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The protein chaperone Ssa1 affects mRNA localization to the mitochondria

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ABSTRACT

Many nuclear-transcribed mRNAs encoding mitochondrial proteins are localized near the mitochondrial outer membrane. A yet unresolved question is whether protein synthesis is important for transport of these mRNAs to their destination. Herein we present a connection between mRNA localization in yeast and the protein chaperone Ssa1. Ssa1 depletion lowered mRNA association with mitochondria while its overexpression increased it. A genome-wide analysis revealed that Ssa proteins preferentially affect mRNAs encoding hydrophobic proteins, which are expected targets for these protein chaperones. Importantly, deletion of the mitochondrial receptor Tom70 abolished the impact of Ssa1 overexpression on mRNAs encoding Tom70 targets. Taken together, our results suggest a role for Ssa1 in mediating localization of nascent peptide-ribosome-mRNA complexes to the mitochondria, consistent with a co-translational transport process.

1. Introduction

The mitochondria of *S. cerevisiae* consist of several hundred proteins, yet very few of them are encoded and synthesized within this organelle. The remaining proteins are encoded in the nucleus, and need to be imported into the mitochondria. Import of these proteins can occur following their complete synthesis in the cytosol (i.e., post-translationally) and the mechanisms for such a process are well-established (reviewed in [1]). Nonetheless, there are numerous evidences for ribosomes and mRNA association with the mitochondria, implying that proteins can be imported while being translated (reviewed in [2,3]). Active ribosomes were found on the mitochondria outer membrane [4,5]. These ribosomes synthesize mitochondrial proteins [6,7] and the translated peptide was found to be important for ribosome association [8]. Importantly, ribosome-associated chaperones were found to support protein import to the mitochondria, consistent with a co-translational process [9–11]. Co-translational import was also deduced from the observation that many mRNAs encoding mitochondrial proteins are localized to the mitochondria's vicinity. A translationally active mRNA was found near the mitochondria of rat hepatocytes [12] and genome-wide analysis of mitochondria isolated from yeast cells revealed association of many mRNAs [13]. This was later confirmed by Fluorescent In Situ Hybridization (FISH) [14] and *in vivo* fluorescent microscopy [15].

The mechanism by which these mRNAs approach the mitochondria (i.e., their transport process) is not clear. mRNAs transport to other cellular destinations occurs by one of two alternative modes: A pre-translational mode, in which the mRNA is transported as a large, translationally-inhibited complex (reviewed in [16,17]), and the repression is relieved only at the destination, when a proper cue is provided [18,19]. Alternatively, a translational process was found to be important for transport to the ER. The emerging nascent chain is recognized by the Signal Recognition Particle (SRP) and the mRNA-Ribosome-Nascent peptide-SRP complex is transported to a receptor on the ER leading to association of many mRNAs and ribosomes with this organelle. Thus, a clear distinction between transport of mRNA prior to its translation and transport while it is translated is participation of the nascent-chain and nascent-chain associated proteins in the latter.

Recently we showed that deletion of a protein receptor from the mitochondria outer membrane results in a decrease in association of many mRNAs [20]. In addition, features from within the coding region [21], and a proper start codon [15] were found to contribute to mitochondria association of some mRNAs. These data suggest that a translational process is important for mRNAs association with mitochondria.

To establish the role of protein synthesis in mRNA transport we tested whether the emerging nascent chain plays a role in transport. This was done by utilizing a nascent-chain associated factor (Ssa1) [22] that is known to support transport of mitochondria pro-

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teins [23] in a manner that involves the mitochondrial Tom70 receptor [24]. Indeed, we show that Ssa1 depletion lead to reduction in association of many mRNAs with the mitochondria, and its overexpression increased association. Its effect correlates with protein features such as hydrophobicity and number of aromatic amino acids, features that are hallmarks for chaperone association. Importantly, Ssa1 impact appears to be dependant on Tom70. These results imply that many mRNAs are translated while *en route* to the mitochondria.

2. Materials and methods

2.1. Yeast growth

For mitochondrial fractionation cells were grown in YP-galactose medium (1% yeast extract, 1% peptone, and 2% galactose). Cells carrying expression plasmids were grown in a defined medium (0.17% yeast nitrogen base and 0.5% ammonium sulfate) with the necessary amino acids.

The following strains were used: *Mat a, ade2-101, his3-delta200, leu2-delta1, ura3-52, trp1-delta63, lys2-801, tom70::HIS3* and its parental strain [24], *Mat a, his3-11,3-15,leu2-3112, trp1Δ1,lys2, ssa1-45 URA3,ssa2::LEU2,ssa3::TRP1,ssa4::LYS2, Mat a, his3-11,3-15,leu2-3112, trp1Δ1,lys2, ssa2::LEU2, ssa3::TRP1, ssa4::LYS2*, and their parental strain [25]. Where indicated. The following plasmids were used: *TEF promoter-SSA1 in pRS416* [26] or its control empty vector, and *Gal1p-Aco1 ORF with MTS with 3 point mutations in the MTS* or with a normal MTS [27].

2.2. Cell fractionation and RNA analyses

Cells were fractionated as previously described [28]. Briefly, cells were grown at 30 °C to an optical density at 600 nm of 1. A sample of the lysate was set a side prior to fractionation (Total), and the remaining lysate was fractionated to a cytosolic fraction and crude mitochondria fraction. In all cases equivalent amounts were analyzed. RNA was purified from each sample and subjected for microarray analysis [28] or Northern analysis [29].

2.3. Statistical analyses

Statistical analyses were performed with SPSS. For independent and normally distributed samples, a t-test was used for determining differences significance. Unpaired samples were analyzed either by Levene's test (for normally distributed) or Mann Whitney test (for non-normally distributed).

3. Results

3.1. Altered Ssa expression affects mRNA association with mitochondria

Ssa1 is one of four highly similar proteins (Ssa1-4) that belong to the Hsp70 family. We tested the impact of depletion of Ssa proteins on mRNA association with mitochondria of *S. cerevisiae*. SSA^+ and a temperature sensitive strain ($ssa1^{ts}ssa2\text{-}4\Delta$) were subjected to a one hour heat shock followed by a crude mitochondria preparation [20]. This fractionation separates the lysate into two samples: A sample that contains mitochondria marker (Por1p, a mitochondria membrane porin), and is devoid of cytosolic marker (Hexokinase 1 (Hxk1p) Fig. 1A. We designate this fraction Mitochondria-containing fraction. The second sample contains Hxk1p and is devoid of Por1, and therefore designated Cytosolic fraction. Importantly, it efficiently separates between mRNAs encoding cytosolic proteins (ACT1, encoding actin) and mRNAs encoding mitochondria proteins (COB, encoding Cytochrome b). The mitochondria-containing fraction also contain ER membranes because significant amounts of SEC61 mRNA, encoding the Sec61 tunnel, and Cue1 protein, which is an ER membrane protein with a role in protein degradation, were found in this fraction (Fig. 1A). The presence of ER membranes may provide additional information regarding the role of Ssa1 in targeting to this organelle. Herein, however, we tested only mRNAs that encode well-established mitochondria proteins (see below), therefore our conculsions are limited to targeting to this organelle. In any case, the co-purification of ER components does not affect our conclusions because all tested mRNAs encode mitochondria proteins, therefore unambiguously associated with the mitochondria moiety and not ER of this fraction. Thus, the impact of Ssa1 on their fractionation pattern is due to changes in their mitochondria association. Notably, by this protocol Ssa1p appears in the cytosolic fraction.

RNA from the mitochondria fraction of SSA^+ and $ssa1^{ts}ssa2\text{-}4\Delta$ was subjected to microarray analysis. Several thousands of genes had signals significantly higher than background (2918 in SSA^+ and 4330 genes in ssa^{ts}) and 2033 genes passed the filtration criteria in both samples. Supplementary Table 1 presents these genes, ranked according to the extent of change in their mitochondria association. For verification of the microarray results by an alternative method, we chose several representative genes: ACO1, ATP2, ADY2, CCP1, CYB2 and POR1, which encode well-established mitochondrial proteins: aconitase, beta subunit of the F1 component, acetate transporter, cytochrome C peroxidase, cytochrome b2 and mitochondria proin, respectively. The changes in association of these mRNAs upon Ssa inactivation was tested by northern analysis and compared to their ranking in the microarray analysis (Fig. 1B, Supplemental Table 2). ACO1, ATP2, ADY2 and CCP1 were among the top 60 affected mRNAs in the microarray analysis, and appeared to be strongly affected from SSA depletion also by the northern analysis (Fig. 1B). While the decrease in mRNA localization is reproducible, it may be considered modest (~two-three fold, see additional examples in Figs. 3 and 4). This suggests that additional chaperones are involved in transport and may partially compensate when Ssa1 is inactive. Considering the large repertoire of chaperones in *S. cerevisiae* and the overlap in their target proteins [30], this is not unexpected. In addition, POR1 mRNA (encoding mitochondria porin) appeared among those with lowest relative decrease (ranked 1769 of 2033 genes), and the same is observed by northern analysis.

The strain used above is deleted of the highly similar Ssa2-4 proteins and contain an *ts* allele of Ssa1. To get insight into the specificity of the impact, we tested the fractionation pattern in a strain that contain a normal Ssa1 allele and is deleted of Ssa2-4 genes (Fig. 1C). In this strain ATP2 and ACO1 showed the same fractionation pattern as in the SSA^+ strain. These mRNAs appeared unaffected also when assayed in a $ssa1^{ts}ssa2\text{-}4\Delta$ strain that was not subjected to a temperature shift but maintained at the permissive temperature (30 °C) (Fig. 1C). These results reveal that at least for these mRNAs the changes in fractionation are due to Ssa1 inactivation. Interestingly, ADY2 mRNA appeared to be affected in the $ssa2\text{-}4\Delta$ strain and in the $ssa1^{ts}$ in the permissive temperature, indicating of some specificity in Ssa proteins function in targeting to the mitochondria.

To further establish the role of Ssa1 in mRNA localization, we utilized a strain that overexpresses Ssa1 and tested the mitochondria association of several mRNAs (Fig. 1D). We focused on mRNAs with a relatively low association under normal conditions (Supplemental Table 2), as the impact of Ssa1 will be more apparent on them (TIM23, ADH3 and IDH1, which encode Translocase of the Inner Mitochondrial membrane, mitochondrial alcohol dehydrogenase isozyme III and subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, respectively). In all cases the ratio

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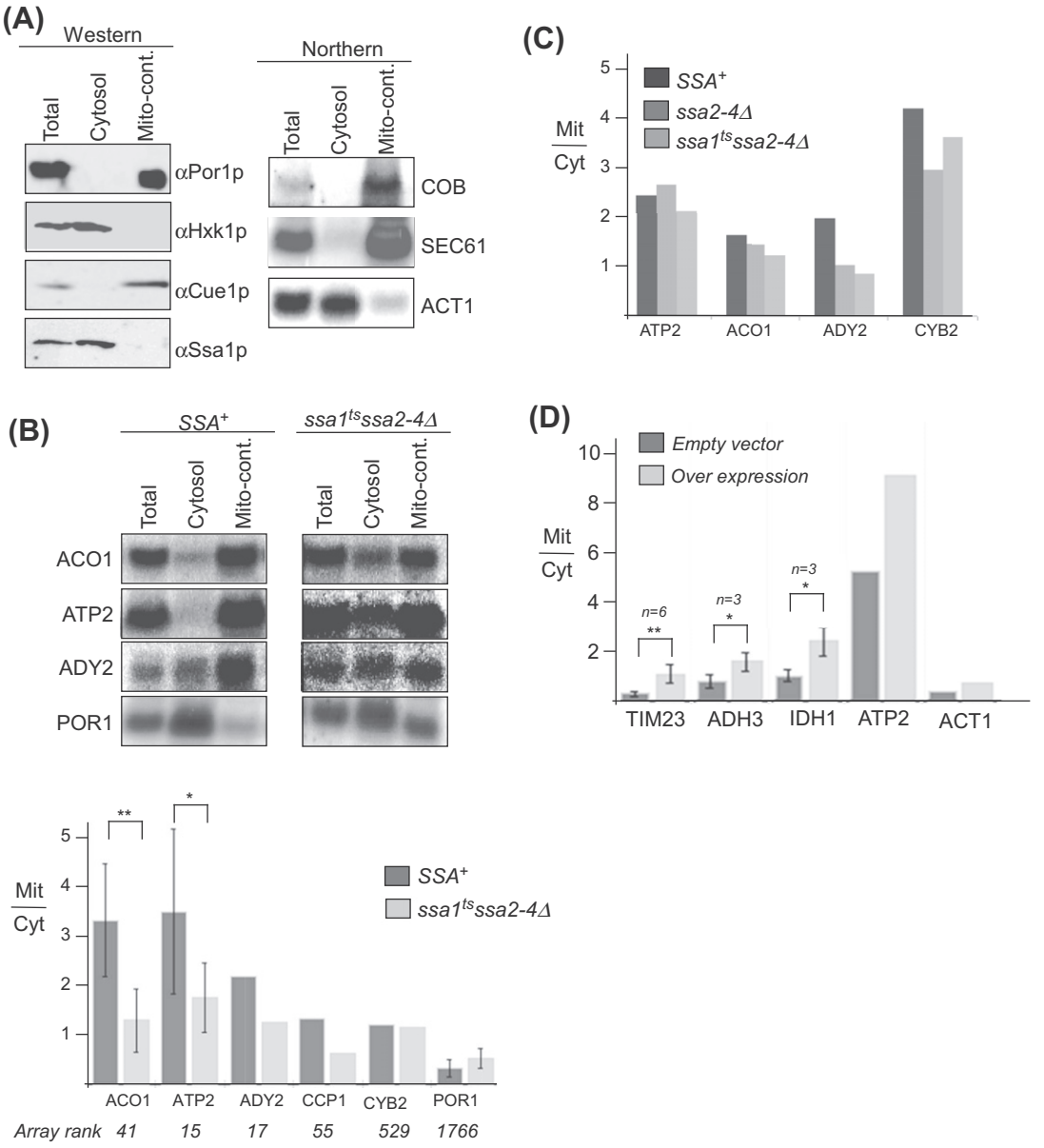

Fig. 1. Ssa1 is involved in mRNA localization to the mitochondria. (A) Fractionation assessment: Cells were subjected to a crude-mitochondria fractionation procedure and equal amounts were collected before fractionation (Total) and from the Cytosolic (Cytosol) and Mitochondria-containing (Mito-cont.) fractions. The protein markers were tested by western analyses: Por1p – a mitochondria membrane porin , Hxk1p – cytosolic hexokinase 1, Cue1p – ER membrane protein with a role in protein degradation. The following marker mRNAs were tested by northern analyses: COB – encoding mitochondria cytochrome b, SEC61 – encoding the Sec61 ER tunnel and ACT1 – encoding actin. (B) Ssa1 depletion: Cells (either *SSA*$^{+}$ or *ssa1*^{ts}*ssa2-4Δ*) were grown at 30 °C and shifted to 37 °C for a one hr shock. Cells were fractionated after the heat shock, RNA was extracted from each fraction and equal amounts were subjected to northern analysis with the indicated probes. All tested mRNAs encode well-established mitochondria proteins: ACO1 encodes aconitase, ATP2 ancodes beta subunit of the F1 component, ADY2 – acetate transporter, CCP1 – cytochrome C peroxidase, CYB2 – cytochrome b2 and POR1 mitochondria porin. Error bars represent the S.E.M. of three independent fractionation experiments. $^{*}P$-value $\leqslant 0.1$, $^{**}P$-value $\leqslant 0.05$. Quantitation values appear in Supplementary Table 2. The ranking of each gene in the microarray analysis (Supplementary Table 1) is shown (strongest decrease was ranked 1 and lowest 2033). (C) Specificity of Ssa proteins: Fractionation procedure was performed on the following strains: *SSA*$^{+}$, expressing normal Ssa proteins and maintained at the permissive temperature; *ssa2-4Δ*, deleted of *SSA2-4* genes and expressing a normal Ssa1 protein; *ssa1*^{ts}*ssa2-4Δ*, expressing a *ts* Ssa1 allele yet maintained at the permissive temperature. The fractionation pattern of the indicated mRNAs was analyzed by northern assay. (D) Ssa1 overexpression: Cells were transformed with a plasmid expressing Ssa1 from a strong promoter or an empty vector and the mitochondria association of the indicated mRNAs was tested by northern analysis. TIM23 encodes Translocase of the Inner Mitochondrial membrane, ADH3 encodes mitochondrial alcohol dehydrogenase isozyme III, IDH1 encodes Subunit of mitochondrial NAD (+)-dependent Isocitrate Dehydrogenase, ATP2 encodes beta subunit of the F1 component and ACT1 encodes actin. Error bars represent the S.E.M. and the number of experimental repeats (n) is indicated. $^{*}P$ value $\leqslant 0.1$, $^{**}P$ value $\leqslant 0.05$.

between the mitochondria-containing and cytosolic fractions (M/C) was improved when Ssa1 was over expressed, ranging from 2.7 fold for IDH1 up to 4 fold for Tim23. Even the association of ATP2, an mRNA that under normal conditions is highly associated with mitochondria (>85% association, Supplementary Table 2), was improved upon overexpression of Ssa1. Taken together, these results support the notion that Ssa1 is involved in mRNA targeting to the mitochondria.

3.2. Characteristics of mis-localized mRNAs

To identify the basis for Ssa proteins impact on mRNA association, we first analyzed whether the most affected mRNAs (top 5%) encode proteins with similar function. Applying the GO Term finder tool implemented in Saccharomyces Genome Database (SGD) revealed that the most affected mRNAs are highly enriched for genes encoding ER associated proteins (P value 1.2×10^{-3}), plasma

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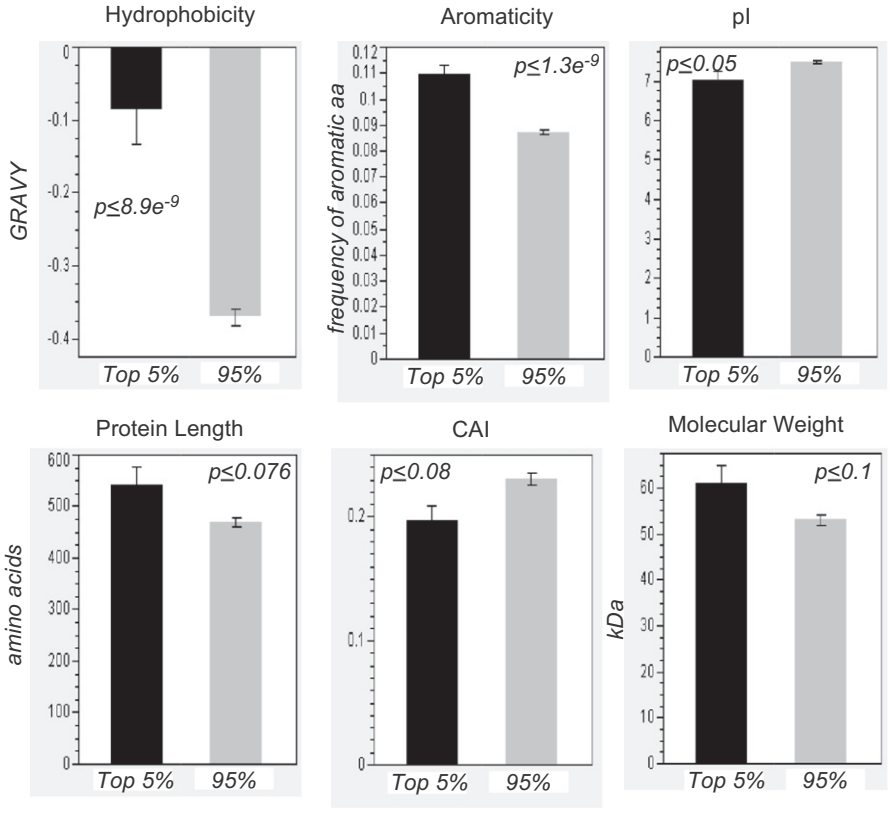

Fig. 2. Properties of proteins encoded by the affected mRNAs. The indicated physical features of proteins encoded by mRNAs with strongest decrease in mitochondria association (Top 5%) were compared to the rest of the mRNAs (95%). P-values are indicated for each feature.

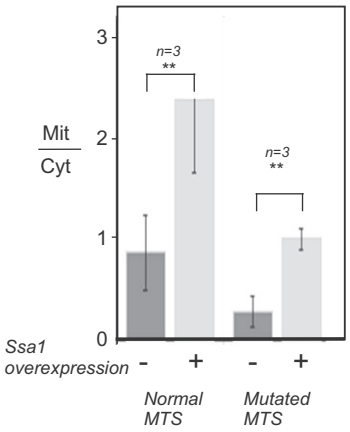

Fig. 3. Ssa1 improves mitochondria association of mRNA with a defective MTS. Cells were transformed with plasmids expressing either Aco1p with its normal MTS (Normal) or Aco1p containing three point mutations in its MTS (Mutated) which decrease its protein and mRNA association with mitochondria . The effect of Ssa1 overexpression on their mitochondria association was tested. Error bars represent the S.E.M. of three independent experiments. $^{**}P$ value $\leqslant 0.05$.

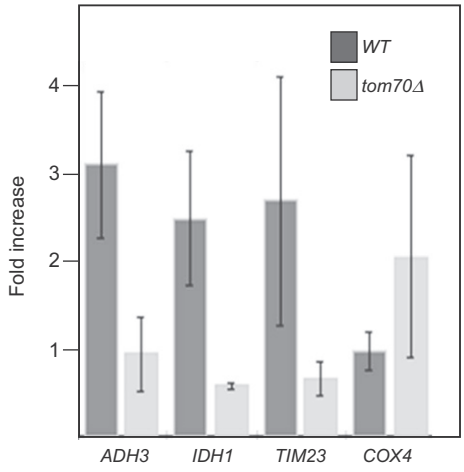

Fig. 4. Ssa1 effect is exerted through the mitochondria protein receptor Tom70. Plasmids overexpressing Ssa1 or its empty vector control were introduced into *tom70Δ* and changes in mRNA association were tested for the indicated mRNAs. Error bars represent the S.E.M. of three independent experiments.

membrane proteins (P value 8.5×10^{-4}) and mitochondrial proteins (P value 9.7×10^{-5}). These results reveal that Ssa proteins are involved in targeting mRNAs to the ER and mitochondria. A role for Ssa in mRNA localization to either of these organelle was never observed. Herein we focus on the role in mitochondria localization.

We tested whether the affected mRNAs share a common sequence motif, by applying the MEME and DRIM algorithms on the 3′ UTRs of the mRNAs [31,32]. No significant enrichment was found among these mRNA. We therefore searched whether the proteins encoded by the affected mRNAs have similar physical features (Fig. 2). Protein size, pI and Codon Adaptation Index values appeared similar between the 5% of most affected mRNAs and the remaining 95% of mRNAs. Frequency of aromatic amino acids and hydrophobicity of the protein (GRAVY scores), however, were significantly different between these groups. Thus, Ssa1 impact is preferentially exerted on a subset of mRNAs that encode hydrophobic proteins or proteins with relatively high number of aromatic amino acids. This preference is consistent with studies that identified proteins that interact with Ssa1 or its bacterial homolog [30,33].

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In vitro studies have shown that Ssa1 binds the mitochondria targeting sequence (MTS) with relation to its amphipaticity [34]. We therefore analyzed whether features of this sequence correlate with the genome-wide impact of Ssa. The features that were examined include the hydrophobic moment (μHδ), the maximal hydrophobicity (Hmax) of the hydrophobic face of the helical structure and the number of positively charged residues [35]. We did not find any correlation between the extent of impact and any of these parameters. To experimentally test if Ssa1 role is exerted through the MTS we tested the impact of over expression on an mRNA (ACO1 mRNA) with MTS that includes 3 point mutations in positive amino acids: K9P, R10I and R14C [27]. This mutation severely reduces ACO1 mRNA association with mitochondria (Fig. 3 and [20]). Yet, Ssa1 overexpression improved the localization of this mRNA to the same extent as the mRNA encoding the normal aconitase protein (Fig.3).

3.3. Tom70 is important for Ssa-mediated localization

In vitro studies have shown that Ssa1 enhances protein import to the mitochondria through interaction with the protein receptor Tom70 [24], which is localized on the mitochondria outer membrane. We therefore tested whether deletion of Tom70 lowers the impact of Ssa1 on mRNA localization. A plasmid overexpressing Ssa1 was introduced into *tom70Δ*, and mitochondria association of several mRNAs was tested by northern analysis (Fig. 4). While in wild-type cells Ssa1 overexpression led to increased association of TIM23, ADH3 and IDH1, this impact was significantly reduced in *tom70Δ*. These mRNAs encode proteins that are imported by Tom70p [36,37]. As a control we tested COX4 mRNA, which encodes a mitochondrial protein (Subunit IV of cytochrome c oxidase) that is imported in a Tom70-independent manner [36]. As can be seen in Fig. 4, we did not observe a reduction in Ssa1 impact on COX4 localization. These results suggest that Ssa1 role on localization of some mRNAs is mediated through interaction with Tom70 receptor.

4. Discussion

Localization of mRNAs to the activity site of their encoded protein is a widely observed phenomenon, identified from bacteria through human. The data presented herein indicate that mRNA localization to yeast mitochondria is mediated (at least in part) by a protein chaperone (Ssa1). While the role of protein chaperone might be exerted through a direct interaction with the RNA, we find this very unlikely; Ssa1 was never shown to directly interact with RNA and we did not find enrichment to RNA motif among the target mRNAs. On the other hand, Ssa1 is known to be associated with many proteins, and we found that the affected mRNAs share common features within their encoded proteins. This suggests that Ssa1 role is exerted through the translated protein and not the mRNA. Impact on mRNA localization through the encoded protein is most easily explained by binding of Ssa1 to its protein targets while they are translated (i.e., as part of a ribosome-mRNA complex), as was suggested for the ribosome-associated factors RAC and NAC [11,38]. A plausible model will pose that translation of mRNAs start throughout the cytoplasm and once the protein emerges from the ribosome exit tunnel it associates with Ssa1. The enrichment for hydrophobic domains (Fig. 2), and the observation that Ssa1 can support targeting of aconitase with a defective MTS (Fig. 3), suggests that Ssa1 interacts with regions throughout the protein and not only with the N-terminal MTS. While in contact with the emerging protein, Ssa1 support targeting to the mitochondria through interaction with the mitochondria-outer membrane protein Tom70. This interaction, first demonstrated *in vitro* [24], suggests that Tom70 can associate with Ssa1 while the latter is in contact with the nascent chain; i.e., while the mRNA is translated. Overall, Ssa1 serves as a connector between Tom70 and the emerging protein and thereby support mRNA localization to the mitochondria.

Ssa1 is unlikely to remain associated with the translated protein or the mitochondria once the protein import started as we did not detect it at the membrane fraction (Fig. 1). Anchoring to the mitochondria probably occurs by other mitochondria-associated proteins. A prime candidate for this is Puf3, which is localized to the outer membrane of the mitochondria [39] and support mitochondria association through interaction with motifs in the 3′ UTR [15,40]. Anchoring through interaction with 3′ UTRs seems preferable as it will not interfere with the translation process of the ORF.

The main role of Ssa is in processes related to protein folding, such as preventing premature folding or correcting an inappropriate fold. These roles are exerted on almost half of the yeast proteome [30], and affect proteins that function at the cytosol, nucleus or the ER. It is therefore clear that Ssa by itself cannot account for the proper targeting of mRNAs encoding mitochondria protein. Specificity is likely to be obtained by other factors, which recognize unique features of these proteins or mRNAs. For example, the protein receptor Tom20 recognize elements on the MTS and may therefore anchor MTS-containing cargos to the mitochondria while non-MTS cargos will be rejected [20]. Another protein that likely to support proper targeting is Puf3, which recognize sequences at the 3′ UTR of mRNAs encoding mitochondria proteins, and thereby anchor only the appropriate mRNAs to the mitochondria [40]. We speculate that many mRNAs are targeted to the mitochondria through the function of Ssa1, yet only those that contain proper signals remain anchored to this organelle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.11.025.

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