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Nonconventional localizations of cytosolic aminoacyl-tRNA synthetases in yeast and human cells

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ABSTRACT

By definition, cytosolic aminoacyl-tRNA synthetases (aaRSs) should be restricted to the cytosol of eukaryotic cells where they supply translating ribosomes with their aminoacyl-tRNA substrates. However, it has been shown that other translationally-active compartments like mitochondria and plastids can simultaneously contain the cytosolic aaRS and its corresponding organellar ortholog suggesting that both forms do not share the same organellar function. In addition, a fair number of cytosolic aaRSs have also been found in the nucleus of cells from several species. Hence, these supposedly cytosolic-restricted enzymes have instead the potential to be multi-localized. As expected, in all examples that were studied so far, when the cytosolic aaRS is imported inside an organelle that already contains its *bona fide* corresponding organellar-restricted aaRSs, the cytosolic form was proven to exert a nonconventional and essential function. Some of these essential functions include regulating homeostasis and protecting against various stresses. It thus becomes critical to assess meticulously the subcellular localization of each of these cytosolic aaRSs to unravel their additional roles. With this objective in mind, we provide here a review on what is currently known about cytosolic aaRSs multi-compartmentalization and we describe all commonly used protocols and procedures for identifying the compartments in which cytosolic aaRSs relocalize in yeast and human cells.
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1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) constitute a family of ubiquitous enzymes present in the three kingdoms of life and essentially known for transfer RNA (tRNA) aminoacylation. Indeed, they are required for the ligation of the 20 standard proteinogenic amino acids (aa) to their cognate tRNAs [1]. tRNA aminoacylation and protein synthesis occur in the cytosol of all organisms but unlike bacteria, eukaryotes have membrane-enclosed structures compartmentalizing their cytosol. Among these compartments, mitochondria in all eukaryotes and also chloroplasts in plants synthesize proteins by translating organellar mRNAs [2]. Hence, a compartmentalized set of the 20 aaRSs is expected in each translationally-active compartment. Since no gene encoding mitochondrial aaRSs was found in any of the mitochondrial DNA sequenced so far, the nuclear genome has to encode both full sets of cytosolic and organellar aaRSs. However, while this is true for the set of 20 cytosolic aaRSs, a full set of 20 additional separate genes encoding organellar aaRSs has never been found in any of the nuclear genomes sequenced so far [3–5]. Both sets of genes can usually be easily distinguished based on phylogenetic analyses. These studies show that a majority of the mitochondrial aaRSs are of bacterial descent but that the α-proteobacterial endosymbiotic origin of the mitochondrial aaRSs has largely been lost because of gene replacements, intranuclear gene duplications and divergence or horizontal gene transfers [6,7].

In plants, the evolution of the different aaRSs seems more complicated. Monocot and dicot have either at least 1 gene encoding a given aaRS for each protein-synthesizing compartment or 2 genes with one gene encoding the cytosolic and the second both the mitochondrial and the chloroplastic aaRS [8]. Surprisingly, both have one instance where only one gene seems to encode all 3 activities: isoleucyl-tRNA synthetase (IleRS) for monocot and glutaminyl-tRNA synthetase (GlnRS) for dicot [5,8,9]. The same is true for diatoms and brown algae in which a single gene seems to encode all 3 arginyl-tRNA synthetases (ArgRSs) [5]. In many instances, the mechanism that allows expression of the organellar aaRS from a gene that also encodes the cytosolic isoform is unknown or ill defined [10,11]. For the sake of brevity, from now on we will use the three letters code for each amino acid mentioned.

Genes encoding several mitochondrial aaRSs are also missing in metazoan and fungal genomes and various strategies are used by these organisms to produce the mitochondrial aaRS from an apparently missing gene. Many of them are encoded by the same exact gene that encodes the cytosolic form, except that the mRNA that will be translated into the mitochondrial isoform contains an additional 5′-extension for the mitochondrial targeting sequence (MTS). The synthesis of these 2 proteins from the same gene is achieved through different mechanisms: (i) alternative transcription start as shown for the yeast ValRS and HisRS mRNAs [12,13]; (ii) alternative splicing as shown for example for the human LysRS [14]; (iii) alternative translation initiation at two different AUG start codons within the same mRNA such as for the human GlyRS [15]; or (iv) alternative transcription start at a non-canonical start codon such as for the yeast AlaRS [16]. The complete list of cytosolic and organellar aaRSs generated from a single gene is presented in Table 1.

Eukaryotes, with the exception of a few parasites that possess a mitochondrial GlnRS encoded by the same gene as the cytosolic GlnRS, use a different strategy to compensate for the ubiquitous absence of the gene encoding mitochondrial GlnRS [41]. This widespread absence of a mitochondrial GlnRS is an illustration of the evolutionary origin of mitochondria that very likely originated from an α-proteobacterial endosymbiont that was generating glutaminyl-$tRNA^{Gln}$ (Gln-$tRNA^{Gln}$) not by direct charging of $tRNA^{Gln}$ by a GlnRS but by a tRNA-dependent transamidation pathway, metazoans and fungi kept the endosymbiont's route for mitochondrial Gln-$tRNA^{Gln}$ formation [42]. This tRNA-dependent transamidation pathway requires first glutamylation of mitochondrially-encoded $tRNA^{Gln}$ ($_{mt}tRNA^{Gln}$) by a mitochondrial non-discriminating GluRS and subsequent transamidation of the charged glutamate into glutamine by a mitochondrial tRNA-dependent amidotransferase (AdT) [9,28,43,44]. However, the two enzymes that sustain this pathway seem to have species-specific features. In human cells, it is the mitochondrial GluRS that charges both the $_{mt}tRNA^{Glu}$ and the $_{mt}tRNA^{Gln}$ [44], whereas in the yeast *Saccharomyces cerevisiae* a pool of the cytosolic GluRS is imported into the mitochondria to carry out glutamylation of $_{mt}tRNA^{Gln}$ [28]. Even if metazoans and fungi both belong to the opisthokonta group, their trimeric mitochondrial AdTs differ by one subunit, the classical bacterial-like GatC subunit found in metazoan AdTs is replaced by a fungi-specific GatF subunit [45].

In yeast, there is only one CysRS gene (Supp. Table 1) suggesting that it encodes both the cytosolic and mitochondrial isoforms. However, the mitochondrial CysRS has yet not been experimentally characterized. Along the same line, *S. cerevisiae* genome has two genes, *AIM10* and *YHR020W* that encode for proteins showing similarities to prokaryotic (*AIM10*) and eukaryotic (*YHR020W*) ProRSs (Supp. Table 1). While there are no experimental proofs that *AIM10* encodes a protein with ProRS activity, its deletion leads to a respiratory-deficient yeast cell showing that it required for

Table 1
List of dual-localized cytosolic aaRSs identified so far.

Organism	Localisation	aaRS	Techniques used	Mechanism	References
Hs	Mitochondrial	GlyRS	IF, CF	Alternative transcription start	[15,17]
		LysRS	FM	Alternative mRNA splicing	[14]
	Nuclear	**LysRS**	CM, CF, CE	Phosphorylation	[18,19]
		MetRS	FM, CE, IF, CF	Cell growth signal induced	[19,20]
		PheRS	IF	n.d.	[21]
		TrpRS	CF, IF, IEM	IFN gamma induced	[22–24]
		TyrRS	IF, CM, CF	tRNATyr binding	[25]
		TyrRS	CF, IF	Oxidative stress induced	[26]
Sc	Mitochondrial	AlaRS	G, CF	Non-AUG alternative translation start	[16,27]
		GluRS	FM, G, CF, CM	Arc1p released	[28,29]
		GlyRS1	G	Non-AUG alternative translation start	[30,31]
		HisRS	G, CF	Alternative transcription start	[12,32]
		ValRS	G	Alternative translation start	[13,33]
	Nuclear	**MetRS**	FM, CF, CM	Arc1p released	[29,34]
		TyrRS	FM, CF	NLS characterisation	[25,35]
Tb	Mitochondrial	IleRS	IF, CF	Trans-splicing	[36,37]
		GluRS	IF, CF	Trans-splicing	[37,38]
		GlnRS	IF, CF	Trans-splicing	[37,38]
		ProRS	IF	Trans-splicing	[37]
Pf	Apicoplast	AlaRS	IF	n.d.	[39]
		CysRS	FM, IF	Alternative mRNA splicing	[40]
		GlyRS	IF	n.d.	[39]
		ThrRS	IF	n.d.	[39]

aaRSs in bold are enzymes that relocalize stricto sensu from cytosol to the organellar compartment. *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Tb*, *Trypanosoma brucei*; *Pf*, *Plasmodium falciparum*; CM, confocal microscopy; IF, immunofluorescence; CF, cell fractionation; FM, epifluorescence microscopy; CE, capillary electrophoresis; IEM, immuno-electron microscopy; G, genetic; NLS, nuclear localization signal; n.d., not determined.

mitochondria function [46]. The protein encoded by the *YHR020W* gene has been shown to retain ProRS activity, *in vitro*, and to be essential for cell survival, suggesting that it might be the cytosolic ProRS [47]. The mitoproteome done on purified *S. cerevisiae* mitochondria (performed as described in Section 5; Fig. 1A) shows that cytosolic CysRS is found in the mitoproteome, suggesting that the

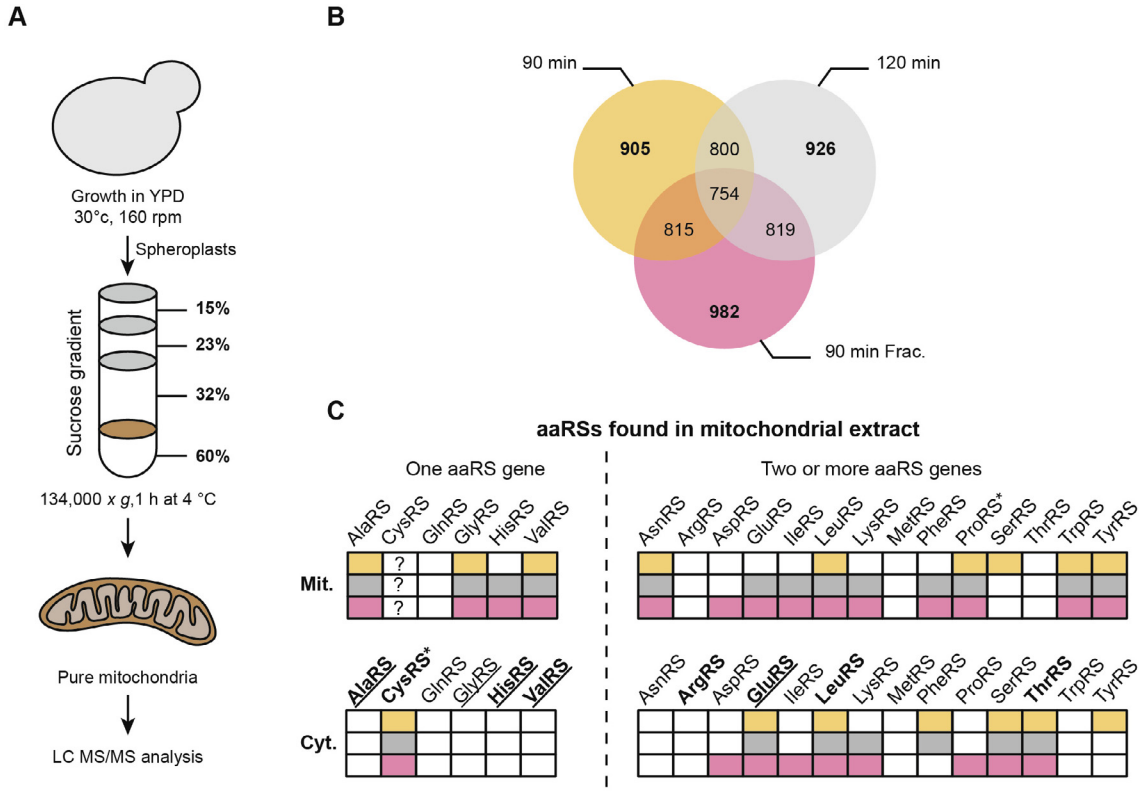

Fig. 1. Mitochondrial localization of cytosolic and mitochondrial aaRSs. (A) Schematized representation of yeast mitochondria preparation. The procedure we followed was that described by Meisinger and coworkers [48]. Mitochondria are recovered from the 60%-32% interphase. (B) LC MS/MS analysis of the mitoproteome. After separation, pure yeast mitochondria were subjected to LC MS/MS analysis with 3 different injection gradients (90 min, 120 min and 90 min Frac, see Section 5 for details). Number of total proteins identified in each samples are represented in bold. Proteins found shared between conditions are also showed. (C) Mitochondrial (top) and cytosolic (bottom) forms of aaRSs identified in mitochondrial extract by LC MS/MS analysis. Colors are corresponding to samples in panel B. Cytosolic aaRSs predicted to have an MTS by at least 1 predictor (see Table 5) are in bold. Yeast cytosolic aaRSs previously shown experimentally as mitochondria dual-localized are underlined (see Table 1). No mitochondrial specific CysRS identify in yeast. ∗ indicates putative aaRSs. See Supplemental Table 1 for yeast aaRSs genes name.

missing mitochondrial CysRS could be compensated by mitochondrial import of the cytosolic ortholog (Fig. 1C). The *AIM10* gene product was found in the different mass-spectrometry analyses (Fig. 1C and Supp. Table 2), confirming its mitochondrial localization, but further experimental proofs that this protein is a functional ProRS are still needed. Interestingly, the *YHR020W* gene product was also found in one of the mass-spectrometry data set we obtained (Fig. 1C and Supp. Table 2), suggesting that the cytosolic ProRS might be dual-localized. In addition, depending on the sample injection time used prior to mass spectrometry, 6–8 additional other cytosolic aaRSs were found in the mitoproteome. Interestingly, each of these mitochondria-imported cytosolic aaRSs has a mitochondrial ortholog encoded by a separate gene. Nonetheless, the deletion of any of these genes encoding the *bona fide* mitochondrial aaRS leads to a respiratory deficiency (SGD website source). This means that the cytosolic aaRS orthologs, imported into mitochondria, cannot compensate for the loss of the mitochondrial ones and thus suggests that they have other roles than cognate $_{mt}$tRNA aminoacylation.

Relocalization of cytosolic aaRSs is not restricted to compartments in which their presence is required to supply protein synthesis with aa-tRNAs. Indeed, some cytosolic aaRSs have been localized in the nucleus both in human and *S. cerevisiae* (Table 1). These and other proteins of the translation machinery were first observed while analyzing the protein content of *Xenopus* oocytes and has since also been observed in mammalian and yeast cells [20,29,49–51]. Their proposed function in the nucleus is that by charging only the mature tRNAs, these aaRSs are in charge of a quality-control step of the tRNA maturation process prior to their export to the cytoplasm [49,50]. More recent reports show other functions for these nuclear pools of human and yeast aaRSs that do not require their tRNA-charging capacities. For example, under oxidative stress a portion of the human TyrRS relocalizes to the nucleus where it activates E2F1, a transcription factor that up-regulates the expression of DNA damage repair genes [26]. Likewise, a pool of the human MetRS relocalizes into the nucleoli of proliferating cells to regulate rRNA biogenesis [20]. In the yeast *S. cerevisiae*, a pool of MetRS relocalizes into the nucleus to regulate transcription of genes encoding subunits of respiratory complexes [29]. More broadly, a previous bioinformatics analysis identified 16 yeast cytosolic aaRSs as harboring a putative nuclear localization signal (NLS) suggesting that these enzymes could enter the nucleus to ensure additional functions other than translation [52]. However the MetRS, experimentally shown to relocalize into the nucleus was not identified by this bioinformatics study. This highlights the limits of the bioinformatics prediction algorithms to identify targeting sequences, hence the difficulties to predict in which additional compartments these aaRSs could eventually relocalize. However, the scientific community that studies these enzymes has come to the consensus that any cytosolic aaRSs could potentially be multi-localized.

Defining what we mean by multi-localized aaRSs is not an easy task given the variety of strategies that organisms use to relocalize the same protein in several compartments. In the present review, we will define a multi-localized aaRS as a cytosolic aaRS that is found in at least 2 different compartments and for which the organellar isoform is produced from the same gene as the cytosolic one through the various strategies that have been detailed in the previous paragraphs (see also Table 1).

The mechanisms regulating these multi-localizations have been mostly studied for aaRSs that belong to multi-synthetase complexes (MSCs) found in all eukaryotic species studied so far. They are composed of 2–9 cytosolic aaRSs interacting with 1–3 cytosolic anchors called aminoacyl-tRNA synthetase-interacting multifunctional proteins (AIMPs) [53–59]. It has been proposed and experimentally verified for some of the MSC-interacting cytosolic aaRSs that these MSCs are reservoirs for releasable aaRSs that can relocalize to other subcellular compartments to exert nonconventional functions [29,60,61].

In this review, we gather the various methods and procedures that have been used to address the localization of individual cytosolic aaRSs (Fig. 2). Some are biochemical (organelle purification),

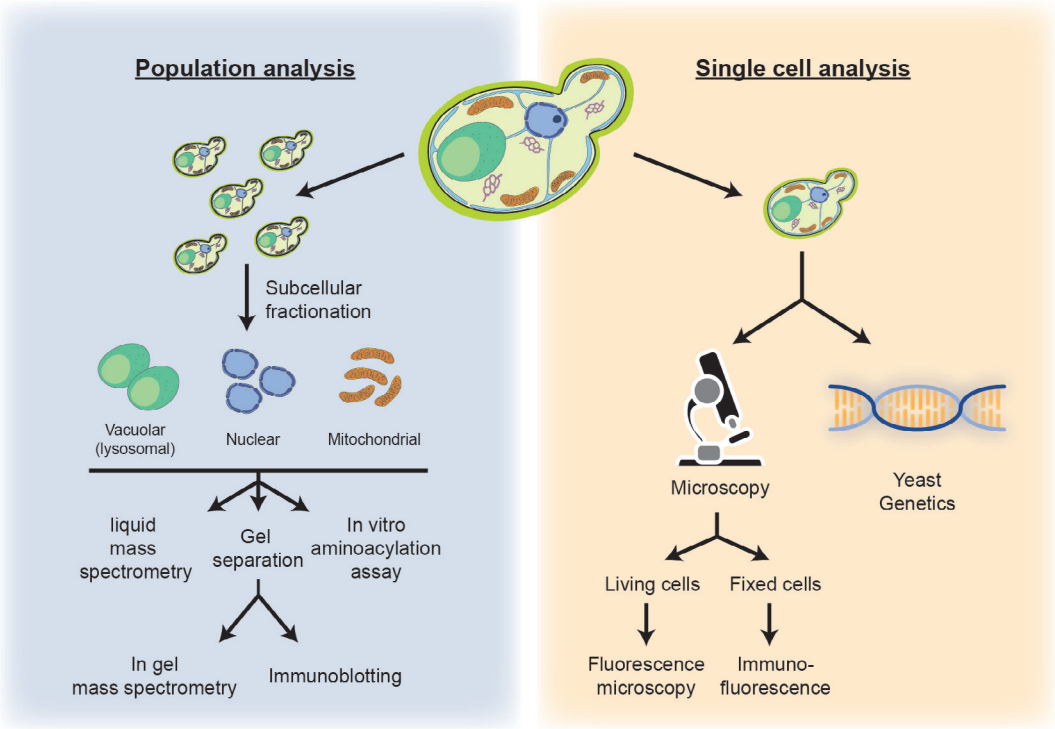

Fig. 2. Different strategies commonly used for localizing aaRSs in yeast cells. All techniques can be used on mammalian cells except those specific to yeast genetics.

genetic (isolation of mutations preventing mitochondrial import, not described in this review) and others are from cell biology (immunofluorescence on fixed cells or GFP-tagged proteins on living cells). The localization of each aaRS in various organisms together with the techniques that were used to localize them are described in Table 1. We also mention the predicted organellar import signals (MTS and NLS) that we found in yeast and human aaRSs. All the methods described in this review have their advantages and drawbacks, and the purpose of this review is to help researchers defining which techniques or procedures should be used to localize a given cytosolic aaRSs. We have focused mainly on techniques used on yeast and human cells although we mention work done on other organisms.

2. Antibodies: A fundamental tool for aaRSs localization

To ascertain the hitherto unreported localization of pools of cytosolic aaRSs pool in a compartment can be done by various methods, but immunodetection will be almost inevitable. Apart from genetics and tagging one's favorite aaRS with a fluorescent protein, all techniques rely on the use of antibodies because of their high degree of specificity and sensitivity. The key step in confirming the localization of a cytosolic aaRS in a compartment with accuracy and reproducibility is to choose the specific antibodies against the marker protein of the compartment of interest. The number of antibodies currently available has expanded in the last years, however published articles often do not report critical parameters (such as the origin of antibodies, the immunogen used to raise the antibodies...), which sometimes compromises the reproducibility of the results. Hence there is a push to require for at least, minimal reporting standards about the use of antibodies [62,63]. In Table 2, we provide an exhaustive list of proteins that were used as compartment markers for both immunofluorescence and Western blot, in studies that report the subcellular localization of an aaRS. More controls used for yeast cells can be found in the *Current Protocols in Cell Biology* paper by Rieder and Emr [64]. Immunodetection of aaRSs, like for any other protein, has so far been done on either native and unmodified aaRS or on tagged enzymes. Table 3 lists the few antibodies that were raised against native aaRSs and that have been used to determine their subcellular localizations. Since antibodies directed against native aaRSs can unpredictably cross-react with other proteins and be less sensitive than expected, most of the immunodetections of aaRSs where performed on tagged-proteins using anti-tag antibodies that usually are highly specific and sensitive. The main limit of this approach is that adding a tag at one extremity of the aaRS can potentially influence the intracellular localization of the chimeric enzyme [65]. Table 4 lists all the tags that have been fused so far to specific aaRSs of different organisms, which extremity of the protein was tagged, which antibody and what approach were used to detect the tagged aaRSs.

Table 2
Proteins used as controls for compartment staining and for confirming organellar localization of aaRSs.

Compartment	Application	Organism	Protein	Antibody source	References
Cytosol	WB	*Hs*	Tubulin beta	n.a.	[18]
			Hsp90 alpha	c.a.	[20]
		Sc	Pgk1	c.a.	[29]
			Gut2	c.a.	[32]
		Tb	eEF1A	n.a.	[36]
Nucleus	WB	*Hs*	Lamin B1	c.a.	[18,20]
			YY1	c.a.	[20]
		Sc	Hta2	c.a.	[29]
			Nop1	c.a.	[29]
Nucleolus	IF	*Hs*	Nucleolin	c.a.	[20]
Mitochondria	WB	*Sc*	Por1	c.a.	[29]
			Atp2	n.a.	[32]
			Rip1	n.a.	[32]
			Cit1	n.a.	[32]
	IF	*Tb*	mHSP70	n.a.	[36,37]
Apicoplast	IF	*Pf*	ACP	h.	[39,40]

WB, western blot; IF, immunofluorescence; *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Tb*, *Trypanosoma brucei*; *Pf*, *Plasmodium falciparum*; n.a., not available; c.a., commercially available; h., homemade.

Table 3
Antibodies used for localizing untagged aaRSs.

Organism	Application	aaRS	Antibody	aaRS antigen	References
Hs	IF	ArgRS	h.: rabbit	His-tagged 72 N-terminal residues of *Hs*ArgRS	[20]
		Glu-ProRS	h.: rabbit	His-tagged peptide from D677 to E884 of *Hs*EPRS	[20]
		GlnRS	h.: rabbit	His-tagged 236 N-terminal residues of *Hs*GlnRS	[20]
		LysRS	c.a.; rabbit	n.d	[18]
		MetRS	h.: rabbit	Full-length denatured His-tagged *Hs*MetRS	[20]
		PheRS	h.; rabbit	Endogenous PheRS from sheep liver	[21]
		TyrRS	h.: rabbit	n.d	[25,26]
	WB	TyrRS	h.: rabbit	n.d	[25,26]
		TrpRS	h.: rabbit	Full-length *Hs*TrpRS	[22]
Sc	WB	GluRS	c.a.; rabbit	His-tagged full-length *Sc*GluRS	[29]
		HisRS	h.; mouse	Full-length *Sc*HisRS	[32]
		MetRS	c.a.; rabbit	His-tagged full-length *Sc*MetRS	[29]
PF	WB, IF	CysRS	h.; rabbit	KLH conjugate 14 C-terminal residues of *Pf*CysRS	[40]

WB, Western Blot; IF, Immunofluorescence; *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Pf*, *Plasmodium falciparum*; n.d., not described; c.a., commercially available; h., homemade.

Table 4
Protein-tags used for localizing aaRSs.

Organism	Tag	Tagged aaRS	Tag position	Application	Antibody	References
Hs	GFP	ArgRS	C-ter	CM		[66]
		AsnRS	C-ter	CM		[66]
		LysRS	C-ter; N-ter	CM, FM		[19,66]
		MetRS	C-ter; N-ter	CM, FM		[19,66]
	V5 epitope	GlyRS	C-ter	IF, WB	c.a.	[15]
Sc	GFP	MetRS	C-ter; N-ter	FM, CM		[29,34]
		Nter-GluRS	C-ter	CM		[28]
		GluRS	N-ter	FM, CM		[34]
		TyrRS	C-ter	FM		[35]
	V5 epitope	GlnRS	C-ter	WB, FM	c.a.	[67]
	6× His	AlaRS	C-ter	WB	c.a.	[27]
	c-Myc	TyrRS	C-ter	WB	c.a.	[35]
Tb	V5 epitope	CysRS	C-ter	IF	c.a.	[37]
		GluRS	C-ter	IF	c.a.	[37]
		GlyRS	C-ter	IF	c.a.	[37]
		IleRS	C-ter	IF	c.a.	[37]
		MetRS	C-ter	IF	c.a.	[37]
		ProRS	C-ter	IF	c.a.	[37]
		SerRS	C-ter	IF	c.a.	[37]
		ValRS	C-ter	IF	c.a.	[37]
	3× HA	IleRS	C-ter	IF, WB	n.d.	[36]
Pf	GFP	AlaRS	C-ter	IF, WB	c.a.	[39]
		CysRS	C-ter	IF, WB	c.a.	[40]
		GlyRS	C-ter	IF, WB	c.a.	[40]
		ThrRS	C-ter	IF, WB	c.a.	[39]
	3× HA	AlaRS	C-ter	IF, WB	c.a.	[39]
		GlyRS	C-ter	IF, WB	c.a.	[39]
		ThrRS	C-ter	IF, WB	c.a.	[39]

Hs, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Tb*, *Trypanosoma brucei*; *Pf*, *Plasmodium falciparum*; CM, confocal microscopy; FM, fluorescence microscopy; IF, immunofluorescence; WB, western blot; c.a., commercially available; n.d., not described.

3. Prediction and analysis of aaRSs with organellar import signal

Among the many bioinformatic predictors of protein localizations, we chose 3 to predict the presence of an MTS and 3 to predict NLSs [68]. We used these predictors regardless of the organismal origin of the aaRSs analyzed. Moreover, to gather as much information as possible we tried the "Euk-mPloc2.0" website (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) which predicts whether an aaRS might be localized to the mitochondria, the nucleus, the cytosol or extracellularly without predicting any targeting sequence of any kind [69].

3.1. Mitochondrial targeting sequence prediction

MTS were predicted using TPpred2.0 (http://tppred2.biocomp.unibo.it/tppred2) [70], TargetP1.1 (http://cbs.dtu.dk/services/TargetP/) [71,72] and MitoFates (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi) [73]. Query sequences should be given in the FASTA format, and depending on the MTS predictor, between 1 and 20 sequences can be analyzed at once. These predictors give back a mitochondrial probability score, the MTS sequence, the putative cleavage site that follows the MTS and the N-terminal peptide resulting from the cleavage. Additionally, MitoFates gives the mitochondrial processing enzymes responsible for maturation of the imported proteins. (Supp. Tables 3a and 3b). The MitoFates predictor was used for fungal, metazoan and plant proteins, while TargetP1.1 was used for non-plant organisms with no cut-off and with cleavage site prediction.

Submitting the yeast cytosolic aaRSs sequences to these predictors resulted in the HisRS having a strong probability of harbouring an MTS, the Cys-, Glu- and Val-RS a good probability and the Arg-, Leu- and Thr-RS only a low probability (Table 5). The predictions have been partially validated since the relocalization of cytosolic His-, Glu- and Val-RS has been experimentally described (Table 1). The good probability of CysRS of having an MTS suggests that it is the cytosolic CysRS that aminoacylates the mitochondrial-encoded $tRNA^{Cys}$ since there is no gene encoding a mitochondrial CysRS.

For human aaRSs, only the mitochondrial LysRS and GlyRS are encoded by the same gene as the cytosolic form. Mitochondrial LysRS originates from alternative splicing [14], and the mitochondrial GlyRS *via* an alternative start site [15,17]. Analyzing the remaining human aaRSs by the predictors shows that only the CysRS might contain an MTS, however, this cytosolic aaRS has never been described to localize to mitochondria (Table 5).

3.2. Nuclear localization signal prediction

To identify NLS, we used NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/) [74], SeqNLS (http://mleg.cse.sc.edu/seqNLS/) [75], and NLS-Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) [76] predictors. For all predictors, we used default settings. We added a 60 amino-acids N- or C-terminal cut-off for the predictions by NLS-Mapper since it is the only one able to predict bipartite NLSs which are usually found in the 60 first or last amino acids of a sequence. We therefore only kept bipartite NLSs of aaRSs fitting this criteria This program is better suited for prediction purposes since instead of a simple "nuclear or not nuclear" output, it ranks protein localization on a scale from exclusively nuclear (8–10), partially nuclear (7–8), dual-localized cytosol-nucleus (3–5) or exclusively cytosolic (1–2). Supplemental Tables 3a and 3b list the results obtained with these 3 predictors for human and yeast aaRSs.

Schimmel and Wang, in their first attempt at identifying NLSs in the 20 yeast cytosolic aaRSs using the programm PSORT II, showed that Ala-, Gly-, Pro-, **Asn**-, **Asp**-, **Gln**-, **Glu**-, **His**-, **Ile**-, **Leu**-, **Lys**-, **Pheβ**-, **Ser**-, **Tyr**- and **Val**-RS harbor at least one SV40

Table 5
Predictions of mitochondrial targeting and nuclear localization signals among *S. cerevisiae* and human cytosolic aaRSs.

		MTS predictors[1]						Localization predictor Euk-mPloc2.0		NLS predictors[2]							
		TPpred2.0		TargetP1.1		MitoFates				NLStradamus		SeqNLS		NLS Mapper (Monopartite)		NLS Mapper (Bipartite)	
		Yeast	Human	Yeast	Human	Yeast	Human	Yeast	Human	Yeast	Human	Yeast	Human	Yeast	Human	Yeast	Human
Class I	**ArgRS**	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C/M	C	n.p.	n.p.	n.p.	1	n.p.	n.p.	1	1
	CysRS	0.771	0.776	n.p.	n.p.	0.539	n.p.	C	C	1	1	1	2	n.p.	n.p.	1	4
	GluRS	0.896		0357		n.p.		C		1		2		n.p.		1	
	GlnRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	n.p.	1	2	1	n.p.	n.p.	2
	IleRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	n.p.	2	2	1	1	1	2
	LeuRS	n.p.	n.p.	0.538	n.p.	n.p.	n.p.	C	C	2	1	2	2	n.p.	n.p.	1	2
	LysRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	1	2	1	n.p.	n.p.	2	2
	MetRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	n.p.	1	1	1	n.p.	1	1	3
	TrpRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	n.p.	1	1	1	n.p.	1	1
	TyrRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C/N	C/E	1	n.p.	2	2	1	1	2	2
	ValRS	0.871	n.p.	n.p.	n.p.	0.996	n.p.	C/M	C	1	1	1	2	n.p.	n.p.	n.p.	1
Class II	**AlaRS**	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C/M	C	n.p.	n.p.	2	1	n.p.	n.p.	1	1
	AsnRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	n.p.	1	1	n.p.	2	1	2
	AspRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	n.p.	2	1	n.p.	n.p.	2	1
	GlyRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	n.p.	n.p.	1	2	n.p.	n.p.	n.p.	1
	HisRS	0.556	n.p.	0.846	n.p.	0.996	n.p.	C/M	C/M	1	n.p.	1	n.p.	1	n.p.	3	2
	PheRS1	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	n.p.	n.p.	1	1	n.p.	1	n.p.	2
	PheRS2	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	n.p.	n.p.	1	2	1	n.p.	1	2
	ProRS	n.p.		n.p.		n.p.		C		n.p.		2		n.p.		n.p.	
	SerRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C	C	1	1	1	2	n.p.	1	1	1
	ThrRS	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	C/M	C	n.p.	1	2	2	1	n.p.	2	3
	GluProRS		n.p.		n.p.		n.p.		C		1		2		1		1

n.p.: not predicted; for NLS sequence details see Supp. Table 3a.
[1] MTS scores are probabilities of the considered protein to have an MTS.
[2] NLS scores are numbers of NLS sequences identified in each aaRS protein. C, cytosolic; M, mitochondrial; N, nuclear; E, extracellular.

T-antigen-like NLS or bipartite NLS (aaRSs in bold type have been found both in our study and by Schimmel and Wang) [49,77]. Using the aforementioned websites, we could classify the yeast aaRSs in three groups, those with a strongly probability of having an NLS (**Asn**-, **Asp**-, Cys-, **Gln**-, **Glu**-, **His**-, **Ile**-, **Leu**-, **Lys**-, **Pheβ**-, **Ser**-, Trp- and **Tyr**-RS), those likely to have one (Ala-, Met-, Thr- and **Val**-RS) and those unlikely to have one (Arg-, Gly-, Pheα- and Pro-RS) (Table 5). We see a high overlap between the published results and our own suggesting the robustness of the methods used. It is noteworthy that the MetRS missing from the original list was predicted by NLS-Mapper and then by SeqNLS. Despite the fact that these bioinformatics studies highlight the presence of NLSs in many cytosolic aaRSs, only 2 have been experimentally confirmed: the MetRS [29] and the TyrRS (3 bioinformatically found NLSs and one confirmed [35]). This emphasizes the need to use different algorithms to identify NLSs and that they are not yet sufficiently accurate to not require experimental confirmation.

Similar NLS prediction analysis has been done on human cells (Table 5). Using the aforementioned websites, we could classify the human aaRSs in three groups, those with a strong probability of having an NLS (Cys-, **GluPro**-, **Leu**-, **Lys**-, Met-, **Pheβ**-, **Ser**-, Thr- and **Val**-RS), those likely to have one (Ala-, Arg-, **Asn**-, **Asp**-, **Gln**-, Gly-, **Ile**-, Pheα-, Pheβ, Trp- and **Tyr**-RS) and the ones unlikely to have an NLS (**His**RS).

The fact that NLS predictions for yeast and human aaRSs converge seem to reinforce the hypothesis that this localization is true and not artifactual. The one notable exception is HisRS which is most likely nuclear in yeast and most unlikely in human. Since the nuclear localizations of the MetRS, TyrRS and IleRS have been determined experimentally, we can speculate that either some of their functions are shared and would have been present in their common ancestor, or that they exert different roles suggesting that the functions were acquired after the opisthokonta splitting (Table 5).

4. Subcellular fractionation

Subcellular fractionation can be divided into 3 different techniques according to the precision of the separation and the organelle that one wants to purify: crude membranes fractionation, nuclear purification and mitochondrial purification.

4.1. Crude membranes fractionation from yeast cells

This technique allows the separation of membranes from the cytosol (S100) and yields 2 separate membrane fractions (P13 and P100). To analyze the subcellular distribution of yeast aaRSs we performed a subcellular fractionation adapted from the protocol published by Bonangelino and collaborators [78].

- Yeast cells are inoculated in the morning or the previous evening depending on the generation time in the appropriate medium (YPD or SC dropout) on a rotary shaker at 200 rpm at 30 °C.
- In the evening, the cells are diluted in 100 mL of the same medium to reach an OD_{600nm} of about 0.5 in the morning. 40 units of OD_{600nm} are transferred to a 50 mL Falcon tube. From here on out, all steps are performed on ice and centrifugations are performed at 4 °C.
- The cell suspension is centrifuged for 5 min at 5250*g* at 4 °C to pellet the cells. For cells grown on SC dropout medium, add 1 mL of YPD to facilitate the pelleting.
- Cells are washed twice in 20 mL of ice cold fractionation buffer (20 mM Hepes/KOH, pH 6.8, 50 mM CH_3COOK, 10 mM $MgCl_2$, 250 mM Sorbitol, 10 mM NaN_3).
- Cells are then resuspended in 800 μL of fractionation buffer (with protease inhibitor cocktail cOmplete ULTRA Tablets, EDTA-free from Roche™) and transferred into a 15 mL cold Corex®II centrifuge tube (ref. 1-8441-15) containing 650 μL of glass beads (acid washed 0.25–0.5 mm diameter beads).

- The tube is vortexed 6 times for 30 s at full speed to lyse cells.
- The lysat is transferred to a 1.5 mL microfuge tube and centrifuged at 4 °C for 5 min at 500*g* in a fixed-angle rotor to clarify it.
- The S5 supernatant is carefully transferred to a fresh microfuge tube and centrifuged at 4 °C for 10 min at 13,000*g* in a fixed-angle rotor.
- The pellet (P13), which mainly contains the plasma membrane, the endoplasmic reticulum, the nucleus, the vacuole and mitochondria, is kept on ice until the end of the experiment.
- The supernatant (S13) is transferred in a 1.5 mL ultracentrifugation microfuge tube (Beckman Coulter® ref. 357448) for further centrifugation at 4 °C for 1 h at 100,000*g* in an ultracentrifuge using a fixed rotor.
- The supernatant (S100), which contains cytosol and vesicles, is carefully transferred to a fresh 1.5 mL microfuge tube. The pellet (P100) contains Golgi and endosomes.
- P13 and P100 are subsequently resuspended in fractionation buffer in the same volume as the S100 fraction.

4.2. Purification of nuclei and preparation of nuclear protein extracts

Purification of nuclei needs to be performed quickly to avoid passive diffusion of small proteins through nuclear pores. Moreover, the composition of buffers is critical for maintaining osmotic balance to prevent nuclear proteins from leaving the nucleoplasm fraction.

4.2.1. Purification of nuclei from yeast cells

This method is an adaptation from the original protocol of Stephanie E. Rieder and Scott D. Emr [79].

- Yeast cells from 2 L of culture in rich or synthetic medium, depending on auxotrophic markers or studied conditions, are harvested at 0.7 OD_{600} and washed once with water to ensure that all traces of medium are eliminated.
- The wet pellet is weighed (approximately 7 g/2 L of culture). Pellet is resuspended in 100 mM Tris H_2SO_4, 10 mM DTT in a volume of 2 mL/g of wet cells and incubated 20 min at 30 °C with gentle agitation. The purpose of this step is to weaken the cell wall.
- The mixture is centrifuged 4 min at 3500*g* and the pellet is washed with 1.1 M sorbitol to keep osmotic pressure, 100 mM K_2HPO_4/KH_2PO_4, pH 7.4 using 7 mL of buffer per g of cells and resuspended with the same buffer.
- Zymolase 20T is added to the mixture (5 mg/g of pellet) and incubated during 45 min at 30 °C with gentle agitation (80 rpm), to digest the cell wall.

All following steps are performed at 4 °C, to avoid protein degradation. Approximately 3.5 g of wet cells should be settled by falcon 50 (two falcons 50 for 2 L of culture at 0.7 OD_{600nm}). Note that all the subsequent steps are detailed for the treatment of 1 falcon 50 out of the two necessary to treat the whole 2 L culture.

- The spheroplasts (cells without their wall) are centrifuged 4 min at 3500*g*, gently resuspended in 1.1 M sorbitol, 100 mM K_2HPO_4/KH_2PO_4, pH 7.4 to remove the zymolase and centrifuged again 4 min at 3500*g*.
- The pellet is resuspended in 20 mL 1.1 M sorbitol, 100 mM K_2HPO_4/KH_2PO_4, pH 7.4 and loaded on a 6 mL cushion containing 30 mM sorbitol, 5% (v/v) ficoll 400, with protease inhibitors. The spheroplasts are centrifuged 10 min at 4000*g*.
- The pellet is resuspended into 15 mL of ice cold ficoll 20% (v/v), 20 mM K_2HPO_4/KH_2PO_4, pH 7.4, 1 mM $MgCl_2$ and protease inhibitors, and the spheroplasts are broken by osmotic shock in potter by 20 S in maximum 5 min. The ficoll keeps the osmotic pressure between the medium and the nuclei, to avoid nuclear protein from diffusing out of the nucleus.
- The lysate is incubated 10 min on ice to let nuclei cool down prior to subsequent centrifugation during 5 min at 13,000*g*.
- The supernatant is collected and immediately centrifuged again 10 min at 13,000*g*. At this step, nuclei cannot pellet because of the viscosity of the buffer.
- The supernatant is then loaded on a ficoll gradient containing 20 mM K_2HPO_4/KH_2PO_4 pH 7.4, 1 mM $MgCl_2$, protease inhibitors and ficoll 50% (v/v), ficoll 40% (v/v), ficoll 30% (v/v), 6.5 g each in 25 × 89–mm ultracentrifuge tube (Beckman Coulter®, Ultra Clear Tubes, 38.5 mL, ref. 344058). Then, the gradient is ultracentrifuged 1 h at 58,000*g* (Beckman, SW28 rotor, 18,000 rpm).
- After the centrifugation, the 20 and 30% layers containing all the cellular debris are removed. The 40% layer and the interface 40–50% containing the nuclei are harvested by pipetting. Nuclei are also present in the interface 30–40%, but this interface layer is not harvested because it contains too many non-nuclear contaminants.
- To remove the ficoll, the samples are diluted 10 times in 20 mM K_2HPO_4/KH_2PO_4, pH 7.4, 1 mM $MgCl_2$ and centrifuged 10 min at 10,000*g* to pellet nuclei (since the ficoll is diluted, nuclei are able to pellet at 3000*g*).
- Nuclei are finally resuspended in 500 μL of buffer containing 100 mM Tris HCl pH 7.8, 0.1 mM EDTA, 5 mM β-Mercaptoethanol, 1 mM benzamidine, protease inhibitors, and sonicated for 80 s (1 s on/1 s off, 30% amplitude, Bioblock Vibracell) in order to disrupt the nuclear enveloppe.
- After a chilling step on ice to avoid foaming, nuclei are resonicated for 30 s (1 s on/1 s off, 30% amplitude, Bioblock Vibracell).
- The nuclear extracts are ultra-centrifuged 30 min at 100,000*g* to pellet nuclear membranes. The supernatant contains soluble nuclear proteins.

4.2.2. Purification of nuclei from human cells

This protocol is adapted from Jason M. Dahlman and Denis C. Guttridge [80]. Perform all steps at room temperature unless otherwise specified. Volumes are determined for the preparation of 10 samples. All centrifugation was performed in a bench top microfuge. A minimum of 1×10^6 cultured cells are used for this protocol.

- Cell culture media is removed from the plates.
- Cells are gently washed twice with 1 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).
- After the addition of 1 mL of PBS, cells are removed from the polystyrene tissue culture dish using a cell scraper.
- Scraped cells (1 mL) are removed with a 1 mL pipette and placed in a 1.5 mL microcentrifuge tube.
- Cells are centrifuged at 250*g* for 5 min at 4 °C.
- After centrifugation, supernatants are removed and 5 pellet volumes of cytoplasmic extraction buffer (CEB: 10 mM HEPES-KOH, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.25% (v/v) Tergitol-type NP-40 (Sigma-Aldrich), Complete protease inhibitor cocktail, EDTA-free (Roche)) are added to each sample.
- Cellular pellets are dissolved by flicking the tubes. It is important to minimize the amount of bubbles generated while flicking. After adding the cytoplasmic extraction buffer, let samples stand on ice for 1–3 min before resuspending and make sure that cellular pellets are completely dissolved.
- Resuspended pellets are incubated on ice for 5 min and then, centrifuged at 650*g* for 4 min at 4 °C.

- After centrifugation, supernatants, containing the cytoplasmic proteins, are carefully transferred to new 1.5 mL microcentrifuge tubes. While removing the supernatant, take special care not to disrupt the nuclear pellet, which now should take on an opaque appearance. The cytoplasmic extract can be either disposed of or stored at −80 °C.
- 100 μL of cytoplasmic wash buffer (CWB: 10 mM HEPES-KOH, pH 7.6, 60 mM KCl, 1 mM EDTA, Complete protease inhibitor cocktail, EDTA-free (Roche)) is added to the nuclear pellets to remove the excess of NP-40. The 1.5 mL microcentrifuge tubes are gently tapped to dislodge the nuclear pellets. There is no need to resuspend the pellets.
- Centrifugation is repeated at 650*g* for 4 min at 4 °C.
- Supernatants are carefully discarded, once again taking care not to disturb the nuclear pellets.
- 1 pellet volume of nuclear extraction buffer (NEB: 20 mM Tris HCl pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, Complete protease inhibitor cocktail, EDTA-free (Roche)) is added to the 1.5 mL microcentrifuge tubes containing the nuclear pellets and they are resuspended by flicking the tubes until they are completely resuspended. Try to minimize the amount of bubbles you generate while flicking.
- The resuspended nuclear pellets are incubated on ice for 10 min and mixed by flicking every 2 min.
- After the 10 min incubation, the resuspended nuclear pellets are centrifuged at 15,000*g* for 10 min at 4 °C.
- After centrifugation, supernatants, containing the nuclear proteins, are transferred to new pre-chilled 1.5 mL micro centrifuge tubes
- Nuclear extracts are stored at −80 °C.

4.3. Purification of mitochondria and preparation of mitochondrial protein extracts

4.3.1. Purification of mitochondria from yeast cells

To analyze the mitochondrial localization of aaRSs, we performed a mitochondria purification (described below) adapted from the protocol published by Meisinger and collaborators [48].

- All steps are perform at 4 °C unless otherwise specified. To get enough mitochondria it is recommended to use at least 2–3 L of yeast cells grown to log-phase.
- Yeast cells are pelleted by centrifugation at 3000*g* for 5 min and resuspended in distilled water.
- Cells are pelleted again as for nuclei preparation, the wet weight is measured and the pellet is resuspended at a concentration of 2 mL/g of pellet in prewarmed at 30 °C DTT buffer (100 mM Tris-H_2SO_4, pH 9.4, 10 mM dithiothreitol (DTT)).
- After 20 min incubation in a 30 °C shaker at 80 rpm, cells are pelleted at 3000*g* for 5 min and resuspended in 7 mL/g of zymolyase buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4), pelleted again at 3000*g* for 5 min and resuspended at a concentration of 7 mL/g of cells in zymolyase buffer containing 3 mg Zymolyase 20T (Seikagaku Kogyo Co., Tokyo, Japan) per gram wet weight.
- After incubation for 45 min in 30 °C shaker at 80 rpm, cells are pelleted at 3000*g* for 5 min and resuspended in 7 mL/g of fresh zymolyase buffer, pelleted again at 3000*g* for 5 min and resuspended (6.5 mL/g wet weight) in ice-cold homogenization buffer (0.6 M sorbitol, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 0.2% (w/v) BSA (Sigma-Aldrich), and 1 mM of freshly prepared PMSF).
- From this step, it is very important to maintain the lysate, buffers and rotors at 4 °C to prevent proteolysis.
- Samples are poured in a glass Teflon homogenizer and spheroplasts are homogenized with 15 S.
- Samples are then 2-fold diluted with ice-cold homogenization buffer and the lysate is cleared by centrifugation at 1500*g* for 5 min to remove cell debris and nuclei.
- The supernatant is centrifuged at 4000*g* for 5 min, the pellet is discarded and the supernatant is centrifuged again at 12,000*g* for 15 min to isolate the mitochondrial fraction.
- The supernatant is discarded and the mitochondrial pellet is resuspended in 0.5 to 1 mL of SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and the protein concentration is measured using the Bradford method and adjusted to 5 mg/mL.

In order to obtain highly purified mitochondria, firstly prepare sucrose step gradients in Beckman centrifuge tubes (Beckman Coulter®, Ultra Clear Tubes, 13.2 mL, ref. 344059):

- 1.5 mL 60% (w/v) sucrose diluted in EM buffer (1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) is loaded at the bottom of the centrifuge tube.
- Without disturbing the phases, 4 mL 32% (w/v), 1.5 mL 23% (w/v), and 1.5 mL 15% (w/v) sucrose/EM are carefully pipetted stepwise and the tubes are kept at 4 °C.
- Samples are homogenized again in a glass Teflon homogenizer (10–15 strokes) and poured (0.2 to 1 mL) to the sucrose gradient.
- The clarified samples are then centrifuged (Beckman, SW41 Ti rotor) at 134,000*g* for 1 h at 4 °C and the highly purified mitochondria are collected with a Pasteur pipette from the 60–32% sucrose interface.
- Mitochondrial samples are diluted 2-fold in SEM buffer and centrifuged at 12,000*g* for 15 min. Repeat this step to wash the purified mitochondria.
- Pellet can be frozen at −80 °C.

For extraction of soluble mitochondrial proteins, mitochondria are disrupted by sonication (see below) otherwise they are resuspended in SEM buffer and the concentration is adjusted as required.

- Mitochondria pellets are resuspended in 1 vol of lysis buffer (50 mM Tris-HCl, pH 7.2, 1.1 M Sorbitol, 2 mM EDTA; 5 mM β-mercaptoethanol).
- Sonication (4 run of 10 S with a 2 s duration time, amplitude 25%; Vibracell) is performed with a small probe (2 mm diameter) on ice.
- Broken mitochondria are then centrifuged for one hour at 105,000*g* at 4 °C to eliminate unbroken mitochondria and disrupted membranes.
- The supernatant which is recovered, corresponds to soluble mitochondrial proteins and can be frozen at −80 °C.

4.3.2. Obtention of mitoplasts from yeast mitochondria

For generation of mitoplasts, digitonin is used to permeabilize the outer mitochondrial membrane of yeast mitochondria.

- Mitochondria are suspended at a concentration of 1 mg/ml in SEM buffer.
- 250 μg of mitochondria are incubated for 25 min on ice in 250 μl of SEM buffer containing 0.05% to 0.2% digitonin.
- The resulting mitoplasts are harvested by spinning at 14,000 rpm for 10 min and the supernatant is saved for further analysis.
- The mitoplast containing pellet is washed once again with SEM buffer.
- To determine the efficiency of digitonin treatment, 100 μg of mitochondria are treated as above with digitonin, centrifuged,

and the pellet and supernatant fractions separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore). Immunoblots are performed using antibodies specific for cytochrome *c* peroxidase (CCPO) (intermembrane space), TIM23 (inner membrane), delta-1-pyrroline-5-carboxylate dehydrogenase (Put2p) (matrix) and porin (outer membrane).

4.3.3. Purification of mitochondria from human cells

This protocol is adapted from Jason M. Dahlman and Denis C. Guttridge [80] and has been designed to extract mitochondria from HeLa cells. Volumes are adapted to cells grown in 5 flasks of 58 cm^2. All steps are performed on ice or at 4 °C.

- Cell culture growth media from desired cells is removed.
- Cells are gently washed twice with 5 mL of PBS (see Section 4.2.2).
- Cells are removed from the polystyrene tissue culture dish by adding 1 mL of PBS and the cells are scraped gently with a cell scraper.
- Cells are centrifuged for 5 min at 250*g* at room temperature
- The pellet is resuspended in 1 mL of BSA buffer (0.6 M sorbitol, 1 mM EDTA, 20 mM Hepes-KOH, pH 7.6, 300 mM NaCl, Complete protease inhibitor cocktail, EDTA-free (Roche) and 0.3% (v/v) of BSA (1 mg/mL)).
- Cells are then mechanically broken on ice by repeated (at least 80 times) aspiration/backflow using a 1 mL syringe with a G23 needle.
- The lysate is centrifuged twice at 1200*g* for 6 min at 4 °C.
- The supernatant is then centrifuged at 16,000*g* for 40 min at 4 °C.
- Pellet can be frozen at −80 °C.

4.3.4. Obtention of mitoplasts from human mitochondria

For generation of mitoplasts, digitonin is used to permeabilize the outer mitochondrial membrane of human mitochondria.

- Mitochondria-containing pellet is resuspended in 300 μL BSA buffer (0.6 M sorbitol, 1 mM EDTA, 10 mM Hepes-KOH, pH 6.7 and 0.3% (v/v) of BSA (1 mg/mL)) containing 0.2% digitonin (50 μL of a 1 mg/mL stock solution) and incubated 15 min at room temperature.
- 1 mL of BSA buffer is added and the mixture is centrifuged 20 min at 16,000*g* at 4 °C.
- The resulting pellet is washed two times with 1 mL BSA buffer without resuspending the mitoplasts.
- The final mitoplasts-containing pellet can be resuspended in various types of buffers depending on the subsequent analyses that will be performed (TRIzol (Invitrogen) for RNA extraction, Laemmli for SDS-PAGE analysis...) or stored at −80 °C as a wet pellet.

5. Mass spectrometry analysis and identification of cytosolic aaRSs in yeast mitochondrial extracts

We performed yeast mitochondria purification as explained in the Section 4.3.1 and recovered pure mitochondria at the 32–60% interphase from a cushions gradient (Fig. 1A). In order to identify mitochondrial proteins, mitochondria were disrupted (see Section 4.3.1) and soluble proteins were subjected to mass spectrometry (see protocol below). For analysis, we carried out 3 different injections, two of 90 min (one regular and one using restricted mass ranges of 400–800 *m*/*z* and 800–1250 *m*/*z*, termed Frac.) and one of 120 min. These features allowed us to identify 905 (90 min.), 982 (90 min. Frac.) and 926 (120. min) mitochondrial proteins respectively (Fig. 1B). We describe here the detailed procedure for protein digestion and mass spectrometry analysis as well as data analysis.

5.1. Protein digestion solution

- Proteins from mitochondrial extracts are resuspended in 50 mM ammonium bicarbonate.
- After a reduction-alkylation step (5 mM DTT and 10 mM iodoacetamide), proteins are digested overnight with 100 ng of sequencing-grade trypsin (Promega).
- After centrifugation at 12,000*g*, the supernatants are collected in glass inserts and vacuum dried.

5.2. Nano-liquid Chromatography – electrospray Ionization TripleTOF MS/MS Analysis

- Before injection, dried peptides are resuspended in 15 μL of 0.1% (v/v) formic acid. One third of each sample was injected on a NanoLC-2DPlus system (nanoFlexChiP module; Eksigent, ABSciex, Concord, Ontario, Canada) coupled to a TripleTOF 5600 mass spectrometer (ABSciex) operating in positive mode.
- Peptides are loaded with a trap and elute configuration on C18 reverse-phase columns (ChIP C-18 precolumn 300 μm ID × 5 mm ChromXP and ChIP C-18 analytical column 75 μm ID × 15 cm ChromXP; Eksigent).
- Peptides are eluted by using a 5–40% gradient of 0.1% (v/v) formic acid in acetonitrile for 90 or 120 min at a 300 nL/minute flow rate. The TripleTOF 5600 was operated in high-sensitivity data-dependant acquisition mode with Analyst software (v1.6, ABSciex) on a 400–1250 *m*/*z* range.
- To extend the sensitivity, two new injections are carried out using restricted mass ranges, 400–800 *m*/*z* and 800–1250 *m*/*z*. An external calibration is performed before each sample by monitoring 10 peptides of a beta-galactosidase trypsic digest. A discovery "Top20" method is used: up to 20 of the most intense multiply-charged ions (2+ to 5+) are selected for CID fragmentation, with a cycle time of 3.3 s.

5.3. Database search and data analysis

Raw data are first converted to Mascot Generic File format (.mgf) and searched against the yeast *S. cerevisiae* database supplemented by a decoy database (reverse sequences). The database search algorithm used is Mascot (version 2.2, Matrix Science, London, UK) through the ProteinScape 3.1 package (Bruker Daltonics, Leipzig, Germany). Peptide modifications allowed during the search are: N-acetyl (protein), carbamidomethylation(C) and oxidation (M). Mass tolerances in MS and MS/MS is set to 20 ppm and 0.5 Da, respectively. Two trypsin missed cleavages sites is allowed. Peptide identifications obtained from Mascot is validated with a protein FDR < 1%, using the Protein Assessment tool from ProteinScape. Identified proteins are assessed by spectral count.

The aaRSs identified in the mitochondrial extract analyzed by three different injections methods are presented in Fig. 1C and Supp. Table 2. Excepting the Arg-, Met- and Thr-RS (note that there are no genes encoding the mitochondrial CysRS and GlnRS in yeast), we found all the mitochondrial aaRSs in our assay. The absence of these three mitochondrial aaRSs could be due to a strong binding to the mitochondrial membrane that resulted in their loss during the protein extract preparation. Indeed, it has previously been shown that a functional aaRS could be membrane-anchored [81]. In addition to the mitochondrial aaRSs, we identified 10 cytosolic ones (Asp-, Glu-, Ile-, Leu-, Lys-, Phe-, Pro, Ser-, Thr- and Tyr-RS) of which only the cytosolic GluRS has been shown experimentally to be dual-localized [28,29]. Since there is no gene identified for the yeast mitochondrial CysRS, we assume

Table 6
List of markers for specific compartments in mammalian cells and their respective antibodies.

Compartment	Protein marker*	Antibody
Nucleus	Lamin B1	Abcam ab16048
Plasma membrane	Cadherin	Abcam ab6528
Endoplasmic Reticulum	Calnexin	Abcam ab22595
	Calreticulin	Abcam ab2907
	GRP-78	Abcam ab21685
Golgi	GM130	Abcam ab31561
	Giantin	Abcam ab80864
	TGN46	Abcam ab2809
Mitochondria	COX IV-1	Abcam ab14744
	Cytochrome *c*	Abcam ab13575
Endosomes	Early endosome antigen 1	Abcam ab2900
	Ras related protein Rab-5A	Abcam ab18211
	Ras related protein Rab-7a	Abcam ab126712
Lysosomes	LAMP-1	Abcam ab24170
	LAMP-2	Abcam ab18529
Autophagosomes	MAP1A/MAP1B LC3 A	Abcam ab52768
Peroxisomes	Catalase	Abcam ab16771

* Protein marker names are recommended names or short names from the UniProt database.

that the cytosolic form would also be mitochondrial; and we, indeed, found it in the mitochondrial fraction we analyzed. It is now important to confirm the mitochondrial localization of these cytosolic aaRSs and then determine their function in this other compartment.

6. Microscopy analysis and single cell localization

Unlike subcellular fractionation, microscopic analysis allows the study of aaRSs localization at the single cell scale. For more precision, a confocal microscope is needed to set only one focal plan and prevent accumulation of cytosolic fluorescence around the organelle. If the aaRS is fused to GFP, analysis can be performed directly on living cells, and aaRSs used with a GFP-tag are listed in Table 4. To control the aaRS localization on living cells, the need of compartment markers is essential, like DAPI and Mitotracker for nuclear and mitochondrial compartment, respectively. For yeast cells, some additional fluorescent dyes can also be used for fluorescent microscopy on living cells, as CellTracker Blue CMAC (ThermoFischer Scientific, ref. C2110) that is specific for vacuolar lumen (DAPI filter), or FM4-64 (ThermoFischer Scientific, ref. T3166) used in time lapse experiments to stain the plasma membrane, the endosomes and the vacuolar membrane (RFP filter) [82]. For more precision, protein markers can be targeted by specific antibodies: frequently used markers for mammalian intracellular compartments are listed in Table 6. As GFP can possibly perturb the aaRS localization due to its large size, smaller tags (listed in Table 4) have been used for immunofluorescence (IF) study. For IF, a fixation step is essential to allow antibodies penetration in the sample. Next sections present immunofluorescence protocols for both yeast and human cells.

6.1. Immunofluorescence on yeast cells

This technique allows the observation by fluorescent microscopy of the aaRSs of interest on fixed yeast cells.

6.1.1. Fixation steps

- Cells are grown overnight in 20 mL of either complete or synthetic medium (YPD or SC dropout medium) to about 1×10^7 cells/mL (OD_{600nm} = 0.4–0.6); 10 mL culture gives 6 samples.
- 1.25 mL of 1 M K_2HPO_4/KH_2PO_4, pH 6.5 and 1.25 mL of 37% (v/v) formaldehyde are added to 10 mL of overnight culture (10×10^7 cells) and incubated for 2 h at room temperature with gentle shaking every 30 min.
- Cells are centrifuged for 5 min at 3000*g*, washed 3 times with 10 mL SP buffer (1.2 M sorbitol, 0.1 M KPO_4, pH 6.5) and resuspended in 1 mL SP/ME (SP, 20 mM β-mercaptoethanol).
- 10 μL Zymolyase 20T (5 mg/mL) is added and the mixture is incubated 45 min at 30 °C (gentle shacking every 10 min); the amount of Zymolyase needed depends on the strain.
- Cells are centrifuged for 5 min at 2000*g*, washed 3 times with 3 mL SP buffer and resuspended in 0.1 mL SP; after the digestion of the cell wall, the resuspension has to be very gentle to avoid lysis.
- Cells are stored at 4 °C (can be kept for 1–2 weeks).

6.1.2. Incubation with antibodies

The following steps are done on a slide for immunofluorescence (HTC or epoxy, single use slides, diameter 6 mm) at room temperature.

- Slides are washed with ethanol 96% (v/v).
- 20 μL of poly-Lysine (0.1% (w/v) in H_2O) is added to each well and incubated for 1 min.
- The liquid is removed, slides are dried and washed 3 times with 20 μL H_2O; all washing steps are done by adding one drop (with Pasteur pipette) and removing with vacuum without touching the slide.
- Slides are placed in a humid chamber (Petri dish with H_2O-soaked paper), 15 μL of fixed yeast cells are added to each well and incubated for 30 min.
- The excess of liquid is removed very gently and slides are washed twice with PBS 1× (see Section 4.2.2).
- Slides are then incubated for 5 min with 15 μL of PBT (PBS 1×, Triton X-100 0.1% (v/v), NaN_3 0.1% (w/v), BSA 1% (w/v)).
- After removing the excess of liquid, slides are incubated for 30 min at room temperature with 15 μL of primary antibody diluted in PBT, according to manufacturer indications.
- Slides are washed 10 times with PBT, followed by the incubation for 30 min in the dark and at room temperature with 15 μL of secondary antibodies (either Cy-conjugated or Alexa Fluor®-conjugated; ThermoFischer Scientific) diluted in PBT.
- Slides are washed 10 times with PBS.
- Optional: Nuclei can be stained with 15 μL of DAPI (10 μg/mL) and incubated 5 min in the dark. 5 washes with PBS are needed to avoid non-specific background fluorescence.
- Finally, 3 μL of Vectashield® (Vector laboratories) are added and slides are covered with coverslip; fix coverslip on all ends with nail polish.
- Slides can be stored at 4 °C in the dark (can be kept for months) or directly observed by fluorescence microscopy.

6.2. Immunofluorescence on human cells

This technique allows observing the subcellular localization of the aaRSs of interest in fixed mammalian cells on a single cell scale with a fluorescence microscope.

- Cells are grown in complete medium, mostly DMEM (D5796 Sigma) or MEM (M2279 Sigma), supplemented with 10% (v/v) Fetal Calf Serum (FCS).
- On day one of the experiment, the medium of the culture flask is removed, cells are washed with PBS (see Section 4.2.2.) and detached using trypsin (T4049 Sigma).
- Cells are then spotted on 10-well spotslides (Marienfeld Superior, #1216650) to a 70% confluency (for most cell types 4000–5000 cells per spot), let to attach and grown overnight in a cell culture incubator at 37 °C, 5% CO_2.

- After removing the medium, cells are fixed by adding 50 μL of 4% (v/v) formaldehyde (F8775 Sigma) in PBS to the spots and incubated for 20 min at room temperature.
- If Mitotracker (Thermofisher Scientific, M7512) is used to detect mitochondria, Mitotracker staining has to be done on live cells prior to fixation. Therefore, cells are incubated for 15–45 min at 37 °C with Mitotracker diluted in pre-warmed medium (37 °C) to a concentration of 100–500 nM. Cells are then washed once in medium and once in PBS and fixed as described above.
- Cells are then washed once with PBS and permeabilized with 0.2% (v/v) Triton-X 100 (Sigma-Aldrich) in PBS for 10 min at room temperature.
- After one more wash with PBS, unspecific sites are blocked by incubation with 20% (v/v) FCS in PBS for one hour at RT.
- Cells are then incubated with the antibodies raised against the aaRS of interest and marker proteins of the different subcellular compartments in PBS-2% (v/v) FCS for two hours at room temperature or overnight at 4 °C.
- After washing five times with PBS, the fluorophore-conjugated secondary antibodies diluted in PBS-2% (v/v) FCS are added for one hour at room temperature. DAPI can be added to the secondary antibody solution at a concentration of 300 nM.
- Finally, after two final washes in PBS, a drop of pre-warmed (50 °C) elvanol (Poly(vinyl alcohol) – 341584 Sigma-Aldrich) is added to each spot and the slide sealed with a coverslip. Cells are then visualized using a confocal laser scanning microscope and fluorescence can be quantified with the ImageJ software.

7. Concluding remarks

Localizing multi-compartmental aaRSs cannot be done using a one-for-all technique. For example, using fluorescence microscopy to simultaneously visualize in a single cell, the organellar and cytosolic fractions of the same aaRS (or protein in general) is complicated since the fluorescence of the organellar pool will be masked by the fluorescence of the cytosolic portion which is usually more abundant. So far no tools have been developed to overcome this difficulty and the use of confocal microscopy is usually not sufficient when one wants to ascertain the localization of a minor organellar pool of a cytosolic aaRS. Moreover, like for any other protein putatively organellar, it is currently very difficult to distinguish between a lumenal localization of an aaRS and a peripheral localization of the protein on the cytosolic side of the organelle. Being able to make these distinctions is extremely important to be able to study the nonconventional roles of these multi-localized aaRSs and identify the cellular processes to which the organellar pools of these cytosolic aaRSs participate. Also, the possibility that several cytosolic aaRSs could be peripherally associated to membrane compartments other than the mitochondria and the nucleus by interacting either with membrane proteins or directly to lipids has been overlooked so far. For all these reasons, one still has to turn to the isolation of membranes and afterwards to organelle purification to be able to ascertain the relocation inside a given compartment of a fraction of a cytosolic aaRS (summarized in Table 1).

Another difficulty to overcome when working with dual-localized aaRSs is that the cytosolic pool of these proteins is always essential for viability, rendering the study of the functions of the organellar pools of the same aaRS very difficult. In this respect, yeast is a very convenient model especially when one wants to analyze the mitochondrial activity of a cytosolic aaRSs. Since these organelles are only essential for the respiratory metabolism and not when yeast is fermenting, mutations of the cytosolic aaRS gene that lead to only a respiratory deficiency (fermentative growth is unaffected) are targeting residues essential for the mitochondrial activity of the dual-localized aaRS without affecting its cytosolic role. This simple phenotypic screen was successfully used to identify the cryptic and nonconventional MTS of yeast cytosolic GluRS which participates to the mitochondrial transamidation pathway upon mitochondrial import [28]. It was also used to show that mitochondrial forms of His-, Val- and GlyRSs are generated through an alternative translational start [13,31,32].

If confirming that a pool of a cytosolic aaRS can relocate inside an organelle is difficult with the current technologies, identifying the import signals to study the dynamics of these proteins is even more arduous. Import signal predictors still have a low consensual predictive accuracy for these dual-localized aaRSs, mainly because they often contain nonconventional import signals. Identifying the organellar roles of dual-localized aaRSs will necessitate the reinterpretation of previous yeast mutant screens as well as establishing new mutant screens. In humans, the systematic sequencing of genes encoding cytosolic aaRSs of patients with mitochondrial-related diseases and in depth analysis of the localization, protein interactants and activity of the mutant aaRS is essential for identifying the organellar functions of dual-localized aaRSs. By doing so, it was recently shown that the mutation in the cytosolic GlyRS gene that causes type 2D Charcot-Marie-Tooth disease changes the protein binding activity of GlyRS that interferes with a signaling pathway essential for motor neuron survival [83].

These findings also raise many questions, the most important being how cells regulate the distribution of multi-compartmental cytosolic aaRS between the cytosol and the different organelles. One mechanism by which cells manage the subcellular distribution of the various pools of a cytosolic aaRS is by forming the MSCs and triggering release of each cytosolically-anchored aaRS by post-translational modification of the aaRS. Since so far, a maximum of 9 cytosolic aaRSs were found participating to MSCs, there are probably others posttranslational modifications, or other mechanisms that participate in regulating the dynamic compartmentalization of the remaining 11 potentially multi-localized aaRSs. Another important issue concerning the dynamical relocalization of these aaRSs is whether a portion of an existing pool is redistributed or a fraction of the neosynthesized proteins will specifically be deviated on demand towards the organelle? Finally, while in this review we focused on the relocalization of cytosolic aaRSs within the cell, it has to be noted that several human aaRSs can be secreted in the extracellular medium where they also accomplish nonconventional functions [84,85].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2016.09.017.

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