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Saccharomyces cerevisiae* Mhr1 can bind Xho I-induced mitochondrial DNA double-strand breaks *in vivo

Kanchanjunga Prasai¹, Lucy C. Robinson², Kelly Tatchell², and Lynn Harrison^{1,*}

¹Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

²Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

Abstract

Mitochondrial DNA (mtDNA) double-strand break (DSB) repair is essential for maintaining mtDNA integrity, but little is known about the proteins involved in mtDNA DSB repair. Here, we utilize *Saccharomyces cerevisiae* as a eukaryotic model to identify proteins involved in mtDNA DSB repair. We show that Mhr1, a protein known to possess homologous DNA pairing activity *in vitro*, binds to mtDNA DSBs *in vivo*, indicating its involvement in mtDNA DSB repair. Our data also indicate that Yku80, a protein previously implicated in mtDNA DSB repair, does not compete with Mhr1 for binding to mtDNA DSBs. In fact, C-terminally tagged Yku80 could not be detected in yeast mitochondrial extracts. Therefore, we conclude that Mhr1, but not Yku80, is a potential mtDNA DSB repair factor in yeast.

Keywords

Mitochondrial DNA; double strand break repair; Mhr1; Yku80; mitochondrial DNA immunoprecipitation

1. INTRODUCTION

Mitochondria are essential eukaryotic organelles that harbor multiple copies of their own genetic material. Mitochondrial DNA (mtDNA) is organized in nucleoprotein complexes called nucleoids [1] that are tethered to the inner mitochondrial membrane and typically, encode a small number of protein subunits of the oxidative phosphorylation (OXPHOS) system [2]. Because of its close proximity to the site of ROS generation from the mitochondrial respiratory chain, mtDNA is highly susceptible to oxidative damage. Such

*Corresponding author: Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA. Phone: 318-675-4213, FAX: 318-675-4217, lclary@lsuhsc.edu.

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DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

ROS-induced mtDNA damage includes strand breaks, base loss, and base modifications [3]. Progressive accumulation of damaged mtDNA molecules can cause respiratory chain dysfunction that can escalate ROS production, leading to further mtDNA damage. Such a 'vicious cycle' has been associated not only with aging but also with a variety of pathological conditions including diabetes, atherosclerosis, and cancer [4]. To prevent the detrimental outcomes of mtDNA damage, mitochondria have multiple mtDNA repair pathways, primarily base excision repair, mismatch repair, and double-strand break repair (for review see [5]).

DNA double-strand breaks (DSBs) are considered the most deleterious DNA lesions and can be generated by endogenous ROS, exogenous agents like ionizing radiation, as well as from biological processes like DNA replication fork arrest [6]. DSBs in the nuclear genome are repaired primarily by two major DSB repair pathways: homologous recombination (HR) and classical non-homologous end joining (C-NHEJ). HR involves resection of the damaged DNA molecule in the 5'-3' direction, formation of a 3' single-stranded tail, strand invasion and homologous pairing with an undamaged sister chromatid, and finally DNA replication to restore the genetic information in the damaged DNA molecule. (for review see [7]). C-NHEJ on the other hand, repairs DSBs by direct ligation of broken DNA ends and hence does not require a homologous DNA template (for review see [8]). During C-NHEJ, the Ku70/Ku80 heterodimer (Ku complex) binds the DNA ends, protecting the DNA from resection/ degradation and anchoring the DNA ends in close proximity for ligation. The DNA-bound Ku complex then facilitates recruitment of C-NHEJ factors that modify the DNA termini and mediate rejoining of the fragmented DNA molecule. In addition to these predominant nuclear DNA DSB repair pathways, evidence also exists for a minor nuclear DNA DSB repair pathway called alternative non-homologous end joining (A-NHEJ; also known as microhomology-mediated end joining or MMEJ) (for review see [9]). MtDNA DSB repair activities and repair products have been identified in several eukaryotic systems [10–14]. However, unlike the large number of proteins involved in nuclear DNA DSB repair, the factors mediating mtDNA DSB repair are not well characterized and only a handful of proteins have been designated as *bona fide* mtDNA DSB repair factors in the entire Eukaryota domain [15, 16].

The purpose of this study was to identify proteins that are involved in mtDNA DSB repair. We employed the budding yeast *Saccharomyces cerevisiae* as a eukaryotic model system because: (i) genome manipulation is simple and feasible; (ii) mtDNA is not essential for cell viability; and (iii) DNA repair systems are highly conserved from yeast to humans. In this study, we focused on two potential mtDNA DSB repair factors: yeast Ku80 (Yku80) and Mhr1 (mitochondrial homologous recombinase). Yku80 is a component of the yeast Ku (Yku) complex, which functions as a stable Yku70/Yku80 heterodimer during nuclear C-NHEJ [17]. Even though the components of the Yku complex have not been demonstrated to localize to the mitochondrial compartment, genetic evidence has indicated the involvement of the Yku complex in mtDNA DSB repair. The rate of mtDNA deletions, which are considered as products of DSB repair, was reported to be higher in *yku70/yku80* null mutants compared to wild-type cells [18]. This suggests a role for Yku in mtDNA repair [18]. Another potential mtDNA DSB repair candidate is Mhr1, which is located in the mitochondrial inner membrane and matrix subcompartments [19]. *In vitro*, Mhr1 promotes

heteroduplex joint formation between single-stranded DNA and homologous double-stranded DNA [19]. This recombinase activity of Mhr1 has been extensively studied with respect to its involvement in recombination dependent rolling-circle mtDNA replication at the mitochondrial replication origin *ori5* (for review see [20]). However, the role of Mhr1 in repairing general mtDNA DSBs generated in different regions of the mitochondrial genome has not been explored. An important characteristic of a DSB repair protein is its ability to bind at or near the DSBs and it has not been shown that Mhr1 does bind to mtDNA DSBs *in vivo*. To address this, we employed a mitochondrial-targeted restriction endonuclease Xho I (mitoXhoI) to introduce DSBs into the yeast mtDNA. After verifying the presence of Mhr1 in yeast mitochondrial extracts, we determined the ability of Mhr1 to bind to mitoXhoI-induced DSBs. In addition, we examined the localization of Yku80 to yeast mitochondria and compared the degree of Mhr1 binding to mitoXhoI-induced DSBs in a wild-type strain versus an isogenic *yku80* null mutant.

2. MATERIALS AND METHODS

2.1 Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (oligonucleotides) were purchased from Eurofins (Louisville, KY, USA), IDT Inc. (Coralville, Iowa, USA) or the DNA Facility at Iowa State University (Ames, IA, USA). The sequences of the oligonucleotides used in this study are listed in Table 1.

2.2 DNA manipulation and plasmid construction

Plasmid amplification was performed in *Escherichia coli* strain TOP 10 (Life Technologies, Carlsbad, CA, USA). For selection of plasmids, bacteria were grown in LB media containing carbenicillin (100 µg/ml; Life Technologies, Carlsbad, CA, USA). Plasmids were harvested from bacteria and purified using the QIAfilter Plasmid Maxi Kit (QIAGEN GmbH, Hilden, Germany). Restriction enzymes (NEB, Ipswich, MA, USA), T4 DNA ligase (Promega Corporation, Madison, WI, USA) and Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) were used as per the manufacturer's recommendations. Plasmids generated for this work were sequenced by the DNA Facility at Iowa State University to ensure correct reading frame and correct orientation of inserts.

The Xho I coding sequence was amplified from pXhoRM3.OB (provided by New England Biolabs) using the NtermXhoI and CtermXhoI primers, Phusion High-Fidelity DNA Polymerase, and an annealing temperature of 71°C. The insert was purified and digested with PstI and NotI. The plasmid pcKuFlag1-1 [21] was digested with PstI and NotI to remove the *Mycobacterium tuberculosis* Ku coding sequence and the resulting linear vector DNA was ligated with the PstI-NotI digested Xho I coding sequence to generate pMTSXhoIFlag. The Xho I-Flag sequence from pMTSXhoIFlag was amplified with BamXhoI and SalFlag primers using Phusion High-Fidelity DNA Polymerase and an annealing temperature of 65°C. The insert was digested with BamHI and SalI and ligated to the mitochondrial targeting sequence of F₀-ATPase subunit 9 (Su9) of *Neurospora crassa* [24] in the pGAL plasmid. This was done by BamHI and SalI digestion of pGalSu9RFP [21], which removed the red fluorescent protein (RFP) coding sequence, and ligation of this

linearized vector to the BamHI-SalI-digested Xho I-Flag DNA fragment generated pGalSu9XhoI. The plasmids pGalSu9RFP and pGalSu9XhoI contain the *GAL1* promoter and hence the expression of mitochondrial-targeted proteins (RFP and Xho I, respectively) is driven in a galactose-inducible manner. Glucose represses the promoter, while raffinose has no effect on expression.

2.3 Yeast strains, media, and transformation

S. cerevisiae strains used in this study are listed in Table 2 and are congenic to KT1357 [25]. To generate KJP1976, a strain capable of expressing C-terminally Myc-tagged Mhr1 from the endogenous *MHR1* locus, the Myc9 tag and the *kITRP1* selectable marker from plasmid pWZV87 ([28]; a gift from Dr. David S. Gross) were amplified using MHR1Myc9F and MHR1Myc9R primers, Phusion High-Fidelity DNA Polymerase, and an annealing temperature of 70°C. Seventeen nucleotides at the 3' end of the primers (MHR1Myc9F and MHR1Myc9R; Table 1) were complementary to the regions upstream or downstream of the *MYCX9-kITRP1* cassette in pWZV87. The remaining nucleotide sequences in the MHR1Myc9F and MHR1Myc9R primers were homologous to ~40 bp immediately 5' and 3' to the *MHR1* stop codon, respectively. These regions of homology were used to direct integration of *MYCX9-kITRP1* cassette to the 3' end of the *MHR1* gene, eliminating the Mhr1 stop codon and generating a coding region to produce a Mhr1-Myc tagged fusion protein. The PCR product was purified and introduced into the *trp1* mutant strain KT1358 [26]. Transformants containing *MYCX9-kITRP1* cassette were selected in medium lacking tryptophan. Positive transformants were then screened by PCR using MHR1F and MHR1R primers. A positive transformant was mated with KT1357, following which the diploid strain was sporulated and haploid meiotic segregants were isolated by tetrad analysis to obtain KJP1976. The expression of Myc-tagged Mhr1 from KJP1976 was verified by western analysis.

The yeast strain KJP2011 expressing C-terminally Myc-tagged Yku80 from the endogenous *YKU80* locus was engineered as described for KJP1976 except that Yku80Myc9F and Yku80Myc9R primers were used to amplify the Myc9 tag and *kITRP1* selectable marker from pWZV87. Yku80Myc9F and Yku80Myc9R primers also contained nucleotide sequences that were homologous to ~50 bp immediately 5' and 3' to the *YKU80* stop codon, respectively. The purified PCR product was introduced into KT1357 and transformants were selected in medium lacking tryptophan, following which positive transformants were screened by PCR using Yku80F and Yku80R primers. A positive transformant was mated with KT1358 and the diploid strain was sporulated, following which KJP2011 was obtained by tetrad analysis. The expression of Myc-tagged Yku80 from KJP2011 was verified by western analysis.

The *yku80* mutant (KJP2038) and the *mhr1* mutant (KT3898) were constructed by amplifying the G418 cassette from the *yku80* or *mhr1* deletion panel strain [29] with YKU80DelF and YKU80DelR primers or MHR1-F^{LCR} and MHR1-R^{LCR} primers, respectively. The amplification was performed with Phusion High-Fidelity DNA Polymerase, and an annealing temperature of 61.5°C. The purified PCR product was introduced into KJP1976 for the *yku80* null mutant and into KT1112/KT1113 diploid strain

for the *mhr1* null mutant and transformants were selected by their ability to grow in G418 (200 µg/ml; Life Technologies, Grand Island, NY, USA). The absence of the *YKU80* or *MHR1* gene was confirmed by PCR using YKU80DelF and DeltaYKU80R primers or MHR1-F^{LCR} and MHR1-R^{LCR} primers, respectively.

YPD and YPEG contained 1% yeast extract and 2% bacto peptone with 2% glucose or 2% ethanol and 3% glycerol, respectively. Selective media were prepared by combining complete supplement mixture lacking uracil (–Ura) or tryptophan (–Trp; Sunrise Science Products, San Diego, CA, USA) with yeast nitrogen base (Sunrise Science Products) and 2% carbon source.

Yeast transformation was performed by a LiAc procedure as described previously [30] with the following modifications: (i) cells were grown in YPD to OD₆₀₀ of 0.6–0.9, and (ii) the carrier DNA was from salmon testes (Sigma, Saint Louis, MO, USA).

2.4 Spot dilution analysis

Yeast cultures were grown to stationary phase in –Ura raffinose liquid medium if they contained pGalSu9RFP or pGalSu9XhoI, while KJP1976, KT1357 and KT3898 were grown in YPD. The cultures were 25-fold serially diluted using sterile water and 6 µl of each dilution applied to a –Ura galactose plate for strains containing pGalSu9RFP or pGalSu9XhoI, or YPD and YPEG for KJP1976, KT1357 and KT3898. Cells were grown at 30°C for 48 h. The colonies on the –Ura galactose plate from strains containing pGalSu9RFP or pGalSu9XhoI were then replica-plated to YPEG prior to growth at 30°C for 36 h.

2.5 Determination of percentage of p⁺ colonies

Determination of the percentage of p⁺ colonies was performed as described previously [21]. Briefly, stationary phase KT1357 cultures harboring pGalSu9XhoI pre-grown in –Ura raffinose were diluted in fresh –Ura raffinose liquid medium to a final concentration of OD₆₀₀ of 0.04. The cultures were grown at 30°C for 3 h on a rotary shaker (200 rpm), after which 2% galactose or 2% raffinose was added. The cultures were then returned to the rotary shaker and aliquots were removed at the indicated time points. Cells were counted using a hemocytometer and serially diluted in sterile water to plate ~200 cells on –Ura glucose agar plates. The colonies were counted and replica-plated to YPEG, following which YPEG-positive colonies were counted and the percentage of p⁺ colonies was calculated using the following formula: % of p⁺ colonies = (number of colonies on YPEG/ number of colonies on –Ura glucose) × 100

2.6 Immunoblot analysis

Total cell extracts were prepared by lysing yeast cells with glass beads in trichloroacetic acid as described previously [31]. Mitochondrial extracts were prepared from yeast cells using the Yeast Mitochondria Isolation Kit according to manufacturer's instructions (Bio Vision, Milpitas, CA, USA). Protein extracts were subjected to electrophoresis through 4–20% tris-glycine gradient gels (Life Technologies, Carlsbad, CA, USA) and transferred to 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc., Germany). Membranes were probed

with the following antibodies: (i) anti-Flag M2 primary antibody (1:2000; Sigma, Saint Louis, MO, USA) and a sheep anti-mouse secondary horseradish peroxidase antibody (1:8000; Amersham Biosciences, Buckinghamshire, UK); (ii) anti-Cox4 (1:2000; MitoSciences, Eugene, OR, USA) primary antibody or anti-Pgk1 (1:30000; Invitrogen, Frederick, MD, USA) primary antibody and a goat anti-mouse secondary horseradish peroxidase antibody (1:20000 and 1:31250, respectively; Bio-rad, Hercules, CA, USA); and (iii) anti-cMyc primary antibody (1:6000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a goat anti-mouse secondary horseradish peroxidase antibody (1:30000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

For all immunoblots, antibody binding was detected by incubation with chemiluminescent substrate (ECL-Prime; GE Healthcare, Buckinghamshire, UK) and visualized using autoradiography. Bound antibody was removed from blots with stripping buffer (Thermo Scientific, Rockford, IL, USA) prior to subsequent re-probing.

2.7 Total DNA isolation

Isolation of yeast total DNA was performed either using the Yeastar Yeast Genomic DNA kit (Zymo Research, Irvine, CA), or as described previously [32] with slight modifications. Briefly, cells aliquoted at indicated time points were lysed with glass beads for 5 min in lysis buffer and phenol/chloroform/isoamylalcohol (25:24:1; Fisher Scientific, Fair Lawn, NJ, USA). The pellet obtained after ethanol precipitation was resuspended in TE and treated with DNase-free RNase for 1 h at 37°C. A final DNA precipitation was performed with ice-cold absolute ethanol in the presence of ammonium acetate. The DNA pellet was resuspended in TE and quantitated using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA).

2.8 Real-time quantitative PCR (qPCR)

Real-time qPCR was performed as described previously [21]. The monomeric form of the budding yeast mitochondrial genome contains two Xho I restriction sites, located at 23224 bp (upstream Xho I site) and 51370 bp (downstream Xho I site). Amplification across the upstream and downstream Xho I sites was performed using XhoIupF/R and XhoIdownF/R primer sets, respectively. The COX2F/R primer set was used to amplify a region of *COX2* in the mtDNA, and the ACT1F/R primer set was utilized to amplify a region of *ACT1* in the nDNA.

2.9 Southern analysis

Southern analysis was performed to demonstrate mtDNA fragmentation by sonication. The sonicated DNA-protein complex (see Section 2.10) was treated with proteinase K (50 µg/ml; Roche Diagnostics, Indianapolis, IN, USA) at 65°C for 16 h. DNA was then extracted twice with phenol/chloroform/isoamylalcohol (25:24:1; Amresco LLC, Solon, OH, USA) and precipitated with ice-cold absolute ethanol in the presence of sodium acetate. The purified DNA was dissolved in TE, electrophoresed through a 1% agarose gel, and transferred by capillary action [33] to a nylon membrane (Hybond-N; Amersham Biosciences, Buckinghamshire, UK). The membrane was hybridized with two ³²P-labeled mtDNA probes. ³²P-labeled mtDNA probes were generated by PCR with [α -³²P] dCTP and mtDNA

specific primer sets: ProbeXhoIupF/R primer set amplified a 548 bp mtDNA segment located ~2 kb 5' to the upstream Xho I site, whereas ProbeCOX2F/R primer set amplified a 511 bp mtDNA segment that includes part of the *COX2* gene. Hybridization was performed overnight in 50X Denhardt's solution, 20× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.5% SDS, 10% dextran sulfate, and 0.1 mg/ml salmon testes single-stranded DNA. The membrane was washed twice with 6× SSC and 0.1% SDS for 15 min at 65°C, once with 2× SSC and 0.1% SDS for 15 min at 65°C, once with 2× SSC and 0.5% SDS for 30 min at 50°C, and once with 0.5× SSC and 0.5% SDS for 30 min at 50°C, following which the ³²P signal was visualized by autoradiography using Konica SRX-101A Medical Film Processor (Tokyo, Japan).

2.10 mtDNA immunoprecipitation (mtDNA IP)

mtDNA IP was performed as described previously [21] with minor modifications. Briefly, yeast cells containing pGalSu9XhoI were grown in raffinose or galactose for 2 h. DNA was crosslinked to proteins by the addition of formaldehyde (1% final concentration). Cells were harvested, resuspended in lysis buffer and lysed with glass beads by vigorous shaking. The sample was sonicated to obtain a mean DNA length of ~0.3 kb. After centrifugation at 4°C, the supernatant containing soluble DNA-protein complexes was mixed with 1.5 µg of anti-cMyc antibody or mouse IgG1 (Sigma, Saint Louis, MO, USA) for 16 h at 4°C. Dynabeads protein G (50 µl; Life Technologies, Oslo, Norway) was added to the DNA-protein-antibody complex and mixed for 16 h at 4°C. DNA-protein complexes were eluted and the immunoprecipitated DNA was purified. The purified immunoprecipitated DNA was dissolved in TE and subjected to real-time qPCR using RegionIF/R, RegionIIF/R, RegionIIIF/R, and COX2F/R primer sets. A standard curve specific for each amplicon was used to determine the amount of mtDNA present in each immunoprecipitate and background signal obtained from IgG1 control was subtracted. The net immunoprecipitate so obtained was used to calculate percentage of input immunoprecipitated using the following formula: % Input immunoprecipitated = (Amount of mtDNA in net IP/ Amount of mtDNA in total input) × 100; where total input represents total amount of DNA-protein complex used in the immunoprecipitation.

2.11 Statistics

Data are presented as mean ± standard deviation (SD) of three independent experiments. Statistical comparisons were performed with ANOVA followed by *post hoc* analysis using Tukey's multiple comparison test. Differences are considered significant at $P < 0.05$.

3. RESULTS

3.1 Galactose-inducible system for expression of mitochondrial-targeted proteins

To deliver proteins to yeast mitochondria, we utilized the N-terminal 69 amino acids of F₀-ATPase subunit 9 (Su9) of *Neurospora crassa* as a mitochondrial targeting sequence (MTS). This sequence has previously been used to target proteins to yeast mitochondria [21, 24]. The plasmid pGalSu9RFP [21] contains the Su9 MTS in frame with RFP (Figure 1A), and induction of the *GALI* promoter with galactose results in the expression of a fusion protein in which the C-terminus of Su9 is linked to the N-terminus of RFP. We have previously

demonstrated that induction of pGalSu9RFP with galactose results in localization of RFP to yeast mitochondria [21]. Similarly, galactose-mediated induction of pGalSu9XhoI (Figure 1B) results in expression of a fusion protein in which the N-terminus of Xho I is connected to Su9, while its C-terminus is tagged with two Flag epitopes.

3.2 Mitochondrial-targeted Xho I (mitoXhoI) cleaves mtDNA and decreases mitochondrial respiration

The ~85.8 kb monomeric form of yeast mtDNA [34] contains two Xho I restriction sites, which are located at 23224 bp and 51370 bp, respectively (Figure 2A). To determine expression of mitoXhoI, KT1357 containing pGalSu9XhoI were grown in the presence of galactose and aliquots were taken at increasing time intervals to isolate total cell extracts. The extracts were subjected to western analysis using anti-Flag antibody to detect expression of mitoXhoI. As shown in Figure 2B, mitoXhoI was expressed within 1 h of galactose treatment and expression increased over 8 h.

Next, the mtDNA-cleaving activity of mitoXhoI was assessed using PCR. Introduction of a DSB at the Xho I sites decreases the amount of PCR product generated using XhoIupF/R and XhoIdownF/R primer sets, which amplify across the upstream and downstream Xho I sites, respectively. KT1357 containing pGalSu9XhoI was grown in the presence of galactose for up to 8 h, during which cells were harvested to obtain total DNA. Real-time qPCR was performed to amplify across the upstream and downstream Xho I sites. The mitochondrial gene *COX2* and the nuclear gene *ACT1* were used as controls for mtDNA and nuclear DNA (nDNA) amplifications, respectively. The *COX2* mitochondrial gene was selected because it is situated > 20 kb away from either Xho I site. As shown in Figure 2C, the normalized mtDNA/nDNA ratio across both Xho I restriction sites decreased substantially within 1 h of galactose treatment, indicating that digestion of both restriction sites occurred within 1 h of plasmid induction. The degree of cleavage increased with time such that after 2 h, ~80% of both Xho I sites were cleaved. In contrast, amplification across a region of *COX2* did not fluctuate significantly over the 8h time period. Therefore cleavage by mitoXhoI was specific for the Xho I sites in the mtDNA.

Cleavage at both Xho I restriction sites in the mtDNA can result in the loss of an ~28.1 kb mtDNA fragment that contains genes encoding essential components of the OXPHOS machinery. To determine if expression of mitoXhoI compromises mitochondrial respiration, KT1357 containing pGalSu9XhoI or pGalSu9RFP were serially diluted and spotted on galactose agar medium to induce expression of the mitochondrial-targeted proteins. Cells carrying pGalSu9XhoI grew slowly on galactose medium, whereas cells containing pGalSu9RFP grew normally (Figure 2D, top panel). The colonies were then replica plated to YPEG, a medium containing ethanol and glycerol that requires functional electron transport chain activity for metabolism; hence, only respiratory competent yeast cells can grow on the medium. Although cells carrying pGalSu9RFP grew well on YPEG, cells containing pGalSu9XhoI formed small or no colonies (Figure 2D, lower panel), indicating a loss of mitochondrial respiratory activity. The mitoXhoI-induced respiration defect was quantitated by growing cells containing pGalSu9XhoI in raffinose or galactose liquid medium for increasing time intervals. Cells were then plated on solid medium containing glucose,

following which the colonies were replica plated to YPEG to determine the percentage of ρ^+ colonies. We observed ~60% decrease in the percentage of ρ^+ colonies after 30 min of growth in galactose (Figure 2E). Even though expression of mitoXhoI and its mtDNA-cleaving activity was not detectable during the first 30 minutes of galactose induction (Figures 2B and 2C), it should be noted that during total cell extract and total DNA isolation procedures, cells and cell pellets were processed in ice-cold conditions, which is expected to stop further protein production and mtDNA cleavage. However, during the ρ^+ colony formation assay cells were processed (counted, serially diluted, and plated) at room temperature, which took ~10–15 min. In addition, glucose repression of the *GAL1* promoter takes time [35] following cell plating. Therefore, it is likely that during the processing time the cells were continuing to produce mitoXhoI protein and the mitoXhoI was able to catalyze mtDNA cleavage and hence petite formation. As shown in Figure 2E, the degree of petite formation increased progressively with time such that by 4 h, almost all colonies were respiratory deficient. In contrast, colonies obtained from cells grown with the raffinose control sugar essentially maintained a ρ^+ phenotype. Subsequent experiments were performed following a 2 h galactose induction of pGalSu9XhoI since ~80% cleavage of the Xho I sites was observed at this time point.

3.3 MitoXhoI and Mhr1, unlike Yku80, localize to mitochondria

KT1357 was genetically manipulated to generate KJP1976, which expresses Myc-tagged Mhr1 (Mhr1-9XMyc) from the *MHR1* genetic locus. A deletion in the *MHR1* gene in *S. cerevisiae* was generated (KT3898) to compare to KJP1976 to make sure the addition of the 9xMyc tag did not destroy the function of Mhr1. During the production of this mutant, G418-resistant diploid colonies were sporulated and tetrads were analyzed for G418 segregation. It was determined that all G418-resistant spore clones failed to grow on YPEG solid media, which indicates a petite mutant phenotype likely due to the loss of mtDNA. To determine the respiratory competency of KJP1976, a spot dilution test was performed. As can be seen in Figure 3, KJP1976 has a ρ^+ phenotype, indicating Mhr1-9XMyc is functional.

Following transformation with pGalSu9XhoI, KJP1976 was grown in medium containing raffinose or galactose for 2 h, and total cell extracts were prepared. Mitochondrial extracts were also prepared from 2 h galactose treated cells. Western analysis not only confirmed localization of Myc-tagged Mhr1 and Flag-tagged mitoXhoI to yeast mitochondria, but also verified galactose-dependent expression of mitoXhoI (Figure 4A).

To determine if Yku80 localizes to yeast mitochondria, we engineered a yeast strain (KJP2011) capable of expressing Myc-tagged Yku80. In a previous study [36], Yku80 fused to 13 Myc peptides was found to be functional. Hence, we generated a strain (KJP2011) where Yku80 fused to only 9 Myc peptides (Yku80-9XMyc) was expressed from the *YKU80* genetic locus. KJP2011 containing pGalSu9XhoI was grown in raffinose or galactose for 2 h, following KT1357 KJP1976 KT3898. Total cell extracts and mitochondrial extracts were prepared. Western analysis revealed galactose-dependent expression of mitoXhoI, which localized to mitochondria (Figure 4B). Interestingly, we could only detect Yku80 in the total cell extracts; Yku80 was not seen in the mitochondrial extracts.

3.4 mtDNA cleaving activity of mitoXhoI in KJP1976 and KJP2038

Mammalian mitochondria contain a truncated form of Ku80, which is absent in a cell line that lacks Ku80 mRNA expression [37]. Evidence has also shown that nuclear NHEJ competes with HR, and loss of Ku80 or Ku70 in mammalian cells increases nuclear HR [38, 39]. Pierce et al [39] put forward the hypothesis that the binding of the Ku heterodimer to the two ends of a DSB could prevent recombination proteins such as Rad51 obtaining access to the DNA ends, blocking repair by HR. In *S. cerevisiae*, it was also determined that the presence of Yku results in the lethality of a *sae2 sgs1* mutant due to the blockage of end-processing of replication-associated DSBs [40]. Therefore, models in *S. cerevisiae* have been proposed in which Yku-bound DNA ends block DNA resection and hence nuclear HR [40]. Even though western analysis of strain KJP2011 provided no evidence that Yku80 is present in mitochondria, we wanted to ensure that the *YKU80* gene product is not competing with Mhr1 during mtDNA DSB repair. Therefore, we deleted the *YKU80* gene in KJP1976, and the resulting *yku80* null strain, KJP2038, as well as KJP1976 were transformed with the mitoXhoI expression plasmid.

Strains KJP1976 and KJP2038 were grown in raffinose or galactose for 2 h, following which cells were harvested to isolate total DNA. To confirm that cleavage of the Xho I restriction sites occurs only following mitoXhoI expression in these strains, real-time qPCR was performed. As shown in Figures 5A and 5B, the amplification of a region of *COX2* was similar when the strains were grown in raffinose or galactose. However, decreased PCR amplification across both Xho I sites was only found in DNA from galactose treated cultures. This indicates the Xho I sites were cleaved and the degree of mtDNA cleavage at both restriction sites was comparable in strains KJP1976 and KJP2038.

3.5 Mhr1 binds near mitoXhoI-induced DSBs

The restriction endonuclease Xho I recognizes the palindromic sequence 5'-CTCGAG-3' in double-stranded DNA (Figure 6A) and cleaves after the first cytosine, resulting in the production of 5' 4-nucleotide single-stranded complementary ends. To determine if Mhr1 binds near mitoXhoI-induced DSBs and to determine whether Yku80 alters Mhr1 binding to mtDNA DSBs, we performed an mtDNA immunoprecipitation (IP) assay. KJP1976 and KJP2038 containing pGalSu9XhoI grown in raffinose or galactose for 2 h were treated with formaldehyde, following which the DNA crosslinked to protein was sonicated. Southern analysis of sonicated DNA with mtDNA specific probes revealed that the average length of mtDNA fragments was ~0.3 kb (Figure 6B). The immunoprecipitation of DNA-protein complexes was carried out either with anti-cMyc antibody or the IgG1 control. Real-time qPCR of purified immunoprecipitated DNA was performed using *COX2*, region I-, region II-, or region III-specific primer sets (Figure 6A). The distance of the amplicons from the upstream Xho I site at regions I and II is 404 bp and 169 bp, respectively, whereas the distance of the region III amplicon is 105 bp from the downstream Xho I site. The nucleotide sequence in region IV near the downstream Xho I site (Figure 6A) consists of extensive stretches of adenine and thymine. This prevented design of qPCR primers within 900 bp of the downstream Xho I site at region IV. As shown in Figure 6C, Mhr1 binds to *COX2* sequences in KJP1976 and KJP2038, and this interaction occurred irrespective of the status of mitoXhoI expression in either strain. However, there was a significant increase in

the degree of Mhr1 binding to regions I, II and III following growth in galactose compared to growth in raffinose for KJP1976 and KJP2038 (Figure 6D–F). Binding to regions I–III was comparable in both strains.

4. DISCUSSION

MHR1 was first identified as a gene required for mitochondrial recombination that occurs after mating between yeast cells [41]. The finding that Mhr1 promotes heteroduplex joint formation between single- and homologous double-stranded DNA *in vitro* [19] supports its *in vivo* role in mtDNA recombination. Several studies have explored the recombinase activity of Mhr1 in mediating recombination-dependent rolling circle replication [19, 42, 3] at *ori5*, an active mtDNA origin of replication possessing inherent DSBs [44, 45]. However, a potential role of Mhr1 as a ‘common’ mtDNA DSB repair factor has not been thoroughly investigated. Because of its abundance in the mitochondrial fraction and its ability to mediate homologous pairing *in vitro* [19], we hypothesized that Mhr1 is a general mtDNA DSB repair factor. In this study, we have provided evidence that Mhr1 does bind to DNA near DSBs induced in mtDNA at regions other than *ori5*, which supports the idea that Mhr1 is involved in mtDNA DSB repair.

DSBs can be lethal. Following their occurrence, DSBs are recognized and bound by DSB repair proteins in order to restore genomic integrity. Indeed, one of the hallmarks of a classical DSB repair protein is its ability to bind at or near DNA ends. To understand if Mhr1 possesses this characteristic, we explored its ability to bind to mtDNA DSBs. For this, we employed a mitochondrial-targeted Xho I (mitoXhoI) to generate DSBs in the mitochondrial genome. Expression of mitoXhoI was achieved from a galactose-inducible expression system. After confirming expression and mtDNA cleaving activity of mitoXhoI, we determined the binding ability of Mhr1 to mitoXhoI-induced DSBs. Results from mtDNA IP revealed that Mhr1 binds near the Xho I restriction sites at a significantly higher level following induction of mitoXhoI expression compared to the uninduced condition. In contrast, the binding of Mhr1 to the control region *COX2* was comparable between galactose and raffinose treatment groups. The basal binding of Mhr1 to the *COX2* gene occurs presumably because Mhr1 is a component of mitochondrial nucleoids [46], which are nucleoprotein complexes consisting of mtDNA molecule(s) and a diverse set of proteins involved in mtDNA maintenance [1]. Taken together, these results clearly demonstrate that Mhr1 binds preferentially at or near the sites where the mtDNA DSBs occur.

Yku80 is a *bona fide* mammalian Ku80 homolog [47]. The essential nature of Yku80 in C-NHEJ has been previously demonstrated. Using an *in vivo* plasmid rejoining assay, it was shown that *yku80* null mutants were as defective as *yku70*, *yku80* double mutants in repairing restriction endonuclease (EcoRI, Xho I, or PstI) induced DSBs [47]. Following association of Yku80 with Yku70, the Yku heterodimer binds DNA ends [48] to mediate nuclear DNA DSB repair via C-NHEJ, particularly in G1-phase haploid yeast cells when a homologous DNA template is unavailable for recombinational repair [49]. With regard to its role in mtDNA DSB repair, indirect evidence exists for the involvement of the Yku complex in suppressing mtDNA deletions [18]. Interestingly, loss of the MRX complex, which is also implicated in mitochondrial DSB repair, decreased the deletion frequency as would be

expected if the complex is involved in inaccurate repair [18]. Previous studies have established that Rad50 and Mre11, two of the proteins of the MRX complex, are part of the yeast mitochondrial proteome [50], whereas there is no published data demonstrating Yku80 localizes to yeast mitochondria. To determine if Yku80 localizes to the mitochondrial compartment, we isolated the mitochondrial fraction from a yeast strain capable of expressing C-terminally Myc-tagged Yku80 from the endogenous *YKU80* locus. Western analyses revealed the presence of Yku80 in total cell extracts but not in mitochondrial extracts, indicating that C-terminally Myc-tagged Yku80 does not significantly localize to yeast mitochondria. It is possible that the increase of mtDNA deletions by loss of Yku70 and Yku80 [18] could be due to an increase in cellular stress and disruption of mitochondrial genome maintenance because of the other functions of Yku70/Yku80. Ku70 in mammalian cells is involved in suppressing apoptosis by binding Bax in the cytosol [51, 52] and the Yku70/Yku80 complex is also situated at telomeres in *S. cerevisiae* [53]. It is also possible that a C-terminally truncated form of Yku80 is present in the mitochondria and is involved in DSB repair. In mammalian cells, a C-terminally truncated version of Ku80 has been shown to localize to mammalian mitochondria [37]. Since we added a Myc epitope to the C-terminus of Yku80, a C-terminally truncated form of Yku80 would not have been detected in this study. It should, however, be noted that the C-terminus of Yku80 is required for C-NHEJ in *S. cerevisiae* [54]; hence, the presence of a C-terminally truncated form of Yku80 in yeast mitochondria would not likely assist in mtDNA DSB repair. Nonetheless, there is still the possibility that an alternate version of Yku80 may localize to yeast mitochondria. To understand if any form of Yku80 is involved in mtDNA DSB repair, we deleted the *YKU80* gene and compared the degree of Mhr1 binding to mitoXhoI-induced DSBs in *yku80* null mutants versus wild-type cells. Previous studies [55, 56] have demonstrated competition between the HR and C-NHEJ pathway for nuclear DNA DSBs and evidence exists in *S. cerevisiae* that the Yku heterodimer can block DNA end processing at replication-associated DSBs and hence HR [40]. Therefore, we hypothesized that if the *YKU80* gene product participates in mtDNA DSB repair, deletion of the *YKU80* gene would increase the availability of mtDNA DSBs and hence increase Mhr1 binding to mtDNA DSBs. Data from the mtDNA IP assay revealed a comparable amount of Mhr1 binding near mtDNA DSBs between the wild-type strain and the *yku80* null mutant, which suggests that the *YKU80* gene product does not compete with Mhr1 for binding to mtDNA DSBs *in vivo*. This observation, together with the lack of Yku80 in yeast mitochondrial extracts, suggests that Yku80 is likely not involved in mtDNA DSB repair under normal growth conditions.

5. CONCLUSIONS

To our knowledge, this is the first study in which Mhr1 has been shown to bind to mtDNA DSBs *in vivo*. This property of Mhr1, together with its homologous pairing activity *in vitro* [19], supports the hypothesis that Mhr1 is a general mtDNA DSB repair factor. Our results also indicate that Yku80 does not compete with Mhr1 for binding to mtDNA DSBs and cannot be detected in yeast mitochondrial extracts. Taken together, this work suggests that Mhr1, but not Yku80, is a general mtDNA DSB repair factor in yeast and we propose that Mhr1-mediated HR is likely the predominant pathway by which yeast cells repair mtDNA DSBs.

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Abbreviations

mtDNA	mitochondrial DNA
DSB	double-strand break
OXPHOS	oxidative phosphorylation
nDNA	nuclear DNA
ROS	reactive oxygen species
HR	homologous recombination
C-NHEJ	classical non-homologous end joining
Yku80	yeast Ku80
Mhr1	mitochondrial homologous recombinase
Yku	yeast Ku
mitoXhoI	mitochondrial-targeted restriction endonuclease Xho I
RFP	red fluorescent protein
–Ura	complete supplement mixture lacking uracil
-Trp	complete supplement mixture lacking tryptophan
Gal	galactose
TCE	total cell extracts
Raff	raffinose

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Highlights

- This study indicates that Mhr1 can bind to DNA in the region near DNA double-strand breaks generated in the mitochondrial DNA by a mitochondrial-targeted Xho I.
- This study suggests that Mhr1 is involved in repair of mitochondrial DNA double-strand breaks.
- This study suggests that Yku80 does not localize to the mitochondria and is unlikely to be involved in mitochondrial DNA repair.

Figure 1.

Schematic representation of the galactose-inducible system for expression of mitochondrial-targeted proteins. The expression cassettes of pGalSu9RFP (**A**) or pGalSu9XhoI (**B**) containing the galactose-inducible promoter (*GAL1 P*) and downstream fusion protein coding sequence is shown. Su9 = F₀-ATPase subunit 9 of *N. crassa*, RFP = red fluorescent protein, XhoI = Xho I restriction endonuclease

Figure 2.

Expression and activity of mitoXhoI in KT1357. (A) The ~85.8 kb budding yeast mtDNA monomer has two Xho I restriction sites, located at ~23.2 kb and ~51.4 kb, respectively. Arrows flanking the Xho I restriction sites represent qPCR primers that amplify across the sites. Arrows within *COX2* represent qPCR primers that amplify a region of *COX2*. (B) KT1357 carrying pGalSu9XhoI was grown in galactose for the indicated time points to obtain total cell extracts, which were subjected to western analysis and probed with anti-Flag (to detect mitoXhoI) or anti-Pgk1 antibody. The immunoblot shown is representative of three independent experiments. Pgk1, phosphoglycerate kinase, was used as a loading control. (C) KT1357 containing pGalSu9XhoI was grown in galactose (Gal) for the indicated times, following which total DNA was isolated and the mtDNA/nDNA ratio was

determined using real-time qPCR for specific mtDNA regions. The mtDNA/nDNA ratio obtained at different time points for each mtDNA region was normalized to the 0 h galactose sample. Data are represented graphically as mean $\pm$ SD from three independent experiments. **(D)** KT1357 containing pGalSu9XhoI or pGalSu9RFP were serially diluted and applied to a –Ura galactose plate, following which cells were grown for 48 h and the colonies were imaged (top panel). The colonies were then replica plated to YPEG and imaged after ~36 h (bottom panel). **(E)** Cells harboring pGalSu9XhoI were grown in media containing raffinose (Raff) or galactose (Gal) for the indicated times, following which cells were plated on –Ura glucose media. The colonies were then replica plated to YPEG and the percentage of ρ^+ colonies was determined. Data are represented graphically as mean $\pm$ SD from three independent experiments.

Figure 3.

The addition of the Myc tag to Mhr1 does not result in a petite phenotype. KT1357 expresses wild-type Mhr1, while KJP1976 expresses Mhr1-9XMyc. KT3898 is a strain deleted for *MHR1* and is used for comparison. Stationary phase cultures grown in YPD were diluted and a spot dilution test performed on YPD and YPEG. Only KT3898 is respiratory deficient.

Figure 4.

Expression of mitoXhoI in KJP1976 and KJP2011. **(A)** Mitochondrial extracts (Mito) were prepared from KJP1976 carrying pGalSu9XhoI grown in the presence of galactose (Gal) for 2 h, and total cell extracts (TCE) were prepared after growth in either raffinose or galactose for 2 h. The extracts were probed with anti-Flag (to detect mitoXhoI), anti-cMyc (to detect Mhr1), anti-Cox4, or anti-Pgk1 antibody. An image representative of three independent experiments is shown. Cox4, cytochrome C oxidase subunit 4, is a mitochondrial protein. Pgk1 is used as a cytosolic marker and is a loading control for total cell extracts. **(B)** KJP2011 carrying pGalSu9XhoI was grown in the presence of raffinose or galactose for 2 h to obtain total cell extracts (TCE) and mitochondrial extracts (Mito). The extracts were subjected to western analysis and probed with anti-Flag (to detect mitoXhoI), anti-cMyc (to detect Yku80), anti-Cox4 or anti-Pgk1 antibody. An image representative of three independent experiments is shown.

Figure 5.

Xho I restriction sites in KJP1976 and KJP2038 are cleaved only following induction of mitoXhoI expression. Strains KJP1976 and KJP2038 express Mhr1-9XMyC, but KJP2038 is deleted in the *YKU80* gene. Total DNA was isolated from KJP1976 (A) or KJP2038 (B) carrying pGalSu9XhoI grown in raffinose (Raff) or galactose (Gal) for 2 h and the mtDNA/nDNA ratio was determined at the indicated mtDNA regions. The mtDNA/nDNA ratio obtained from the 2 h galactose sample was normalized with respect to the 2 h raffinose sample. Data are represented graphically as mean $\pm$ SD from three independent experiments and * represents $P < 0.05$ between the indicated samples.

Figure 6.

Following induction of mitoXhoI expression (Gal samples), Mhr1 has increased binding to mtDNA near the Xho I restriction sites. (A) Schematic representation of upstream and downstream Xho I sites depicting the location of qPCR primers (arrows) used for quantitating immunoprecipitated mtDNA. Xho I cleaves the palindromic sequence 5'-CTCGAG-3' after the first cytosine (site represented by closed triangles) resulting in a staggered break containing 4 base overhang. Cleavage of both Xho I sites results in the removal of ~28.1 kb mtDNA segment. (B) KJP1976 carrying pGalSu9XhoI was grown in medium containing raffinose (Raff) or galactose (Gal) for 2 hours, following which formaldehyde crosslinked DNA-protein complexes were sonicated to shear cellular DNA. DNA-protein crosslinks were reversed and the purified DNA was subjected to southern

analysis. Binding of mtDNA-specific radioactive probes to the sonicated DNA samples was visualized by autoradiography. (C–F) KJP1976 and KJP2038 harboring pGalSu9XhoI were grown in raffinose or galactose for 2 h, following which DNA-protein complexes were crosslinked with formaldehyde, sheared by sonication and immunoprecipitated with either anti-Myc antibody or IgG1 control. Crosslinks were reversed and purified immunoprecipitated DNA samples were quantitated by real-time qPCR using primers specific for the indicated mtDNA region. Each graph represents data as mean $\pm$ SD from three independent experiments. * denotes $P < 0.05$ between the indicated samples.

Table 1

Oligonucleotides used in this study

Name	Sequence (5'-3')	Purpose	Reference
BamXhoI	AAAAAAGGATCCGCATTGGATCTAGCCGAATACG	Plasmid construction	This study
SalFlag	AAAAAAGTCGACTCACTTGTCGTCATCGTCC	Plasmid construction	[21]
NtermXhoI	AAAAAACTGCAGGCATTGGATCTAGCCGAA TACGACCGC	Plasmid construction	This study
CtermXhoI	AAAAAAGCGGCCGCACGCGGATGCGACTCTGC	Plasmid construction	This study
COX2F	AAAGTTGATGCTACTCCTGGTAGA	Real-time qPCR	[22]
COX2R	CATGACCTGTCCACACAAC	Real-time qPCR	This study
ACT1F	GTATGTGTAAAGCCGGTTTTG	Real-time qPCR	[23]
ACT1R	CATGATACCTTGGTGTCTTGG	Real-time qPCR	[23]
XhoIupF	GAGTAGAATCTGTAAAAAGATTATTTC	Real-time qPCR	This study
XhoIupR	TTAGTTCGACGGTTGTGCATATAATT	Real-time qPCR	This study
XhoIdownF	ATATGGGGTTATGGATTTCGTTC	Real-time qPCR	This study
XhoIdownR	AAATAGGTATGAACCTCGAGTA	Real-time qPCR	This study
RegionIF	TTTATAATGGTAGAGTAAAAATTGTACC	Real-time qPCR	This study
RegionIR	CACCTTTTACAAATGAACCATCAC	Real-time qPCR	This study
RegionIIF	AGACGCCTCTGGTTATCTAAG	Real-time qPCR	This study
RegionIIR	TACTGATCTCATTGGATTATACC	Real-time qPCR	This study
RegionIIIF	TATTACTTCATATGGGGTTATGG	Real-time qPCR	This study
RegionIIIR	AAATAGAGGTAGGAGGGAGGA	Real-time qPCR	This study
ProbeXhoIupF	CCATTATCATCTATTAGGCAC	Southern analysis	This study
ProbeXhoIupR	TTTCTTGTAGTCTCTGAGGATC	Southern analysis	This study
ProbeCOX2F	ATAACTCCTTGCTTCATACC	Southern analysis	This study
ProbeCOX2R	AAGGTGTGGTACATCATTC	Southern analysis	This study
YKU80DelF	CAAAGATTAGCAGTTGGAGGG	<i>YKU80</i> deletion	This study
YKU80DelR	CTTACCGAGCCTAAGTTGG	<i>YKU80</i> deletion	This study
DeltaYKU80R	GCTTAATCTTCCGTTAGCAGG	Deletion verification	This study
MHR1Myc9F	CGAATCACATCCTACAGACGAGCGGAAG TGTCTTCCAGTCCGGTTCTGCTGCTAG	<i>MHR1-MYCX9</i> tagging	This study
MHR1Myc9R	GTTTTTTTTTCTACCTGCATTTGTATATCTAA TGACGAAGCGTTCCTCGAGGCCAGAAGAC	<i>MHR1-MYCX9</i> verification	This study
MHR1F	GGTGACAAGGACAAACATTGG	<i>MHR1-MYCX9</i> verification	This study
MHR1R	AAGGTGAGGAGGATAAACG	<i>MHR1-MYCX9</i> verification	This study
MHR1-F ^{LCR}	GCTGTAGAGAACTTTCTCAG	<i>MHR1</i> deletion & verification	This study
MHR1-R ^{LCR}	GTTTCAAGTCACACAACAGC	<i>MHR1</i> deletion & verification	This study
Yku80Myc9F	TGAAGCGCGGTGAACAACACAGTAGGGGAAGTCC AAACAATAGCAATAATTCCGGTTCTGCTGCTAG	<i>YKU80-MYCX9</i> tagging	This study
Yku80Myc9R	CTCTTTAACTGTGGTGACGAAAACATACTCAAA GGATGTTAGACCTTTTCTCGAGGCCAGAAGAC	<i>YKU80-MYCX9</i> tagging	This study
Yku80F	CATAGGCGAAGTAACAACAG	<i>YKU80-MYCX9</i> verification	This study

Name	Sequence (5'-3')	Purpose	Referencece
Yku80R	GGTGTAAGGAGGAAAAGC	<i>YKU80-MYCX9</i> verification	This study

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Table 2

Yeast strains used in this study

Name	Genotype	Reference
KT1357	<i>MATα ura3 leu2 his3 trp1</i> ; mitochondrial genotype: ρ^+	[25]
KT1358	<i>MATα ura3 leu2 his3 trp1</i> ; mitochondrial genotype: ρ^+	[26]
KJP1976	<i>MATα ura3 leu2 his3 trp1 MHR1-MYCX9:TRP1</i> ; mitochondrial genotype: ρ^+	This study
KJP2011	<i>MATα ura3 leu2 his3 trp1 YKU80-MYCX9:TRP1</i> ; mitochondrial genotype: ρ^+	This study
KJP2038	<i>MATα ura3 leu2 his3 trp1 MHR1-MYCX9:TRP1 yku80::KAN-MX</i> ; mitochondrial genotype: ρ^+	This study
KT3898	<i>MATα ura3 leu2 his3 mhr1 ::kanMX</i> ; mitochondrial genotype: ρ^-	This study
KT1112	<i>MATα ura3 leu2 his3</i> ; mitochondrial genotype: ρ^+ (congenic to KT1357)	[27]
KT1113	<i>MATα ura3 leu2 his3</i> ; mitochondrial genotype: ρ^+ (congenic to KT1358)	[25]