

# The intracellular location of two aminoacyl-tRNA synthetases depends on complex formation with Arc1p

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**In yeast, two aminoacyl-tRNA synthetases, MetRS and GluRS, are associated with Arc1p. We have studied the mechanism of this complex formation and found that the non-catalytic N-terminally appended domains of MetRS and GluRS are necessary and sufficient for binding to Arc1p. Similarly, it is the N-terminal domain of Arc1p that contains distinct but overlapping binding sites for MetRS and GluRS. Localization of Arc1p, MetRS and GluRS in living cells using green fluorescent protein showed that these three proteins are cytoplasmic and largely excluded from the nucleus. However, when their assembly into a complex is inhibited, significant amounts of MetRS, GluRS and Arc1p can enter the nucleus. We suggest that the organization of aminoacyl-tRNA synthetases into a multimeric complex not only affects catalysis, but is also a means of segregating the tRNA-aminoacylation machinery mainly to the cytoplasmic compartment.**

**Keywords:** aminoacyl-tRNA synthetase/ARC1/GluRS/  
MetRS/tRNA

## Introduction

Aminoacyl-tRNA synthetases are required for the faithful translation of the genetic code, as they catalyse charging of tRNAs with their cognate amino acids (Martinis *et al.*, 1999a,b; Ibba and Söll, 2000). The 20 different aminoacyl-tRNA synthetases, one for each amino acid, are divided into two classes (I and II) according to the sequence and structure of their catalytic domains, which are usually conserved in all kingdoms (Francklyn *et al.*, 1997; Woese *et al.*, 2000). They are also characterized by idiosyncratic tRNA-binding domains that are attached to, or inserted in, the class-defining catalytic core (Ibba *et al.*, 1997). When compared with the prokaryotic synthetases, the corresponding enzymes from yeast or higher eukaryotes often contain extensions to the N- or C-termini, which are not essential for catalytic activity (Mirande, 1991; Kisselev and Wolfson, 1994; Reed and Yang, 1994; Weygand-Durasevic *et al.*, 1996). Their function is not

always clear. In some cases, they serve as non-specific tRNA-binding platforms that facilitate the association of the synthetases with their cognate tRNAs (Whelihan and Schimmel, 1997; Wang and Schimmel, 1999; Cahuzac *et al.*, 2000; Frugier *et al.*, 2000). In other cases, these eukaryote-specific appendices are required for assembly into multisynthetase complexes (Rho *et al.*, 1996, 1999; Agou and Mirande, 1997; Kim *et al.*, 2000a), although catalytic domains may also be responsible for the formation of these complexes (Kim *et al.*, 2000a). Higher eukaryotes are indeed characterized by the presence of a supramolecular multienzyme complex containing nine aminoacyl-tRNA synthetases and three non-catalytic polypeptides, p43 (pro-EMAPII), p38 and p18 (Filonenko and Deutscher, 1994; Quevillon and Mirande, 1996; Norcum and Warrington, 1998; Quevillon *et al.*, 1999). The function of this complex is still unknown. It is possible that p43 could modulate the activity of the enzymes, as is shown to be the case for human ArgRS (Park *et al.*, 1999).

We previously identified in yeast a smaller complex of aminoacyl-tRNA synthetases consisting of methionyl-tRNA (MetRS) and glutamyl-tRNA (GluRS) synthetases, as well as the protein Arc1p, the yeast homologue of p43 (Simos *et al.*, 1996; Quevillon *et al.*, 1997). Arc1p associates with the two enzymes both *in vivo* and *in vitro*. Furthermore, the catalytic efficiency of the *in vitro* reconstituted complexes of MetRS or GluRS with Arc1p is substantially increased compared with the monomeric enzymes (Simos *et al.*, 1998; Deinert *et al.*, 2001). This stimulation is due to the increased affinity of the complexes for the cognate tRNAs and requires the C-terminal part of Arc1p. Indeed, this part of Arc1p harbours a tRNA-binding domain (TRBD), which is conserved in p43 as well as other proteins (Kleeman *et al.*, 1997; Morales *et al.*, 1999; Kaminska *et al.*, 2000). Surprisingly, in mammals this domain can also function as a cytokine (Knies *et al.*, 1998; Wakasugi and Schimmel, 1999; Behrendorf *et al.*, 2000), possibly linking the progression of apoptosis to the inhibition of protein translation (Weiner and Maizels, 1999). Recently, the structure of the TRBD of p43 has been solved, showing that part of it adopts the OB fold, an oligonucleotide-binding structural motif (Kim *et al.*, 2000b; Renault *et al.*, 2001). In yeast, Arc1p is required for optimal cell growth and is essential for viability in the absence of the tRNA nuclear export factor Los1p (Simos *et al.*, 1996).

Apart from their established role in tRNA aminoacylation, aminoacyl-tRNA synthetases are also implicated in several other cellular processes such as mitochondrial RNA splicing or transcriptional, as well as translational, regulation (reviewed in Martinis *et al.*, 1999a,b). Recently, aminoacyl-tRNA synthetases have also been implicated in nuclear tRNA export as inhibition of tRNA-aminoacylation causes accumulation of mature tRNAs

inside the nucleus of *Xenopus* oocytes (Lund and Dahlberg, 1998; Simos and Hurt, 1999). The requirement of tRNA-aminoacylation for efficient nuclear tRNA export was also observed in yeast cells (Sarkar *et al.*, 1999; Grosshans *et al.*, 2000). These observations indicate that aminoacyl-tRNA synthetases can enter the nucleus. Cell fractionation and biochemical experiments have demonstrated the presence of tRNA-aminoacylation activities in the nuclear compartment of higher eukaryotic cells (Lund and Dahlberg, 1998; Nathanson and Deutscher, 2000). However, direct intranuclear localization by microscopic methods has been performed for only a few aminoacyl-tRNA synthetases in mammalian cells (Kisselev and Wolfson, 1994; Popenko *et al.*, 1994; Ko *et al.*, 2000), and for one enzyme in yeast cells (Azad *et al.*, 2001).

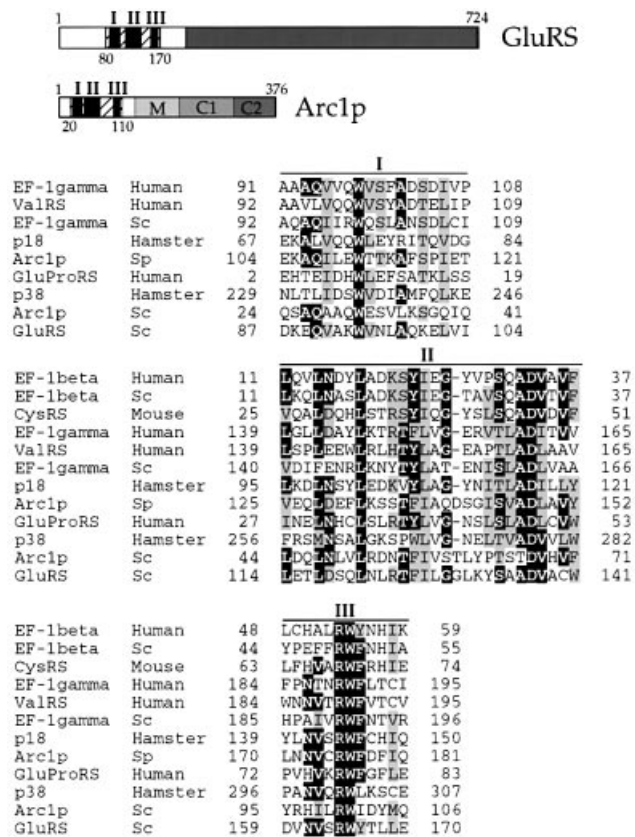
In order to understand the molecular details of the assembly of the Arc1p–MetRS–GluRS complex, we have expressed and purified from yeast truncated versions of all three components. Our results show that the formation of the complex *in vivo* requires the N-terminally appended non-catalytic domains of the synthetases, both of which interact with overlapping sites on the N-terminal domain of Arc1p. Furthermore, localization of these three components demonstrated that all of them can be located in the nucleus only if they are not assembled in a complex. Therefore, association of eukaryotic aminoacyl-tRNA synthetases into a multimeric complex provides a means of regulating their subcellular distribution.

## Results

### A functional link between the N-terminally appended domain of GluRS and Arc1p

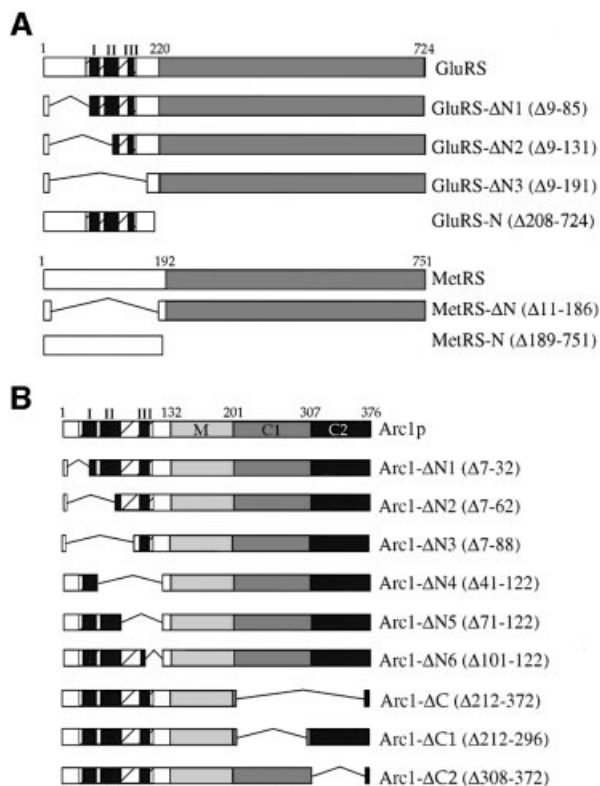
The structural genes for yeast cytoplasmic MetRS and GluRS, *MES1* and *YGL245W* (from now on called *GUS1*), respectively, encode proteins that contain N-terminal extensions when compared with the corresponding *Escherichia coli* enzymes. In the case of MetRS, the 185 residue long N-terminal domain is not homologous to known proteins and can be removed without affecting the activity or stability of monomeric MetRS (Walter *et al.*, 1989). In the case of GluRS, sequence alignment reveals an N-terminal extension of 210 amino acids, a part of which (residues 87–170) is homologous to several other proteins (Figure 1 and data not shown). These polypeptides include the N-terminal parts of GluRSs from other species as well as other aminoacyl-tRNA synthetases and proteins involved in translation, such as the  $\beta$  and  $\gamma$  subunits of the eukaryotic elongation factor EF1B and the C-terminal regions of p18 and p38, the two non-catalytic components of the mammalian multisynthetase complex (Koonin *et al.*, 1994; Quevillon and Mirande, 1996; Quevillon *et al.*, 1999). Strikingly, this domain is also present in the N-terminal region of Arc1p (amino acids 24–106) and its closely related homologue from *Schizosaccharomyces pombe* (*S.pombe* Arc1p).

To study the function of the appended N-terminal regions of GluRS and MetRS in the formation of the Arc1p complex, we generated truncation mutants as shown schematically in Figure 2A. Three different N-terminal deletion constructs were made in the case



**Fig. 1.** Top: schematic representation of the *S.cerevisiae* GluRS and Arc1p protein sequences. In GluRS the grey box represents the catalytic domain, while in Arc1p it represents the different domains as indicated. In both proteins the hatched box represents a conserved motif. The parts of the motif corresponding to the homology blocks shown below are marked as black bars numbered I–III. The M, C1 and C2 domains of Arc1p correspond to amino acids 132–201, 202–307 and 308–376, respectively. Bottom: alignment of three homologous sequence blocks (I–III) from the  $\beta$  and  $\gamma$  subunits of the human and *S.cerevisiae* (Sc) EF-1B, mouse cysteinyl-tRNA synthetase (CysRS), human valyl-tRNA synthetase (ValRS), the non-catalytic components of the Chinese hamster ovary multisynthetase complex p18 and p38, human bifunctional glutamyl-prolyl-tRNA synthetase (GluProRS), *S.cerevisiae* glutamyl-tRNA synthetase (GluRS) and *S.cerevisiae* and *S.pombe* (Sp) Arc1p. Identical residues are shown white on a black background, while partially conserved residues are in grey boxes. The numbers of the first and last residue in each block are indicated. Alignment was performed using the ClustalW program.

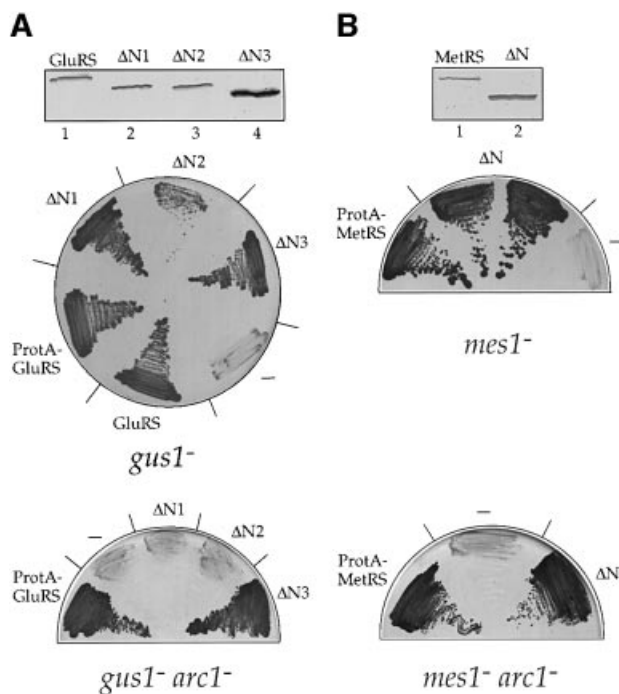
of GluRS: GluRS- $\Delta$ N1 (lacking residues 9–85), GluRS- $\Delta$ N2 (lacking residues 9–131) and GluRS- $\Delta$ N3 (lacking residues 9–191). In the case of MetRS, one mutant (MetRS- $\Delta$ N) lacking the entire N-terminal part (residues 11–186) was constructed. Subsequently, full-length MetRS, GluRS and the N-terminal deletion constructs were tagged with protein A (ProtA) to facilitate their detection and purification. All the truncated constructs were stably expressed and functional, as shown by their ability to complement the corresponding *gus1* and *mes1* null strains (Figure 3). Only in the case of GluRS- $\Delta$ N2 did the cells grow more slowly, while all other mutants complemented to wild-type levels. This shows that the N-terminal appended domains of both GluRS and MetRS are not required for cell growth and, consequently, aminoacylation activity. However, since GluRS- $\Delta$ N3 and



**Fig. 2.** Schematic representation of deletion mutants used in this study. (A) Deletion mutants of GluRS and MetRS. White and grey boxes indicate the non-catalytic N-terminal domains and the catalytic domains of the enzymes, respectively. The hatched boxes represent the conserved motif in the N-terminal domain of GluRS and the vertical black bars the homology blocks shown in Figure 1. (B) Deletion mutants of Arc1p. The N-terminal (N), middle (M) and C-terminal (C1 and C2) domains of Arc1p are indicated by different shades of grey. The hatched box in the N-domain represents the conserved motif also found in GluRS and the vertical black bars the homology blocks shown in Figure 1. The M, C1 and C2 domains of Arc1p correspond to amino acids 132–201, 202–307 and 308–376, respectively. The position of each deletion is represented by a line. Numbers refer to amino acid residues. Numbers in parentheses indicate the deleted amino acids.

MetRS-ΔN are expressed in higher amounts than the corresponding full-length proteins (Figure 3), it is possible that the reduced functionality of the N-terminally truncated proteins is compensated for by overproduction.

We then tested whether the deletions in the N-terminal domains of GluRS and MetRS affect cell growth when the expression of Arc1p is simultaneously abolished. For this reason we constructed haploid yeast strains in which the deletion of the chromosomal copies of either *MES1* or *GUS1* genes was combined with the disruption of the *ARC1* gene. As shown in Figure 3A, GluRS-ΔN3 lacking the entire N-domain could still complement the double-disrupted *gus1<sup>-</sup> arc1<sup>-</sup>* strain. However, GluRS-ΔN1 or GluRS-ΔN2, which lacked parts of the N-domain, could not rescue these cells, exhibiting a synthetic lethal relationship with Arc1p. This demonstrates a functional link between the N-domain of GluRS and Arc1p. Since both GluRS-ΔN1 and GluRS-ΔN2 contain the catalytic domain of GluRS, which is sufficient for survival (as in GluRS-ΔN3), these results also indicate that the shortened

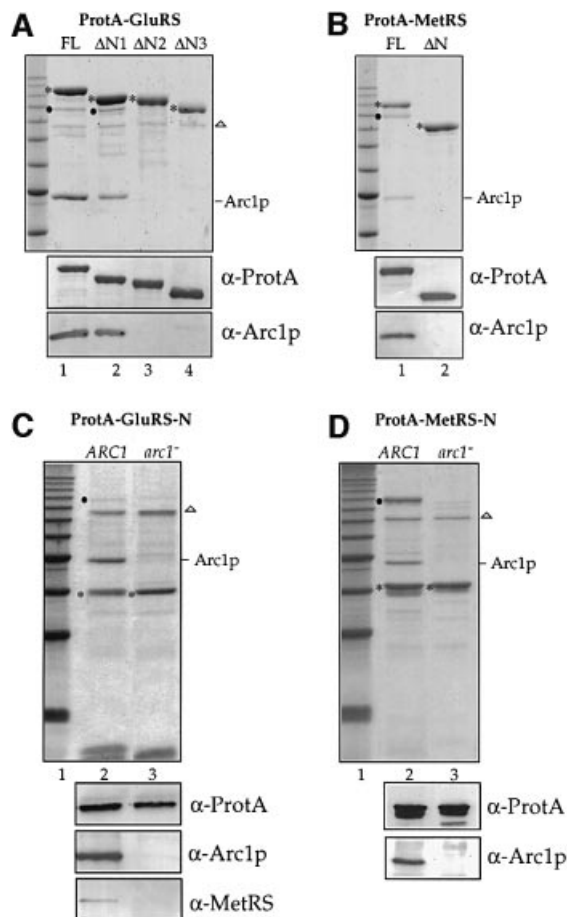


**Fig. 3.** Genetic analysis of the N-terminal truncation mutants of (A) GluRS and (B) MetRS. Upper panels: western blotting analysis of extracts from cells expressing ProtA-tagged full-length or N-terminally truncated forms of GluRS (A, lanes 1–4) and MetRS (B, lanes 1 and 2) in the strains *gus1<sup>-</sup>* and *mes1<sup>-</sup>*, respectively, using an anti-ProtA antibody. Equal amounts of extracts were loaded in each lane. Middle panels: complementation of the knock-out strains *gus1<sup>-</sup>* (A) and *mes1<sup>-</sup>* (B) by constructs expressing full-length or N-terminally truncated ProtA-tagged GluRS or MetRS, respectively, as indicated. Lower panels: complementation of the double knock-out strains *gus1<sup>-</sup> arc1<sup>-</sup>* (A) and *mes1<sup>-</sup> arc1<sup>-</sup>* (B) by constructs expressing full-length or N-terminally truncated ProtA-tagged GluRS or MetRS, respectively, as indicated. Complementation is shown by the ability to lose the pURA3-GUS1 (A) or pURA3-MES1 (B) plasmids and grow in the presence of 5-FOA. – indicates transformation with an empty plasmid.

N-domain exhibits a dominant-negative phenotype when Arc1p is missing (see Discussion). The double-disrupted *mes1<sup>-</sup> arc1<sup>-</sup>* strain was equally well complemented by full-length MetRS or MetRS-ΔN, showing that the complete removal of the N-domain of MetRS can be tolerated even in the absence of Arc1p (Figure 3B).

### **The N-terminally appended domains of MetRS and GluRS are both necessary and sufficient for binding to Arc1p**

To find out whether the appended N-domains of GluRS and MetRS are involved in the formation of the Arc1p–MetRS–GluRS complex, the ProtA tagged truncation mutants were purified by IgG–Sepharose affinity chromatography from total cell extracts of yeast cells expressing the corresponding mutants. This analysis revealed that when the N-terminal domains of GluRS and MetRS are missing, binding to Arc1p and formation of the ternary complex are abolished (Figure 4A, lanes 3 and 4, and B, lane 2). However, in the case of ProtA–GluRS-ΔN1, association with Arc1p and MetRS still persists, showing that the non-conserved first 85 residues of GluRS do not participate in complex formation (Figure 4A, lane 2). The apparent stoichiometry of the



**Fig. 4.** The N-domains of MetRS and GluRS are both essential and sufficient for stable association with Arc1p. Affinity purification by IgG-Sepharose chromatography of (A) the full-length (FL) ProtA–GluRS and the three N-terminal truncated forms expressed in the *gus1*<sup>-</sup> strain; (B) the full-length (FL) ProtA–MetRS and the N-terminal truncated form expressed in the *mes1*<sup>-</sup> strain; (C) the N-terminal domain of GluRS (ProtA–GluRS-N) from wild-type (*ARC1*) and *arc1*<sup>-</sup> strains; (D) the N-domain of MetRS (ProtA–MetRS-N) from wild-type (*ARC1*) and *arc1*<sup>-</sup> strains. Upper panels: analysis of the column eluates by SDS–PAGE and Coomassie Blue staining. The first lane from the left contains the 10 kDa ladder molecular weight markers (the stronger band corresponds to 50 kDa). The asterisks indicate the positions of the ProtA–fusion proteins. The black dots indicate the position of co-purifying MetRS (A and C) or GluRS (B and D). The identity of the protein bands corresponding to GluRS and MetRS in (A) and (B) was confirmed by mass spectroscopic analysis. The position of co-purifying Arc1p is indicated on the right. The protein band (which is indicated by a triangle) at 75 kDa corresponds to an irrelevant contaminant. Lower panels: western blotting analysis of the eluates using anti-ProtA, anti-Arc1p or anti-MetRS antibodies as indicated. Only the relevant parts of the blots are shown.

complexes isolated from crude extracts deviates from the 1:1:1 ratio for GluRS, MetRS and Arc1p that is observed upon *in vitro* reconstitution of the Arc1p–GluRS–MetRS complex from purified components (Deinert *et al.*, 2001). This difference is most likely due to the dilution of the extracts and to the limited proteolysis that preferentially removes the N-terminal appended domains of the enzymes.

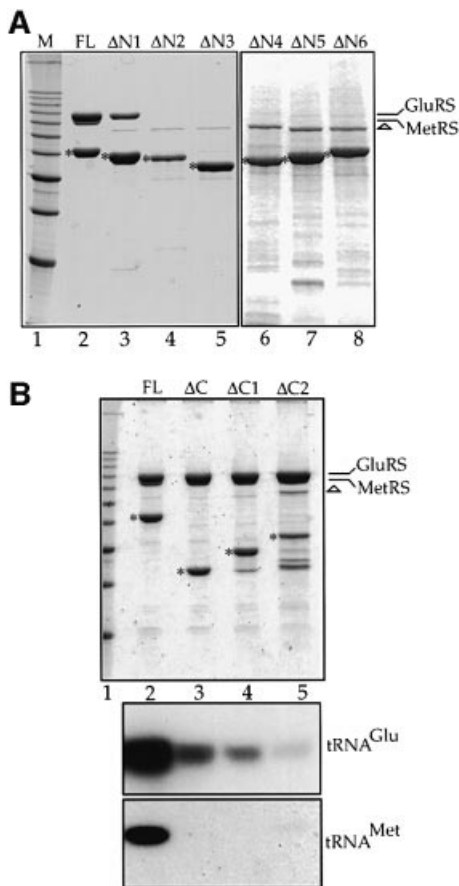
To determine whether the N-domains of MetRS and GluRS (MetRS-N and GluRS-N, respectively, shown schematically in Figure 2A) are sufficient for binding to Arc1p, they were tagged with ProtA, expressed in

wild-type and *arc1*<sup>-</sup> cells, and isolated by affinity chromatography. As shown in Figure 4C (lane 2), ProtA–GluRS-N co-purified with both Arc1p and MetRS. The specificity of this interaction is shown by the fact that both Arc1p and MetRS were absent when GluRS-N was purified from an *arc1*<sup>-</sup> strain (Figure 4C, lane 3). This also shows that no direct interaction takes place between the N-domain of GluRS and MetRS, i.e. these two proteins can only assemble into the complex via Arc1p. Similar results were also obtained for ProtA–MetRS-N: this domain also co-purified specifically with Arc1p and GluRS (Figure 4D). Furthermore, the stoichiometry of the co-purified complexes suggests that Arc1p binds more efficiently to the N-domain of GluRS than to that of MetRS. Taken together, our data demonstrate that the N-terminally appended domains of MetRS and GluRS are necessary and sufficient for binding to Arc1p. In the case of GluRS, the Arc1p binding site was mapped to the conserved part of the N-domain, between amino acids 86 and 207.

#### **The N-domain of Arc1p harbours distinct but overlapping binding sites for MetRS and GluRS**

We have previously demonstrated that the 132 amino acid long N-terminal domain of Arc1p (Arc1-N) can form a stable complex with both MetRS and GluRS *in vivo* as well as *in vitro* (Simos *et al.*, 1998; Deinert *et al.*, 2001). As revealed by the alignment shown in Figure 1, the central part of Arc1-N (residues 24–106) contains a motif also found in GluRS. To reveal the role of this motif in the formation of the Arc1p–GluRS–MetRS complex, we introduced serial deletions in the N-domain of Arc1p and constructed the mutants Arc1-ΔN1–Arc1-ΔN6, which are shown schematically in Figure 2B. These truncated Arc1p forms were tagged with ProtA, expressed in the *arc1* null strain and purified by affinity chromatography from total cell extracts of yeast cells expressing the corresponding mutants. As expected, full-length ProtA-tagged Arc1p co-purified with GluRS and MetRS (Figure 5A, lane 2). Deletion of the first 32 residues of Arc1p caused dissociation of MetRS but not of GluRS, which was still recovered with Arc1-ΔN1 (Figure 5A, lane 3). Western blot analysis using an antibody against MetRS confirmed that MetRS was indeed completely absent from the ProtA–Arc1-ΔN1 eluate (data not shown). In all other cases (Arc1-ΔN2–Arc1-ΔN6), the deletions caused dissociation of both GluRS and MetRS, as revealed by their absence from the corresponding eluates (Figure 5A, lanes 4–8). These data show that an intact N-terminal domain of Arc1p is required for binding to MetRS. However, the first part of this domain (residues 7–32) is dispensable for the Arc1p–GluRS interaction. Therefore, we conclude that the N-domain of Arc1p contains distinct but overlapping binding sites for MetRS and GluRS. Furthermore, the Arc1p–GluRS interaction is mediated by domains sharing the same conserved sequence motif, which may, accordingly, represent a dimerization element.

The biochemical evidence for the interaction sites on Arc1-N was also confirmed genetically using the previously isolated synthetic lethal yeast strains *slk117* and *slk88* (Simos *et al.*, 1996). Strain *slk117*, which carries a mutation in the *MES1* gene and lacks full-length Arc1p, could not be complemented by any of the Arc1p N-domain



**Fig. 5.** The N-domain of Arc1p contains distinct but overlapping binding sites for MetRS and GluRS, while an intact C-domain is only required for tRNA binding. IgG-Sepharose affinity purification of (A) ProtA-tagged full-length (FL) Arc1p (lane 2) and six versions carrying truncations at the N-terminal domain (lanes 3–8) expressed in the *arc1<sup>-</sup>* strain and (B) ProtA-tagged full-length (FL) Arc1p (lane 2) and three versions carrying complete or partial deletions of the C-terminal domain (lanes 3–5) expressed in the *arc1<sup>-</sup>* strain. (A and B, upper panel): analysis of the eluates by SDS-PAGE and Coomassie Blue staining. The asterisks indicate the positions of the ProtA-fusion proteins. The positions of co-purifying GluRS and MetRS are indicated on the right. The apparent lack of stoichiometry in (B), lanes 3–5, is due to the co-migration of MetRS and GluRS. The protein band at 75 kDa (indicated by a triangle) corresponds to an irrelevant contaminant. Lane 1, molecular weight markers (10 kDa ladder; 30–120 kDa). (B, lower panels) Northern blot analysis of co-purifying RNAs using radioactive probes against tRNA<sup>Glu</sup> and tRNA<sup>Met</sup>. Only the relevant part of the autoradiographs is shown.

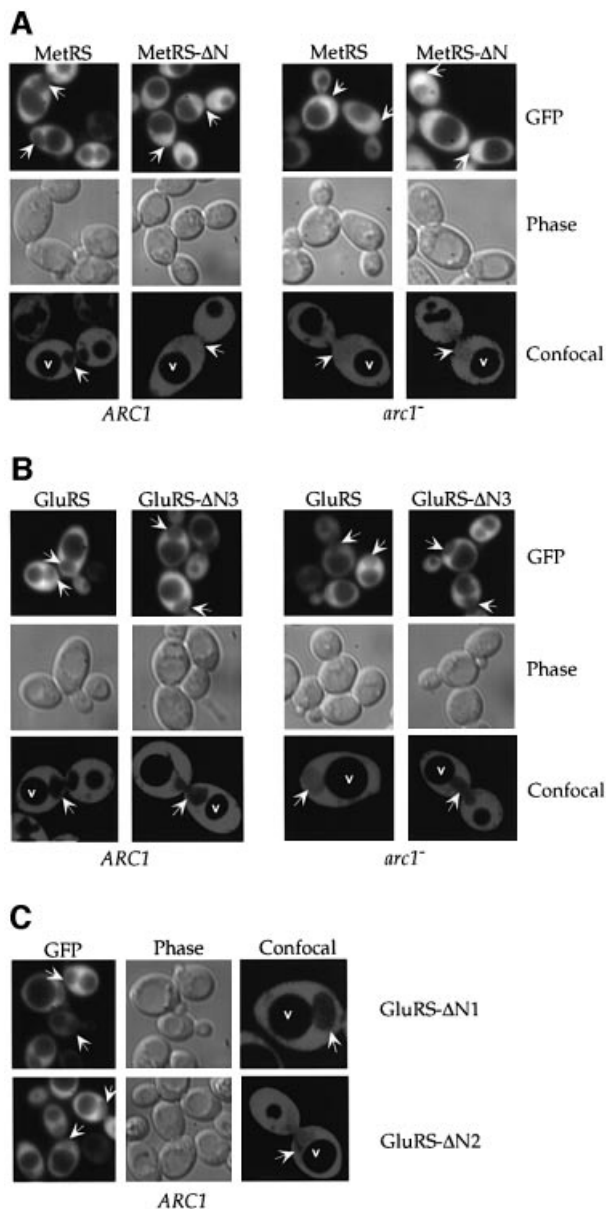
deletion mutants. On the other hand, strain *slk88*, which carries a defective GluRS and also lacks Arc1p, survived when transformed by Arc1- $\Delta$ N1 but not by any of Arc1- $\Delta$ N2–Arc1- $\Delta$ N6 (data not shown). Therefore, the lack of biochemical interaction correlates with the lack of complementation and vice versa.

While the N-domain of Arc1p is involved in the formation of the Arc1p–GluRS–MetRS ternary complex, its C-terminal domain (residues 201–376) has been shown to mediate broad-specificity binding of free Arc1p to several tRNA species and high-specificity binding of the Arc1p–MetRS–GluRS complex to the cognate tRNAs (Simos *et al.*, 1998; Deinert *et al.*, 2001). According to the homology between Arc1p and the mammalian protein EMAPII, part of the C-domain of Arc1p (residues

203–303; marked C1 in Figure 2B) contains a distinct structural motif called the OB fold. The OB fold is also part of the anticodon binding domain of three aminoacyl-tRNA synthetases and, interestingly, represents the core structure of the prokaryotic structure-specific tRNA-binding protein Trbp111 (Swairjo *et al.*, 2000). To find out whether the tRNA-binding properties of Arc1p are due to the presence of this OB fold, we prepared Arc1- $\Delta$ C1 and Arc1- $\Delta$ C2, two constructs in which either the region corresponding to the OB-fold (C1) or the rest of the Arc1p C-domain (residues 308–372; C2) was deleted, respectively (see Figure 2B). These ProtA-tagged mutant forms as well as full-length Arc1p and Arc1- $\Delta$ C (which lacks the entire C-domain) were purified by affinity chromatography. As shown in Figure 5B, in all four cases similar amounts of MetRS and GluRS were co-purified. However, analysis of the co-purifying tRNAs showed that not only removal of the whole C-domain but also deletion of either C1 or C2 caused a dramatic reduction in the amounts of cognate tRNA<sup>Glu</sup> and tRNA<sup>Met</sup> that bound to the Arc1p–MetRS–GluRS complex. This shows that the OB fold subdomain (C1) is not sufficient for stable tRNA binding, but that the presence of the C2 subdomain is also required. Furthermore, the inability of Arc1p to bind tRNA does not influence at all the protein–protein interactions between Arc1p and the two tRNA-aminoacylation enzymes.

#### **Formation of the Arc1p–MetRS–GluRS complex causes nuclear exclusion of its components**

Aminoacyl-tRNA synthetases are normally found in the cytoplasm. However, recent results have suggested the existence of minor nuclear pools of these enzymes, which may be required for the charging of the newly synthesized tRNAs before nuclear export (Lund and Dahlberg, 1998; Nathanson and Deutscher, 2000; Azad *et al.*, 2001). To study the intracellular distribution of the Arc1p–MetRS–GluRS complex and its individual components, we tagged MetRS, GluRS, Arc1p and their described truncation mutants with green fluorescent protein (GFP) and expressed them in yeast. All the GFP-tagged constructs were expressed normally when checked by western blotting analysis and were functional because they could complement the corresponding mutants (data not shown). Furthermore, the N-terminal GFP tag did not influence the integrity of the Arc1p ternary complex because the GFP-tagged enzymes co-purified with Arc1p as efficiently as the untagged native ones (data not shown). The cells expressing the GFP-tagged proteins were examined by conventional fluorescence microscopy as well as by confocal laser scanning microscopy in order to eliminate the contribution of cytoplasmic fluorescence to the nuclear signals. As shown in Figure 6 and summarized in Table I, GFP–GluRS and GFP–MetRS were detected exclusively in the cytoplasm of *ARC1* cells, with distinct nuclear and vacuolar exclusion. The same was also true for GFP–Arc1p expressed in *arc1<sup>-</sup>* cells (Figure 7; Table I). We have observed that those mutant proteins that retained the ability to associate into a complex (GFP–GluRS- $\Delta$ N1, GFP–Arc1- $\Delta$ N1, GFP–Arc1-N, GFP–Arc1- $\Delta$ M and GFP–Arc1- $\Delta$ C) exhibited similar localization to the corresponding full-length proteins, with very little or no nuclear signal (Figures 6C and 7; Table I). Strikingly,



**Fig. 6.** Nuclear entry of MetRS and GluRS through deletions in their N-terminal domains or by disruption of the *arc1* gene. Localization by fluorescence and confocal laser scanning microscopy of (A) GFP-MetRS and GFP-MetRS- $\Delta$ N in *mes1*<sup>-</sup> (left panels) and *mes1*<sup>-</sup> *arc1*<sup>-</sup> cells (right panels); (B) GFP-GluRS and GFP-GluRS- $\Delta$ N3 in *gus1*<sup>-</sup> (left panels) and *gus1*<sup>-</sup> *arc1*<sup>-</sup> cells (right panels); (C) GFP-GluRS- $\Delta$ N1 and GFP-GluRS- $\Delta$ N2 in *gus1*<sup>-</sup> cells. Note that GFP-GluRS- $\Delta$ N1 and GFP-GluRS- $\Delta$ N2 cannot be localized in the double-disrupted *gus1*<sup>-</sup> *arc1*<sup>-</sup> cells because the partial deletions of the N-domain of GluRS are lethal in the absence of Arc1p. In all cases, arrows indicate the position of the nucleus in representative cells, as inferred from the phase-contrast fields. In the confocal images, which show sections through the middle of the cells, v indicates the vacuoles and arrows point to the nuclei.

however, when the GFP-tagged enzymes were unable to assemble into the Arc1p-MetRS-GluRS complex, either because of deletions in their N-domains or due to the absence of Arc1p, they could be detected inside the nucleus. In the case of GFP-MetRS- $\Delta$ N or when GFP-MetRS was expressed in *arc1*<sup>-</sup> cells, the nuclear signal was identical to the cytoplasmic signal, suggesting

**Table I.** Localization of GFP-tagged Arc1p, GluRS and MetRS constructs

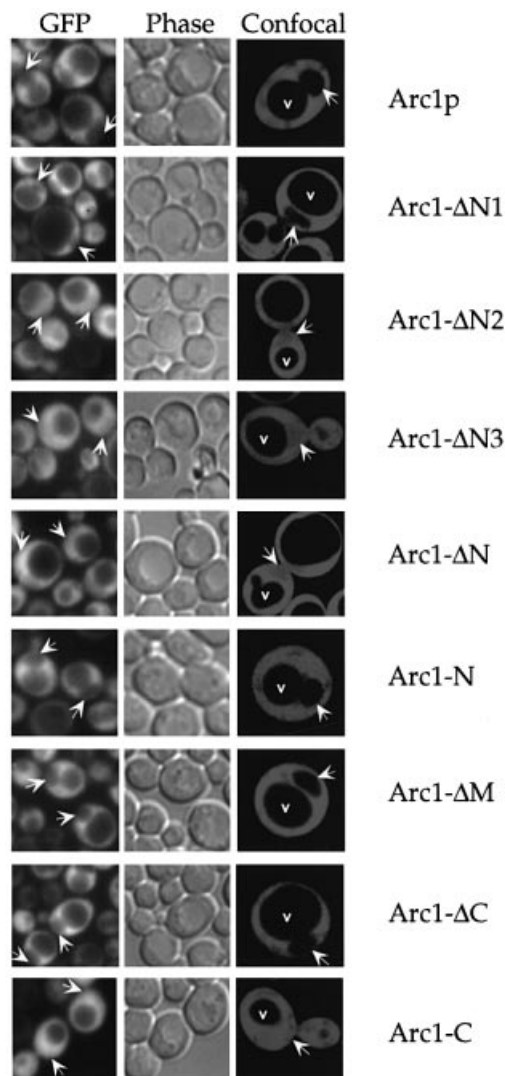
Construct	Strain	Formation of complex	Localization
Arc1p	<i>arc1</i> <sup>-</sup>	+	C
Arc1-N	<i>arc1</i> <sup>-</sup>	+	C
Arc1- $\Delta$ N	<i>arc1</i> <sup>-</sup>	-	C + N
Arc1- $\Delta$ N1	<i>arc1</i> <sup>-</sup>	+	C
Arc1- $\Delta$ N2	<i>arc1</i> <sup>-</sup>	-	C + N
Arc1- $\Delta$ N3	<i>arc1</i> <sup>-</sup>	-	C + N
Arc1-C	<i>arc1</i> <sup>-</sup>	-	C + N
Arc1- $\Delta$ C	<i>arc1</i> <sup>-</sup>	+	C
Arc1- $\Delta$ M	<i>arc1</i> <sup>-</sup>	+	C
MetRS	<i>mes1</i> <sup>-</sup> <i>ARC1</i>	+	C
MetRS	<i>mes1</i> <sup>-</sup> <i>arc1</i> <sup>-</sup>	-	C + N
MetRS- $\Delta$ N	<i>mes1</i> <sup>-</sup> <i>ARC1</i>	-	C + N
MetRS- $\Delta$ N	<i>mes1</i> <sup>-</sup> <i>arc1</i> <sup>-</sup>	-	C + N
GluRS	<i>gus1</i> <sup>-</sup> <i>ARC1</i>	+	C
GluRS	<i>gus1</i> <sup>-</sup> <i>arc1</i> <sup>-</sup>	-	C + n
GluRS- $\Delta$ N1	<i>gus1</i> <sup>-</sup> <i>ARC1</i>	+	C
GluRS- $\Delta$ N2	<i>gus1</i> <sup>-</sup> <i>ARC1</i>	-	C + n
GluRS- $\Delta$ N3	<i>gus1</i> <sup>-</sup> <i>ARC1</i>	-	C
GluRS- $\Delta$ N3	<i>gus1</i> <sup>-</sup> <i>arc1</i> <sup>-</sup>	-	C

C, cytoplasmic signal and very little or no nuclear signal; C + N, cytoplasmic signal plus nuclear signal of similar intensity; C + n, cytoplasmic signal plus weaker nuclear signal.

the presence of a significant nuclear pool of free enzyme (Figure 6A). In the case of GluRS, the effects of the deletions were variable. GFP-GluRS, when expressed in the absence of Arc1p, as well as GFP-GluRS- $\Delta$ N2 could be clearly detected inside the nucleus, although their nuclear signals were not as strong as in the cytoplasm (Figure 6B and C). GFP-GluRS- $\Delta$ N3, on the other hand, despite the fact that it is 26 amino acid residues shorter than GFP-GluRS- $\Delta$ N2, exhibited only very weak nuclear staining (Figure 6B; also see Discussion). Concerning the Arc1p mutants, GFP-Arc1- $\Delta$ N2, GFP-Arc1- $\Delta$ N3, GFP-Arc1- $\Delta$ N and GFP-Arc1-C, all of which lack the binding sites for MetRS and GluRS, displayed nuclear signals (Figure 7). In summary, there was a strong correlation between the inability of the various synthetase or Arc1p mutants to form a complex and their presence inside the nucleus (Table I). These data suggest that since most of the fusion proteins used in these experiments exceed in size the diffusion limit of the nuclear pore complex (~50 kDa), Arc1p, GluRS and MetRS are capable of actively entering the nucleus. However, when organized into the Arc1p-MetRS-GluRS complex, they are excluded from the nucleus. The formation of this complex, which is mediated by the N-terminal domains of its individual components, may therefore trigger cytoplasmic retention or, alternatively, active nuclear export (also see Discussion).

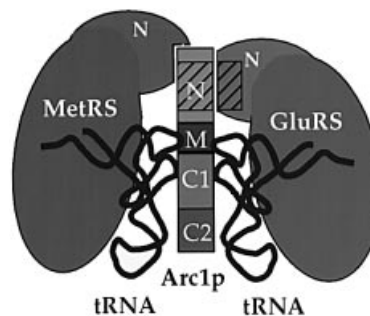
## Discussion

A stable complex is formed in yeast between Arc1p and two aminoacyl-tRNA synthetases, GluRS and MetRS. The results presented in this work, together with our previous findings (Simos *et al.*, 1998; Deinert *et al.*, 2001), suggest the following model for the organization of this complex (Figure 8). Both enzymes interact directly with Arc1p. These protein-protein interactions are mediated, on the



**Fig. 7.** Deletions in the N-terminal domain of Arc1p allow entry into the nucleus. Localization by fluorescence microscopy and confocal laser scanning microscopy of GFP-tagged Arc1p or its various deletion mutants in *arc1<sup>-</sup>* cells as indicated. Arc1-ΔN1, Arc1-ΔN2, Arc1-ΔN3 and Arc1-ΔC are described in Figure 2B. Arc1-ΔN (Δ7–122), Arc1-N (Δ134–372), Arc1-ΔM (Δ134–198) and Arc1-C (Δ7–198) are described in Simos *et al.* (1998). The arrows indicate the position of the nucleus in representative cells, as inferred from the phase-contrast fields. The confocal images show sections through the middle of the cells; v indicates the vacuoles and arrows point to the nuclei.

one hand, by the N-terminally appended domains of the enzymes and, on the other hand, by the N-domain of Arc1p, which contains distinct but overlapping binding sites for the two enzymes. The catalytic domains of the two enzymes are therefore free to associate with, and aminoacylate, the cognate tRNAs. Selection and binding of these tRNAs are facilitated by the TRBD of Arc1p, which would recognize the conserved core ‘elbow’ structure of the tRNA and direct them to the active sites of the enzymes. This requires both the OB-fold subdomain (C1) as well as the eukaryote-specific C-terminal part (C2) of Arc1p. However, it remains unknown whether both cognate tRNAs can bind to the complex at the same time or one at a time. In either case, both enzymes obtain a



**Fig. 8.** A schematic hypothetical model for the organization of the yeast Arc1p–MetRS–GluRS complex. The domains of Arc1p (N, M, C1 and C2) are indicated by different shades of grey. The N-terminal domains of MetRS and GluRS are labelled. The hatched areas represent the conserved motifs present in the N-terminal domains of both Arc1p and GluRS. For details see text.

catalytic advantage when associated with Arc1p, as shown by the *in vitro* reconstitution and analysis of the corresponding complexes (Simos *et al.*, 1998; Deinert *et al.*, 2001).

This interaction model may serve as a simple ‘prototype’ for the mammalian multisynthetase complex. Apart from nine synthetases, this complex contains three non-catalytic components (p43, p38 and p18), all of which share homologies with Arc1p. The TRBD of Arc1p is conserved in p43, which has also been shown to be able to bind to tRNA and stimulate the activity of at least one enzyme of the complex (Quevillon *et al.*, 1997; Park *et al.*, 1999). The N-domain of Arc1p contains a motif that is present in both p18 and p38 as well as in the N-terminal appended domain of the bifunctional synthetase GluProRS, another component of the multisynthetase complex (Quevillon and Mirande, 1996; Quevillon *et al.*, 1999). This domain, originally identified in the glutathione *S*-transferase superfamily, also occurs within human valyl-tRNA synthetase (ValRS), mouse cysteinyl-tRNA synthetase (CysRS), the putative GluRSs from *S.pombe* (ID: O13775) and *Arabidopsis thaliana* (Day *et al.*, 1998), and the β and γ subunits of the eukaryotic elongation factor EF-1B (Koonin *et al.*, 1994; our unpublished results). We have shown here that this motif represents a protein–protein interaction domain responsible for the Arc1p–GluRS association in yeast. This motif appears to function only in heterodimerization as neither GluRS nor the N-domain of Arc1p forms homo-oligomers in solution, as shown previously by their migration in gel-filtration columns (Deinert *et al.*, 2001). Therefore, we also predict that in mammals this motif may play a role in the formation and stabilization of higher order aminoacyl-tRNA synthetase complexes. Indeed, this conserved motif most likely represents the protein interface between human ValRS and the δ subunit of the eukaryotic elongation factor 1 complex (Bec *et al.*, 1994; Janssen *et al.*, 1994). The occurrence of this motif in both tRNA-aminoacylation and protein translation factors may indicate the presence of a network of interactions that brings together and coordinates these two machineries. In several cases, interaction of an aminoacyl-tRNA synthetase with the translation elongation machinery has indeed been shown to offer a catalytic advantage (Reed *et al.*, 1994; Negrutskii *et al.*, 1996, 1999). However,

it may also provide the means for subcellular spatial organization, i.e. co-localization of the tRNA-aminoacylation machinery with the sites of ongoing protein translation (Kisselev and Wolfson, 1994; Stapulionis and Deutscher, 1995; Negrutskii and El'skaya, 1998).

Aminoacylation of the tRNAs that come off the translating ribosomes in the cytoplasm is the major function of aminoacyl-tRNA synthetases. On the other hand, and according to recent data (Lund and Dahlberg, 1998; Nathanson and Deutscher, 2000), at least a minor pool of synthetases has to enter the nucleus in order to aminoacylate the newly synthesized tRNAs as a pre-condition for their nuclear export. Data from the yeast system have also pointed to a link between nuclear tRNA-aminoacylation and tRNA nuclear export. Inhibition of aminoacylation leads to accumulation of mature tRNAs inside the yeast nucleus (Sarkar *et al.*, 1999; Grosshans *et al.*, 2000). Furthermore, there is a synthetic lethal relationship between Arc1p and the yeast nuclear tRNA export receptor Los1p (Hellmuth *et al.*, 1998), and mutations that reduce the nuclear pool of tyrosyl-tRNA synthetase (TyrRS) have recently been shown to impair tRNA nuclear export (Azad *et al.*, 2001). This demonstration of minor intranuclear pools of aminoacyl-tRNA synthetases in both yeast and vertebrates raises the following question: how is this relative distribution of aminoacyl-tRNA synthetases between nucleus and cytoplasm controlled? As demonstrated in several cases of regulated nucleocytoplasmic transport (reviewed in Kaffman and O'Shea, 1999), the answer to this can be the formation of a complex that is actively excluded from the nucleus due to cytoplasmic retention, enhanced nuclear export or masking of nuclear localization signals.

Along these lines, our results show that the formation of the Arc1p-synthetase complex does indeed dictate the predominant subcellular distribution of its components. We have shown that Arc1p, MetRS or GluRS can enter the nucleus only upon the condition that the Arc1p-MetRS-GluRS complex has been disrupted, i.e. only as free proteins. Disruption of the complex was achieved either by removing the N-terminal domains of the enzymes or Arc1p in various combinations. In all cases except one (GFP-GluRS- $\Delta$ N3), a correlation was observed between the inability to form the complex and the occurrence of a significant nuclear pool (Table I). The most likely explanation of our results is that Arc1p, MetRS and GluRS contain nuclear localization signals that can drive at least a certain amount of these proteins inside the nucleus. Such signals have indeed been predicted in the case of Arc1p and GluRS (Schimmel and Wang, 1999). However, when the proteins are organized into a complex, as is the case under wild-type steady-state conditions, they are found only or predominantly in the cytoplasm. The observation that only very small amounts of GFP-GluRS- $\Delta$ N3 enter the nucleus, despite the lack of interaction with Arc1p, may be due to the fact that the longer truncation in this mutant removed a putative nuclear localization signal together with the Arc1p interaction domain. An alternative explanation is that the structural integrity of this mutant is affected, although this seems unlikely as this mutant is both stably expressed and functional *in vivo*. In the same vein, GluRS- $\Delta$ N2 may be less functional than GluRS- $\Delta$ N3 (Figure 3A) due to the

former's increased nuclear import and corresponding depletion of its cytoplasmic pool (Figure 6C). However, it should be mentioned that most of the conditions that disrupt the Arc1p ternary complex and subsequently lead to a change in the subcellular distribution of its components do not affect significantly the levels of *in vivo* aminoacylation or the rate of nuclear export of the cognate tRNAs (our unpublished data). This is not unexpected, as despite the increased nuclear pools of GluRS and MetRS, which should not disturb nuclear aminoacylation and efficient tRNA export, the major parts of GluRS and MetRS remain in the cytoplasm and can therefore aminoacylate efficiently the bulk of tRNA.

The formation of a ternary complex that is excluded from the nucleus represents a simple way of controlling the relative subcellular distribution of Arc1p, GluRS and MetRS. At equilibrium, and depending on the relative affinities between the components of the complex and their cellular abundance, a certain pool will be non-associated and therefore free to enter the nucleus, while the rest would be found in the cytoplasm as part of the ternary complex. Interference with complex formation would then disrupt the equilibrium in favour of dissociation and subsequent nuclear entry, as we have observed experimentally. The identification of the nuclear localization sequences of Arc1p, MetRS and GluRS, as well as the nature of the nuclear exclusion mechanism of this and other related tRNA-aminoacylation complexes, are certainly interesting subjects for future research.

## Materials and methods

### Yeast strains, media and plasmids

The yeast strains RS453 and *arc1*<sup>-</sup> have been described previously (Simos *et al.*, 1996). The following previously described plasmids were used: pUN100 (*CEN/ARS, LEU2*) (Elledge and Davis, 1988); pRS314 (*CEN/ARS, TRP1*), pRS315 (*CEN/ARS, LEU2*), pRS316 (*CEN/ARS, URA3*) (Sikorski and Hieter, 1989); pASZ11 (*CEN/ARS, ADE2*) (Stotz and Linder, 1990); pRS315-P<sub>NOP1</sub>-GFP, pRS315-P<sub>NOP1</sub>-ProtA (Hellmuth *et al.*, 1998); pUN100-MES1, pPP1-ProtA-GluRS (Simos *et al.*, 1996); pEMBLyex4-His<sub>g</sub>-MetRS (Deinert *et al.*, 2001). Plasmid pASZ11-P<sub>NOP1</sub>-GFP was constructed by subcloning the NOP1::GFP cassette from pRS315-P<sub>NOP1</sub>-GFP. Yeast cells were grown in rich YPD medium or synthetic SDC medium containing the necessary amino acids and nutrients. For counterselection of cells containing *URA3* plasmids, 5-fluoroorotic acid (FOA; Toronto Research Chem.) was used at 1  $\mu$ g/ml.

### Construction of strains carrying deletions of the chromosomal genes coding for MetRS and GluRS

Deletion of the chromosomal genes coding for GluRS and MetRS (*GUS1* and *MES1*, respectively) was achieved by PCR-mediated single-step targeted integration. The selectable marker was the *S.pombe his5*<sup>+</sup> gene, which can substitute the *S.cerevisiae HIS3* gene and allow growth of the RS453 strain on media lacking histidine (Longtine *et al.*, 1998). This gene was amplified by PCR from plasmid pFA6a-His<sub>3</sub>MX6 using 70 nucleotide long primers that had 5'-ends (50 nt) annealing to the upstream or downstream regions of the GluRS or MetRS open reading frames and 3'-ends (20 nt) annealing to the marker gene. The products of the PCR reactions were used to transform the diploid strain RS453 and select for His<sup>+</sup> transformants. The integration of the *his5*<sup>+</sup> gene in the correct loci and generation of *gus1::his5*<sup>+</sup> or *mes1::his5*<sup>+</sup> heterozygotes were confirmed by PCR. Sporulation and tetrad dissection of the heterozygous diploid cells carrying the null alleles of *MES1* and *GUS1* demonstrated 2:2 segregation for viability and histidine auxotrophy, showing as expected that the products of *MES1* and *GUS1* are essential. To construct haploid cells harbouring a *gus1* or *mes1* knockout allele and rescued by plasmid-encoded GluRS or MetRS (shuffle strains), the heterozygous *gus1::his5*<sup>+</sup> or *mes1::his5*<sup>+</sup> strains were transformed with pRS316-URA3-GluRS or pRS316-URA3-MetRS, respectively, sporu-



lated and tetrads dissected. Haploid *gus1::his5<sup>+</sup>* or *mes1::his5<sup>+</sup>* cells complemented by the corresponding wild-type genes on the *URA3*-containing plasmid exhibited no growth defect but died on 5-FOA-containing plates unless co-transformed with a plasmid encoding functional parts of GluRS or MetRS, respectively.

### Miscellaneous procedures

Expression of the fusion proteins transformed in yeast cells was checked by western blotting of whole-cell extracts using polyclonal antibodies against the GFP moiety (Clontech), rabbit PAP (Dako) to detect the ProtA tag or antibodies against Arc1p (Simos *et al.*, 1996). Affinity purification of ProtA fusion proteins was performed essentially as described previously (Simos *et al.*, 1996, 1998). The localization of the GFP-tagged proteins in living yeast cells was examined in the fluorescein channel of a Zeiss Axioskop fluorescence microscope. Images were obtained with a Xillix Microimager CCD camera and processed with Improvison Openlab 1.7 software.

Confocal laser scanning microscopy was performed using a Leica TCS MP confocal microscope. Series of 0.5  $\mu\text{m}$  sections were recorded. The images shown in Figures 6 and 7 correspond to the sections approximately in the middle of the cells.

Protein concentrations were determined using the Protein Assay reagent from Bio-Rad. SDS-PAGE, DNA manipulations (restriction digests, PCR amplifications, etc.), RNA extraction and northern analysis were performed according to standard protocols. Detection of tRNAs on northern blots was conducted using the following end-labelled synthetic DNA oligonucleotides complementary to tRNA specific sequences: tRNA<sup>Glu(UUC)</sup>, 5'-CGGTCTCCACGGTAAAGC-3'; elongator tRNA<sup>Met(CAU)</sup>, 5'-TCGACCTTCAGAT-3'.

### Supplementary data

Owing to space limitations, cloning and tagging of GluRS and MetRS by ProtA and GFP as well as the construction of plasmids expressing ProtA- and GFP-tagged truncated forms of MetRS, GluRS and Arc1p are described in detail in the Supplementary data, available at *The EMBO Journal Online*.

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