Supplemental Information

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Supplemental Figures

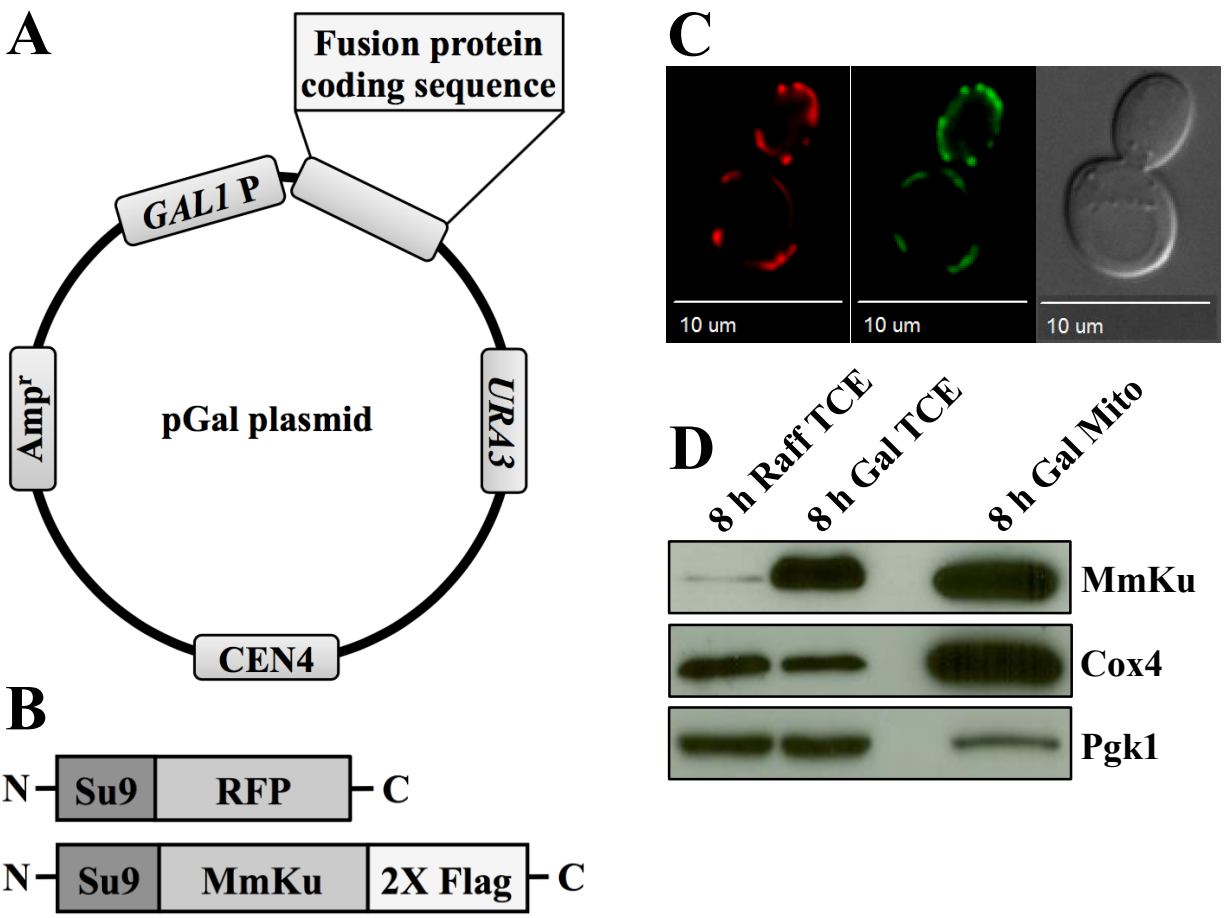

Figure S1. Galactose (Gal)-inducible expression of mitochondrial-targeted proteins. (**A**) Schematic representation of pGal plasmid. *GAL1*P: Gal-inducible promoter; Amp^r: ampicillin/carbenicillin resistance gene; *URA3*: gene encoding orotidine-5'-phosphate decarboxylase; CEN4: centromere from chromosome IV. (**B**) Schematic representations of fusion proteins expressed from two pGal plasmids: Su9 is fused to the N-terminus of both RFP and MmKu; MmKu also has 2 Flag epitopes at the C-terminus. (**C**) pGalSu9RFP was introduced into KT1112, and RFP expressed in the presence of Gal was visualized using fluorescence microscopy (left panel). Mitochondria in live cells were stained with MitoTracker® Green FM and visualized under the fluorescence microscope (middle panel). The red and green fluorescence signal in the same cell localized to the same mitochondrial structures. While images were being captured by microscopy the yeast cells moved and this prevented a merged image from the same yeast cell. The corresponding image to the right is DIC. Images shown are representative of 3 independent experiments. (**D**) Mitochondrial extracts (Mito) were obtained from cells carrying pGalSu9MmKu grown in the presence of Gal for 8 h, and total cell extracts (TCEs) were prepared from cells harboring the same plasmid grown either in raffinose (Raff) or Gal for 8 h. The extracts were probed with anti-Flag, anti-Cox4, or anti-Pgk1 antibody. An image representative of 2 independent experiments is shown. Cox4, cytochrome C oxidase subunit 4, is a mitochondrial marker. Pgk1, phosphoglycerate kinase, is used as a cytosolic marker for mitochondrial extracts and as a loading control for TCEs. The presence of a faint band of Pgk1 in the lane containing mitochondrial extracts shows a low level of cytosolic contamination.

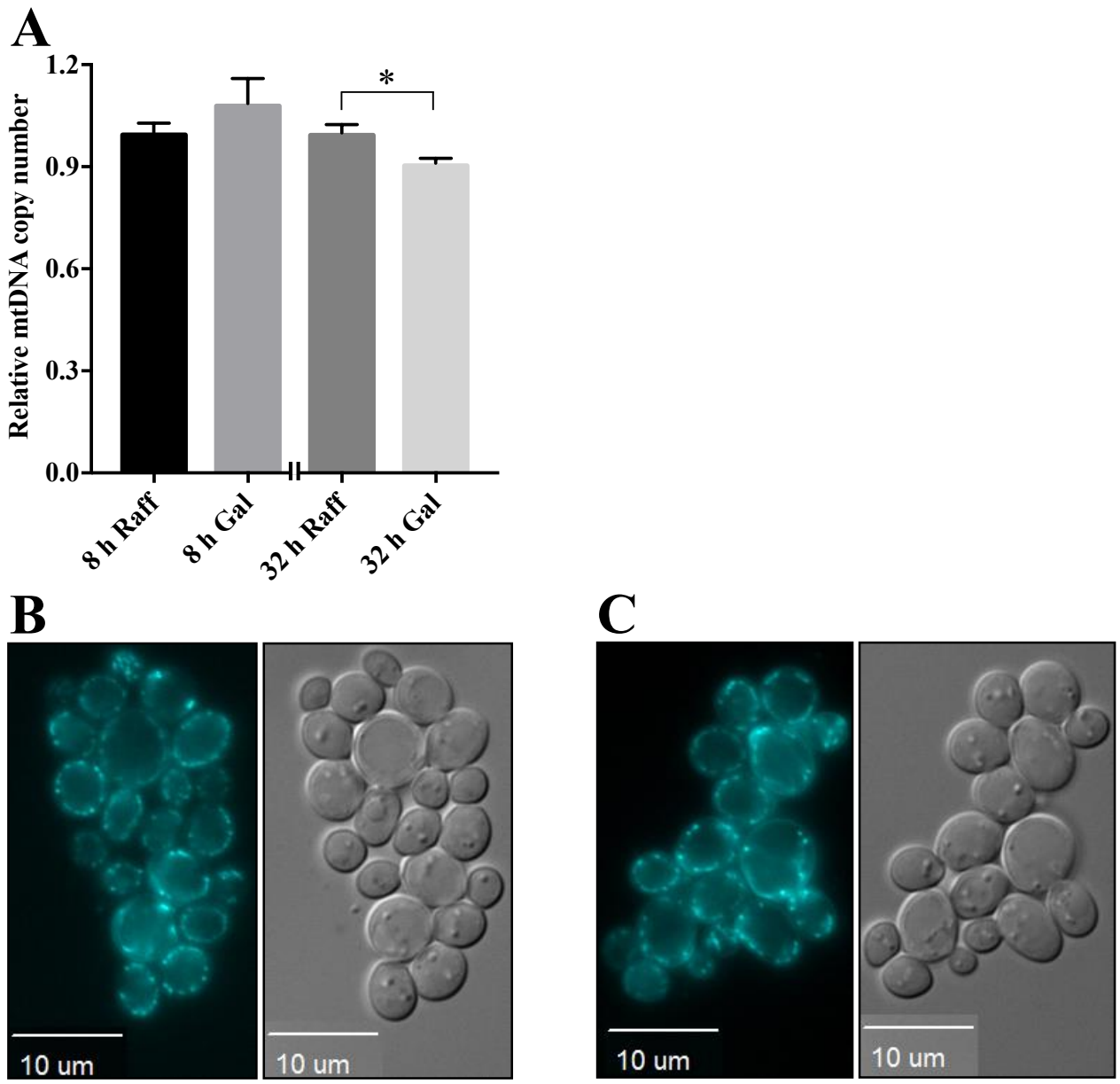

Figure S2. Comparison of mtDNA content in cells containing pGalSu9RFP grown in Raff or Gal. (**A**) Total DNA was isolated from KT1112 carrying pGalSu9RFP grown in Raff or Gal for 8 h or 32 h, following which real-time qPCR was performed to determine the relative mtDNA copy number. Data are represented graphically as mean ± SD from three independent experiments. * represents $P < 0.05$. (**B**) Cells containing pGalSu9RFP were grown in Raff for 32 h, treated with DAPI at 30°C for 30 min, and visualized using fluorescence microscopy. The corresponding image to the right is DIC. (**C**) Cells carrying pGalSu9RFP grown in Gal for 32 h underwent the same procedure as described in Figure S2B. Images shown are representative of three independent experiments.

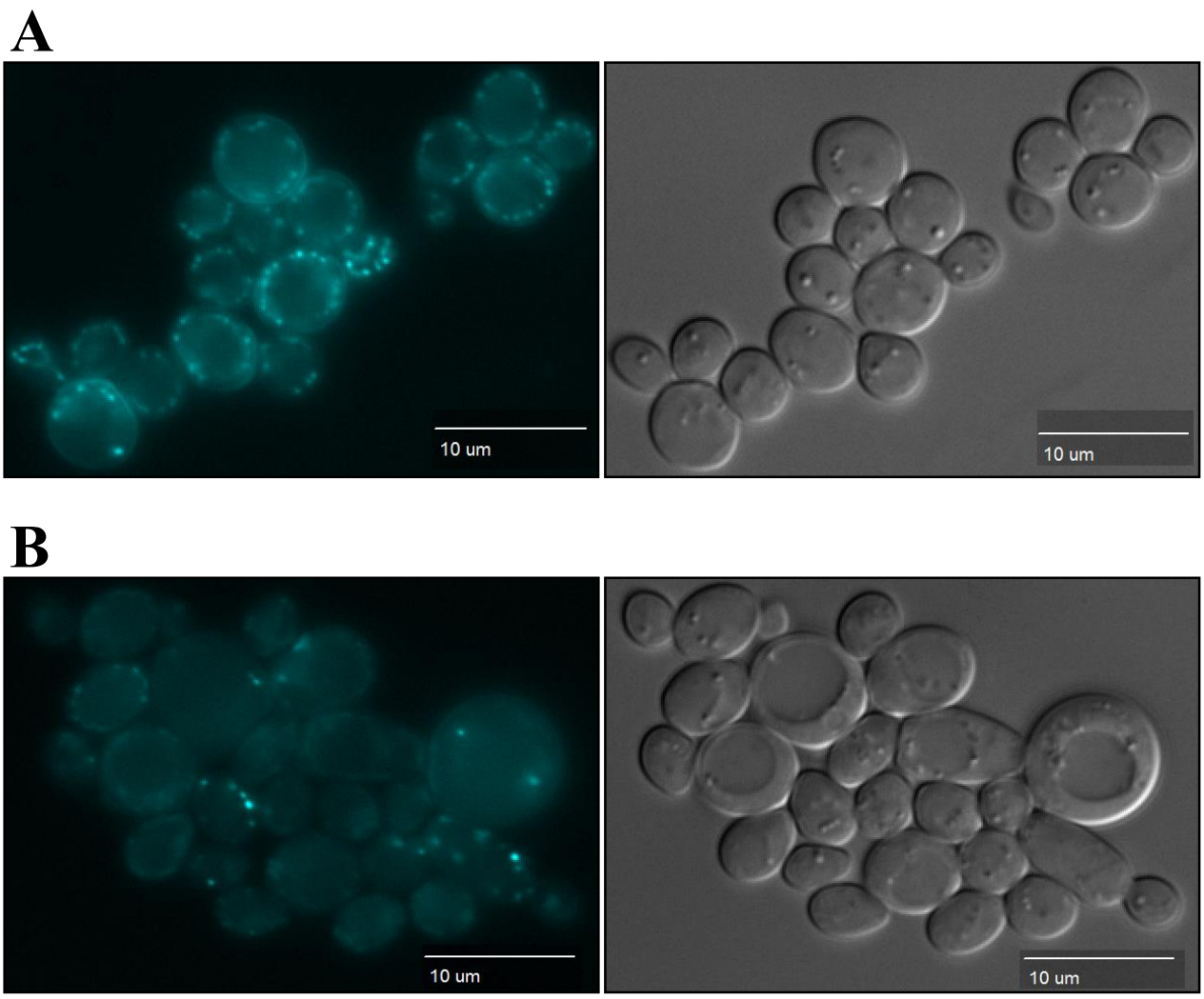

Figure S3. Mitochondrial-targeted MmKu triggers mtDNA depletion. (**A**) Cells containing pGalSu9MmKu were grown in Raff for 32 h. Following treatment with DAPI at 30°C for 30 min, cells were visualized using a fluorescence microscope (left panel). The corresponding image to the right is DIC. (**B**) Cells containing pGalSu9MmKu grown in Gal for 32 h underwent the same procedure as described in Figure S3A. Images shown are representative of three independent experiments. DAPI staining experiments were performed on live yeast cells. During such vital staining procedure, localization of DAPI into the nucleus is somewhat restricted (1). The preferential mtDNA staining by DAPI could be due to: (i) the negative mitochondrial membrane potential towards the matrix side, which may electrophoretically pull positively charged DAPI to make it readily available for binding to mtDNA; (ii) DAPI preferentially binds to AT regions of DNA and yeast mtDNA has high AT content [GC content of yeast mtDNA is 17.1% (2)].

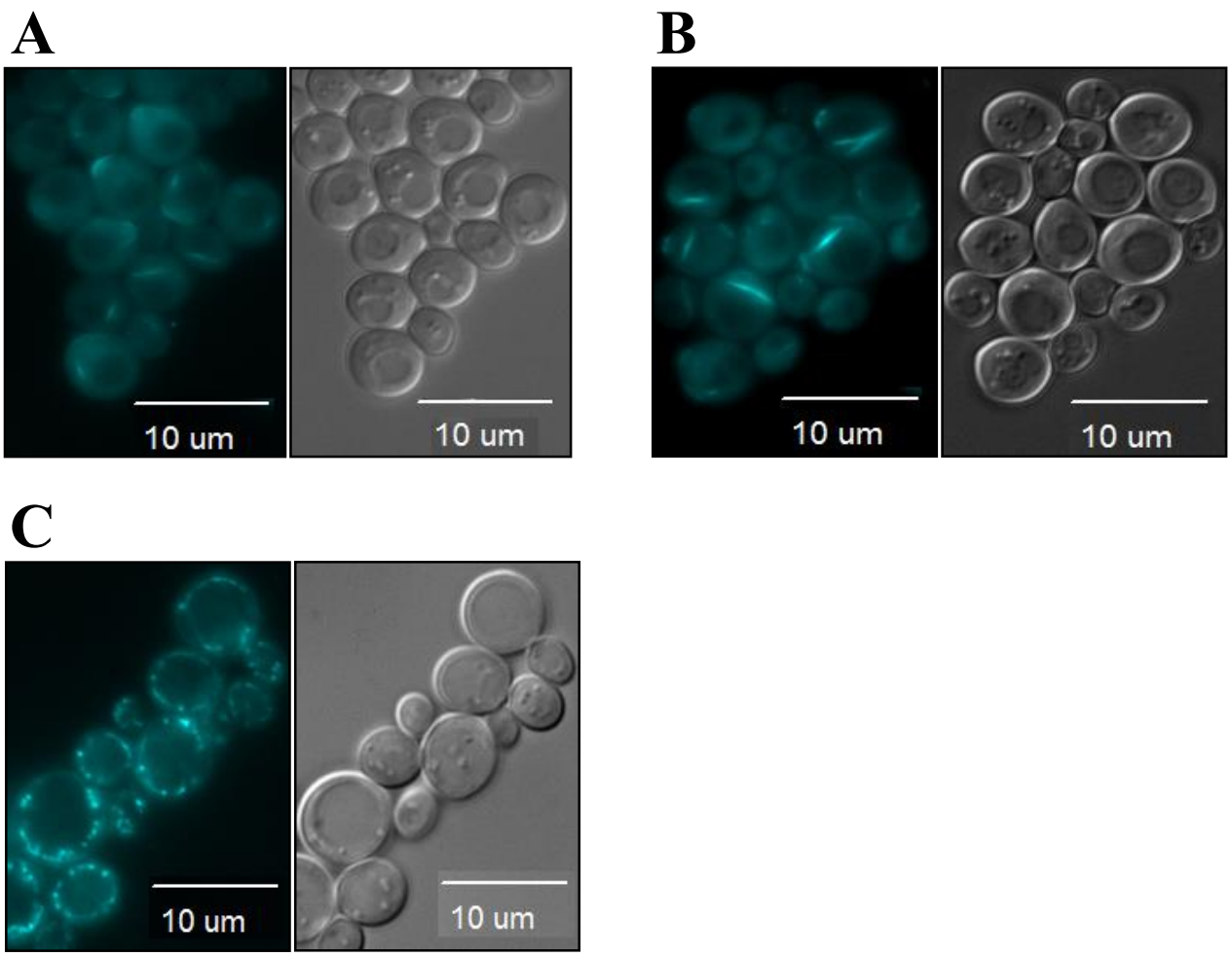

Figure S4. Verification for the lack of mtDNA in ρ^0 cells generated following MmKu expression. (**A**) ρ^0 colonies obtained by expressing mitochondrial-targeted MmKu (Table 1) were grown in synthetic complete medium containing Raff. After treating with DAPI at 30°C for 30 min, cells were visualized using a fluorescence microscope (left panel). The corresponding image to the right is DIC. Representative images from an MmKu-induced ρ^0 colony are shown. (**B**) KT1177 (ρ^0 strain) and (**C**) KT1112 (ρ^+ strain) following the same DAPI staining procedure. Images shown are representative of two independent experiments.

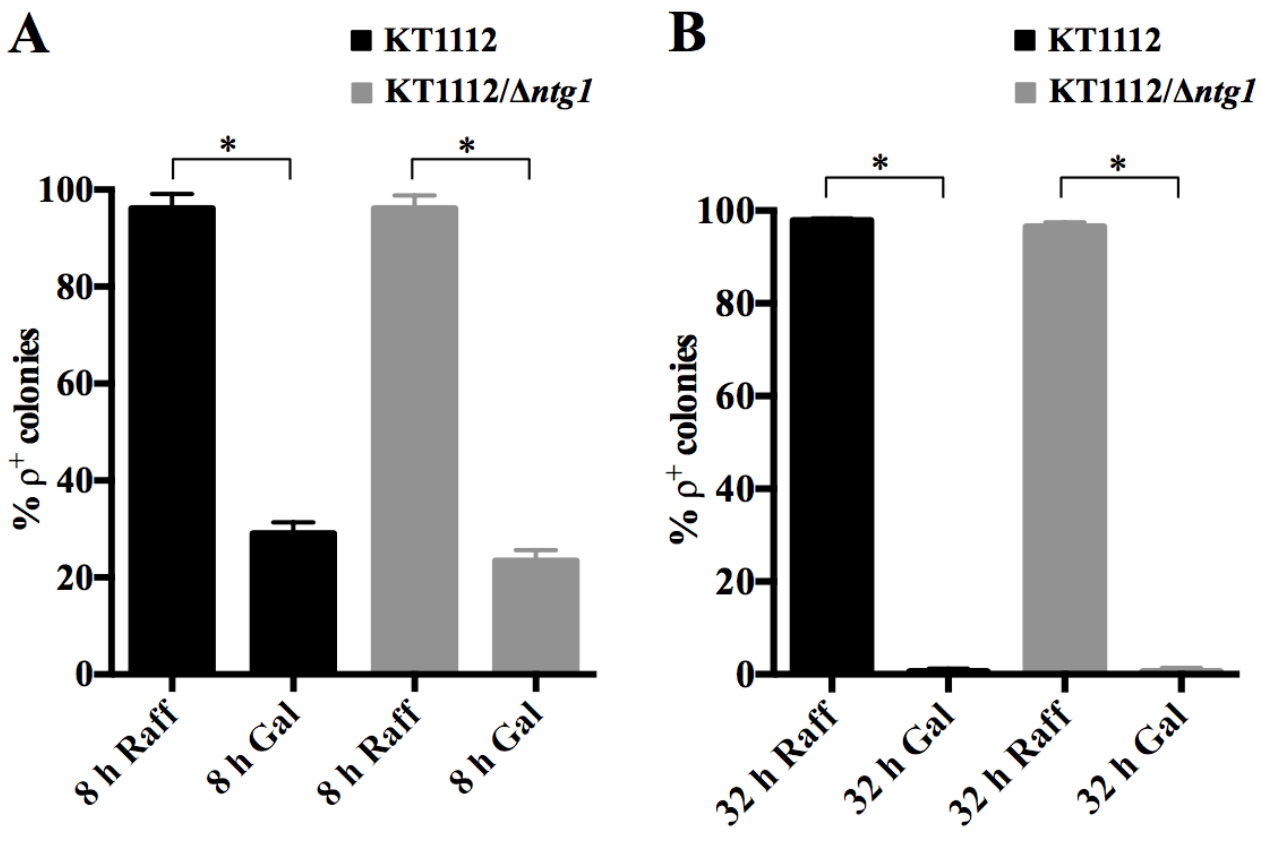

Figure S5. Comparison of the percentage of ρ^+ colonies between KT1112 and KT1112/Δ*ntg1*. (**A**) KT1112 and KT1112/Δ*ntg1* containing pGalSu9MmKu were grown at the same time in media containing Raff or Gal for 8 h and plated on –Ura glucose (–Ura D). (**B**) The cultures were then diluted at 8 h and grown for another 24 h after which the cells were plated on –Ura D. Colonies on –Ura D were replica-plated to YPEG and the percentage of ρ^+ colonies was calculated. The average percentage of ρ^+ colonies from three independent experiments are represented graphically. Error bars represent SD and * denotes $P < 0.05$.

A

Experiment number	Normalized signal across the DSB region at *ori5*	
	KT1112	KT1112/Δ*ntg1*
1	2.34	2.7
2	2.41	2.65
3	1.75	1.95

B

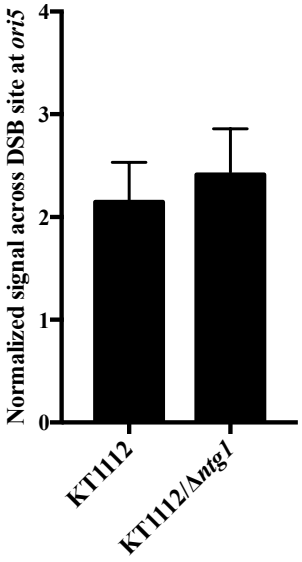

Figure S6. PCR from KT1112 and KT1112/Δ*ntg1* DNA using primers encompassing the region where DSBs are induced in *ori5*. KT1112 and KT1112/Δ*ntg1* were grown in media containing Gal for 5 h following which cells were harvested to isolate total DNA. End-point PCR was performed from total DNA using ORI5AcrF/ORI5R primer set and XhoIUpInF/R primer set. The signal of the PCR product obtained from ORI5AcrF/ORI5R primer set was normalized with that obtained from XhoIUpInF/R primer set. The values for the normalized signal across the DSB region of *ori5* from three independent experiments are presented in a table (**A**) and the mean ± SD of the three experiments are shown graphically (**B**).

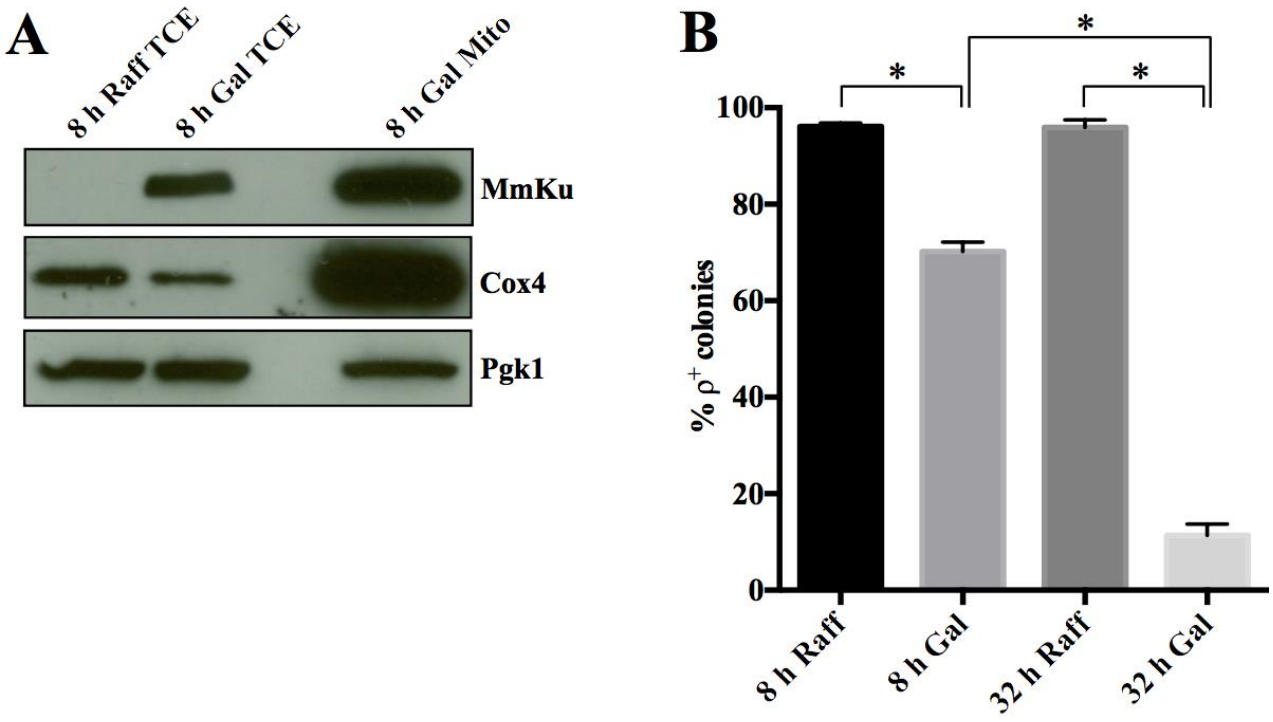

Figure S7. Expression and activity of mitochondrial-targeted MmKu from KT1112 pRS305MmKu. (**A**) Mitochondrial extracts (Mito) were obtained from KT1112 pRS305MmKu grown in Gal for 8 h, and total cell extracts (TCE) were obtained from the same strain grown in Raff or Gal for 8 h. The extracts were subjected to immunoblot analysis, following which the blot was probed with anti-Flag, anti-Cox4 or anti-Pgk1 antibody. An image representative of two independent experiments is shown. (**B**) The same yeast strain was grown in media containing Raff or Gal for 8 h and cells were plated on –Leu D. The cultures were then diluted at 8 h and grown for another 24 h following which the cells were plated on –Leu D. Colonies on –Leu D were replica-plated to YPEG and the percentage of ρ^+ colonies was determined. The average percentage of ρ^+ colonies from three independent experiments is represented graphically. Error bars represent SD and * denotes $P < 0.05$.

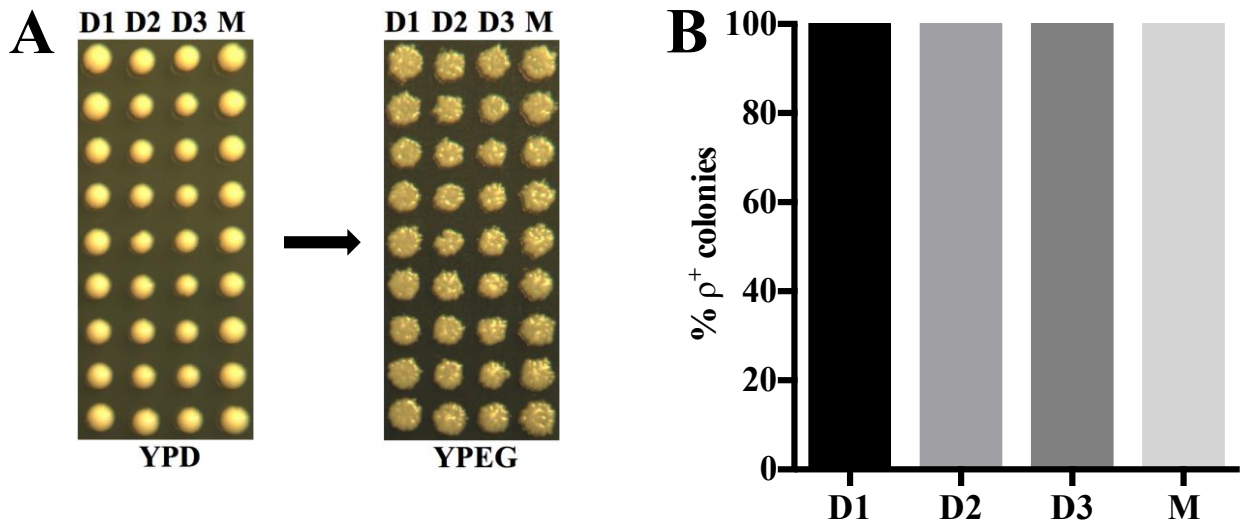

Figure S8. Pedigree analyses of the parental strain KT1112 reveal respiration competent phenotype in all colonies from mother and daughter generations. (**A**) The procedure described for KT1112 pRS305MmKu pedigree (Figure 4A) was repeated for KT1112. (**B**) The data from 4 independent experiments for KT1112 pedigree were pooled to determine the percentage of ρ^+ colonies for each daughter generation and the mother generation.

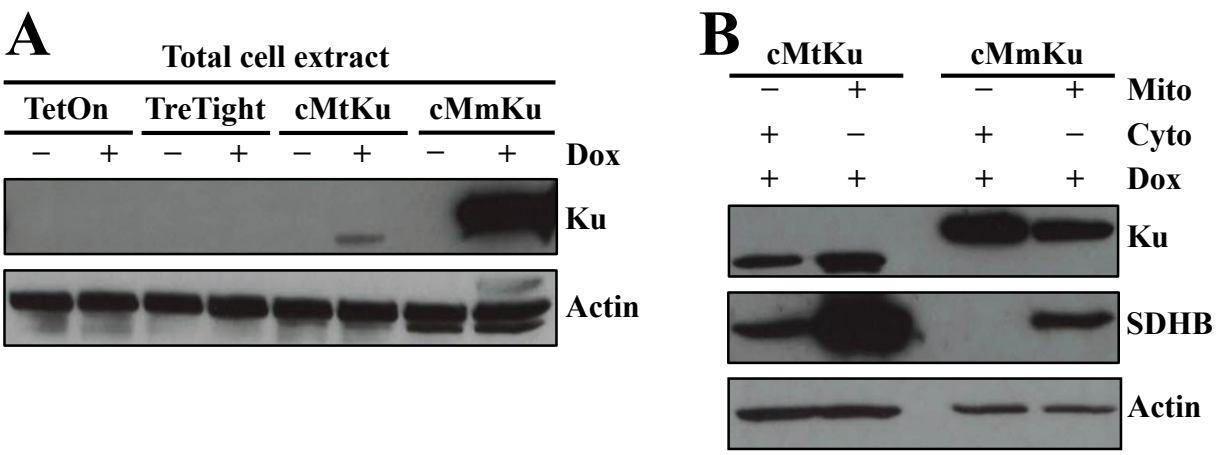

Figure S9. Expression of mitochondrial-targeted bKu proteins in human MCF7 cell lines. (**A**) MCF7 cell lines were incubated in the presence or absence of Dox for 48 h, following which total cell extracts were isolated. The extracts were probed with anti-Flag or anti-Actin antibody. An image representative of two independent experiments is shown. TetOn: MCF7 Tet-On; TreTight: MCF7 TreTight; cMtKu: MCF7 cMtKu; cMmKu: MCF7 cMmKu. (**B**) Mitochondrial extracts (Mito) and cytosolic extracts (Cyto) and were obtained from cMtKu and cMmKu cell lines following treatment with Dox for 48 h. 35 µg of each extract from cMtKu and 3.5 µg of each extract from cMmKu were used for western analysis and the membranes were probed with anti-Flag, anti-SDHB or anti-Actin antibody. An image representative of two independent experiments is shown. SDHB, succinate dehydrogenase complex iron sulfur subunit B, is a mitochondrial marker. A part of the cytosolic bKu signal may be accounted for by mitochondrial contamination of the cytosolic fraction, as evident from the presence of SDHB in the MtKu cytosolic extract. The presence of actin in the mitochondrial fractions was expected, as actin has been identified in human mitochondrial nucleoids (3,4).

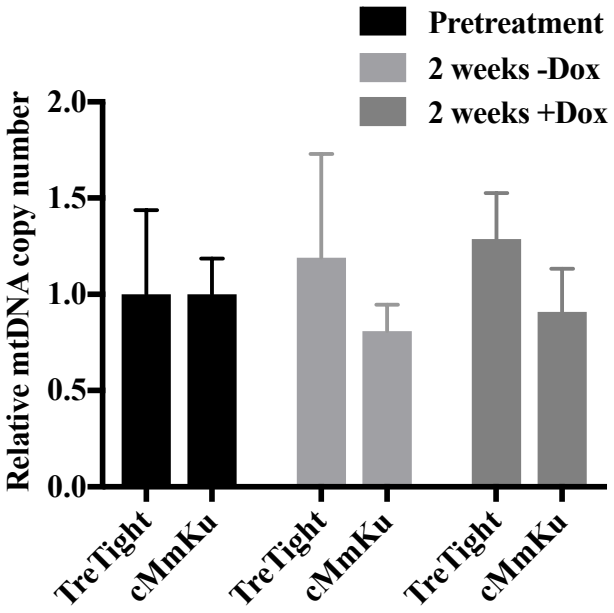

Figure S10. Determination of relative mtDNA copy number of human MCF7 cells grown in ρ^0 medium. MCF7 TreTight and MCF7 cMmKu cells lines were grown in ρ^0 medium (medium containing pyruvate and supplemented with uridine) according to the timeline shown in Figure 5A. Total DNA was then isolated to determine the relative mtDNA copy number. The relative mtDNA copy number of each cell line in the treatment group (– or +Dox) was normalized to the respective cell line in the pretreatment group. Data are represented graphically as mean ± SD from three independent experiments.

Supplemental Tables

Table S1. Oligonucleotides used in this study

Name	Sequence (5'-3')	Purpose	Reference
MM26	AAAAAGCGGCCGCGGACTTGGCAGCTTGTTTG	Plasmid generation	This study
MM27	AAAAAGTCGACCGCTCCATCTGGAAGGGTTC	Plasmid generation	This study
Flag6	GGCCGCAGACTACAAGGACGATGACGACAAGG-ACTACAAGGACGATGACGACAAGTGACC	Plasmid generation	This study
Flag7	GGCCGGTCACTTGTCGTCATCGTCCTTGTAGTCC-TTGTCGTCATCGTCCTTGTAGTCTGC	Plasmid generation	This study
Ku40	AAAAAGCTAGCATGTCCGTCCTGACGCCGCTG	Plasmid generation	This study
Ku41	AAAAAAAGCTTCTATGCGGCCCCATTCAGATCCTCTTCTG	Plasmid generation	This study
KE1	AAAAACTGCAGCGAGCCATTTGGACGGGTTCG	Plasmid generation	This study
Ku30	AAAAAGCGGCCGCCGGAGGCGTTGGGACGTTTG	Plasmid generation	This study
Su9-Nterm	AAAAAACTGCAGATCTATGGCCTCCACTCGTGTCCTCG	Plasmid generation	This study
RFP-Cterm	AAAAAAAGATCTCTACAGGAACAGGTGGTGGCGGCCC	Plasmid generation	This study
BamMmKu	AAAAAAGGATCCCGCTCCATCTGGAAGGGT	Plasmid generation	This study
SalFlag	AAAAAAGTCGACTCACTTGTCGTCATCGTCC	Plasmid generation	This study
Gal2	AAAAAAACTAGTATCACGAGGCCCTTTCGTCTTC	Plasmid generation	This study
Gal3	AAAAAACTCGAGCGTATATATACCAATCTAAGTCTGTGC	Plasmid generation	This study
RFP1	TCGATCTCGAACTCGTG	Plasmid sequencing	This study
MM9	TCCAGGAAGTAGCTGCGG	Plasmid sequencing	This study
MM10	GTCATGGTGGTGCATACCC	Plasmid sequencing	This study
RS1	ATACGACTCACTATAGG	Plasmid sequencing	This study
RS2	ACAGGAAACAGCTATGAC	Plasmid sequencing	This study
NTG1F	CACTGTCTCAGTCTATTGGGAGG	KT1112/Δ*ntg1* generation	This study
NTG1R	GCCCGTGGTATCGTTAGATGTCG	KT1112/Δ*ntg1* generation	This study
deltaNTG1R	GAGGCAACCGTTACAGGAATGG	*NTG1* deletion verification	This study
Y2Oli2	CATGACCCATAGCTTCC	End-point PCR	This study
Y2Oli7	GCTGCACTATAAGATAGG	End-point PCR	This study
XhoIF	TGATGGTTCATTTGTAAAAGG	End-point PCR	This study
XhoIR	AATCATTACTGATCTCATTGG	End-point PCR	This study
ORI5AcrF	GGTATATAATGAAGATCTATTAGAACC	End-point PCR	This study
XhoIUpInF	CAGGACCTAGTGTAGATTTAG	End-point PCR	This study
XhoIUpInR	CTCGTATAAGATTGGGTCAC	End-point PCR	This study
ACT1F	GTATGTGTAAAGCCGGTTTTG	Real-time qPCR	(5)
ACT1R	CATGATACCTTGGTGTCTTGG	Real-time qPCR	(5)
COX1F	CTACAGATACAGCATTTCCAAGA	Real-time qPCR	(5)
COX1R	GTGCCTGAATAGATGATAATGGT	Real-time qPCR	(5)
ORI5F	CAGAGCACACATTTGTTAATATTTAATAA	Real-time qPCR	(6)
ORI5R	CCCGGATATCTTCTTGTTTATC	Real-time qPCR	(6)
Human18SF	AGCCATGCATGTCTAAGTACGCACG	Real-time qPCR	(7)
Human18SR	CAAGTAGGAGAGGAGCGAGCGACCA	Real-time qPCR	(7)
Human-mtDNAF	CAGGAGTAGGAGAGAGGGAGGTAAG	Real-time qPCR	(7)
Human-mtDNAR	TACCCATCATAATCGGAGGCTTTGG	Real-time qPCR	(7)

Table S2. Quantitation of MmKu and Cox4 in total cell extracts

Time and treatment	MmKu/Pgk1	Cox4/Pgk1
8 h Raff	0.004 ± 0.001	0.449 ± 0.031
8 h Gal	0.852 ± 0.181*	0.348 ± 0.155
32 h Raff	0.001 ± 0.003	0.264 ± 0.095
32 h Gal	0.765 ± 0.102**	0.051 ± 0.033**

Levels of MmKu and Cox4 from immunoblots (Figure 2D) were quantitated. The levels were normalized to Pgk1 and represented as mean ± SD. * and ** denote $P < 0.05$ for protein level in Gal culture compared to Raff culture at 8 h and 32 h, respectively.

Supplemental Materials and Methods

DNA manipulation and plasmid construction

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.

The pTRE-Tight vector system (Clontech, Mountain View, CA, USA) was used to express the MmKu and MtKu in MCF7 Tet-On cells (Clontech, Mountain View, CA, USA). PCR with primers MM26 and MM27, Phusion High-Fidelity DNA Polymerase and pDUBmKu (8) was performed according to manufacturer's recommendations using an annealing temperature of 70°C. This produced a DNA fragment containing the MmKu coding sequence, minus the translational start and stop codons, with a SalI restriction site at the 5' end and a NotI restriction site at the 3' end. The DNA was digested with SalI and NotI and ligated into the SalI-NotI sites of the pCMV/myc/mito pShooter vector (Invitrogen, ThermoFisher Scientific) to generate pcmKu. This positioned the mitochondrial targeting sequence from human cytochrome C oxidase subunit VIII in-frame and upstream of the MmKu coding sequence. To tag the MmKu protein with two Flag epitopes, the Flag6 and Flag7 oligonucleotides were annealed by heating to > 90°C and cooling slowly to room temperature, prior to ligation into the NotI site of pcmKu to generate pcmKuFlag. The coding sequence for the mitochondrial-targeted MmKu Flag-tagged protein was then amplified using primers Ku40 and Ku41, Phusion High-Fidelity DNA Polymerase, pcmKuFlag and an annealing temperature of 65°C. The PCR-generated DNA fragment was then digested with NheI and HindIII, and ligated into the NheI-HindIII sites of pTRE-Tight to generate pTREcmKuFlag.

The MtKu coding sequence was amplified using Phusion High-Fidelity DNA Polymerase, KE1 and Ku30 primers, pKuEnls (9) and an annealing temperature of 70°C. The MtKu DNA fragment was digested with PstI and NotI and ligated into the PstI-NotI sites of pCMV/myc/mito pShooter to generate pcKumyc. This positioned MtKu downstream of the mitochondrial targeting sequence. The Flag tag was added to the 3' terminus of the MtKu coding sequence by ligating the Flag6-Flag7 double-stranded DNA molecule into the NotI site of pcKumyc to generate pcKuFlag1-1. PCR with Phusion High-Fidelity DNA Polymerase, Ku40 and Ku41 primers and pcKuFlag1-1 generated a DNA fragment that was digested with NheI and HindIII. This fragment was ligated into the NheI-HindIII sites of pTRE-Tight to generate pTREctKuFlag.

The pGal plasmid (originally called pJB9) is a low copy number plasmid constructed by incorporating the *GAL1* promoter directly into a yeast centromere-containing shuttle plasmid YCp50 (10) (Supplemental Figure S1A). The *GAL1* promoter in the plasmid allows expression of a downstream coding region in the presence of Gal. Expression is repressed by glucose, and growth in Raff medium neither induces nor represses the promoter. The mitochondrial targeting sequence of F_0-ATPase subunit 9 (Su9) of *Neurospora crassa* was used to target proteins to yeast mitochondria (11). To express mitochondrial-targeted red fluorescent protein (RFP), the Su9-RFP coding sequence was amplified from B1642 [(12); a generous gift from Dr. Paul W. Doetsch, Emory University, Atlanta, GA, USA] using Su9-Nterm and RFP-Cterm primers. The fragment was digested with BglII and introduced into the pGal plasmid at the BamHI site to generate pGalSu9RFP.

For expression of mitochondrial-targeted MmKu containing two Flag epitopes in yeast cells, pGalSu9RFP was digested with BamHI and SalI to remove the RFP coding sequence, resulting in the generation of pGalSu9. The MmKuFlag coding region from pcmKuFlag was amplified using BamMmKu and SalFlag primers. The product was digested with BamHI and SalI, and ligated to pGalSu9 to generate pGalSu9MmKu.

A yeast integrating plasmid for expression of mitochondrial-targeted MmKu was constructed by amplifying the sequence *GAL1*P-Su9-MmKu-Flag from pGalSu9MmKu using Gal2 and Gal3 primers.

The product was digested with SpeI and XhoI, and ligated to SpeI and XhoI digested pRS305 (13) to generate pRS305MmKu.

The coding sequence in each plasmid was sequenced by the DNA Facility at Iowa State University to ensure correct reading frame and correct orientation of inserts.

mtDNA Immunoprecipitation (mtDNA IP)

Following the initial culture set up, yeast cells containing pGalSu9MmKu were grown in Gal for 5 h. DNA was crosslinked to proteins by the addition of formaldehyde to a final concentration of 1%. 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 precleared with Dynabeads protein G (45 µl; Life Technologies, Oslo, Norway) for 16 h at 4°C with mixing. The supernatant obtained from the preclearing step was treated with 1.5 µg of anti-Flag M2 antibody or mouse IgG1 (Sigma, Saint Louis, MO, USA) for 16 h at 4°C. Dynabeads (40 µl) were then added to the DNA-protein-antibody complex and mixed for 16 h at 4°C. The mixture of Dynabeads and DNA-protein-antibody complexes were washed, in succession, with lysis buffer, high salt lysis buffer, wash buffer, and TE. DNA-protein complexes were eluted from the Dynabeads twice with elution buffer at 65°C for 30 min. Crosslinks were reversed at 65°C for 16 h in the presence of proteinase K (50 µg/ml; Roche diagnostics, Indianapolis, IN, USA). The solution containing the DNA was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1; Amresco LLC, Solon, OH, USA) and the DNA precipitated with ice-cold absolute ethanol in the presence of glycogen (20 µg; Affymetrix, Santa Clara, CA, USA). The purified immunoprecipitated DNA was dissolved in 60 µl TE, of which 2 µl was subjected to real-time qPCR using *ori5* specific primers (ORI5F and ORI5R) and *COX1* specific primers. A standard curve specific for each amplicon was used to determine the amount of mtDNA present in each immunoprecipitate and background signal obtained from the IgG1 control was subtracted. The net immunoprecipitate obtained was used to calculate the percentage of input immunoprecipitate (IP) using the following formula:

% Input IP = (Amount of mtDNA in net IP/ Amount of mtDNA in total input) x 100

Total input represents the total amount of DNA-protein complex used in the immunoprecipitation.

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