Supplementary Information

Plasmid and yeast strain construction

The eIF1A surface residues were visualized in the human eIF1A NMR structure (Battiste et al., 2000) using Swiss-PdbViewer (Guex and Peitsch, 1997) . After compilation of a multiple sequence alignment among eIF1A homologs from *S. cerevisiae* (accession NP_013987), *H. sapiens* (accession 1D7Q_A), *C. elegans* (accession NP_500650), *D. melanogaster* (accession AAF44294) and *T. aestivum* (accession P47815) using ClustalW (Thompson et al., 1994), residues which were both conserved and surface-accessible were selected for mutagenesis. *TIF11* mutant alleles were constructed by fusion PCR using p3499, containing WT *TIF11-FL*, as template and the following primers. To construct $RKKVW_{66\text{-}70}AAAVA$, CHA34 (5' GTTGGGAAGGGCGATCGGTGCGG 3') and CHA33 (5' GCTCACTCATTAGGCACCCCAGGC 3') were the upstream and downstream primers, respectively, and CHA73 and CHA74 (listed in Table S1 of Supplementary information) were the mutagenic primers. For $DEAR_{98\text{-}101}AAAA$, CHA34 and CHA33 were again employed but CHA67 and CHA68 replaced CHA73 and CHA74. Likewise, the pairs of mutagenic primers indicated in Table S1 for each mutant replaced CHA73 and CHA74 in the scheme described above for constructing $RKKVW_{66\text{-}70}AAAVA$. The fusion PCR products were inserted between the *EcoRI* and *SalI* sites of YCplac111. The entire subcloned fragments of all mutant constructs were confirmed by DNA sequencing. Yeast strains harboring the mutant constructs were constructed from strains H2809 and H3582 by plasmid shuffling. Media were prepared as described (Sherman et al., 1974).

Plasmid p4385 was constructed from p1780-IMT by switching the *URA3* marker to *TRP1* using one-step gene conversion (Cross, 1997) with the *SmaI* fragment isolated from pUT11.

Polysome profiles and analysis of 43S complexes in vivo.

HCHO crosslinking, WCE preparation and sucrose density gradient analysis was performed as described by (Nielsen et al., 2004). For analysis of 43S complexes, 700 μL fractions were collected, mixed with 6X loading dye [300 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 0.6% (w/v) bromophenol blue, 60% (v/v) glycerol and 600 mM β-mercaptoethanol], and 20 μL samples were separated on 4-20% Tris-Glycine polyacrylamide gels (Criterion, BioRad). After electroblotting to nitrocellulose membranes, membranes were probed with polyclonal antibodies against the appropriate initiation factors and 40S ribosomal proteins. Antibodies against eIF3b, eIF3i, eIF5, eIF2γ (Phan et al., 1998), eIF2α (Dever et al., 1992), eIF1A (Olsen et al., 2003) and eIF1 (Valasek et al., 2004) have been described. FLAG antibody was obtained commercially from Sigma. RPS22 antibody (generated in rabbit) was kindly provided by Jan van't Riet and RPS2 antibody (also generated in rabbit) was kindly provided by Jon Warner.

***lacZ* reporter assays**

Briefly, cells were grown in uninducing (U) medium (SC-U) for 6h, or in inducing (I) medium (SC-UIV) for 2.5h after which SM was added to 0.5 μg/mL for 6h, reaching a final OD_{600} of ~2.0 in both conditions. WCEs were prepared and β-galactosidase assays performed as described previously (Moehle and Hinnebusch, 1991). Specific activity is

reported as nmol ONPG (*o*-nitrophenyl-β-D-galactopyranoside) cleaved min^{-1} mg^{-1}. All values reported are means (± SE) from 3 replicate cultures, each assayed in duplicate.

Preparation of recombinant eIF1A proteins

Unlabeled eIF1A proteins were prepared using the protocol for eIF1 purification as described previously (Algire et al., 2002), with an additional purification step over a HiTrap heparin column as described for eIF1A. TAMRA-labeled wild type eIF1A was prepared as described previously (Maag and Lorsch, 2003).

In vitro analyis of eIF1A ribosome binding

The K_ds for eIF1A proteins and 40S subunits in the presence of 1 μM eIF1 were determined by fluorescence anisotropy competition experiments as previously described (Maag and Lorsch, 2003). Factors used in 43S•mRNA assembly kinetics (40S subunits, eIF1, eIF2, [^{35}S]-Met-$tRNA_i^{Met}$ and mRNA) were prepared as described (Algire et al., 2002). Native gel assays for the formation of 48S were performed as described previously (Maag et al., 2005). The final concentrations of the components were 0.5 nM ^{35}S-Met-$tRNA_i$, 1 μM eIF1, 1-10 μM eIF1A (depending on the K_d for the mutant factor), 1 μM mRNA and 10 nM 40S subunits. At the specified time, reactions were quenched by chasing with 200nM unlabeled TC, which completely blocked further binding of labeled TC prior to gel loading (data not shown). The amount of pre-bound TC that dissociated in the presence of chase was found to be insignificant over the course of an experiment (data not shown).

Toe-printing analysis of scanning/AUG recognition

48S complexes were assembled in reactions containing: 3 pmol 40S subunits, 9 pmol eIF2, 9 pmol eIF3, 5 pmol eIF4F, 10 pmol eIF4A, 10 pmol eIF4B, 10 pmol eIF1, 75 pmol eIF1A (recombinant yeast or human), 5 pmol Met-$tRNA_i^{Met}$, 2 pmol β-globin mRNA, 20 mM Tris-HCl pH 7.5, 100 mM potassium acetate, 2.5 mM $MgCl_2$, 1 mM DTT, 0.25 mM spermidine, 1 mM ATP, 0.2 mM GTP and 2U/μL ribonuclease inhibitor (RNAseOUT, Invitrogen) in a 40 μL volume. Assembly, primer extension and polyacrylamide gel analysis of products were performed as described (Pestova et al., 1998; Pestova and Kolupaeva, 2002).