Tamura et al., http://www.jcb.org/cgi/content/full/jcb.200603087/DC1

Supplemental results

Tetrad analysis shows that Tam41 is not an essential yeast protein

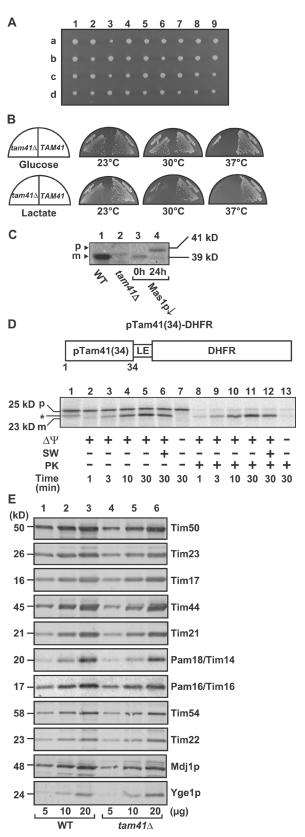
Disruption of the TAM41 gene was performed as follows. A DNA fragment containing Candida glabrata HIS3 gene was amplified by PCR using pCgHIS3 (Kitada et al., 1995) as a template with primers 5'-GATTGAGCTAATAATTTGAAT-TAATAGGAGCTGCTTTTTACTTTGATATATCCT-GAAGTTGTTGTAAAACGACGGCCAGT-3' and 5'-TTGATTTACTAATACGGCTGAGCAAAAGTCTCT-GAGTATTCTTTAATGTGAACCCTTTTGCACAGGAAA-CAGCTATGACC-3'. The amplified DNA fragment, flanked by 40 base pairs of the sequences upstream and downstream of the TAM41 gene, was introduced into the WT diploid strain W303-AB, and His⁺ transformants were selected. Disruption of the TAM41 gene on one of the two chromosomes was confirmed by PCR, and the resulting strain was named TAM41/tam41 Δ . When we subjected TAM41/tam41 Δ to tetrad analysis, all of the four spores grew normally on YPD at 23°C (Fig. S1 A), and the haploid *tam41* disruption mutant ($tam41\Delta$) was obtained.

Temperature-sensitive growth of the tam41 Δ strain

Growth of the strain ($tam41\Delta$) with chromosomal *TAM41* deletion was tested on both fermentable (SCD) and nonfermentable (SCLac) media at various temperatures. $tam41\Delta$ cells showed slow growth at elevated temperature (37°C) as compared with that at 23°C, and the temperature-sensitive growth was more prominent on SCLac than on SCD (Fig. S1 B).

Figure S1. Additional characterization of Tam41. (A) One of the two chromosomal TAM41 genes in a yeast diploid strain, W303, was disrupted, the diploid cells were sporulated, and nine different asci were dissected. The four spores recovered from each ascus were allowed to germinate and to grow on YPD for 45 h at 23°C. (B) The yeast tam41 disruption mutant strain carrying pRS316-Tam41 (TAM41) or pRS316 (tam41) was grown on SCD(-Ura) (Glucose) and SCLac(-Ura) (Lactate) at 23, 30, and 37°C. (C) Yeast G10 strain, in which the MAS1 gene for MPP is under the control of GAL10 promoter (Mas1px; Geli et al., 1990), was cultivated in YPGal medium at 30°C (lane 3) or YPD medium at 30°C for 24 h to deplete MPP (lane 4), and total cell extracts were prepared. Total cell extracts were also prepared for W303-1A (WT) and $tam 41\Delta$ strains cultivated in YPD medium at 23°C (lanes 1 and 2). Proteins in the extracts were analyzed by SDS-PAGE and immunoblotting with anti-Tam41 antibodies. p, precursor form; m, mature form. (D) The radiolabeled fusion protein pTam41(34)-DHFR (as schematically shown in the top panel) were incubated with isolated yeast mitochondria (D273-10B) at 30°C for indicated times with or without $\Delta\Psi$. The mitochondria were then subjected to osmotic swelling (SW) and further treated with or without 100 μ g/ml PK for 30 min on ice. The mitochondria or mitoplasts were reisolated by centrifugation, and proteins were analyzed by SDS-PAGE and radioimaging. Asterisk indicates the form possibly starting from the second Met (residue 22) of the fusion protein. (E) Mitochondria were isolated from WT and $tam 41\Delta$ cells grown at 23°C in YPD medium. Indicated amounts of mitochondrial proteins were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against the indicated proteins.





In vivo conversion of the 41-kD precursor form of Tam41 to the 39-kD mature form To confirm that the 41-kD precursor form of Tam41 is converted to the 39-kD mature from in vivo, we analyzed the apparent molecular mass of endogenous Tam41 by immunoblotting with anti-Tam41 antibodies before and after depletion of Mas1p, a subunit of matrix processing peptidase (MPP; Fig. S1 C). Endogenous Tam41 migrated as a 39-kD protein (Fig. S1 C, lane 1), whereas it exhibited a 41-kD form after inhibiting the presequence cleavage by depletion of Mas1p (Fig. S1 C, lane 4).

The N-terminal 34 residues of the Tam41 precursor functions as a mitochondrial targeting signal

The N-terminal possible presequence of the Tam41 precursor was tested for its ability to direct a nonmitochondrial protein to mitochondria in vitro. For this purpose, a fusion protein (pTam41[34]-DHFR) consisting of residues 1–34 of the Tam41 precursor and mouse DHFR was synthesized with reticulocyte lysate and was incubated with isolated yeast mitochondria. pTam41(34)-DHFR ("p") was converted to the smaller form ("m"), which was resistant to proteinase K treatment, in a $\Delta\Psi$ -dependent manner (Fig. S1 D), indicating that residues 1–34 of the Tam41 precursor can function as a mitochondrial targeting signal.

The gene for pTam41(34)-DHFR was constructed as follows. A DNA fragment for the N-terminal 34 residues of Tam41 was amplified from pRS316-Tam41 by PCR using primers 5'-CCGGATCCATAATTTGAATTAATAGGAGCTGCTTT-3' and 5'-GGCTCGAGCCCCTCCTTTATTTGAGTACTCAGAAG-3'. The amplified DNA was digested with BamHI and XhoI and introduced into pUC119/DHFR (Yamano et al., 2005) to give pUC119/pTam41(34)-DHFR. The BamHI–PstI fragment containing the gene for pTam41(34)-DHFR was subcloned into pGEM-4z.

The steady-state levels of translocator proteins and MMC proteins did not change by the Tam41 depletion

The steady-state levels of translocator proteins and MMC proteins were examined for WT and $tam41\Delta$ mitochondria by immunoblotting with antibodies against known components of the mitochondrial translocators and MMC proteins (Fig. S1 E).

Sc.Tam41 Ca.XP_720669 Dr.NP_001032196 Mm.NP ⁻ 081170 Hs.AAH15088	-MLRVSENGLRFLLKCHSTNVSMFNRLLSTQIKEGRSSIDDAGIIPDGTINERPNHYI 57 MITKCYVQVFRYRRVCHNTKYSFNSVVSIKWLHSAGSSLSQANSSKTSQSSLTSSGFLYY 60
Sc.Tam41 Ca.XP_720669 Dr.NP_001032196 Mm.NP_081170 Hs.AAH15088	EGITKGSDLDLLEKGIRKTDEMTSNFTNYMYKFHRLPPNYGSN 100 EDPHRYNGSDVSANASETTSTSTAKPVIHSATPYSDKYYQPIISQGAKGFDKLIIPVGFC 120
Sc.Tam41	QLITIDKELQKELDGVMSSFKAPCRFVFGVGSGVFEQAGYSKSHSKP147
Ca.XP_720669	ADNQTVSLEEDKESPIQQDQLQEIVNSFDAPIDVSIGYGSGILPODGYDKDKSTSNNTAN 180
Dr.NP_001032196	MSLPALQNSAVFYRRIINRFPQDFSLAFAYGSAVFRQTGSSQGHMVKN 48
Mm.NP_081170	MALQALHSSGVGLRRILAHFPEDLSLAFAYGSAVYRQAGPS-AHQENP 47
Hs.AAH15088	MALQTLQSSWVTFRKILSHFPEELSLAFVYGSGVYRQAGPS-SDQKNA 47
Sc.Tam41	QIDIILGVTYPSHFHSINMRONPQHYSSLKYFGSEFVSKFQQIG-AGVYFNPFANIN 203
Ca.XP_720669	DSKQIDFMFUVKDCGKFHQENLKONRDHYSIKSLRLIKKVQGT-NGMYFNPFIKIN 234
Dr.NP_001032196	NIDFVFAVDDPVTWHIMNLIENRKHYSFLRFLGPKQISSIQSDYGAGVYFNILVPAE 105
Mm.NP ⁻ 081170	NIDLVFTVDDPVAWHAMILKKNWSHYSFLKLLGPRIISSIQNNYGAGVYFNPLIRCD 104
Hs.AAH15088	MIDFVFTVDDPVAWHSKNLKKNWSHYSFLKVLGPKIITSIQNNYGAGVYYNSLIMCN 104
Sc.Tam41	GHDVKYGVVSMETLLKDIATMNTFYLAGRIQ KPVKILKN - DLRVQYWNQLNLKAAATLA 261
Ca.XP_720669	EKLVKYGVISSKSALMDLSEWHSLYFAGRLQ KPVNFITTN - DPRVKFLNQYNLKNAMTIA 294
Dr.NP_001032196	DRLIKYGVISTDALIDDLHMTTLYVAGRFRYPVRILLQEENGNLSATLGNLKSAVTAS 165
Mm.NP ⁻ 081170	GKLIKYGVISTGTIEDLLHMINLYIAGRLQVKIISVNEDMALRAALDKNLRSAVTAA 164
Hs.AAH15088	GRLIKYGVISTNVLIEDLLNWNNLYIAGRLQXPVKIISVNEDVTLRSALDRNLKSAVTAA 164
Sc.Tam41	KHYTLEKNNNKEDEFOFYKEITALSVA GDIRYKLGGENPDKVNNIVTKNFERFOEYY 318
Ca.XP_720669	IFLIDGEGNSRQATFNEROLYEOITKLSYLGDFRMYIGGENPNKSKNIVAKOFHHFKKLY 354
Dr.NP_001032196	FLMLPESFSEELYLGIAGLSYSGDFRMVFGEDKSKVSNIVKDNMQHFROLY 217
Mm.NP ⁻ 081170	CLMLPESFSEEDLFIEIAGLSYSGDFRMVFGEKSKVLNIVKPNHFRELY 216
Hs.AAH15088	FLMLPESFSEEDLFIEIAGLSYSGDFRMVVGEDKTKVLNIVKPNHHFRELY 216
Sc.Tam41	KPIYKEVVLNDSFYLPKGFTLKNTQRLLLSRISK 352
Ca.XP_720669	EPILQYFIHKNFLIIVDNDPVNRTFKPNLVVNRIKLITGLPLKFRQQLYGRYYEKSIKE 414
Dr.NP_001032196	NRLQECPQVVKFQQGRLEVDKSPEGQFTQLMALPRTLQQQITRLVDRFGKNRDVEE 275
Mm.NP ⁻ 081170	ESILQENPQVVVKNHQQLEIDKSPEGQFTQLMTLPRTLQQQINHIMDPPGRNRDVEE 274
Hs.AAH15088	GSILQENPQVVVKSQQGWLEIDKSPEGQFTQLMTLPKTLQQQINHIMDPPGKNRDVEE 274
Sc.Tam41	SSALQTIKGVFTA GITKSIKUAW 375
Ca.XP_720669	IVIDDHBASECUNTACGTTHERLSQNLTKIISKTIISSITQAIRGLLSA CLFNSIKUAV 476
Dr.NP_001032196	ILLQVAQDPDCGSVVQQGISSIVKSSSITQSAKGIATA CLVKTVSUS
Mm.NP ⁻ 081170	ILLQVAQDPDCGDVVRLAISSIVRPSSIRQSTKGLFTA CMKKSVIUSS 322
Hs.AAH15088	TLFQVAHDPDCGDVVRL
Sc.Tam41	AKKLKSMRRS 385
Ca.XP_720669	AKQIKFWTSKK 487
Dr.NP_001032196	KKLOKMWRGWRRKPA 338
Mm.NP ⁻ 081170	RKLNKWKGWRRKAS 337
Hs.AAH15088	LKLHKMWKGWLRKTS 316

Figure S2. Tam41 from various organisms. Amino acid sequence alignments of Tam41 homologues. Identical residues are shown in black and similar residues in gray. Sc, Saccharomyces cerevisiae; Ca, Candida albicans; Dr, Danio rerio; Mm, Mus musculus; Hs, Homo sapiens.

Table S1. Yeast strains

Strain	Genotype	Source
D273-10B	MAT	
W303	MAT a /MAT ade2/ade2 his3/his3 ura3/ura3 leu2/leu2 trp1/trp1 can1/ can1	R. Rothstein (Columbia University, New York, NY)
W303-1A	MATa ade2 his3 ura3 leu2 trp1 can1	R. Rothstein
G10	MATa ade2 his4 ura3 leu2 trp1 mas1::URA3 GAL-MAS1	Geli et al., 1990
YTY4100	MAT a /MAT_ade2/ade2 his3/his3 ura3/ura3 leu2/leu2 trp1/trp1 can1/can1 TAM41/ tam41::CgHIS3	This study
YTY4101	MAT a /MAT_ade2/ade2 his3/his3 ura3/ura3 leu2/leu2 trp1/trp1 can1/can1 TAM41/ tam41::CgHIS3 [pRS316 (URA3)]	This study
YTY4102	MAT a /MAT_ade2/ade2 his3/his3 ura3/ura3 leu2/leu2 trp1/trp1 can1/can1 TAM41/ tam41::CgHIS3 [pRS316-Tam41 (URA3)]	This study
YTY4110	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM23-FLAG::CgHIS3	This study
YTY4111	MATa ade2 his3 ura3 leu2 trp1 can1 TIM23-FLAG::CgHIS3 tam41::CgTRP1	This study
YTY4112	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM17-FLAG::CgHIS3	This study
YTY4113	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM17-FLAG::CgHIS3 tam41::CgTRP1	This study
YTY4114	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM50-FLAG::CgHIS3	This study
YTY4115	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM50-FLAG::CgHIS3 tam41::CgTRP1	This study
YTY4116	MAT a ade2 his3 ura3 leu2 trp1 can1 TIM21-FLAG::CgHIS3	This study
YTY4117	MAT a ade2 his3 ura3 lev2 trp1 can1 TIM21-FLAG::CgHIS3 tam41::CgTRP1	This study
YTY4118	MAT a ade2 his3 ura3 leu2 trp1 can1 PAM16-FLAG::CgHIS3	This study
YTY4119	MAT a ade2 his3 ura3 lev2 trp1 can1 PAM16-FLAG::CgHIS3 tam41::CgTRP1	This study
YTY4120	MAT a ade2 his3 ura3 leu2 trp1 can1 PAM18 -FLAG::CgHIS3	This study

The results show that the levels of Tim50, Tim23, Tim17, Tim44, Tim21, Pam18, Pam16, Tim54, Tim22, Mdj1p, and Yge1p are similar between wild-type and $tam41\Delta$ mitochondria.

Possible homologues of Tam41

The BLAST searches allowed us to find homologues of Tam41 in various organisms. Their amino acid sequence alignments are shown in Fig. S2. Although only Tam41 proteins from *Saccharomyces cerevisiae* and *Candida albicans* have N-terminal extensions, which probably function as mitochondrial targeting presequences, the other Tam41 homologues are also predicted to be localized to mitochondria (MITOP; http://ihg.gsf.de/ihg/mitoprot.html).

References

Geli, V., M.J. Yang, K. Suda, A. Lustig, and G. Schatz. 1990. The MAS-encoded processing protease of yeast mitochondria. Overproduction and characterization of its two nonidentical subunits. J. Biol. Chem. 265:19216–19222.

Kitada, K., E. Yamaguchi, and M. Arisawa. 1995. Cloning of the *Candida glabrata TRP1* and *HIS3* genes, and construction of their disruptant strains by sequential integrative transformation. *Gene (Amst.)* 165:203–206.

Yamano, K., D. Ishikawa, M. Esaki, and T. Endo. 2005. The phosphate carrier has an ability to be sorted to either the TIM22 pathway or the TIM23 pathway for its import into yeast mitochondria. J. Biol. Chem. 280:10011–10017.