEp13RSC1–3MYC was constructed as previously [16]. The plasmid pRS426GPDpr::HAP4 was constructed as follows: A DNA
Table 1. Strains used in this study.

| Strain   | Genotype                                      | Source                  |
|----------|-----------------------------------------------|-------------------------|
| BY4743   | MATα/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 | Research Genetics       |
| BY4741   | MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0             | Research Genetics       |
| BY-1G nps1-1-105 | MATα nps1-1-105-TAP::LEU2 can1Δ::MFα1pr-HIS3-MFα1pr-URA3 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 | This study              |
| BY-1F nps1-13 | MATα nps1-13 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | This study              |
| BYI-1 rsc1Δ | MATα/rsc1Δ::KanMX4/rsc1Δ::KanMX4 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-2 rsc2Δ | MATα/rsc2Δ::KanMX4/rsc2Δ::KanMX4 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-3 nps1-13 | MATα/nps1-13/nps1-13 his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-7 NPS1-TAP | MATα NPS1-TAP-KanMX4 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | [19]                    |
| BYI-17 nps1-105 | MATα/nps1-105/nps1-105 his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-18 rsc7Δ | MATα/rsc7Δ::KanMX4/rsc7Δ::KanMX4 his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-19 HAP4-HA | MATα/HAP4-6HA::URA3/ HAP4-6HA::URA3 his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-20 NPS1-TAP HAP4-HA | MATα NPS1-TAP-KanMX4 HAP4-6HA::URA3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | This study              |
| BYI-21 nps1-13 HAP4-HA | MATα/nps1-13/nps1-13 HAP4-6HA::URA3/ HAP4-6HA::URA3 his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |
| BYI-22 hap4Δ | MATα/hap4Δ/hap4Δ his3Δ1/Δhis3Δ1 leu2Δ0/Δleu2Δ0 ura3Δ0/Δura3Δ0 met15Δ0/met15Δ0 | This study              |

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fragment containing the HAP4 ORF (1 to +349) harboring BamHI and XhoI sites at the 5′ and 3′ ends, respectively, was generated via PCR using the primers HAP4-F and HAP4-R and genomic DNA as the template. The resulting DNA fragment was subcloned into the corresponding sites of pRS426GPD [17]. The plasmid pBS6HAl-URA3 comprised pBluescript II containing the 6 HA sequence, CYC terminator, and URA3 in that order.

Table 2. Primers.

| Primers | Sequence |
|---------|----------|
| ACT1-RIF | CCAGAGCCTTGGTTCCATCC |
| ACT1-RIR | CGGACATAACGATGTTACCC |
| ATP1-RIF | GGCGGTATTAAGTGCTATT |
| ATP1-RIR | TAGACTCTTTAGAGATGCC |
| ATP16-RIF | AGGCTTTTCCATTTGTAATCC |
| ATP16-RIR | TTGAATTTGAGCTTTGCGG |
| COR1-RIF | TCTCTGAGGTAGGCTTTCAA |
| COR1-RIR | TTCAATTTGAGCGCTTACAG |
| COX6-RIF | ACCTACGGCAATTAGATGT |
| COX6-RIR | AGCTTTGAATAATGCTTCC |
| COX12-RIF | AAGGGCGAGAGTATTGCTCC |
| COX12-RIR | TCTGTAGTGTACATCACGTC |
| HAP4-F | AACAAGGATCCAAATGACCGCAAG |
| HAP4-R | CGGATACTCAGGAAATGTGCTTACAG |
| HAP4-6HA-F | GAGACGCTTGGACAGAGATTCGATTGTTTTTAAAGGTACAGTTGGATGTCCTAGCTACCCGATA |
| HAP4-6HA-R | TTTTCGTTATTGATTTGGTTTCTTGATTTTGCAACATGCTTTGGATGTCCTAGCTACCCGATA |

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Synthetic genetic array (SGA) analysis

SGA analysis was performed basically as described by Tong [18], with some modification. To allow the selection of both MATα and MATα double-mutant strains, we integrated the MFA1pr-HIS3-MFa1pr-URA3 sequence into the CAN1 locus of nps1-105-TAP::LEU2 (BY-1G). This strain was mated with the yeast haploid deletion set (BY4741 background) from Research Genetics (Invitrogen) on rich media; diploids were selected on synthetic complete (SC) medium containing 500 μg/ml G418 but lacking leucine. These diploids were induced to sporulate, and meiotic haploid MATα or MATα double mutants were selected on SC medium containing canavanine and G418, but lacking leucine, arginine, and histidine, or on SC medium containing canavanine and G418, but lacking leucine, arginine, and uracil, respectively. To exclude sporulation-deficient mutants caused by haploinsufficiency, we evaluated the growth of meiotic haploid cells via simultaneous selection on haploid-selection medium (SC-His-Arg+Canavanine or SC-Ura-Arg+Canavanine). To evaluate synthetic lethality/sickness interactions with nps1-105, we selected a his3Δ nps1-105 haploid double mutant as a control query each time and compared the growth level of each haploid double mutant strain with that of the control strain by visual inspection. Double mutants were categorized into three groups according to their growth levels (normal, slow, and no growth) at 28°C. We performed another SGA analysis to confirm the growth levels of the double mutants, which exhibited slow or no growth on both or either mating-type background in our first SGA screening. To strictly confirm reproducibility, we confirmed the growth levels of eight double-mutant progenies (MATα4, MATα4) selected independently from the same parental heterozygous diploid per allele (S1 Table). We selected alleles for which all double-mutant progenies exhibited slow or no growth as those exhibiting synthetic lethality/sickness interactions with nps1-105.

Microscopic analysis

Cells were grown to log phase, washed with HEPES buffer (10 mM HEPES-KOH, pH7.4, containing 5% glucose), and stained with 50 nM Mito-Tracker (Molecular Probes) for 10 min in the dark to visualize mitochondria. To detect intracellular reactive oxygen species (ROS), cells were incubated with 5 μg/ml dihydroethidium (Sigma-Aldrich) for 20 min in the dark. The stained cells were observed under a fluorescence microscope (Olympus BX51).

DNA microarray analysis

Microarray analysis was performed as described previously [19, 20], using the Gene Chip Yeast Genome 2.0 Array (Affymetrix). For RNA preparation, wild-type (WT; BY4743) and nps1-13 (BYI-3) cells pre-grown in YPD medium were inoculated in YPEG medium at a concentration of 1 10⁶ cells/ml and grown to mid-log phase for 4 h. Biotinylated cRNA was prepared from
500 ng of total RNA according to the standard Affymetrix protocol, and 5 μg of cRNA was hybridized for 4 h at 45°C on the GeneChip Yeast Genome 2.0 Array. GeneChips were washed and stained using the Hybridization, Wash, and Stain Kit (Affymetrix). Data were analyzed with Operating Software (GCOS) v1.4, using the Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 500. A given gene was considered induced or repressed when the expression ratio was, respectively, higher or lower than 2.0. Microarray data can be retrieved from Gene Expression Omnibus (GEO) under the accession code GSE66685.

Gene ontology term enrichment analysis
To identify enriched Gene Ontology (GO) terms, we used the Saccharomyces Genome Database Gene Ontology Slim-Mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). To evaluate the significance of GO term enrichment among genes that deletions are responsible for growth defects in combination with nps1-105 or among genes that expression is significantly increased or decreased in nps1-13, we performed a hypergeometric distribution. P-values represent the probability that the given list of genes intersects with any functional category occurs by chance. To test HAP-regulated gene enrichment, we referred to the gene set that transcription level was higher in WT cells than in the hap2Δ and hap4Δ mutants [21].

RNA preparation and quantitative real-time PCR analysis
Total RNA was purified using an RNeasy MiniKit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and a Light Cycler (Roche Applied Science). Primers for the specified genes were validated with standard curves before use. Transcript abundance was normalized to ACT1 transcripts. The PCR primers used in this study are listed in Table 2.

Immunoblotting
Yeast cells were grown to log phase, and cell lysates were thereafter prepared at the appropriate times. Proteins in each cell lysate were resolved by SDS-PAGE, followed by immunoblotting, or processed for immunoprecipitation as described previously [14]. The intensities of protein bands obtained by immunoblotting were measured using the image analyzing software, ImageJ (NIH, USA). The following antibodies were used: anti-Cdc28 (Santa Cruz Biotechnology, Inc), anti-HA (Covance), and anti-TAP (Open Biosystems).

Results and Discussion
1. Screening of null mutations indicated a synthetic growth defect in combination with nps1-105
To understand the scope of functions of RSC complex functions, we performed an SGA analysis by crossing a temperature-sensitive mutant of ATPase subunit, nps1-105, with a collection of 4,847 viable deletion strains. Screening was performed three times, and reproducible candidates were further analyzed by tetrad analysis. As shown in Table 4, 95 gene deletions exhibited either synthetic lethal or slow growth phenotypes in combination with the nps1-105 mutation. Among these genes, 18 overlapped with those previously identified by an SGA screening using rsc7Δ as a query [22]. These 95 genes were categorized into the following five broad classes according to their involvement: (1) chromosome metabolism, (2) translation, (3) mitochondria, (4) general metabolism, and (5) transport. In fact, our Saccharomyces Genome Database GO Slim-Mapper-based analysis revealed that these 95 genes were significantly enriched with
respect to GO terms related to these five broad functional classes (S2 Table). The chromosome metabolism class included members of the Ino80 chromatin-remodeling complex, transcription initiation and elongation complexes, spindle assembly checkpoint, and RNA processing. The deletion of 10 of 18 genes of this class was reported to result in a synthetic growth defect in combination with rsc7Δ, indicating that the RSC complex shares a strong genetic relationship with the processes associated with these genes.

A characteristic feature of the genes identified during our screening, compared with those identified in the previous study with rsc7Δ was the presence of genes within the second “translation” class, particularly those involved in ribosome biogenesis, and within the “mitochondria” class. An earlier genome-wide localization study of RSC revealed that this complex frequently

Table 4. Null mutations conferring growth defects in combination with nps1-105.

| Classesa | Processesa | ORF | Geneb | Descriptionc |
|----------|------------|-----|-------|--------------|
| Chromatin remodeling | YNL059C | ARP5 | Actin-Related Protein |
| | YOR141C | ARP8 | Actin-Related Protein |
| | YLR357W | RSC2 | Remodel the Structure of Chromatin |
| Transcription | YMR091C | NPL6 | Nuclear Protein Localization |
| | YMR091C | RSC7 | |
| | YNR010W | CSE2 | Chromosome Segregation |
| | YGR200C | ELP2 | Elongator Protein |
| | YJL140W | RPB4 | RNA Polymerase B |
| | YJR063W | RPA12 | RNA Polymerase A |
| | YJL168C | SET2 | SET domain-containing |
| | YCR084C | TUP1 | dTMP-Uptake |
| DNA damage repair | YOR258W | HNT3 | Histidine triad Nucleotide-binding |
| Chromatid cohesion | YHR191C | CTF8 | Chromosome Transmission Fidelity |
| Spindle assembly checkpoint | YGR188C | BUB1 | Budding Uninhibited by Benzimidazole |
| | YDR532C | KRE28 | Subunit of a kinetochore-microtubule binding complex |
| RNA processing | YDR378C | LSM6 | Like Sm protein |
| | YNL147W | LSM7 | Like Sm protein |
| | YPR101W | SNT309 | Synthetic lethal to prp NineTeen mutation |
| Nuclear pore | YDL116W | NUP84 | Nuclear Pore |
| Ribosome biogenesis | YLR074C | BUD20 | BUD site selection |
| | YCR047C | BUD23 | BUD site selection |
| | YKR024C | DBP7 | Dead Box Protein |
| | YGR271C-A | EFG1 | Exit From G1 |
| | YFR001W | LOC1 | LOCALization of ASH1 mRNA |
| | YGR159C | NSR1 | Nucleolar protein that binds nuclear localization sequences |
| | YMR142C | RPL13B | Ribosomal Protein of the Large subunit |
| | YHR010W | RPL27A | Ribosomal Protein of the Large subunit |
| | YDL075W | RPL31A | Ribosomal Protein of the Large subunit |
| | YJL189W | RPL39 | Ribosomal Protein of the Large subunit |
| | YHR021C | RPS27B | Ribosomal Protein of the Small subunit |
| Regulation of translation | YKL204W | EAP1 | EIF4E-Associated Protein |
| | YGR162W | TIF4631 | Translation Initiation Factor |
| | YOR302W | | Arginine attenuator peptide, regulates translation of the CPA1 mRNA |

(Continued)
| Classes                  | Processes                        | ORF     | Gene  | Description                                      |
|--------------------------|----------------------------------|---------|-------|--------------------------------------------------|
| Mitochondria             | Mitochondrial translation        | YLR069C | MEF1  | Mitochondrial Elongation Factor                   |
|                          |                                  | YNL005C | MRP7  | Mitochondrial Ribosomal Protein                   |
|                          |                                  | YLR439W | MRPL4 | Mitochondrial Ribosomal Protein, Large subunit     |
|                          |                                  | YBR282W | MRPL27| Mitochondrial Ribosomal Protein, Large subunit     |
|                          |                                  | YCR003W | MRPL32| Mitochondrial Ribosomal Protein, Large subunit     |
|                          |                                  | YPR100W | MRPL51| Mitochondrial Ribosomal Protein, Large subunit     |
|                          |                                  | YBR251W | MRPS5 | Mitochondrial Ribosomal Protein, Small subunit     |
|                          |                                  | YPR047W | MSF1  | Mitochondrial aminoacyl-tRNA Synthetase, Phenylalanine (F) |
|                          |                                  | YPL097W | MSY1  | Mitochondrial aminoacyl-tRNA Synthetase, tyrosine (Y) |
|                          |                                  | YJR113C | RSM7  | Ribosomal Small subunit of mitochondria           |
|                          |                                  | YNR037C | RSM19 | Ribosomal Small subunit of mitochondria           |
|                          | Mitochondria-nucleus             | YOL067C | RTG1  | ReTroGrade regulation                            |
|                          | retrograde regulation            | YGL252C | RTG2  | ReTroGrade regulation                            |
|                          | Mitochondrial RNA processing     | YGR150C | CCM1  | COB and COX1 mRNA maturation                     |
|                          |                                  | YIR021W | MRS1  | Mitochondrial RNA Splicing                        |
|                          | Mitochondrial genome             | YBR194W | AIM4  | Altered Inheritance rate of Mitochondria         |
|                          | maintenance                      | YBR179C | FZO1  | FuZZy Onions homolog                             |
|                          |                                  | YDL198C | GGC1  | GDP/GTP Carrier                                  |
|                          |                                  | YJR144W | MGM101| Mitochondrial Genome Maintenance                  |
|                          | Mitochondrial enzyme             | YAL044C | GCV3  | GlyCine cleaVage                                 |
|                          |                                  | YOR136W | IDH2  | Isocitrate DeHydrogenase                         |
|                          |                                  | YBR221C | PDB1  | Pyruvate Dehydrogenase Beta subunit               |
|                          |                                  | YPL188W | POS5  | PerOxide Sensitive                              |
|                          |                                  | YMR267W | PPA2  | PyroPhosphatase                                  |
|                          |                                  | YJR104C | SOD1  | SuperOxide Dismutase                             |
|                          | Amino acid biosynthesis          | YLR027C | AAT2  | Aspartate AminoTransferase                        |
|                          |                                  | YJL071W | ARG2  | ARGinine requiring                               |
|                          |                                  | YJL088W | ARG3  | ARGinine requiring                               |
|                          |                                  | YER069W | ARG5,6| ARGinine requiring                               |
|                          |                                  | YDR127W | ARO1  | AROmatic amino acid requiring                    |
|                          |                                  | YOR303W | CPA1  | Carbamyl Phosphate synthetase A                   |
|                          |                                  | YAL012W | CYS3  | CYStathionine gamma-lyase                        |
|                          |                                  | YEL046C | GLY1  | GLYcine requiring                                |
|                          |                                  | YDR158W | HOM2  | HOMoserine requiring                             |
|                          |                                  | YER052C | HOM3  | HOMoserine requiring                             |
|                          |                                  | YJR139C | HOM6  | HOMoserine requiring                             |
|                          |                                  | YHL011G | PRS3  | PhosphoRibosylpyrophosphate Synthetase            |
|                          |                                  | YOR184W | SER1  | SERine requiring                                 |
|                          |                                  | YGR208W | SER2  | SERine requiring                                 |
|                          |                                  | YCR053W | THR4  | THRReonine requiring                             |
|                          | Carbohydrate                     | YBR126C | TPS1  | Trehalose-6-Phosphate Synthase                   |

(Continued)
localizes adjacent to RNA polymerase III (Pol III)-transcribed genes [23], and transcription of the Pol III-transcribed genes SNR6 and RPR1 was reported to be significantly reduced in Rsc4 C-terminal mutant cells [24]. Furthermore, recent studies showed that RSC depletion causes a pronounced decrease in Pol III occupancy and affects nucleosome density [25, 26]. These observations indicate that reduced Pol III-transcribed gene transcription in nps1-105, in combination with the deletion of genes that function in ribosome biogenesis or translation regulation, might have led to synthetic lethality/slow growth. In contrast, the relationships between RSC and genes implicated in mitochondrial function have not yet been well studied. Therefore, we focused on the mitochondrial functions of RSC.

2. rsc mutants exhibit mitochondrial function-defective phenotypes

To investigate whether RSC mutations affected respiratory growth, we examined the growth of rsc mutant strains on a rich medium containing a non-fermentable carbon source (i.e., ethanol and glycerol (YPEG)). To confirm that these phenotypes were not specific to the nps1-105-mutated allele, we employed another temperature-sensitive mutant, nps1-13 [14], and deletion mutants of RSC1, RSC2, and RSC7 in this experiment. nps1-13 contains amino-acid substitutions in the C-terminal bromodomain of Nps1, resulting in reduced interactions between RSC components. In this mutant cells, the existence of functional RSC complexes was estimated to be five times lower than in WT cells [14]. Rsc1 and Rsc2 are highly homologous proteins

| Classes | Processes | ORF | Gene | Description |
|---------|-----------|-----|------|-------------|
| Transport | Endosomal transport, protein targeting | YJL204C | RCY1 | ReCyCling |
| | | YLR025W | SNF7 | Sucrose NonFermenting |
| | | YPR002C | SNF8 | Sucrose NonFermenting |
| | | YCL008C | SLP22 | Sterile Pseudoreversion |
| | | YBR127C | VMA2 | Vacuolar Membrane ATPase |
| | | YEL027W | VMA3 | Vacuolar Membrane ATPase |
| | | YJR102C | VPS25 | Vacuolar Protein Sorting |
| | | YPL065W | VPS28 | Vacuolar Protein Sorting |
| | | YHR060W | VMA22 | Protein that is required for vacuolar H+-ATPase (V-ATPase) function |
| | | YKL119C | VPH2 | Vacuolar pH |
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contained in distinct RSC complexes. Deletion of either RSC1 or RSC2 does not affect viability, but double deletion of these genes is lethal. The quantity of Rsc2 is 10-fold higher than that of Rsc1 [27]. As shown in Fig 1A, nps1-13 and rsc2Δ cells exhibited impaired growth on a YPEG plate at 30°C. At 35°C, a semi-permissive temperature for growth of all evaluated rsc mutants except rsc7Δ on YPD, none of these rsc mutants grew on the YPEG plate. The data suggest that these rsc mutations induced a defect(s) in respiratory growth. Among the five rsc mutants used in this experiment, rsc2Δ cells exhibited the most severe growth defect on YPEG, suggesting a functional difference between Rsc1-containing (Rsc1-RSC) and Rsc2-containing RSC (Rsc2-RSC) complexes in respiratory growth regulation, with Rsc2-RSC playing a major role in this process. To examine this point, we over-expressed RSC1 in rsc2Δ, and then assessed cell growth on the YPEG plate. As shown in Fig 1B, on the YPEG plate, the growth of rsc2Δ cells harboring RSC1 in a high-copy vector was indistinguishable from that of WT cells, suggesting that RSC complexes containing either Rsc1 or Rsc2 act redundantly to regulate respiratory growth.

Next, we used fluorescence microscopy to observe mitochondrial morphology in RSC mutants stained with Mito-Tracker (Fig 1C). In WT cells, mitochondria appeared as tubular networks distributed near the cell cortex. In contrast, mitochondria in nps1-13 and rsc2Δ cells frequently aggregated to form one or two spots. Mitochondrial aggregation was observed in the cells at all cell-cycle stages. The frequencies of nps1-13 and rsc2Δ cells containing aggregated mitochondria were 13.5% and 12.6%, respectively. Aggregated mitochondria were also observed in rsc7Δ cells, albeit at a lower frequency (9.1%). In contrast, little aggregation was observed in WT cells (<10⁻⁴).

The yeast mitochondrial genome is subject to spontaneous mutations that result in a loss of mitochondrial DNA (mtDNA). Cells that have lost mtDNA form small colonies, termed “petite”, on YPD medium. A defect in respiratory function is known to enhance mtDNA loss. To examine the stability of mtDNA in the rsc mutant cells, we measured the frequencies of petite formation in each strain cultured in YPD to the early-stationary phase. As shown in Fig 1D, all rsc mutants formed petite colonies at higher frequencies than did WT cells; especially, the petite frequencies of nps1-13 and rsc2Δ were three-fold to four-fold higher than that of WT cells, indicating that the defective RSC complex induced mitochondrial genome instability.

These results indicate that the RSC complex plays important roles relevant to mitochondrial respiratory function. Mitochondrial dysfunction also leads to the accumulation of reactive oxygen species (ROS). As shown in Fig 1E, the rate of ROS accumulation was approximately two-fold higher in nps1-13 cells than in WT cells. Among the rsc mutants used in this study, nps1-13 and rsc2Δ exhibited the most severe phenotypes. Given that the functional RSC contents in these strains were estimated to be approximately 5-fold to 10-fold lower than those in WT cells, the former may require a larger amount of RSC under respiratory conditions relative to fermentation conditions.

3. Global transcription analysis of nps1-13 grown under respiratory conditions

To further understand the function of RSC in respiratory growth, we performed a microarray analysis to compare global gene expression profiles between WT and nps1-13 mutant cells grown on YPEG. From valid data on 5,558 genes in WT and nps1-13 cells, 219 and 345 genes in nps1-13 were found to be up- and down-regulated, respectively, above or below the two-fold threshold (S3 Table). A GO SlimMapper analysis of these up- or down-regulated genes revealed that the frequencies of down-regulated genes in the mitochondrial and mitochondrial envelope categories were significantly higher than the general frequency (P-values = 0.022 and
Fig 1. *rsc* mutants exhibit phenotypes defective in mitochondrial function. (A) *rsc* mutants exhibit growth defects on medium containing a non-fermentable carbon source. Five-fold serial dilutions of individual strains (WT (BY4743), *nps1-105* (BYI-17), *nps1-13* (BYI-3), *rsc1Δ* (BYI-1), *rsc2Δ* (BYI-2), and *rsc7Δ* (BYI-18)) were grown to log phase in YPD medium, spotted on YPD and YPEG plates, and incubated at the indicated temperatures for 3 days. (B) Overexpression of *RSC1* suppresses the growth defect of *rsc2Δ* on YPEG. Five-fold serial dilutions of exponentially growing individual strains (WT (BY4743) carrying YEp13 (WT/vector) and *rsc2Δ* (BYI-2) carrying YEp13 (*rsc2Δ*/vector) or YEp13*RSC1-3MYC (*rsc2Δ*/RSC1)) were spotted on YPD and YPEG plates and incubated at 30°C for 3 days. (C) *rsc* mutants contain mitochondria with irregular morphologies. WT (BY4743), *nps1-13* (BYI-3), *rsc2Δ* (BYI-2), and *rsc7Δ* (BYI-18) cells were grown to log phase in YPD medium, stained with Mito-Tracker, and observed under a fluorescence microscope. Numerals on the right sides of panels represent the percentages of cells containing aggregated mitochondria among total cells. All *P*-values were calculated using the two-tailed chi-square test (*n* = 50 cells; **P** < 0.05; ***P** < 0.005). (D) *rsc* mutations enhance mitochondrial DNA loss. WT (BY4743), *nps1-105* (BYI-17), *nps1-13* (BYI-3), *rsc2Δ* (BYI-2), and *rsc7Δ* (BYI-18) cells were plated on YPEG medium; three independent colonies were later picked and separately grown to stationary phase in YPD medium. Two hundred cells from each culture were plated on YPD plates and incubated at 30°C for 3 days. To assess the frequency of petite cells, we counted the total number of cells and the number of petite cells on each plate. Data are presented as the means ± SEM of three replicates. (E) *nps1-13* cells accumulate reactive oxygen species. WT (BY4743) and *nps1-13* (BYI-3) cells harboring pRS426 (WT/vector and *nps1-13*/vector, respectively) or pRS426*GPDpr::HAP4* (WT/HAP4 and *nps1-13/HAP4*, respectively) were grown to log phase in SD-Ura medium, shifted to YPEG medium, and incubated at 30°C with shaking. On the indicated days, portions of the cells were separated, stained with dihydroethidium, and examined under a fluorescence microscope. The experiment was repeated three times (*n* = 300). Data are presented as the means ± SEM.

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This result suggests that RSC is required for the expression of these genes. The mitochondrion-related genes with down-regulated expression in nps1-13 are listed in Table 5. Of these 71 genes, 14 were grouped in the "respiration" category and considered closely relevant to the major phenotypes of rsc mutants described in the previous section. It was especially interesting to find that 7 of these 14 respiration genes were targets of the HAP complex (P-value = 0.038). This HAP complex is a transcription factor composed of Hap2, Hap3, Hap4, and Hap5 and plays a pivotal role in respiratory gene regulation [10]. In addition to the “respiration” group genes, UPS2 in the “organization” group and YMR31 in the “translation” group were also defined as Hap4 target genes. Moreover, although the DNMI, MRPS5, and MEF1 genes, indicated with asterisks in Table 5, do not contain the Hap4-binding sequence in their promoters, expression of these genes has been reported to be controlled by the HAP complex [21]. These results suggest the possibility that RSC might act with the HAP complex to regulate its target genes.

To validate these microarray results, we performed quantitative real-time PCR for HAP complex target genes, ATP1, ATP16, COR1, COX6, and COX12. Consistent with the array data, induction of these genes in nps1-13 during growth on YPEG was lower than that observed in WT cells (Fig 3).

4. RSC interacts with Hap4 to regulate the expression of respiratory genes

The reduced expression of HAP-regulated genes in nps1-13 suggested the possibility that this mutation affected Hap4 expression because HAP complex activity is proportional to the Hap4 subunit level [28]. To verify the level of Hap4, we constructed strains expressing HA-tagged Hap4 and examined the Hap4-HA content by Western blotting. As described previously, the Hap4 level increases upon shifting cells from a medium containing glucose to medium

![Fig 2. Comparison of genes up- or down-regulated genes in nps1-13, grouped using the GO SlimMapper with respect to cellular component. Only the GO terms that appeared in more than 6% of the up- and down-regulated genes are listed. For the remaining GO terms, no significant differences were observed between the frequencies of affected genes and the general frequency. All P-values were obtained using the hypergeometric test (**P < 0.05).](doi:10.1371/journal.pone.0130397.g002)
Table 5. Functional grouping of mitochondria-related genes down-regulated in *nps1-13*.

| ORF      | Gene | logFC(*nps1-13/WT*) | Description                                                                 |
|----------|------|---------------------|-----------------------------------------------------------------------------|
| **Respiration** |      |                     |                                                                             |
| YBL045C  | COR1 | -1.396528328        | CORe protein of QH2 cytochrome c reductase                                 |
| YBL099W  | ATP1 | -1.704958554        | ATP synthase                                                                |
| YDL004W  | ATP16| -2.557231151        | ATP synthase                                                                |
| YDR079W  | PET100| -1.019022029       | PETite colonies                                                             |
| YGL018C  | JAC1 | -1.038732523        | J-type Accessory Chaperone                                                  |
| YGR029W  | ERV1 | -1.109214546        | Essential for Respiration and Viability                                     |
| YGR101W  | PCP1 | -1.703295695        | Processing of Cytochrome c Peroxidase                                       |
| YHR051W  | COX6 | -1.230934459        | Cytochrome c Oxidase                                                        |
| YKL055C  | OAR1 | -1.606497792        | 3-Oxoacyl-[Acyl-carrier-protein] Reductase                                  |
| YLR038C  | COX12| -1.257634276        | Cytochrome c Oxidase                                                        |
| YLR164W  | SHH4 | -1.286480017        | SDH4 Homolog                                                                |
| YLR295C  | ATP14| -1.136379747        | ATP synthase                                                                |
| YMR267W  | PPA2 | -1.556696229        | PyroPhosphatase                                                             |
| YPL270W  | MDL2 | -1.283689019        | MultiDrug resistance-Like                                                   |
| **Metabolism** |      |                     |                                                                             |
| YCL064C  | CHA1 | -1.326318344        | Catabolism of Hydroxy Amino acids                                           |
| YDR305C  | HNT2 | -1.300467492        | Histidine triad Nucleotide-binding                                           |
| YER019W  | ISC1 | -1.104381351        | Inositol phosphopSphingolipid phospholipase C                               |
| YER069W  | ARG5,6| -1.052067786       | ARGinine requiring                                                          |
| YER183C  | FAU1 | -1.26019198        | Folic Acid Utilization                                                      |
| YPL030W  | AGX1 | -1.11633377        | Alanine:Glyoxylate aminotransferan(X)ferase                                 |
| YGL059W  | PKP2 | -1.169211802       | Protein Kinase of PDH                                                        |
| YGR102C  | GTF1 | -3.704929047       | Glutamyl Transamidase subunit F                                             |
| YHR171C  | MSN1 | -1.09387038        | Mitochondrial aminoacyl-tRNA Synthetase, Methionine                         |
| YJL005W  | CYR1 | -1.436163151       | CYclic AMP Requirement                                                      |
| YJL130C  | URA2 | -1.171207794       | URAcet requiring                                                            |
| YJR051W  | OSM1 | -1.664915789       | OSMotic sensitivity                                                          |
| YKL094W  | YUJ3 | -1.339639536       | Monoglyceride lipase (MGL)                                                   |
| YLL027W  | ISA1 | -1.608964228       | Iron Sulfur Assembly                                                        |
| YMR002W  | MIC17| -1.27675268        | Mitochondrial Intermembrane space Cysteine motif protein                    |
| YNL009W  | IDP3 | -1.957974411       | Isocitrate Dehydrogenase, NADP-specific                                      |
| YNL104C  | LEU4 | -1.326612984       | LEUcine biosynthesis                                                        |
| YNL318C  | HXT14| -1.082556945       | HeXose Transporter                                                          |
| YOL045W  | PSK2 | -1.053181566       | Pas domain-containing Serine/threonine protein Kinase                       |
| YPL091W  | GLR1 | -1.926695216       | Cytosolic and mitochondrial glutathione oxidoreductase                      |
| **Organization** |      |                     |                                                                             |
| YBR179C  | FZO1 | -1.630212553       | FuZzy Onions homolog                                                        |
| YHR194W  | MDM31| -1.336269739       | Mitochondrial Distribution and Morphology                                   |
| YIL062C  | ARC15| -1.22799372       | ArP2/3 Complex subunit                                                      |
| YLL001W*| DNMI*| -2.739316701       | DyNaMin-related                                                             |
| YLR168C  | UPS2 | -1.281524891       | UnProceSsed                                                                 |
| YNL26W   | SAM50| -1.00279983        | Sorting and Assembly Machinery                                              |
| YPL029W  | SUV3 | -1.011994907       | SUPpressor of Var1                                                          |
| **Chromosome metabolism** |      |                     |                                                                             |
| YDL164C  | CDC9 | -1.486943089       | Cell Division Cycle                                                         |
| YKL113C  | RAD27| -1.62562909        | RADiation sensitive                                                         |

(Continued)
containing a non-fermentable carbon source [10]. This induction was not affected by the nps1-13 mutation (Fig 4A). Next, we examined whether Nps1 physically interacted with Hap4 by performing a co-immunoprecipitation experiment using a strain expressing both Nps1-TAP and Hap4-HA. As indicated in Fig 4B, Nps1-TAP was detected in immunoprecipitates prepared using an anti-HA antibody, indicating that Nps1-TAP and Hap4-HA physically interacted in vivo. These results suggest that RSC might function together with the HAP complex to induce a set of respiratory genes. However, it is also possible that each complex is independently recruited to the promoter of a target gene through an interaction with some other factor(s) such as histone acetyltransferase or histone deacetylase. Further analysis is required to understand the mechanisms of gene regulation.

### Table 5. (Continued)

| ORF      | Gene  | logFC(nps1-13/WT) | Description                                      |
|----------|-------|-------------------|-------------------------------------------------|
| YMR167W  | MLH1  | -1.615848689      | MutL Homolog                                     |
| YOL042W  | NGL1  | -1.252177233      | Putative endonuclease                            |
| YPL155C  | KIP2  | -1.646124618      | Kinesin related Protein                          |

**Translation**

| ORF      | Gene  | logFC(nps1-13/WT) | Description                                      |
|----------|-------|-------------------|-------------------------------------------------|
| YBR251W* | MRPS5*| -1.148985411      | Mitochondrial Ribosomal Protein, Small subunit   |
| YDR077W  | SED1  | -1.621599493      | Suppression of Exponential Defect                |
| YER153C  | PET122| -1.221689033      | Pctl7e colonies                                  |
| YFR049W  | YMR31 | -1.09117704       | Yeast Mitochondrial Ribosomal protein            |
| YHR070W  | TRM5  | -1.201399879      | Transfer RNA Methyltransferase                   |
| YHR189W  | PTH1  | -1.133275954      | Peptidyl-tRNA Hydrolase                          |
| YLR069C* | MEF1* | -1.015712709      | Mitochondrial Elongation Factor                  |
| YMR158W  | MRPS8 | -1.052102798      | Mitochondrial Ribosomal Protein, Small subunit   |
| YNL227C  | JJ1   | -1.094508612      | J-protein (Type III)                             |
| YOL141W  | PPM2  | -1.031449449      | Protein Phosphatase Methyltransferase            |
| YOR048C  | RAT1  | -1.205794524      | Ribonucleic Acid Trafficking                     |
| YOR188W  | MSB1  | -1.4223978        | Multicopy Suppressor of a Budding defect         |
| YOR335C  | ALA1  | -1.034833773      | ALAnyl-tRNA synthetase                           |
| YPL005W  | AEP3  | -1.086709933      | ATPase ExPression                                |
| YPL082C  | MOT1  | -1.191021182      | Modifier of Transcription                        |

**Other**

| ORF      | Gene  | logFC(nps1-13/WT) | Description                                      |
|----------|-------|-------------------|-------------------------------------------------|
| YDL040C  | NAT1  | -1.987231644      | N-terminal AcetylTransferase                     |
| YLR090W  | XDJ1  | -1.238779632      | Putative chaperone                               |
| YOL099W  | ZEO1  | -1.128791113      | ZEOcin resistance                                |
| YPR095C  | SYT1  | -1.015582931      | Suppress of ypt3                                 |

**Unknown**

| ORF      | Gene  | logFC(nps1-13/WT) | Description                                      |
|----------|-------|-------------------|-------------------------------------------------|
| YGR021W  |       | -2.127560354      | Putative protein of unknown function            |
| YHL014C  | YLF2  | -1.665232462      | protein of unknown function                      |
| YKR070W  |       | -1.259135277      | Putative protein of unknown function            |
| YMR221C  | FMP42 | -1.013995342      | Found in Mitochondrial Proteome                 |
| YNL122C  |       | -2.052330445      | Putative protein of unknown function            |
| YPR097W  |       | -1.622035952      | Protein that contains a Phox homology (PX) domain|

Underlined genes are target of transcription factor HAP complex. Genes with asterisk do not contain HAP complex binding site, but their expression is under the control of the complex. [21]
To understand the relationship between the RSC and HAP complex, we first determined whether the hap4Δ mutant exhibits similar phenotypes as those induced by mitochondrial dysfunction in rsc mutants. The hap4Δ mutant also exhibited growth defects on a medium containing a non-fermentable carbon source and a high frequency of mtDNA loss (S1 Fig). Next, we examined whether the respiratory defect phenotypes of rsc mutants could be relieved by the overexpression of HAP4. For this experiment, we constructed a high-copy plasmid carrying HAP4 that was expressed under the control of the GPD promoter (pRS426GPDpr::HAP4). As shown in Fig 5A and 5B, defective growth of nps1-13 and rsc2Δ on YPEG plates and enhanced formation of petite nps1-13 colonies were alleviated by the overexpression of HAP4. In contrast, little recovery was observed with regard to the increased accumulation of ROS in nps1-13 cells (Fig 1E). These results suggest the involvement of RSC in the transcriptional activation of a set of respiratory genes, together with the HAP complex. However, the results also indicate that RSC might interact with factors other than the HAP complex to regulate mitochondrial function.

In conclusion, our results are the first to show the relevance of the RSC to mitochondrial function. Our findings show that cells require higher amounts of RSC under respiratory condition, compared with fermentable condition, indicating that RSC may orchestrate the expression of genes required for mitochondrial function together with transcription factors other than the HAP complex. Identification of these factors should elucidate the regulation of...
respiration and mitochondrial development. Mitochondrial dysfunction has been linked to a range of pathologies, including cancer (reviewed in [29]). As described, high frequencies of mutations in the components of human chromatin-remodeling complexes have been identified in human cancers; however, the molecular mechanisms underlying the carcinogenic effects of these mutations are largely unknown [30, 31]. In this context, further analysis of the effects of RSC on mitochondrial function should facilitate a better understanding of the functions of mammalian ATP-dependent chromatin remodelers in carcinogenesis.

Fig 4. Nps1 physically interacts with Hap4. (A) nps1-13 mutation does not affect Hap4 expression. HAP4-HA (BYI-19) and nps1-13 HAP4-HA (BYI-21) cells were grown to log phase in YPEG or YPL medium for the times indicated in the figure, after which whole-cell extracts were prepared. Proteins in the extract were separated by SDS-PAGE, and Hap4-HA was detected by immunoblotting. The densities of immunoblot bands labeled with anti-HA were normalized to those labeled with anti-Cdc28 and indicated as a bar graph of values relative to the value of WT cells grown for 0 h in YPD, which was set at "1". Data are presented as the means ± SEM (n = 3). (B) Nps1-TAP physically interacts with Hap4-HA. BYI-20 (NPS1-TAP-KanMX4 HAP4-6HA::URA3) cells were grown to log phase in YPD medium and subsequently shifted to YPEG medium, where they were maintained for 3 h. Immunoprecipitates prepared from cell lysates with anti-HA antibody were subjected to immunoblotting with anti-TAP and anti-HA antibodies. The densities of immunoblot bands stained with anti-TAP in lanes 2 and 3 were normalized with those of bands stained with anti-HA and indicated as a bar graph of values relative to the value of YPD, which was set at "1". Data are presented as the means ± SEM (n = 3).

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Supporting Information

S1 Fig. hap mutant exhibits phenotypes caused by mitochondrial dysfunction. (A) A hap4Δ mutant exhibits growth defect on medium containing a non-fermentable carbon source. Five-fold serial dilutions of individual strains (WT (BY4743), hap4Δ (BYI-22), and nps1-13 (BYI-3)) were grown to log phase in YPD medium, spotted on YPD and YPEG plates, and incubated at the indicated temperatures for 3 days. (B) hap4Δ mutation enhances mitochondrial DNA loss. WT (BY4743), hap4Δ (BYI-22), and nps1-13 (BYI-3) cells were plated on YPEG; three independent colonies were subsequently picked, pre-cultured in SD-Ura medium, and separately grown in YPD medium. On the indicated days, 200 cells from each culture were plated on YPD plates and incubated at 30°C for 3 days. Data are presented as the means ± SEM (n = 3).

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Fig 5. Overexpression of HAP4 alleviated the respiratory defect of nps1-13. (A) Effect of HAP4 overexpression on nps1-13 growth on YPEG. WT (BY4743) and nps1-13 (BYI-3) cells harboring pRS426 (WT/v and nps1-13/v, respectively) or pRS426GPDpr::HAP4 (WT/HAP4 and nps1-13/HAP4, respectively) were grown to log phase in SD-Ura medium, spotted on YPD and YPEG plates in serial five-fold dilutions and incubated at 30°C for 3 days. (B) Effect of HAP4 overexpression on petite nps1-13 colony formation. The strains described in (A) were plated on YPEG; three independent colonies were subsequently picked, pre-cultured in SD-Ura medium, and separately grown in YPD medium. On the indicated days, 200 cells from each culture were plated on YPD plates and incubated at 30°C for 3 days. Data are presented as the means ± SEM (n = 3).

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Supporting Information

S1 Fig. hap mutant exhibits phenotypes caused by mitochondrial dysfunction. (A) A hap4Δ mutant exhibits growth defect on medium containing a non-fermentable carbon source. Five-fold serial dilutions of individual strains (WT (BY4743), hap4Δ (BYI-22), and nps1-13 (BYI-3)) were grown to log phase in YPD medium, spotted on YPD and YPEG plates, and incubated at the indicated temperatures for 3 days. (B) hap4Δ mutation enhances mitochondrial DNA loss. WT (BY4743), hap4Δ (BYI-22), and nps1-13 (BYI-3) cells were plated on YPEG; three independent colonies were subsequently picked and grown separately in YPD medium to stationary phase. Two hundred cells from each culture were plated on YPD and incubated at 30°C for 3 days. To assess the frequency of petite colonies, we counted the total number of viable cells and the number of petite colonies on each plate. Data are presented as the means ± SEM of three replicates.

(TIF)

S1 Table. Reproducibility of the SGA analysis.

(XLSX)

S2 Table. GO terms enriched among the 95 positive genes in terms of biological process.

(XLSX)

S3 Table. Differential DNA microarray data of nps1-13 vs. BY4743.

(XLSX)
Author Contributions
Conceived and designed the experiments: YI FY MU MY ET. Performed the experiments: YI FY MY MN YC KO MS MK RH MY. Analyzed the data: YI FY MU MY ET. Contributed reagents/materials/analysis tools: YI FY MN MU MY MK RH ET. Wrote the paper: ET MY MU.

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