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The protein encoded by the *MFT1* gene is a targeting factor for mitochondrial precursor proteins, and not a core ribosomal protein

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Abstract Yeast cells harboring *mft1* mutations are compromised in mitochondrial protein targeting, and Mft1p has previously been identified as a ribosomal protein. However, two genes, *PLC2* and *YML062C*, are present in the *MFT1* locus, and we show that *mft1* mutant cells are compromised in the function of the cytosolic protein encoded by *YML062C*. The ribosomal protein (YS3a) is actually encoded by the tightly linked *PLC2* gene, and does not play a role in targeting proteins to the mitochondria.

Key words: Mitochondria; Ribosomal protein; Protein targeting

1. Introduction

Ribosomes in the cytosol translate proteins bound for many different intracellular destinations. Targeting factors that ensure the fidelity of nascent polypeptide sorting to subcellular compartments can be associated directly with the ribosome: a signal-recognition particle (SRP) and nascent-polypeptide-associated complex (NAC) can interact with the ribosome during the early stages of the translation of some polypeptides to initiate their sorting [1–3].

In addition to the coupling of translation and sorting of proteins to the endoplasmic reticulum mediated by SRP, there may be a link between translation and protein sorting to other organelles, such as the mitochondria [4]. Mitochondrial targeting sequences are almost always found at the extreme amino-terminus of a precursor protein [5,6], and could therefore be recognised as soon as they emerge from the ribosome. Such a scenario predicts that ribosome-associated factors would assist in the targeting of proteins to the mitochondria. A genetic screen of yeast mutants uncovered at least three *MFT* genes encoding factors involved in the early stages of protein sorting to the mitochondria. One of these, the *mft1-1* mutant, has been characterised and the corresponding locus cloned from the yeast genome [7]. One of the genes at this locus has been identified as encoding a protein associated with yeast ribosomes [8,9], raising the exciting possibility of a direct link between translation and protein import into the mitochondria.

Here we show by genetic analysis of several mutant alleles that the genuine *MFT1* gene is *YML062C*. *YML062C* encodes a 52 kDa targeting factor found in the cytosol. The ribosomal protein previously designated Mft1p [7–9] is instead encoded by the *PLC2* gene, which sits tail-to-tail on chromosome XIII with *YML062C*. In accordance with recently proposed nomenclature, and to avoid confusion, we suggest that the targeting factor encoded by the genuine *MFT1* gene be called Mft52p, and that the ribosomal protein (previously referred to as Mft1p) be called YS3a.

2. Materials and methods

2.1. Expression of the proteins encoded by PLC2 and YML062C

To construct specific expression plasmids, the *YML062C* open reading frame was amplified from yeast genomic DNA by PCR. Amplification of the *YML062C* open reading frame was primed with the oligonucleotides 5′-G GCG GGA TCC ATG GCT CTG TCA CAA AAA CAA ATA G-3′ and 5′-CAG CGG ATC CAA GCT TGC ATT ATA CGT GGT CAT TT-3′, ligated into the plasmid pQE9 (Qiagen) for sequencing, and subcloned into the yeast expression vector YPGE [10]. Monoclonal antibodies recognising the protein encoded by *YML062C* were produced from mice immunised with the hexahistidine-tagged protein. The *PLC2* open reading frame was amplified with the primers 5′-GGC GGA TCC ATC ATG GCT GTT GGT AAG-3′ and 5′-GAC CAC AAG CTT AGT TTC CAA GAC TTC ATC C-3′, removing the natural stop codon. The PCR product was subcloned into the yeast expression vector pYX233 (R and D Systems, Inc.) to introduce the haemagglutinin epitope at the carboxy-terminus of the protein encoded by *PLC2*.

2.2. Subcellular fractionation

Yeast cells were grown in semi-synthetic lactate media to mid-log phase, and converted to spheroplasts with Zymolyase [11]. The cytosolic and ribosomal fractions were prepared according to Cartwright et al. [12], and mitochondria were purified on Nycodenz gradients [13]. For large-scale preparation of ribosomes, yeast cells were grown in semi-synthetic galactose media to mid-log phase and harvested. The cells were disrupted with a French press (10 min at 10 ton/m^2) and 80S ribosomes were purified as described by Takakura et al. [8]. In some control experiments, ribosomes were extracted with 0.5 M potassium acetate according to Lauring et al. [1], or isolated from cells pretreated with cycloheximide [14]. Two-dimensional gel electrophoresis analysis of the purified ribosomes has been described [15]. Protein concentration was determined by Coomassie blue staining [16], and 100 μg protein was typically used per lane for immunoblot analysis.

2.3. Deletion of YML062C

Disruption of the *YML062C* gene was as described by Ito et al. [17], but a Klenow-treated fragment of the *LEU2* gene (not the *URA3* gene) was inserted into the *Eco*RV site in the *YML062C* gene. This disrupts the sequence at the codon corresponding to R^{188} in the mutant cells (*mft1-2*). To construct a deletion mutant (*mft1-3*), the *YML062C* open reading frame was amplified by PCR as described above, and ligated into the plasmid pQE9 (Qiagen). The PCR-amplified DNA fragment in pQE9 was digested with *Pst*I and *Eco*RV, and

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Abbreviations: MFT, mitochondrial fusion protein targeting factor; NAC, nascent-polypeptide-associated complex; SRP, signal recognition particle; PCR, polymerase chain reaction; *YML062C*, yeast open reading frame 062 on chromosome 13 (M), left of centromere on the Crick strand

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a *Pst*I-*Xba*I (blunt) fragment of the *LEU2* gene was ligated into the plasmid, deleting the sequence after the codon corresponding to L^{75}. The *YML062C*::*LEU2* fragment was transformed into the yeast strain SEY2102 to generate the *mft1-3* strain YTHB1 (*MAT*a, *ura3, leu2, his4, suc2, gal2, YML062C*::*LEU2*).

2.4. Miscellaneous

Hexahistidine-tagged Mft52p was purified from *E. coli* M15 (Qiagen) and used to immunise mice for production of monoclonal antibodies. The *mft1-1* allele of *YML062C* was amplified with Expand Polymerase (Boehringer Mannheim). Three independent clones were sequenced completely (Monash Sequencing). DNAsis software (Hitachi) was used for sequence alignments and secondary structure predictions.

3. Results and discussion

3.1. Two open reading frames in the MFT1 locus

Yeast cells carrying the *mft1-1* mutation suffer defects in protein targeting to the mitochondria, which leaves the mutant cells temperature-sensitive for growth. By complementation cloning, the *MFT1* gene was mapped to a 4.2 kb region of chromosome XIII [7]. This portion of chromosome XIII carries two open reading frames: the *PLC2* gene and *YML062C* [17]. The arrangement of the *PLC2* and *YML062C* genes on chromosome XIII is shown in Fig. 1.

3.2. Subcellular localisation of the protein encoded by YML062C

Large-scale mapping of ribosomal proteins has assigned Mft1p as a core protein of the yeast ribosome [8,9]. To determine which gene from the *MFT1* locus encodes the ribosomal protein, we raised antibodies to the proteins encoded by *PLC2* and *YML062C*. Antisera raised to the product of the *YML062C* gene recognise a 52 kDa protein in the cytosol of yeast cells (Fig. 2A, lane 2), and as previously reported [12] little if any of the 52 kDa protein could be detected in the immunoblots of purified yeast ribosomes (Fig. 2A, lane 1). To establish the subcellular location of the protein encoded by the *PLC2* gene, yeast cells expressing an epitope-tagged version of the *PLC2*-encoded protein were analysed by immunoblotting. The monoclonal antibody recognises the 29 kDa epitope-tagged protein as a component of the yeast ribosome (Fig. 2A, lane 1). Analysis of yeast ribosomal proteins by 2D gel immunoblots (Fig. 2B) revealed that the protein encoded by *PLC2* corresponds to the ribosomal protein previously called Mft1p [8]. The 29 kDa protein is not extracted from the ribosomes with high salt (data not shown; see Section 2), and should therefore be considered a core ribosomal protein.

A human gene encoding ribosomal protein S3a was recently cloned [18,19] and sequence similarity suggests that the *PLC2* gene encodes the yeast homolog of S3a (Fig. 2C). S3a is required in binding of eIF2-GTP.Met-$tRNA^{fmet}$ to form a preinitiation complex for protein synthesis [20]. There is no evidence that S3a has any organelle-specific function or would be involved in mitochondrial protein targeting. According to the proposed nomenclature for yeast ribosomal proteins, the protein encoded by *PLC2* should be referred to as YS3a [9]. We conclude that *PLC2* encodes ribosomal protein YS3a, and that the *YML062C* gene encodes a 52 kDa factor found in the cytosol of yeast cells.

3.3. The genuine MFT1 gene

Although the protein encoded by PLC2 had been referred to as Mft1p, the very slow growth phenotype of Δ*plc2* cells is inconsistent with the phenotype of *mft1-1* mutant cells and it has been suggested that the *mft1-1* mutation is more likely located in *YML062C* [17]. To which gene in the *MFT1* locus does the *mft1-1* mutation map?

While the original *mft1-1* mutant [7] shows a strong temperature-sensitive phenotype (Fig. 3A), there is no temperature-sensitive phenotype associated with disruptions of either the *PLC2* gene or the *YML062C* gene (*mft1-2* in Fig. 3B) [17]. The *mft1-2* mutants carry a disruption of the *YML062C* gene that truncates the 52 kDa open reading frame after amino acid R^{188}.

Secondary structure predictions suggest that the protein encoded by *YML062C* has a striking four-helix region at the carboxy-terminus (Fig. 3C), and recent protease mapping of the protein encoded by *YML062C* has revealed a protease-sensitive site between these putative domains [12]. Disruption of the *YML062C* gene at the internal *Eco*RV site would merely truncate the protein at the putative inter-domain region. A complete deletion of the *YML062C* gene (Fig. 3A, *mft1-3*), so that neither of the putative domains of the protein can be expressed, leaves haploid yeast with a temperature-sensitive phenotype consistent with the original *mft1-1* mutants (Fig. 3B).

To be sure that the *YML062C* gene is the site of the *mft1-1* mutation, diploid yeast were produced that carried both recessive *mft1-1* and *mft1-3* mutations. The two mutations behave as alleles of the same gene, leaving the diploid cells temperature-sensitive for growth (Fig. 3D). To determine the nature of the *mft1-1* mutation in *YML062C*, we amplified the mutant gene with a proofreading polymerase and determined the DNA sequence. A (C to A) conversion results in the replacement of the codon for Tyr^{128} (TAC) with a stop codon (TAA). As a result, the open reading frame *YML062C* is truncated in the middle of the N-terminal domain of Mft52p in the *mft1-1* mutant cells.

The *YML062C* gene is the genuine *MFT1* gene, and encodes a 52 kDa cytosolic factor involved in the delivery of proteins to the mitochondria [7,12]. According to convention for naming components of the mitochondrial protein targeting pathway [21], we suggest the protein encoded by the *MFT1*/*YML062C* gene be referred to as Mft52p.

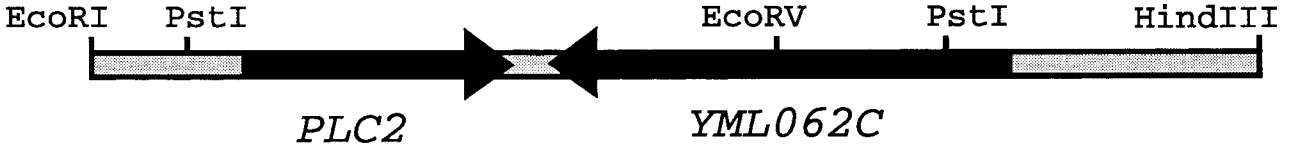

Fig. 1. Arrangement of the *PLC2* and *YML062C* genes on chromosome XIII. The 4.2 kb fragment of DNA from chromosome XIII that complements the *mft1-1* cells is shown. Restriction enzyme sites used in this work are indicated.

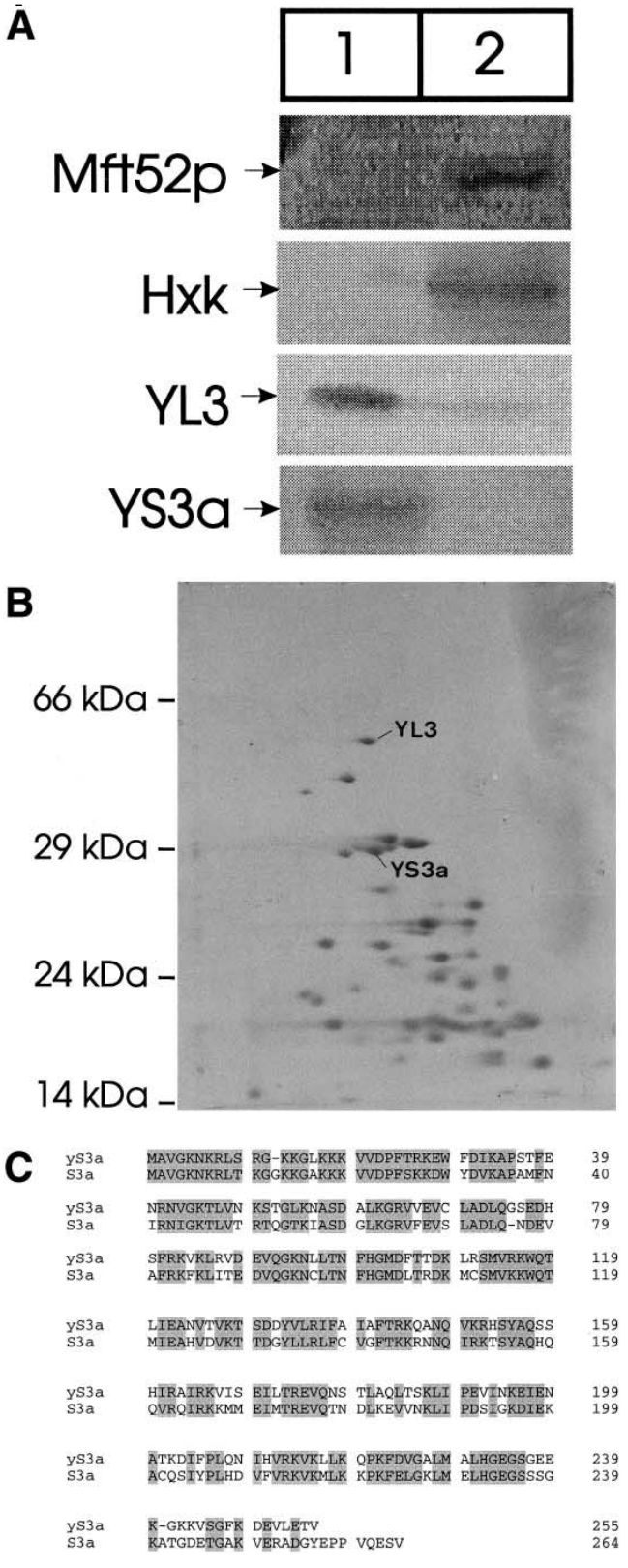

Fig. 2. The *PLC2* gene encodes the yeast homologue of ribosomal protein S3a. A: Ribosomal (lane 1) and cytosolic (lane 2) fractions were prepared from yeast cells and analysed by SDS-PAGE and immunoblotting. Antisera raised to the product of the *YML062C* gene (Mft52p), the cytosolic protein hexokinase (Hxk), ribosomal protein YL3 or the HA epitope engineered at the carboxy-terminus of the protein encoded by *PLC2* (YS3a) were used to decorate the immunoblot. B: Purified ribosomes were analysed by 2D gel elecrophoresis and proteins were stained with Coomassie blue. Immunoblotting of a replica gel enabled identification of ribosomal protein YL3 [8] and the protein encoded by *PLC2* (YS3a). The antibodies recognising YL3 were a kind gift from Jonathon Warner. C: Sequence alignment of the protein encoded by the *PLC2* gene (YS3a) and mammalian protein S3a. Similar amino acids are shaded.
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membrane [23,24]. The *mft1* mutants defined a component acting at an early stage in the protein targeting pathway, and it was therefore of interest when it seemed that the gene mutated in *mft1* cells encoded a ribosomal protein. It is now clear that Mft52p, the protein encoded by the genuine *MFT1* gene is not a core ribosomal protein. Yeast ribosomal protein YS3a is encoded instead by the tightly-linked *PLC2* gene (and *PLC1* on chromosome XII encodes an almost identical isoform of YS3a) [7,25].

The apparent discrepancy in the phenotypes of the disruption (*mft1-2*) and deletion (*mft1-3*) mutants relates to the two-domain structure predicted for the Mft52p protein. Our preliminary structural analysis of recombinant Mft52p purified from *E. coli* suggests that the protein does have two domains [12], and expression of the N-terminal domain alone can complement the growth defects of *mft1-3* cells (T. Beilharz, unpublished). In respect of its structure, Mft52p shares some similarity with the 54 kDa subunit of SRP: both proteins are predicted to have a two-domain structure, characterised by a carboxy-terminal bundle of helices of similar length [26]. In the case of the 54 kDa subunit of SRP, it is this carboxy-terminal domain which binds signal sequences of secretory proteins, to facilitate their delivery to the endoplasmic reticulum [27].

The proposed function of Mft52p in binding to nascent mitochondrial precursor proteins implies that it might transiently associate with translationally active ribosomes [7,12]. However, preliminary characterisation of ribosomes isolated from cycloheximide-arrested yeast cells suggests that while there is a defined set of ribosome-associated proteins locked on to these 80S ribosomes, no detectable amounts of Mft52p or SRP can be isolated with ribosomes arrested in translation (T. Beddoe, unpublished). The proportion of free ribosomes

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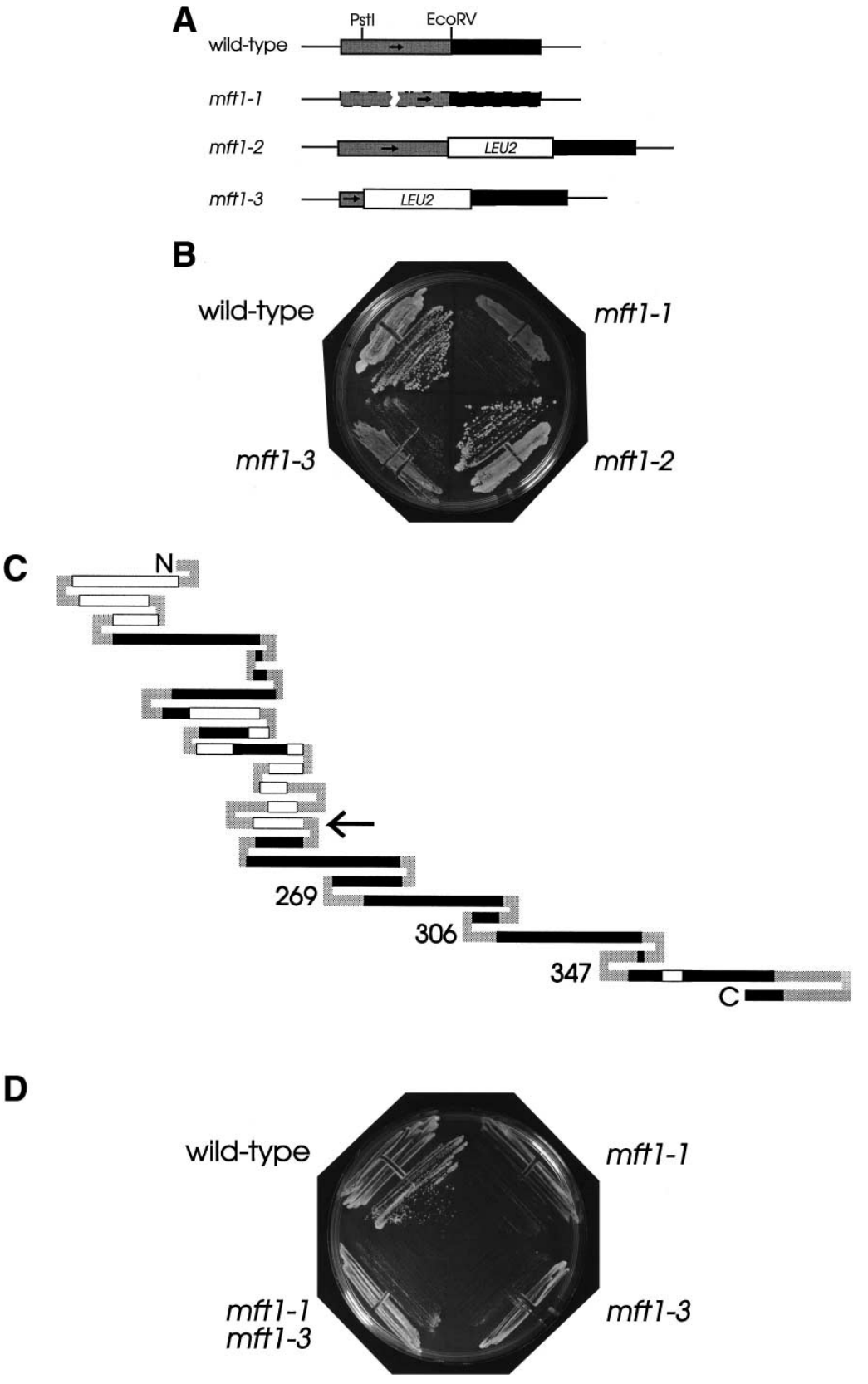
Fig. 3. Loss of Mft52p function leaves yeast cells temperature-sensitive for growth. A: The *YML062C* gene was disrupted by insertion of the *LEU2* gene into the *Eco*RV site (*mft1-2*), or by replacement of the *YML062C* gene from *Pst*I-*Eco*RV with the *LEU2* gene (*mft1-3*). B: Cells were plated on rich medium+2% glucose (YPD) and grown for 2 days at 37°C. Genotypes of the cells are as follows: wild type (SEY2102: *MAT*α, *ura3, leu2, his4, suc2, gal2*), *mft1-1* (*MAT*α, *ura3, leu2, his4, suc2, gal2, mft1-1*), *mft1-2* (*MAT*a, *ura3, leu2, his4, suc2, gal2, YML062C::LEU2*) and *mft1-3* (*MAT*a, *ura3, leu2, his4, suc2, gal2, YML062C* Δ*LEU2*). The *mft1-1* mutants were from Jinnie Garrett and Scott Emr. C: Secondary structure model for Mft52. Predicted helices are shown in black, β-sheet in white and turns in grey. The arrow indicates the position equivalent to the *Eco*RV site in the *YML062C* gene. D: The *mft1-1* cells were crossed with a *mft1-3* haploid strain and diploid (*mft1-1/mft1-3*) cells isolated, and tested for growth on YPD at 37°C.

3.4. Translation and protein targeting

Several pieces of evidence have suggested a link between protein synthesis and protein targeting to the mitochondria [4]. Co-translational import is an efficient means to translocate precursor proteins across the mitochondrial membranes [22] and 80S ribosomes can associate with the mitochondrial outer



that can be caught in the act of carrying targeting factors such as Mft52p and SRP is curiously small. Whatever the mechanism by which Mft52p assists the delivery of mitochondrial precursor proteins, Mft52p is not a core ribosomal protein.

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