

The human *SLC25A33* and *SLC25A36* genes of solute carrier family 25 encode two mitochondrial pyrimidine nucleotide transporters\*

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**Running title:** Human mitochondrial carriers for pyrimidine (deoxy)nucleotides

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**Keywords:** liposome; membrane transport protein; mitochondrion; mitochondrial carrier; pyrimidine nucleotide; SLC25 family; SLC25A33; SLC25A36

**Background:** SLC25A33 and SLC25A36 are two human uncharacterized proteins encoded by the mitochondrial carrier *SLC25* genes.

**Results:** Recombinant SLC25A33 and SLC25A36 transport cytosine, uracil and thymine (deoxy)nucleotides with different efficiency.

**Conclusions:** SLC25A33 and SLC25A36 are mitochondrial transporters for pyrimidine (deoxy)nucleotides.

**Significance:** SLC25A33 and SLC25A36 are essential for mitochondrial DNA and RNA metabolism; other two members of the SLC25 superfamily responsible for 12 monogenic diseases were thoroughly characterized.

## ABSTRACT

The human genome encodes 53 members of the solute carrier family 25 (SLC25), also called the mitochondrial carrier family, many of which have been shown to transport inorganic anions, amino acids, carboxylates, nucleotides and coenzymes across the inner mitochondrial membrane, thereby connecting cytosolic and matrix functions. Here two members of this family, SLC25A33 and SLC25A36 have been thoroughly characterized biochemically. These proteins were overexpressed in bacteria and reconstituted in phospholipid vesicles. Their

transport properties and kinetic parameters demonstrate that SLC25A33 transports uracil, thymine and cytosine (deoxy)nucleoside di- and tri-phosphates by an antiport mechanism and SLC25A36 cytosine and uracil (deoxy)nucleoside mono-, di- and tri-phosphates by uniport and antiport. Both carriers also transported guanine but not adenine (deoxy)nucleotides. Transport catalyzed by both carriers was saturable and inhibited by mercurial compounds and other inhibitors of mitochondrial carriers to various degrees. In confirmation of their identity, (i) SLC25A33 and SLC25A36 were found to be targeted to mitochondria and (ii) the phenotypes of *Saccharomyces cerevisiae* cells lacking *RIM2*, the gene encoding the well characterized yeast mitochondrial pyrimidine nucleotide carrier, were overcome by expressing SLC25A33 or SLC25A36 in these cells. The main physiological role of SLC25A33 and SLC25A36 is to import/export pyrimidine nucleotides into and from mitochondria, i. e. to accomplish transport steps essential for mitochondrial DNA and RNA synthesis and breakdown.

The human genome harbours as many as 53 *SLC25* genes encoding a superfamily of transport proteins called solute carrier 25 (SLC25) family or

mitochondrial carrier family (MCF) (1, 2). The members of this family are characterized by three tandem repeated sequences of about 100 amino acids, each being folded into two transmembrane  $\alpha$ -helices and containing a distinct signature motif (PROSITE PS50920, PFAM PF00153, and IPR00193). In recent years many of these transporters have been investigated at the molecular and biochemical level. They transport inorganic anions, amino acids, carboxylic acids, nucleotides and coenzymes across the inner mitochondrial membrane and, in a few cases, across other membranes (1, 3-5). The importance of mitochondrial carriers is demonstrated by their wide distribution in all eukaryotes, their role in numerous metabolic pathways and cell functions, and the identification of several diseases caused by alterations of their genes (1, 2, 6-8). However, despite the research efforts of many laboratories, the transport properties and the substrate(s) transported by about twenty human mitochondrial carriers remain, as yet, unknown.

The two human MCs SLC25A33 and SLC25A36, encoded by the *SLC25A33* and *SLC25A36* genes, respectively, are the close relatives of the *Saccharomyces cerevisiae* Rim2p. Deletion of *RIM2* causes total loss of mtDNA and lack of growth on non-fermentative carbon sources (9). Furthermore, Rim2p has been demonstrated, by direct assays in reconstituted liposomes, to transport all pyrimidine ribo- and deoxyribo-nucleotides with similar efficiency as well as guanine ribo- and deoxyribo-nucleotides but not adenine nucleotides (10). Most information concerning SLC25A33, which was previously named PNC1, derives from studies in human cells in which PNC1 was silenced or overexpressed. PNC1 overexpression enhanced cell size, mitochondrial TTP levels and diminished ROS (reactive oxygen species), whereas its knockdown caused depletion of mtDNA, reduced oxidative phosphorylation, cell size and mitochondrial UTP levels, and increased ROS levels (11-13). Furthermore, analysis of nucleotide flow in cells with downregulation of PNC1 and treated with labeled uridine or thymidine revealed a slower mitochondrial uptake of uridine triphosphate and a slower release of thymine nucleotides to the cytoplasm (13). Finally, it has recently been found that *Drosophila* S2R<sup>+</sup> cells, silenced for drim2 (the *Drosophila melanogaster* homolog of the *S.*

*cerevisiae* Rim2p), contained markedly reduced pools of both purine and pyrimidine dNTPs in mitochondria, whereas cytosolic pools were unaffected (14).

Until now the biochemical characterization of purified PNC1 has been addressed in a single publication in which the recombinant SLC25A33, reconstituted into liposomes, was shown to transport pyrimidine nucleoside triphosphates with preference for UTP (11). However, in this study only a limited number of potential substrates have been tested and, for example, it is unknown whether nucleoside mono- and di-phosphates are transported by SLC25A33.

The high homology existing between SLC25A33 and SLC25A36 has suggested that these two members of the MCF might have similar functions (1, 2). However, the substrate(s) transported by SLC25A36 have not yet been discovered and its function is still completely elusive.

This study reports the identification and functional characterization of SLC25A36 as well as a much more in depth characterization of SLC25A33 transport activity as compared to that reported by Floyd et al. (11). The *SLC25A33* and *SLC25A36* genes were overexpressed in *E. coli*, and the gene products were purified and reconstituted into liposomes. Recombinant SLC25A33 was shown to transport uracil, thymine and cytosine (deoxy)nucleoside di- and tri-phosphates and SLC25A36 cytosine and uracil (deoxy)nucleoside mono-, di- and tri-phosphates. Both SLC25A33- and SLC25A36-catalyzed transports were saturable and inhibited by mercurial compounds. The green fluorescent protein (GFP) fused to SLC25A36 was found to be targeted to mitochondria as previously shown for SLC25A33 (11). Furthermore, expression of SLC25A33 or SLC25A36 proved to restore the phenotypes of *S. cerevisiae* cells lacking *RIM2* encoding the yeast well characterized mitochondrial pyrimidine nucleotide carrier (10). As pyrimidine (deoxy)nucleotide transporters, SLC25A33 and SLC25A36 are essential for the synthesis and breakdown of mitochondrial DNA and RNA by providing the precursors and removing the products of these processes.

## EXPERIMENTAL PROCEDURES

*Materials*—Radioactive compounds were supplied from PerkinElmer Life Sciences. All (deoxy)nucleotides were obtained from Sigma.

*Sequence Search and Analysis*—Protein and genomic data bases ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) were screened with the protein sequence of SLC25A33 (accession number NP\_115691.1), and SLC25A36 (accession number NP\_001098117.1), using BLASTP and TBLASTN. The amino acid sequences were aligned with ClustalW (version 1.8).

*Construction of Expression Plasmids*—The coding sequences of SLC25A33 and SLC25A36 (accession numbers NM\_032315.2 and NM\_001104647.1, respectively) were amplified by PCR from human testis and brain cDNA, respectively. The oligonucleotide primers were synthesized corresponding to the extremities of the coding sequences, with additional *NdeI* and *HindIII* (for SLC25A33) or *BamHI* and *EcoRI* (for SLC25A36) restriction sites as linkers. The amplified products were cloned into the pRUN (SLC25A33) and pMW (SLC25A36) vectors for expression in *Escherichia coli*.

The RIM2-pRS42H plasmid was constructed by cloning a DNA fragment consisting of the 381 bp upstream the RIM2 open reading frame (ORF), the RIM2 ORF and the 352 bp downstream the RIM2 ORF (amplified from *S. cerevisiae* genomic DNA by PCR using primers with additional BamHI and SacI sites) into the episomal vector pRS42H. For the preparation of the SLC25A33-pRS42H and SLC25A36-pRS42SH plasmids, a chimera consisting of the 381 bp upstream the RIM2 ORF, the coding sequence of SLC25A33 or SLC25A36 with a 6 His-tag before the termination codon and the 352 bp downstream of the RIM2 ORF was constructed. Each of the three fragments was amplified using long primers consisting of the sequences corresponding to the extremities of the template to be amplified and an additional sequence corresponding to the extremity of the fragment to be linked with. After amplification and purification, the three fragments were mixed together and used in an overlapping PCR. First 5 cycles of PCR were run without primers to generate the complete overlapping template; then forward primers (first 25 nucleotides of the chimera with Hind III site) and reverse primers (last 25 nucleotides of the chimera with

SacI site) were added and further 25 cycles of PCR were run. The two chimeras containing either the coding sequence of SLC25A33 or SLC25A36 were checked for correct overlapping and sequence errors; then they were digested with *HindIII* and *SacI* and cloned into the pRS42H plasmid. All the plasmids, prepared as above, were transformed into *E. coli* DH5 $\alpha$  cells. Transformants were selected on 2X YT plates containing ampicillin (100  $\mu$ g/ml) and screened by direct colony PCR and by restriction digestion of purified plasmids. The sequences of the inserts were verified.

*Bacterial expression and purification of recombinant SLC25A33 and SLC25A36*—The expression of recombinant proteins was carried out at 37°C in *E. coli* strain BL21(DE3) (15, 16). Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient (17) and washed at 4°C, first with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0), then twice with a buffer containing 3% (w/v) Triton X-114, 1 mM EDTA and 10 mM PIPES-NaOH pH 7.0, and lastly with TE buffer pH 7.0. Finally, the recombinant SLC25A33 and SLC25A36 were solubilized in 1.2% (w/v) lauric acid and diluted 1:3 with 3% (w/v) Triton X-114, 10 mM PIPES-NaOH pH 7.0 and 1 mM EDTA in the presence (SLC25A36) or absence (SLC25A33) of 20 mM Na<sub>2</sub>SO<sub>4</sub>. Eventual small residues were removed by centrifugation (20800 x g for 20 min at 4°C).

*Reconstitution of SLC25A33 and SLC25A36 into liposomes*—The recombinant proteins in lauric acid were reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column of Amberlite beads (Fluka), as previously described (18, 19) with some modifications. The composition of the initial mixture used for reconstitution was 50  $\mu$ l of purified protein (about 20  $\mu$ g of SLC25A33 or 30  $\mu$ g of SLC25A36), 60  $\mu$ l of 10% Triton X-114, 100  $\mu$ l of 10% phospholipids (L- $\alpha$ -phosphatidylcholine from egg yolk, Sigma) in the form of sonicated liposomes, 10 mM substrate except where otherwise indicated, 0.6 mg (SLC25A33) or 1 mg (SLC25A36) cardiolipin, 10 mM PIPES-NaOH, pH 7.0 (SLC25A33) or 5 mM MES-NaOH / 5 mM PIPES-NaOH, pH 6.25 (SLC25A36) and water to a final volume of 700  $\mu$ l. After vortexing, this mixture was recycled 13-fold

through an Amberlite column (3.2 x 0.5 cm) pre-equilibrated with a buffer containing 10 mM PIPES-NaOH at pH 7.0 (SLC25A33) or 5 mM MES-NaOH / 5 mM PIPES-NaOH at pH 6.25 (SLC25A36) and the substrate at the same concentration as in the starting mixture.

*Transport measurements*—External substrate was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 50 mM NaCl and 10 mM PIPES-NaOH at pH 7.0 for SLC25A33 (buffer A) or 50 mM NaCl and 5 mM MES-NaOH / 5 mM PIPES-NaOH at pH 6.25 for SLC25A36 (buffer B). Transport at 25°C was started by adding the indicated labeled substrates to substrate-loaded proteoliposomes (exchange) or to empty proteoliposomes (uniport). In both cases, transport was terminated by addition of 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline, which in combination and at high concentrations inhibit the activity of several mitochondrial carriers rapidly and completely (see, for example, Refs. 20-23). In controls, the inhibitors were added at the beginning together with the radioactive substrate. Finally, the external radioactivity was removed from each sample of proteoliposomes by a Sephadex G-75 column pre-equilibrated with buffer A or buffer B for SLC25A33 and SLC25A36, respectively, and the entrapped radioactivity was measured. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by proteoliposomes after 1.5 min (SLC25A33) or 3 min (SLC25A36) (in the initial linear range of substrate uptake). For efflux measurements, proteoliposomes containing 2 mM substrate were labeled with 5  $\mu$ M radioactive substrate by carrier-mediated exchange equilibration (18, 24). After 40 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 columns pre-equilibrated with buffer A or buffer B for SLC25A33 or SLC25A36, respectively. Efflux was started by adding unlabeled external substrate or buffer alone (buffer A for SLC25A33 and buffer B for SLC25A36) to aliquots of proteoliposomes and terminated by adding the inhibitors indicated above.

*Subcellular localization*—For the subcellular localization of SLC25A36 in Chinese hamster ovary (CHO) cells, these cells were co-transfected with 4  $\mu$ g of mtEBFP/pcDNAI (where EBFP stands for

enhanced blue fluorescent protein) and 4  $\mu$ g of a modified pcDNA3 plasmid containing the coding sequence of SLC25A36 fused with the enhanced green fluorescent protein (EGFP) sequence at the C-terminus (25). EGFP and EBFP fluorescence were detected as described (25).

*Yeast strains, media and growth conditions*—The strains used in this study are all in W303 genetic context (*his3-11,15; ade2-1; leu2-3,112; ura3-1; trp1-1; can1-100*). The wild-type W303  $\rho^{\circ}$  haploid strain was produced as follows: cells were grown at a density of  $1 \times 10^6$  cells/ml on YPD medium for 24 hours, then 0.05 M phosphate buffer pH 6.5 and 50  $\mu$ g/ml etidium bromide were added to 1 ml of culture. This culture was incubated at 28°C for 24 h. Then the cells were washed twice with H<sub>2</sub>O and plated on YPD medium. The lack of  $\rho^{\circ}$  cell growth was assessed on glycerol as carbon source and the absence of mtDNA by DAPI staining. *RIM2* $\Delta$  diploid strain (*RIM2/RIM2::kanMX*) was generated using the PCR-mediated gene disruption technique (26) by replacing one of the two wild-type *RIM2* copies with the *kanMX* cassette in wild-type W303 diploid strain (EUROSCARF). This strain was sporulated to obtain the wild-type and the *RIM2* $\Delta$  haploid strains. To obtain *RIM2* $\Delta$  strain containing mtDNA, the *RIM2* $\Delta$  haploid strain was transformed with the *RIM2*-pRS42H, *SLC25A33*-pRS42H or *SLC25A36*-pRS42H plasmid, and crossed with the wild-type haploid strain of opposite mating type to obtain the diploid strains. Cell crossing was generated by bringing near the cells on plate with the needle of the Singer micromanipulator. The generation of zygotes was usually produced in 2 hours. All the transformed *RIM2* $\Delta$  diploid strains were used for tetrad dissection in order to obtain transformed *RIM2* $\Delta$  haploid strains containing mtDNA (named *RIM2* $\Delta$  + *RIM2*, *RIM2* $\Delta$  + A33, *RIM2* $\Delta$  + A36).

Wild-type, wild-type  $\rho^{\circ}$  and deletion strains were grown at 28°C in rich medium containing 1% bactopectone and 1% yeast extract, supplemented with 2% glucose (YPD) or 3% glycerol (YPG). 2.2% bacto agar (Difco) was also present in solid media. In all cases the final pH was adjusted to 4.5. The pre-sporulation medium contained 1% yeast extract, 1% bactopectone, 0.17% yeast nitrogen base, 1% potassium acetate, 0.5% ammonium sulphate and 0.05 M potassium

phthalate pH 5, and the sporulation medium contained 1% potassium acetate pH 7.6.

*Procedures for sporulation and dissection*—After 48 hours of growth on YPD plate at 28°C, diploid strains were inoculated in pre-sporulation medium; after 24 hours, cells were collected, washed two times in H<sub>2</sub>O and inoculated in the sporulation medium. To avoid plasmid loss hygromycin (100 µg/ml) was added to the pre-sporulation medium of the diploid strains containing the plasmid *RIM2*-pRS42H, *SLC25A33*-pRS42H or *SLC25A36*-pRS42H. The formation of tetrads was followed by microscopic observation and diploid strains were treated 18 minutes with cytohelicase (Sigma); the spores of asci were separated with the needle of the Singer micromanipulator. After three days, tetrad analysis was performed using marker selection: *RIM2*Δ spores were geneticin (G418) resistant; transformed *RIM2*Δ spores were hygromycin resistant.

*Microscopy*—The microscope used was a Zeiss Axio Imager Z1 Fluorescence Microscope with AxioVision 4.8 Digital Image Processing System, and the objective len used was 63x Oil. The fluorescence was observed using filter sets for DAPI (365 nm excitation and 445/450 nm emission), DASPMI (550/25 nm excitation and 605/670 nm emission) and GFP (470/40 nm excitation and 525/50 nm emission).

*Measurements of oxygen consumption*—Intact cell respiration was determined at 30°C using an Oxygraph-2 k system (Oroboros, Innsbruck) equipped with two chambers, and the data were analyzed using DatLab software (27). Exponentially growing cultures in YPD or YPG were harvested at an OD<sub>600</sub> of 0.7 to 1, centrifuged at 3,000 x g for 5 min at 4°C, and resuspended in YP at a density of approximately 5 OD<sub>600</sub> units. 50 µl of this suspension correspondig to about 5 × 10<sup>6</sup> cells/ml were added to each chamber containing 2 ml of YP. The chambers were closed and respiration was recorded. The highest rate of respiration was determined by adding 0.5% ethanol and then 2.5-10 µl of 2 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma). The addition of 2 µM antimycin A (Sigma) accounted for non-mitochondrial oxygen consumption.

*Other methods*—Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. The identity of purified SLC25A33 and SLC25A36

was assessed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry of trypsin digests of the corresponding band excised from a Coomassie-stained gel (28, 29). The amount of pure SLC25A33 and SLC25A36 was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard. To assay the protein incorporated into liposomes, the vesicles were passed through a Sephadex G-75 column, centrifuged at 300,000 x g for 30 min and delipidated with organic solvents as described in Capobianco et al. (30). Then, the SDS-solubilized protein was determined by comparison with carbonic anhydrase in SDS gels. The share of incorporated SLC25A33 or SLC25A36 was about 18% of the protein added to the reconstitution mixture. For fluorescence microscopy analyses cells were grown in YPD liquid medium at 28°C; then exponential phase cells were fixed with 1% formaldehyde and treaded with 1 µg/ml DAPI (4',6'-diamidino-2-phenylindole, Sigma) for DAPI staining or treated with 1 mM DASPMI (2-(4-(dimethylamino)styryl)-1-methylpyridinium iodide, Sigma) for DASPMI staining.

## RESULTS

*Subcellular localization of SLC25A36*—Since the presence of MCF proteins is by no means restricted to mitochondrial membranes (7, 31 and references therein) and the subcellular localization of SLC25A36 has not yet been determined, the intracellular localization of SLC25A36 was investigated. CHO cells were transfected with the pcDNA3-SLC25A36-EGFP plasmid, and images were acquired 40-45 hours after transfection. About 35% of cells were transfected, and green fluorescence revealed typical mitochondrial localization of SLC25A36 (Fig. 1). Furthermore, the green fluorescence of the GFP-tagged protein completely overlapped with the blue fluorescence of a mitochondrially targeted BFP co-expressed in the same cells. Thus SLC25A36, which lacks a canonical mitochondrial targeting N-terminal extension, contains in its amino acid sequence the structural information for import into mitochondria in accordance with data available for other mitochondrial carriers (32, 33).

*Bacterial expression of SLC25A33 and SLC25A36*—Reconstitution of recombinant proteins in liposomes is a method frequently used to identify

transport properties of carrier proteins. Therefore, the open reading frames of *SLC25A33* and *SLC25A36* were expressed in *E. coli* BL21(DE3) cells (Fig. 2, lanes 4 and 7, respectively). The gene products accumulated as inclusion bodies and were purified by centrifugation and washing (Fig. 2, lanes 5 and 8, respectively). The apparent molecular masses of the purified proteins were about 36.3 and 36.8 kDa for SLC25A33 and SLC25A36, respectively, in good agreement with their respective molecular masses (calculated values with initiator methionine, 35.374 and 34.281 kDa, respectively). The identities of both recombinant proteins were confirmed by MALDI-TOF mass spectrometry and the yield of the purified proteins was approximately 40 mg per liter of culture for both SLC25A33 and SLC25A36. The recombinant proteins were not detected in bacteria harvested immediately before induction of expression (Fig. 2, lane 2 for SLC25A33), nor in cells harvested after induction but lacking the coding sequence in the expression vector (Fig. 2, lane 3 for SLC25A33 and lane 6 for SLC25A36).

*Functional characterization of SLC25A33 and SLC25A36*—In the search for potential substrates of SLC25A33 and SLC25A36, we based our choice on the fact that SLC25A33 was previously identified as a pyrimidine nucleotide carrier transporter of primarily UTP and both SLC25A33 and SLC25A36 are related to Rim2p, which has been thoroughly characterized and shown to be the pyrimidine nucleotide transporter of mitochondria from *S. cerevisiae* (10). In homoexchange experiments (i.e., with the same substrate inside and outside), liposomes reconstituted with recombinant and purified SLC25A33 catalyzed active [<sup>3</sup>H]UTP/UTP and [<sup>3</sup>H]TTP/TTP exchanges and liposomes reconstituted with recombinant and purified SLC25A36 catalyzed active [<sup>3</sup>H]CTP/CTP and [<sup>3</sup>H]GTP/GTP exchanges (Fig. 3). These homoexchanges were completely inhibited by a mixture of pyridoxal 5'-phosphate and bathophenanthroline. In contrast, despite the long incubation period (i.e. 30 min) both SLC25A33- and SLC25A36-reconstituted liposomes did not catalyze homo-exchanges of NAD<sup>+</sup>, S-adenosyl methionine and arginine (Fig. 3). Importantly, no [<sup>3</sup>H]UTP/UTP and [<sup>3</sup>H]TTP/TTP exchange activities in liposomes reconstituted with SLC25A33 and no [<sup>3</sup>H]CTP/CTP and [<sup>3</sup>H]GTP/GTP exchange activities in liposomes

reconstituted with SLC25A36 were detected when SLC25A33 or SLC25A36 had been inactivated by boiling before incorporation into liposomes, or when liposomes were reconstituted with sarkosyl-solubilized protein from bacterial cells either lacking the expression vector or harvested immediately before induction of expression (results not shown). Furthermore, the above-mentioned homoexchanges were nil using pure liposomes, i.e. without incorporated protein (results not shown).

The substrate specificities of reconstituted SLC25A33 and SLC25A36 were examined in-depth by measuring the rates of [<sup>3</sup>H]UTP and [<sup>3</sup>H]CTP into SLC25A33- and SLC25A36-reconstituted liposomes, respectively, that had been preloaded with various potential substrates (Fig. 4). The highest activities of SLC25A33-mediated [<sup>3</sup>H]UTP uptake into proteoliposomes were found with internal UTP, UDP, TTP and TDP (Fig. 4A). To a lesser extent [<sup>3</sup>H]UTP also exchanged with internal dUTP, CTP, CDP, dCTP, dCDP, GTP, GDP, dGTP, dGDP and ITP. In contrast, the uptake of [<sup>3</sup>H]UTP was negligible or very low in the absence of internal substrate (NaCl present, uniport) or in the presence of the following internal substrates: UMP, dUMP, TMP, CMP, dCMP, ATP, ADP, AMP, dATP, dADP, dAMP, GMP, dGMP, NAD<sup>+</sup>, FAD, coenzyme A, S-adenosyl methionine, phosphate, aspartate, lysine (Fig. 4A) and adenosine 5'-phosphosulfate, cAMP, FMN, thiamine monophosphate, thiamine diphosphate, sulfate, succinate, malate, citrate, carnitine, glutamate, glutamine, arginine, cysteine and glutathione (not shown). The (deoxy)nucleoside mono-phosphates of the U, T and C bases were either not exchanged with external [<sup>3</sup>H]UTP or exchanged at a much lower rate than the corresponding (deoxy)nucleoside di- and tri-phosphates. The latter were transported at approximately the same rate by reconstituted SLC25A33.

In liposomes reconstituted with SLC25A36 (Fig. 4B) [<sup>3</sup>H]CTP exchanged efficiently with the (deoxy)nucleotides of the bases C, U, I and G. In contrast, the SLC25A36-mediated [<sup>3</sup>H]CTP uptake in the presence of internal thymine nucleotides and adenine (deoxy)nucleotides was not significantly higher than that found in the presence of NaCl. Similarly, the amount of radioactivity taken up in the presence of internal NAD<sup>+</sup>, FAD, coenzyme A, S-adenosyl methionine, phosphate, aspartate, lysine

(fig 4B) and adenosine 5'-phosphosulfate, cAMP, FMN, thiamine monophosphate, thiamine diphosphate, sulfate, succinate, malate, citrate, carnitine, glutamate, glutamine, arginine, cysteine and glutathione (not shown) was virtually the same as that taken up by uniport. At variance with the SLC25A33-mediated [<sup>3</sup>H]UTP transport (Fig. 4A), the nucleoside monophosphates of the C, U, I and G bases are clearly transported by reconstituted SLC25A36, although at a lower rate than the corresponding nucleoside tri- and di-phosphates. The deoxynucleotides of C, U and G were transported nearly at the same rate than the corresponding nucleotides.

*Effects of inhibitors on SLC25A33 and SLC25A36 activities*—The effects of other mitochondrial carrier inhibitors on the activities of SLC25A33 and SLC25A36 were examined using [<sup>3</sup>H]UTP and [<sup>3</sup>H]CTP, respectively. Both the [<sup>3</sup>H]UTP/UTP and [<sup>3</sup>H]CTP/CTP exchange reactions catalyzed by reconstituted SLC25A33 and SLC25A36, respectively, were inhibited strongly by pyridoxal 5'-phosphate, bathophenanthroline, bromocresol purple, tannic acid and mercurials (HgCl<sub>2</sub>, mersalyl, *p*-hydroxymercuribenzoate) (Fig. 5). The SLC25A33-mediated [<sup>3</sup>H]UTP exchange was inhibited partially by  $\alpha$ -cyano-4-hydroxycinnamate and N-ethylmaleimide. In contrast, the SLC25A36-mediated [<sup>3</sup>H]CTP exchange was inhibited markedly by  $\alpha$ -cyano-4-hydroxycinnamate and very poorly by N-ethylmaleimide. Furthermore, little inhibition was observed with 1,2,3-benzenetricarboxylate, butylmalonate, phenylsuccinate, hemicholinium-3 and quinine. Notably, bongkreic acid inhibited the activities of SLC25A33 and SLC25A36 partially (36.7 and 40.5%, respectively), whereas carboxyatractyloside had a much lower effect at a concentration (10  $\mu$ M) that completely inhibits the ADP/ATP carrier (34). The inhibitor sensitivity of SLC25A33 and SLC25A36, therefore, resembles that of yeast Rim2p but is not identical.

*Kinetic characteristics of recombinant SLC25A33 and SLC25A36*—In Fig. 6A and 6B, the time-courses of 1 mM [<sup>3</sup>H]UTP or [<sup>3</sup>H]CTP uptake into liposomes reconstituted with SLC25A33 and SLC25A36, respectively, were compared either as uniport (in the absence of internal substrate) or as exchange (in the presence of 10 mM UTP or CTP,

respectively). The exchange reactions catalyzed by both SLC25A33 and SLC25A36 followed first-order kinetics, with isotopic equilibrium being approached exponentially. The rate constants and the initial rates of the exchanges deduced from the time-courses (18) were 0.04 and 0.02 min<sup>-1</sup> and 91 and 44  $\mu$ mol / min x g protein for SLC25A33 and SLC25A36, respectively. In contrast, the uniport uptake of [<sup>3</sup>H]UTP by SLC25A33 was negligible and that of [<sup>3</sup>H]CTP by SLC25A36 was very low. The uniport mode of transport was further investigated by measuring the efflux of [<sup>3</sup>H]UTP or [<sup>3</sup>H]CTP from proteoliposomes preloaded with these compounds, because this experimental approach provides a more sensitive assay for unidirectional transport (18). As shown in Fig. 6C and 6D, in the absence of external substrate both the rate and extent of radioactive substrate efflux was very low from SLC25A33-reconstituted liposomes and substantial from SLC25A36-reconstituted liposomes. Of note, with both reconstituted carriers a rapid and extensive efflux of [<sup>3</sup>H]UTP or [<sup>3</sup>H]CTP occurred upon the addition of external UTP or CTP, respectively. Furthermore, both effluxes, i.e. with and without external substrate, were completely prevented if the inhibitors pyridoxal 5'-phosphate and bathophenanthroline were present from the beginning of the proteoliposome incubation (at time 0).

The kinetic constants of recombinant SLC25A33 and SLC25A36 were determined from the initial transport rate of homoexchanges at various external labeled substrate concentrations, in the presence of a fixed saturating internal substrate concentration of 10 mM (Table 1). The specific activity (V<sub>max</sub>) of SLC25A33 for the UTP/UTP exchange at 25°C was 2-fold greater than that of SLC25A36 for the CTP/CTP exchange, whereas the half-saturation constants (K<sub>m</sub>) of SLC25A33 for external UTP and of SLC25A36 for external CTP were nearly the same and similar to the physiological concentrations of UTP and CTP in human cells (35). Several external substrates were competitive inhibitors of SLC25A33 and SLC25A36, as they increased the apparent K<sub>m</sub> without changing the V<sub>max</sub> of the UTP/UTP and CTP/CTP exchange, respectively (not shown). The inhibitory constants (K<sub>i</sub>) of these compounds for SLC25A33 were: 174  $\pm$  20  $\mu$ M (UTP), 195  $\pm$  23  $\mu$ M

(CTP),  $318 \pm 28 \mu\text{M}$  (GTP) and  $333 \pm 35 \mu\text{M}$  (ITP), and those for SLC25A36 were:  $1.3 \pm 0.2 \text{ mM}$  (UTP),  $224 \pm 19 \mu\text{M}$  (CTP),  $276 \pm 26 \mu\text{M}$  (GTP) and  $181 \pm 25 \mu\text{M}$  (ITP). These results show that UTP and CTP are the best substrates of SLC25A33 and SLC25A36, respectively.

*SLC25A33 and SLC25A36 genes are able to complement the absence of RIM2 in the S. cerevisiae RIM2 null mutant*—To investigate whether the two human genes can replace the Rim2p function *in vivo*, the *RIM2* $\Delta$  haploid strain was transformed with the yeast plasmid pRS42H containing the *SLC25A33* or *SLC25A36* gene under the yeast *RIM2* promoter (see Experimental procedures). The *RIM2* $\Delta$  haploid strain was unable to grow on non-fermentable carbon sources like glycerol (see next section), because this strain loses mtDNA at very high frequency, as visualised with DAPI staining (Fig. 7A and Ref. 9). As a consequence, in order to perform a gene complementation assay, it was necessary to reintroduce the mtDNA in the *RIM2* $\Delta$  strain transformed with *SLC25A33* or *SLC25A36*. First we verified whether the *RIM2* $\Delta$  haploid strain transformed with the *S. cerevisiae RIM2* gene could reintroduce the mtDNA. To this end this transformed strain was crossed with the wild type W303 haploid strain, so that, during meiosis, the mtDNA segregated in all four spores, including the two *RIM2* $\Delta$  haploid strains. After sporulation and tetrad analysis, the transformed *RIM2* $\Delta$  haploid strains (named *RIM2* $\Delta$  + *RIM2*) were selected; these strains were able to grow on glycerol (not shown). Furthermore, when stained with DAPI the *RIM2* $\Delta$  + *RIM2* strain was found to contain mtDNA (Fig 7A), showing that *RIM2* is essential to maintain mtDNA. To check whether stabilization of mtDNA in the *RIM2* $\Delta$  + *RIM2* strain was due to the presence of *RIM2* gene, a plasmid loss assay was performed and the resulting cells without plasmid were devoid of mtDNA (not shown).

Having verified that the presence of the *RIM2* gene in *RIM2* $\Delta$  cells reintroduced the mtDNA, we applied the same procedure to investigate the effect of complementing *RIM2* $\Delta$  cells with *SLC25A33* or *SLC25A36*. Fig. 7A shows that the *S. cerevisiae RIM2* $\Delta$  strains upon transformation with the *SLC25A33* or *SLC25A36* gene, crossing with the wild-type strain, sporulation

and tetrad dissection (named *RIM2* $\Delta$  + A33, *RIM2* $\Delta$  + A36 strains, respectively) contained mtDNA, proving that both the two human genes are able to maintain mtDNA as the *S. cerevisiae RIM2* gene does. The stabilization of mtDNA in *RIM2* $\Delta$  + A33 and *RIM2* $\Delta$  + A36 was due to the presence of the human genes because (i) after the loss of plasmid containing *SLC25A33* or *SLC25A36*, these strains were unable to grow on glycerol, and (ii) no mtDNA was observed when the *RIM2* $\Delta$  strain was transformed with the empty plasmid (data not shown). To verify whether the mitochondria were functional, all the strains under investigation were incubated with DASPMI, a vital dye which stains only the membranes of energized mitochondria with a membrane potential positive outside (36). As shown in Fig 7B, the mitochondrial membranes of the *RIM2* $\Delta$  strain as well as that of the wild type rho<sup>o</sup> strain were not stained by DASPMI; by contrast, the mitochondrial membranes of the *RIM2* $\Delta$  + *RIM2*, *RIM2* $\Delta$  + A33, *RIM2* $\Delta$  + A36 strains were stained as the wild type strain, indicating that the mitochondria of the latter strains are functional. Furthermore, the mitochondrial morphology of the various *S. cerevisiae* strains was investigated by using mito-gfp, a mitochondria-targeted green fluorescent protein (37). The *RIM2* $\Delta$  strain transformed with mito-gfp exhibited a fragmented mitochondrial morphology similar to that of wild type rho<sup>o</sup> cells (Fig 7C). In contrast, the *RIM2* $\Delta$  + *RIM2*, *RIM2* $\Delta$  + A33, *RIM2* $\Delta$  + A36 strains transformed with mito-gfp displayed a typical tubular mitochondrial morphology, as the wild-type strain. These results demonstrate that the human genes *SLC25A33* and *SLC25A36* are able to complement the absence of *RIM2* in *S. cerevisiae* cells restoring mtDNA, the mitochondrial membrane potential and the tubular mitochondrial morphology.

In another set of experiments the mitochondrial-dependent oxygen consumption of the wild-type, *RIM2* $\Delta$ , *RIM2* $\Delta$  + *RIM2*, *RIM2* $\Delta$  + A33 and *RIM2* $\Delta$  + A36 strains was measured in a high resolution oxygraph. With the exception of the *RIM2* $\Delta$  strain grown in YPD, all the other strains were grown in YPD (Fig. 8A) or YPG (Fig. 8B), and respiration was determined in YP medium. The oxygen consumption rate of the wild-type strain was  $0.57 \text{ nmol O}_2 \times \text{min}^{-1} \times 10^6 \text{ cells}$  (Fig. 8A) and  $1.61 \text{ nmol O}_2 \times \text{min}^{-1} \times 10^6 \text{ cells}$  (Fig. 8B), values which

are close to those measured by other investigators under similar conditions (38,39). As shown in Fig. 8A and 8B, independently from the fermentative or non-fermentative carbon source used for growth, the expression of *RIM2* or each of the two human genes in *RIM2* $\Delta$  cells restored respiration to wild-type or higher levels. Virtually the same large differences between the wild-type, *RIM2* $\Delta$ , *RIM2* $\Delta$  + *RIM2*, *RIM2* $\Delta$  + A33 and *RIM2* $\Delta$  + A36 were found when measuring the highest rate of respiration in the presence of the uncoupler CCCP; indeed, the CCCP-stimulated respiration / basal respiration ratio was about 2.1 for all the strains grown both in YPD and YPG.

*SLC25A33 and SLC25A36 are able to rescue the lack of growth of the S. cerevisiae RIM2 null mutant*—The lack of growth of *RIM2* $\Delta$  cells on glycerol was largely restored by expressing *SLC25A33*, *SLC25A36* or, as a control, *RIM2* in mtDNA-containing *RIM2* $\Delta$  cells both in solid (Fig. 9A) and liquid (Fig. 9B) YPG medium. In contrast, when the *RIM2* $\Delta$  cells were transformed with the empty vector, no growth restoration was observed (not shown). Similar results were obtained using ethanol and pyruvate as non-fermentative carbon sources, instead of glycerol, in solid and liquid media (data not shown).

## DISCUSSION

In humans several proteins have been identified at the molecular and biochemical level as mitochondrial carriers for adenine nucleotides (1, 2 and references therein) but none of them transported pyrimidine ribo- or deoxy-nucleotides at any appreciable rate. These nucleotides are synthesized in the cytosol by the *de novo* pathway (absent in the mitochondria), and are required inside the mitochondrial matrix for DNA and RNA synthesis. Mitochondria contain the nucleotide salvage pathway which synthesizes nucleotides from the corresponding nucleosides that are transported into the organelles by the equilibrative nucleoside transporters hENT1 and hENT3 (40, 41). As the contribution of the salvage pathway to mtDNA synthesis is insufficient in quiescent cells and even more in cycling cells (42-44), pyrimidine nucleotides must be transported from the cytosol to the mitochondrial matrix. In the past a partial purification from human mitochondria of a carrier that displayed an efficient transport activity for

dCTP has been achieved (45). Furthermore, in studies with isolated mitochondria evidence for a specific and saturable transporter for thymidine monophosphate has been provided (46). However, the molecular nature of these carriers has not been defined.

The results of this study including the transport properties and kinetic characteristics of recombinant *SLC25A33* and *SLC25A36*, together with the localization to mitochondria of *SLC25A36*, presented here, and *SLC25A33*, reported previously (11), demonstrate that these proteins are mitochondrial transporters for pyrimidine ribo- and deoxy-nucleotides. These carriers share 59.8% identical amino acids. However, it is not possible to make reliable assumptions on the substrate specificity or on the transport modes on basis of the amino acid similarity. Therefore, we decided to analyze the biochemical properties of both proteins in a reconstituted system. Similar to other members of the MCF, *SLC25A33* and *SLC25A36* appear as inclusion bodies after recombinant synthesis in *E. coli*. Such inclusions, however, are advantageous since they allow the purification of the heterologously expressed carriers by centrifugation and washing.

Our direct transport measurements show that *SLC25A33* and *SLC25A36* possess a similar substrate specificity. Indeed, both carriers transport pyrimidine nucleotides although with different efficiency. They also transport guanine and inosine nucleotides, but none of the many other compounds tested including adenine (deoxy)nucleotides and dinucleotides. However, *SLC25A33* and *SLC25A36* differ in a number of transport properties. Unlike *SLC25A36*, *SLC25A33* does not transport nucleoside monophosphates. This carrier transports (deoxy)nucleoside di- and tri-phosphates at about the same rate with the following order of efficacy UXP > TXP > CXP. The best substrates of *SLC25A36* are the cytosine (deoxy)nucleoside mono-, di- and tri-phosphates. In comparison with the cytosine (deoxy)nucleotides, the uracil nucleotides are transported by reconstituted *SLC25A36* less efficiently and the thymine nucleotides virtually not at all. According to our kinetic data these large differences in the rate of pyrimidine nucleotide transport can be accounted for by the higher  $K_m$  and lower  $V_{max}$  values of *SLC25A36* for uracil and thymine nucleotides than for cytosine nucleotides. The guanine (deoxy)nucleotides, which are also transported by both *SLC25A33* and *SLC25A36*, have  $K_m$  similar

to those of the respective best substrates but much lower  $V_{max}$  values. Furthermore, in contrast to SLC25A33 and the great majority of mitochondrial carriers which are obligatory exchangers (1, 47), SLC25A36 catalyzes uniport besides exchange of substrates. In this respect, SLC25A36 resembles the carriers for phosphate, glutamate and carnitine/acylcarnitine, which are also capable of mediating uniport at a much lower rate than exchange (48-50).

The substrate specificity of SLC25A33 and SLC25A36 is distinct from that of the about 30 members of the MCF characterized until now. Furthermore, SLC25A33 and SLC25A36 do not exhibit significant sequence homology with any other MC greater than the homology existing among the different members of the MCF. It is therefore likely that SLC25A33 and SLC25A36 are the only pyrimidine nucleotide carriers of the SLC25 family.

As pyrimidine nucleotide carriers, SLC25A33 and SLC25A36 are essential for a number of major processes occurring in the mitochondria, i. e. the synthesis and breakdown of DNA and the various types of RNA including the RNA primers necessary for the initiation of DNA replication and repair. In these processes the pyrimidine (d)NTPs are the precursors of DNA and RNA synthesis and the pyrimidine (d)NMPs are the products of their breakdown. The biochemical properties of recombinant SLC25A33 [that does not transport (d)NMPs] indicate that the main function of this transporter is to catalyze the exchange of cytosolic pyrimidine (d)NTPs for intramitochondrial pyrimidine (d)NDPs. The latter can be produced inside the mitochondria by the intramitochondrial enzymes that convert pyrimidine (d)NMPs to the corresponding (d)NDPs (51,52). According to Floyd et al. (11) and Favre et al. (12) SLC25A33 is induced by the insulin-like growth factor signaling pathway to mTor and its expression is higher in transformed fibroblasts, cancer cell lines and primary prostate cancer than in normal tissues. It is therefore likely that SLC25A33 is operative mainly in cells which grow very rapidly. As regard to SLC25A36, on the basis of our transport measurements the primary physiological function of this carrier [that transports cytosine and uracil (deoxy)nucleoside mono-, di- and tri-phosphates] is probably to catalyze uptake of pyrimidine (d)NTPs into the mitochondrial matrix in exchange for

internal pyrimidine (d)NMPs or, to a lesser extent, (d)NDPs. This exchange may play an important role in mitochondrial nucleic acid metabolism both in quiescent and cycling cells, in which the contribution of the salvage pathway is significant and minimal, respectively (42-44). It should be noted that the SLC25A36-mediated import of pyrimidine (d)NDPs in exchange for (d)NMPs should be relatively minor as compared to the uptake of (d)NTPs in exchange for (d)NMPs for thermodynamic reasons. Even if transported into mitochondria in exchange for (d)NMPs, (d)NDPs would recycle across the membrane exchanging with external (d)NTPs, thus accomplishing a net (d)NTPs<sub>out</sub>/(d)NMPs<sub>in</sub> exchange. Furthermore, SLC25A36 catalyzes an uniport transport mode besides exchange. The rate of uniport is much lower than that of the exchange. However, the uniport reaction may be necessary under certain conditions, for example during early development when mitochondrial biogenesis and the request for (d)NTPs is particularly high.

Several important findings *in vivo*, i. e. the rescue in *S. cerevisiae* *RIM2Δ* cells of mtDNA, mitochondrial respiration, mitochondrial membrane potential and growth on glycerol and other respiratory substrates, strongly support SLC25A33 and SLC25A36 controlling the uptake of pyrimidine (deoxy)nucleotides into mitochondria. Indeed, these *RIM2Δ* cell phenotypes can easily be accounted for by insufficient supply of pyrimidine (deoxy)nucleotides to the mitochondrial matrix, where they are indispensable for the synthesis of mtDNA, its transcription and replication. The altered mitochondrial morphology observed in *RIM2Δ* cells is similar to that exhibited by the wild-type W303  $\rho^o$  cells (fig.7), indicating that this unusual faulty mitochondrial structure is primarily caused by the loss of mtDNA (53). This phenotype of *RIM2Δ* cells is also restored by the *SLC25A33* and *SLC25A36* genes suggesting a complete suppression of the *RIM2Δ* cells defects once the mitochondrial DNA is stabilized by the presence of these human proteins. It cannot be excluded, however, that additional changes in *RIM2Δ* cells may contribute to the observed alteration of the mitochondrial morphology. In this respect, it is worth mentioning that Rim2p has been reported to be needed in iron utilization in mitochondria, for example in Fe-S protein maturation and haem

synthesis (54), and be capable of transporting iron and other divalent metal ions into the mitochondria in co-transport with pyrimidine nucleotides (55). Future studies are warranted to investigate the effects of SLC25A33 and/or SLC25A36 knock-out in mice to provide further insight on the physiological roles of these mitochondrial transporters. It will also be of great interest to investigate whether antiviral and anticancer

nucleoside analogs such as 2',3'-dideoxycytidine, 2',3'-dideoxyinosine and 3'-azido-3'-deoxythymidine, whose toxicity is due to mitochondrial function impairment, are transported by SLC25A33 and/or SLC25A36 from the cytosol, where they are phosphorylated, into the mitochondria where they inhibit the mtDNA polymerase  $\gamma$ .

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## FOOTNOTES

\*This work was supported by grants from the Ministero dell'Università e della Ricerca (MIUR), the National Research Council (CNR), Centro di Eccellenza in Genomica Comparata (CEGBA), Apulia Region, and the Italian Human ProteomeNet no. RBRN07BMCT\_009 (MIUR).

This report is dedicated to Prof. Ernesto Quagliariello on the occasion of the tenth anniversary of his death.

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<sup>3</sup>The abbreviations used are: (d)NTPs, (deoxy)nucleoside triphosphates; (d)NDPs, (deoxy)nucleoside diphosphates; (d)NMPs, (deoxy)nucleoside monophosphates; mtDNA, mitochondrial DNA; MC, mitochondrial carrier; MCF, mitochondrial carrier family

## FIGURE LEGENDS

**FIGURE 1. Subcellular localization of SLC25A36.** CHO cells were transiently co-transfected with pcDNA3 vector carrying the DNA sequence coding SLC25A36 in frame with the GFP DNA sequence and pcDNA1 plasmid carrying the DNA sequence of a mitochondrially targeted BFP (see experimental procedures). Images were acquired by a fluorescence microscope equipped with appropriate filters. SLC25A36-GFP, fluorescence of GFP fused to SLC25A36; mt-BFP, fluorescence of the mtBFP (here shown in red); overlay, merged image of mtBFP fluorescence with SLC25A36-GFP fluorescence.

**FIGURE 2. Expression in *Escherichia coli* and purification of SLC25A33 and SLC25A36.** Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. SLC25A33, lanes 1-5, SLC25A36, lanes 6-8; markers (bovine serum albumin, bovine carbonic anhydrase and cytochrome c) in the left column; lanes 1-4, 6 and 7, *E. coli* BL21(DE3) containing the expression vector, without (lanes 1, 3 and 6), with the coding sequence of *SLC25A33* (lanes 2 and 4) and with the coding sequence of *SLC25A36* (lane 7). Samples were taken immediately before induction (lanes 1 and 2) and 5 h later (lanes 3, 4, 6 and 7). The same number of bacteria was analyzed in each sample. Lanes 5 and 8, purified SLC25A33 (2 µg) and purified SLC25A36 (4 µg) originated from bacteria shown in lanes 4 and 7, respectively.

**FIGURE 3. Homo-exchange activities in liposomes reconstituted with SLC25A33 (A) or SLC25A36 (B).** Transport was initiated by adding the indicated radioactive substrate (final concentration, 0.2 mM) to proteoliposomes preloaded internally with the same substrate (concentration, 10 mM). Where indicated (UTP + i, TTP + i, CTP + i and GTP + i), the radioactive substrate was added together with 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline. The reaction was terminated after 30 min. The values are means  $\pm$  S.D. of at least three independent experiments. Differences between the UTP/UTP and TTP/TTP (A) and the CTP/CTP and GTP/GTP (B) homoexchanges and the NAD<sup>+</sup>/NAD<sup>+</sup>, S-adenosyl methionine/S-adenosyl methionine and arginine/arginine homoexchanges were significant ( $p < 0.01$ , one way Anova and Bonferroni *t*-test). The difference between the UTP/UTP and the TTP/TTP homoexchanges in A and the difference between the CTP/CTP and the GTP/GTP homoexchanges in B were significant ( $p < 0.05$ , one way Anova and Bonferroni *t*-test). Abbreviations: SAM, S-adenosyl methionine.

**FIGURE 4. Dependence on internal substrate of the transport properties of liposomes reconstituted with recombinant SLC25A33 (A) and SLC25A36 (B).** Proteoliposomes were preloaded internally with various substrates (each concentration 10 mM). Transport was started by adding 0.16 mM [<sup>3</sup>H]UTP or 0.2 mM [<sup>3</sup>H]CTP to liposomes reconstituted with SLC25A33 or SLC25A36, respectively. The reaction time was 1.5 min (SLC25A33) and 3 min (SLC25A36). The data are means  $\pm$  S.D. of at least three independent experiments. In (A) differences between the activities of UTP uptake with internal UTP, UDP, dUTP, TTP, TDP, CTP, CDP, dCTP, dCDP, GTP, GDP, dGTP, dGDP and ITP and the activity with internal NaCl and no substrate were significant ( $p < 0.05$ , one way Anova and Bonferroni *t*-test). Differences between the activities of UTP uptake with internal substrate other than those mentioned above and the activity with internal NaCl were not significant ( $p > 0.05$ , one way Anova and Bonferroni *t*-test). In (B) differences between the activities of CTP uptake with internal UTP, UDP, UMP, dUTP, dUMP, CTP, CDP, CMP, dCTP, dCDP, dCMP, GTP, GDP, GMP, dGTP, dGDP, dGMP, ITP, IDP and IMP and the activity with internal NaCl were significant ( $p < 0.05$ , one way Anova and Bonferroni *t*-test). Differences between the activities of CTP uptake with internal substrate other than those mentioned above and the activity with internal NaCl were not significant ( $p > 0.05$ , one way Anova and Bonferroni *t*-test). Abbreviations: CoA, coenzyme A; SAM, S-adenosyl methionine; Pi, phosphate; Asp, aspartic acid; Lys, lysine.

**FIGURE 5. Effect of inhibitors on the [<sup>3</sup>H]UTP/UTP and [<sup>3</sup>H]CTP/CTP exchanges by reconstituted SLC25A33 and SLC25A36, respectively.** Transport was initiated by adding 0.16 mM [<sup>3</sup>H]UTP to SLC25A33-reconstituted liposomes preloaded internally with 10 mM UTP (black bars) or 0.2 mM [<sup>3</sup>H]CTP to SLC25A36-reconstituted liposomes preloaded with 10 mM CTP (hatched bars). The reaction time was 1.5 min (SLC25A33) and 3 min (SLC25A36). Thiol reagents were added 2 min before the labeled substrate; the other inhibitors were added together with the labeled substrate. The final concentrations of the inhibitors were 20 mM (PLP, pyridoxal 5'-phosphate; BAT, bathophenanthroline), 0.2 mM (MER, mersalyl; p-HMB, p-hydroxymercuribenzoate; HgCl<sub>2</sub>), 2 mM (BMA, butylmalonate; BTA, 1,2,3-benzenetricarboxylate; PhSuc, phenylsuccinate), 1 mM (NEM, N-ethylmaleimide; CCN,  $\alpha$ -cyano-4-hydroxycinnamate), 0.4 mM (HEM-3, hemicholinium-3; QUI, quinine), 0.3 mM (BrCP, bromocresol purple), 0.1% (TAN, tannic acid) and 10  $\mu$ M (BKA, bongkrelic acid; CAT, carboxyatractyloside). The extents of inhibition (%) for each carrier from a representative experiment are reported. The data expressed as percentage of residual activity of SLC25A33-mediated [<sup>3</sup>H]UTP/UTP or SLC25A36-mediated [<sup>3</sup>H]CTP/CTP exchange are means  $\pm$  S.E. of at least three independent experiments for each inhibitor and each carrier. The control values for uninhibited [<sup>3</sup>H]UTP/UTP and [<sup>3</sup>H]CTP/CTP exchanges were  $45.9 \pm 2.6$  and  $31.2 \pm 1.5$   $\mu$ mol / min x g protein, respectively.

**FIGURE 6. Kinetics of [<sup>3</sup>H]UTP or [<sup>3</sup>H]CTP transport in liposomes reconstituted with SLC25A33 or SLC25A36, respectively.** Liposomes were reconstituted with recombinant SLC25A33 (A and C) or

SLC25A36 (B and D). A and B, uptake of [<sup>3</sup>H]UTP and [<sup>3</sup>H]CTP. 1 mM [<sup>3</sup>H]UTP in A or 1 mM [<sup>3</sup>H]CTP in B was added to proteoliposomes containing 10 mM UTP in A or CTP in B (exchange, ●) or 10 mM NaCl and no substrate (uniport, ◆). C and D, efflux of [<sup>3</sup>H]UTP and [<sup>3</sup>H]CTP. The internal substrate of proteoliposomes (2 mM UTP in C and 2 mM CTP in D) was labelled with [<sup>3</sup>H]UTP in C and [<sup>3</sup>H]CTP in D by carrier-mediated exchange equilibration. After removal of the external substrate by Sephadex G-75, the efflux of [<sup>3</sup>H]UTP (C) or [<sup>3</sup>H]CTP (D) was started by adding buffer alone (buffer A for SLC25A33 or buffer B for SLC25A36) (◆) or in buffer A 5 mM UTP (●), 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline (□), and 5 mM UTP, 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline (▲) or in buffer B 5 mM CTP (■), 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline (□) or 5 mM CTP, 30 mM pyridoxal 5'-phosphate and 20 mM bathophenanthroline (▲). The values are means of at least three independent experiments.

**FIGURE 7. The *SLC25A33* and *SLC25A36* human genes are able to complement the absence of *RIM2* in *S. cerevisiae* cells rescuing the mtDNA, the mitochondrial membrane potential and the tubular mitochondrial morphology.** Wild-type W303, wild-type W303 rho<sup>o</sup>, *RIM2Δ* and *RIM2Δ* transformed with the *RIM2*-pRS42H, *SLC25A33*-pRS42H or *SLC25A36*-pRS42H plasmid (shown as WT, WT rho<sup>o</sup>, *RIM2Δ*, *RIM2Δ* + *RIM2*, *RIM2Δ* + A33 and *RIM2Δ* + A36, respectively) haploid strains were grown until exponential phase in liquid YPD medium at 28°C, and stained with: DAPI (A), DASPMI (B), mito-gfp (C).

**FIGURE 8. Mitochondrial respiration of wild-type W303, *RIM2Δ*, *RIM2Δ* + *RIM2*, *RIM2Δ* + A33, *RIM2Δ* + A36 strains.** With the exception of the *RIM2Δ* cells, which were grown in YPD, the other cells were grown in YPD (A) or YPG (B), washed, resuspended in YP and tested for oxygen consumption. Respiration rates by the indicated cells were corrected for the oxygen consumption measured in the presence of antimycin A. Data represent mean ± S.D. of three independent experiments. In A and B, differences between the respiratory rates of wild-type, *RIM2Δ* + *RIM2*, *RIM2Δ* + A33 and *RIM2Δ* + A36 cells and the respiratory rate of *RIM2Δ* cells were significant ( $p < 0.01$ , one-way Anova and Bonferroni *t*-test). In A the differences between the respiratory rates of *RIM2Δ* + *RIM2*, *RIM2Δ* + A33 and *RIM2Δ* + A36 cells and the respiratory rate of wild-type cells were not significant ( $p > 0.05$ , one-way Anova and Bonferroni *t*-test). In B the differences between the respiratory rates of *RIM2Δ* + A33 and *RIM2Δ* + A36 cells and the respiratory rate of wild-type cells were significant ( $p < 0.05$ , one-way Anova and Bonferroni *t*-test), whereas the difference between the respiratory rate of *RIM2Δ* + *RIM2* cells and that of wild-type cells was not significant ( $p > 0.05$ , one-way Anova and Bonferroni *t*-test).

**FIGURE 9. Effect of complementing *S. cerevisiae* *RIM2Δ* cells with human *SLC25A33* or *SLC25A36* on growth.** Wild-type W303, *RIM2Δ* and *RIM2Δ* transformed with the *RIM2*-pRS42H, *SLC25A33*-pRS42H or *SLC25A36*-pRS42H plasmid (shown as WT, *RIM2Δ*, *RIM2Δ* + *RIM2*, *RIM2Δ* + A33, *RIM2Δ* + A36, respectively) haploid strains were spotted on solid YPG medium for 48 hours at 30°C as 4-fold serial dilution (A) or were inoculated in liquid YPG medium at 30°C (B). The values of optical density at 600 nm refer to cell cultures after the indicated times of growth. The values are means of three independent experiments. (●), WT; (○), *RIM2Δ*; (◆), *RIM2Δ* + *RIM2*; (□), *RIM2Δ* + A33; and (■), *RIM2Δ* + A36 strains.

**Table 1. Kinetic parameters of recombinant SLC25A33 and SLC25A36.**  $K_m$  and  $V_{max}$  were calculated from double-reciprocal plots of the rates of [ $^3H$ ]UTP, [ $^3H$ ]CTP, [ $^3H$ ]TTP, or [ $^3H$ ]GTP uptake at various concentrations into proteoliposomes containing the same substrates (concentration, 10 mM). The data represent the means  $\pm$  S.D. of at least four independent experiments.

homoexchanges	SLC25A33		SLC25A36	
	$K_m$	$V_{max}$	$K_m$	$V_{max}$
	mM	$\mu\text{mol} / \text{min} \times \text{g protein}$	mM	$\mu\text{mol} / \text{min} \times \text{g protein}$
[ $^3H$ ]UTP/UTP	$0.16 \pm 0.01$	$98.5 \pm 10.2$	$1.19 \pm 0.15$	$30.7 \pm 4.4$
[ $^3H$ ]CTP/CTP	$0.18 \pm 0.02$	$22.5 \pm 3.8$	$0.21 \pm 0.02$	$51.2 \pm 5.7$
[ $^3H$ ]TTP/TTP	$0.08 \pm 0.01$	$50.8 \pm 6.1$	$>2.5$	$< 8$
[ $^3H$ ]GTP/GTP	$0.30 \pm 0.02$	$15.7 \pm 2.4$	$0.23 \pm 0.04$	$23.6 \pm 2.9$

**Figure 1**

SLC25A36-GFP

mt-BFP

overlay

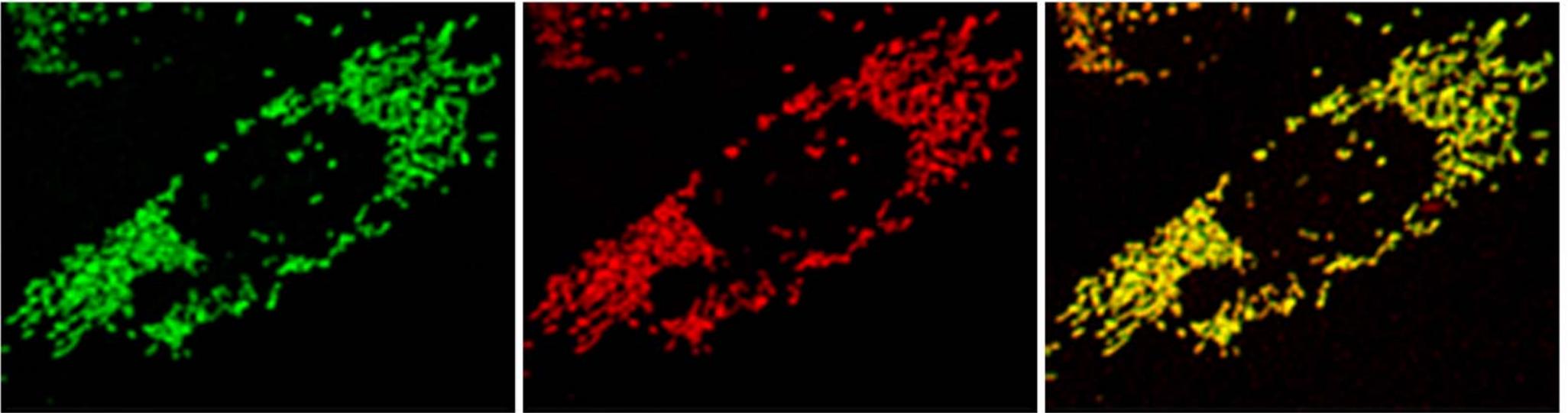


Figure 2

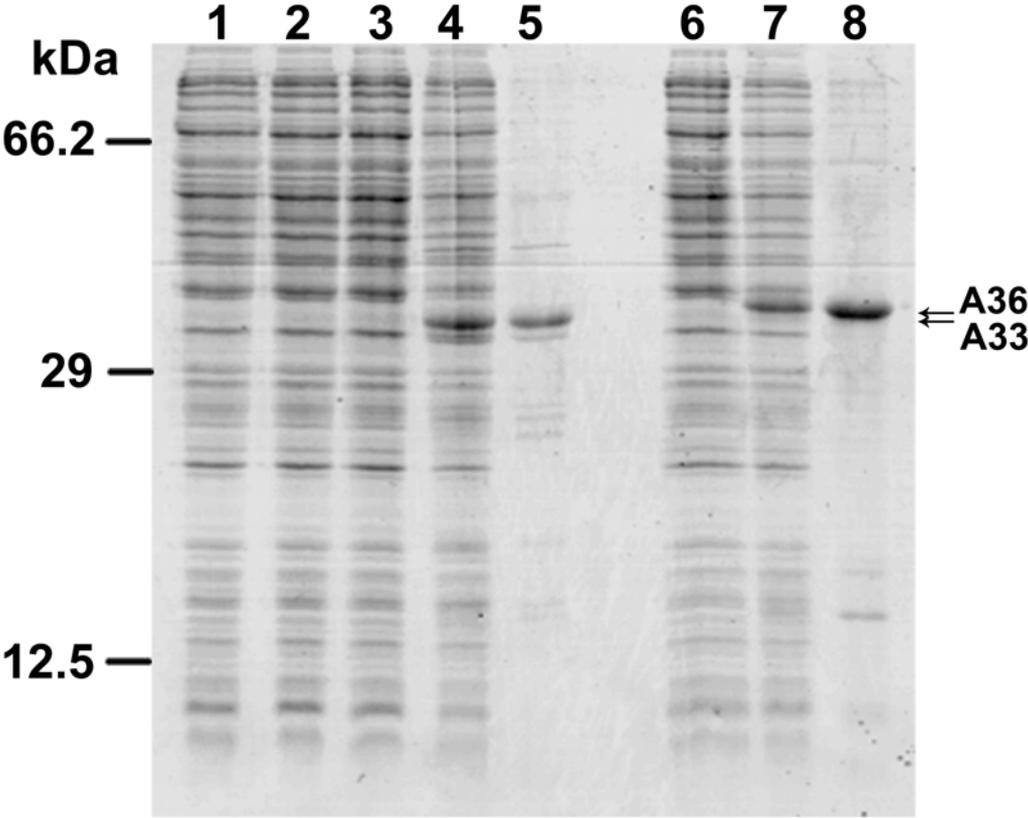


Figure 3

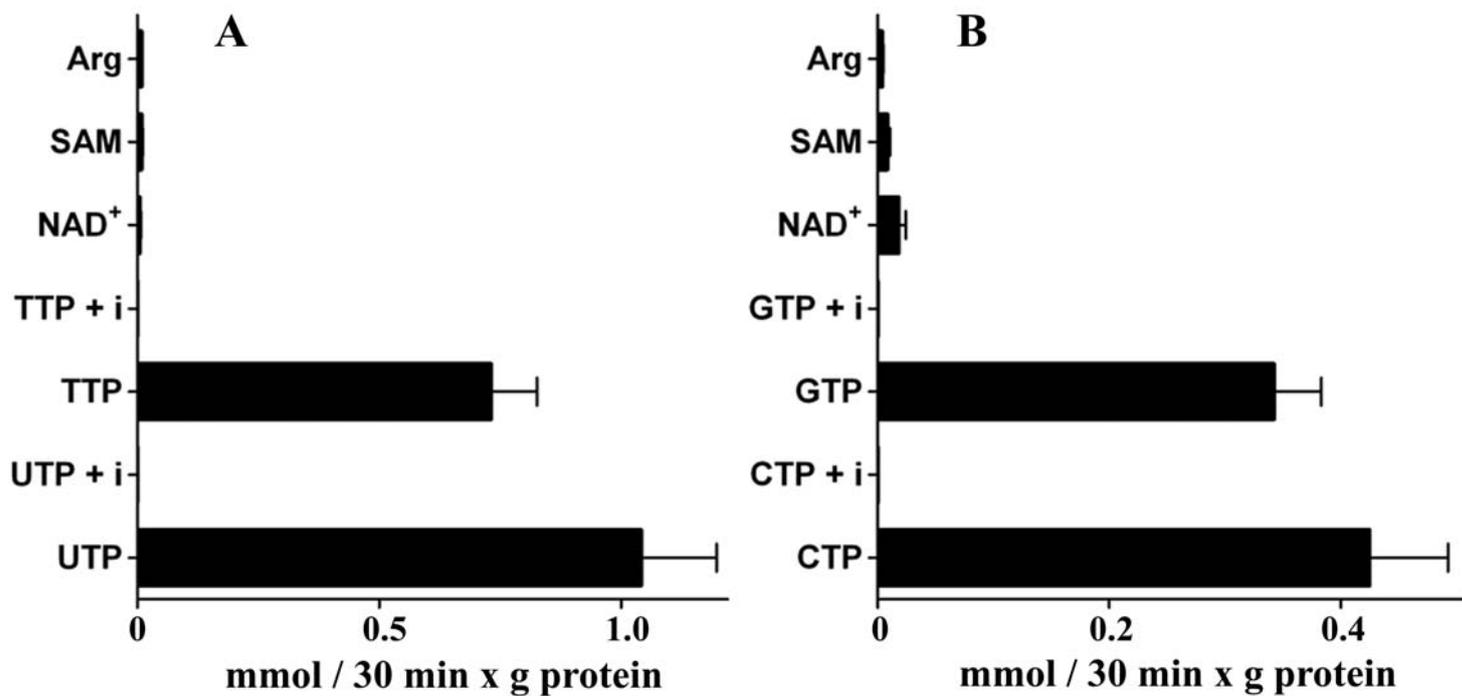


Figure 4

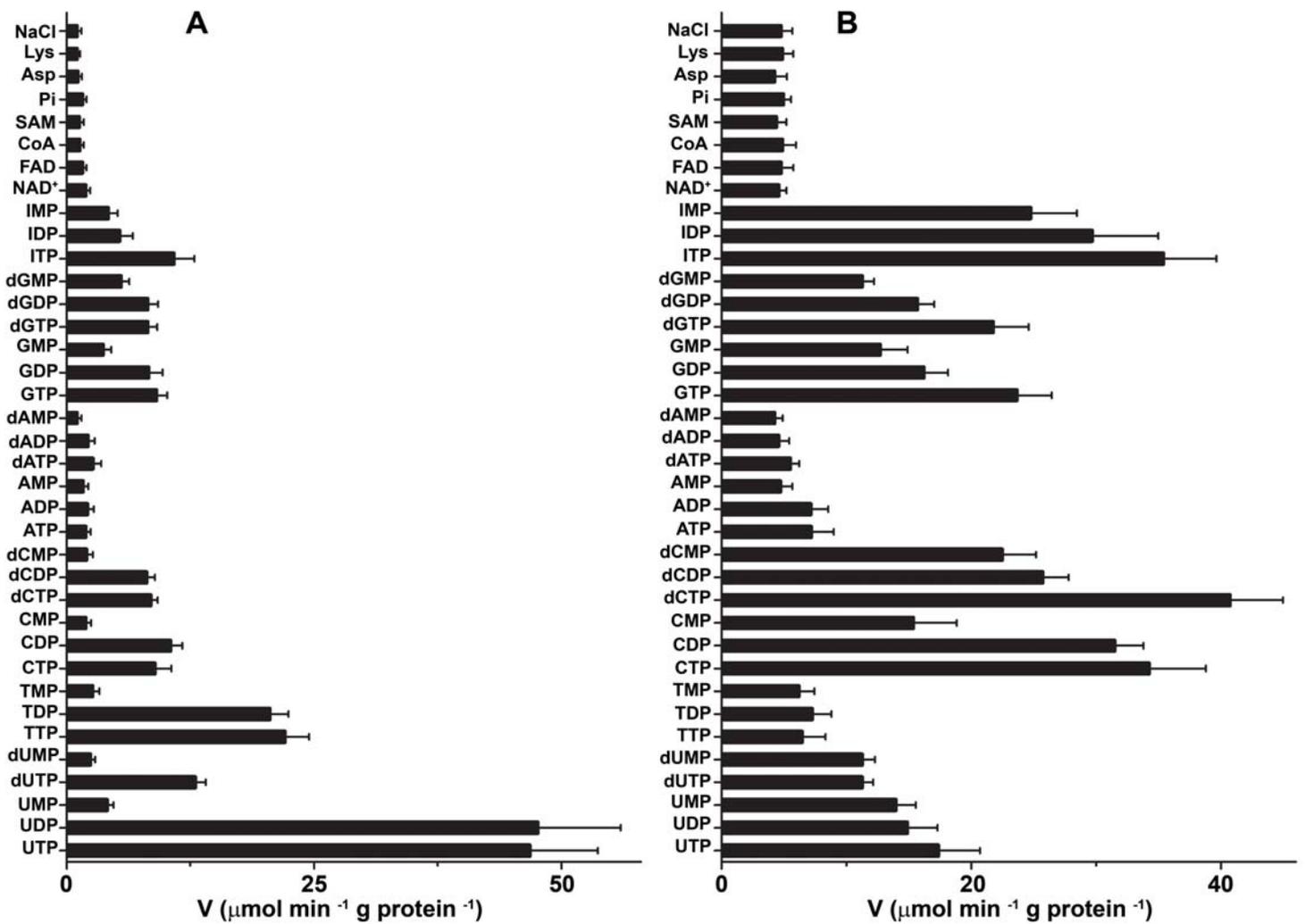


Figure 5

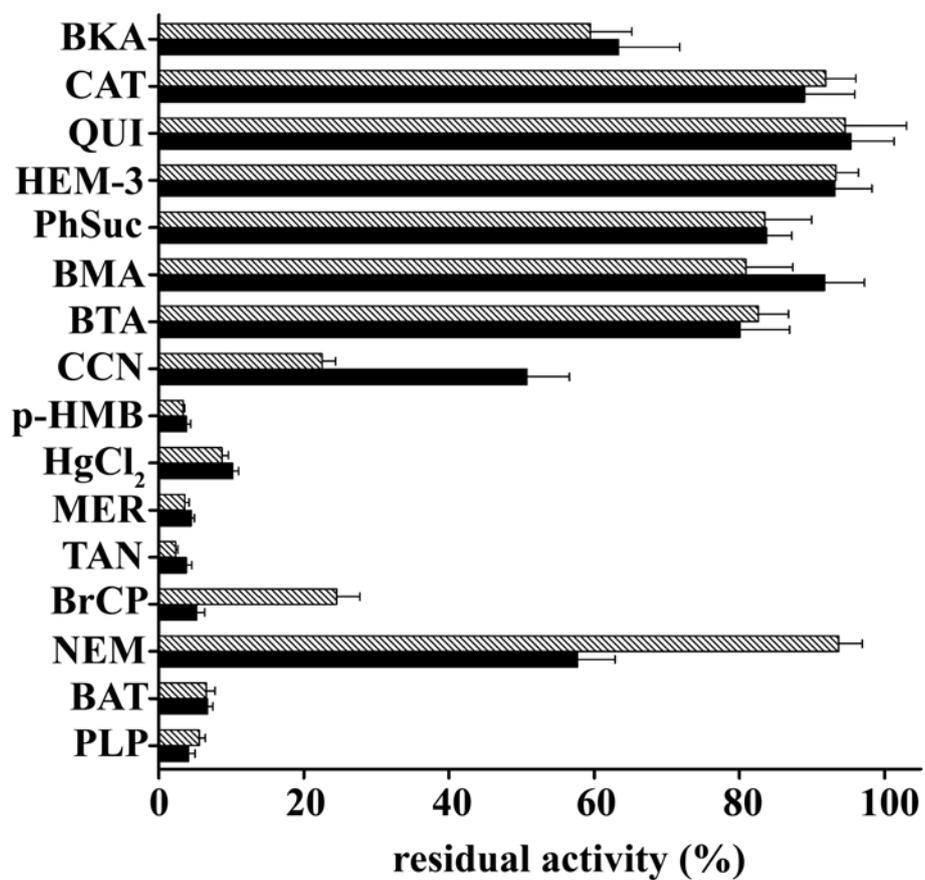


Figure 6

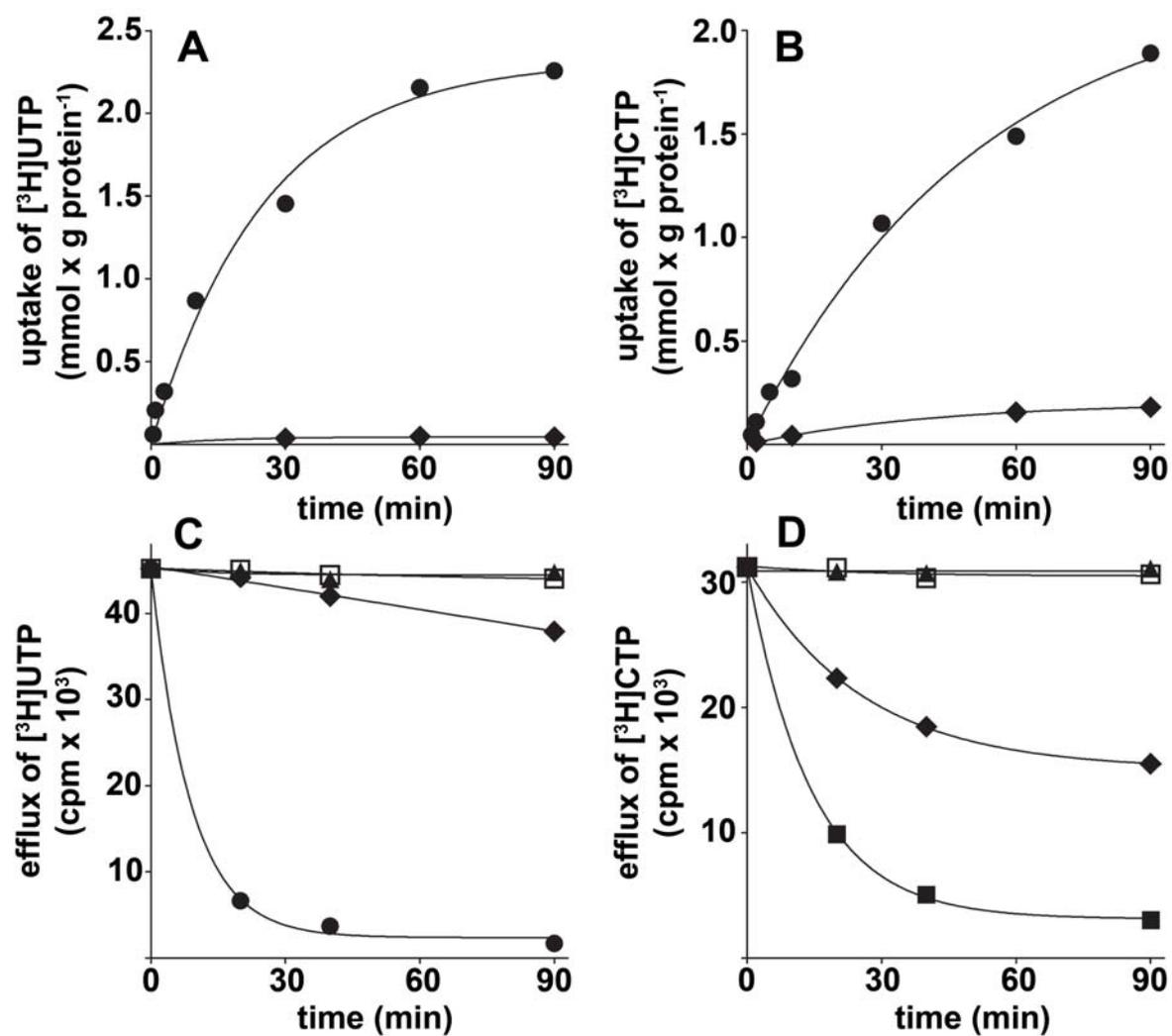


Figure 7

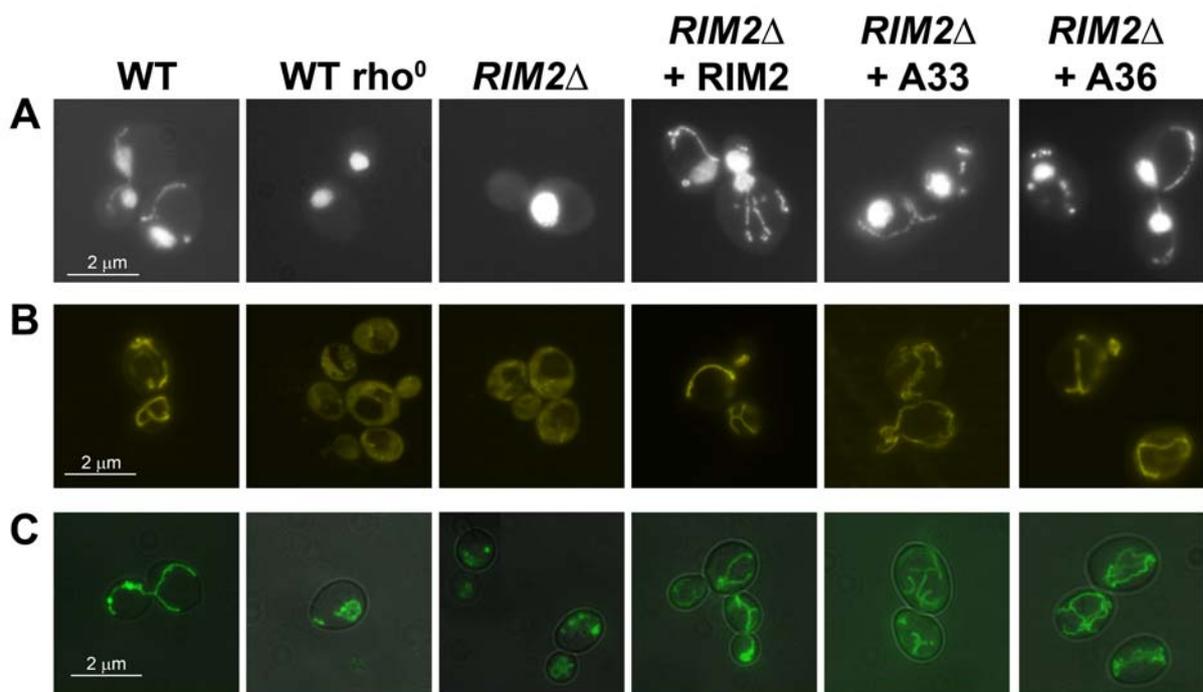


Figure 8

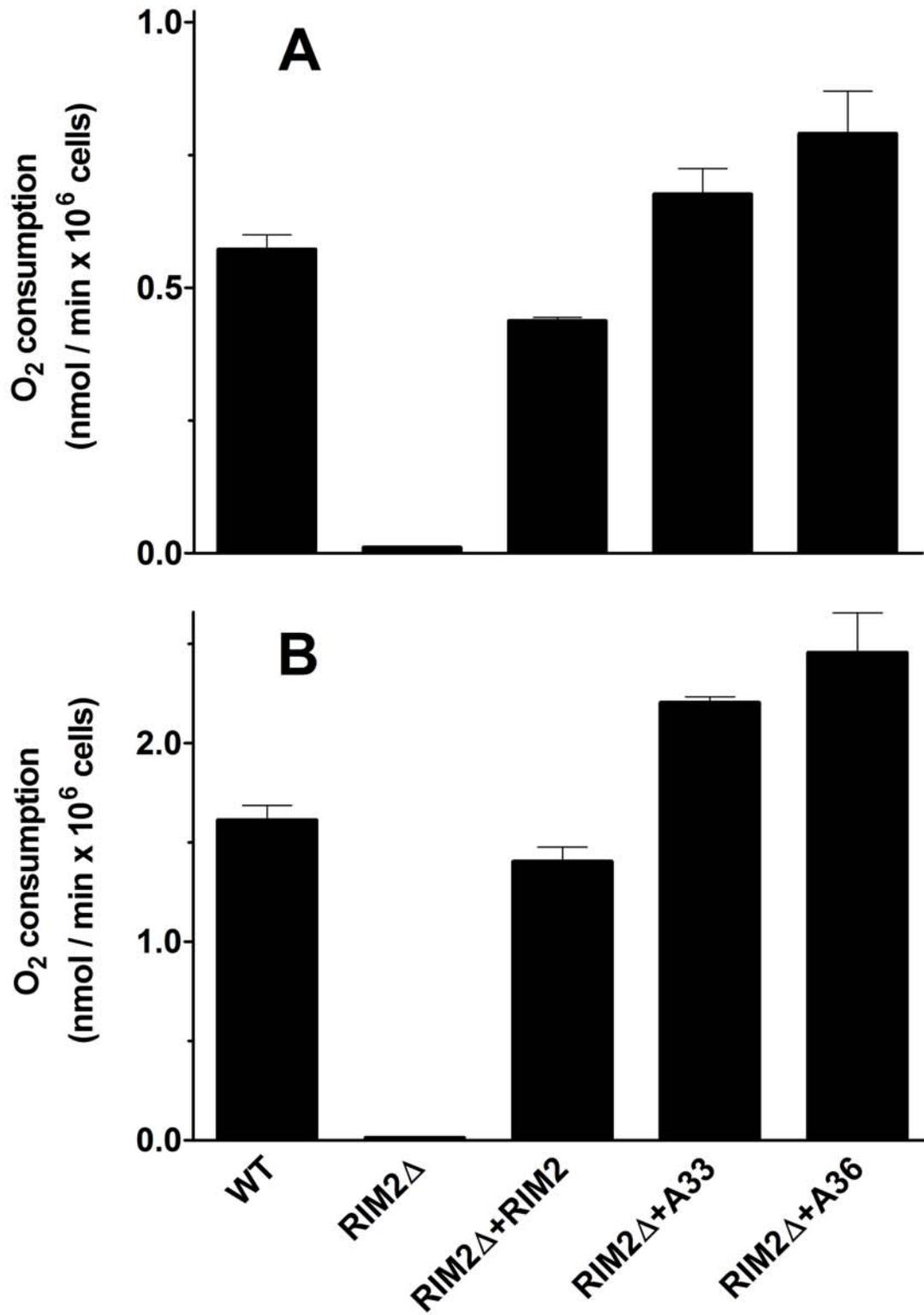
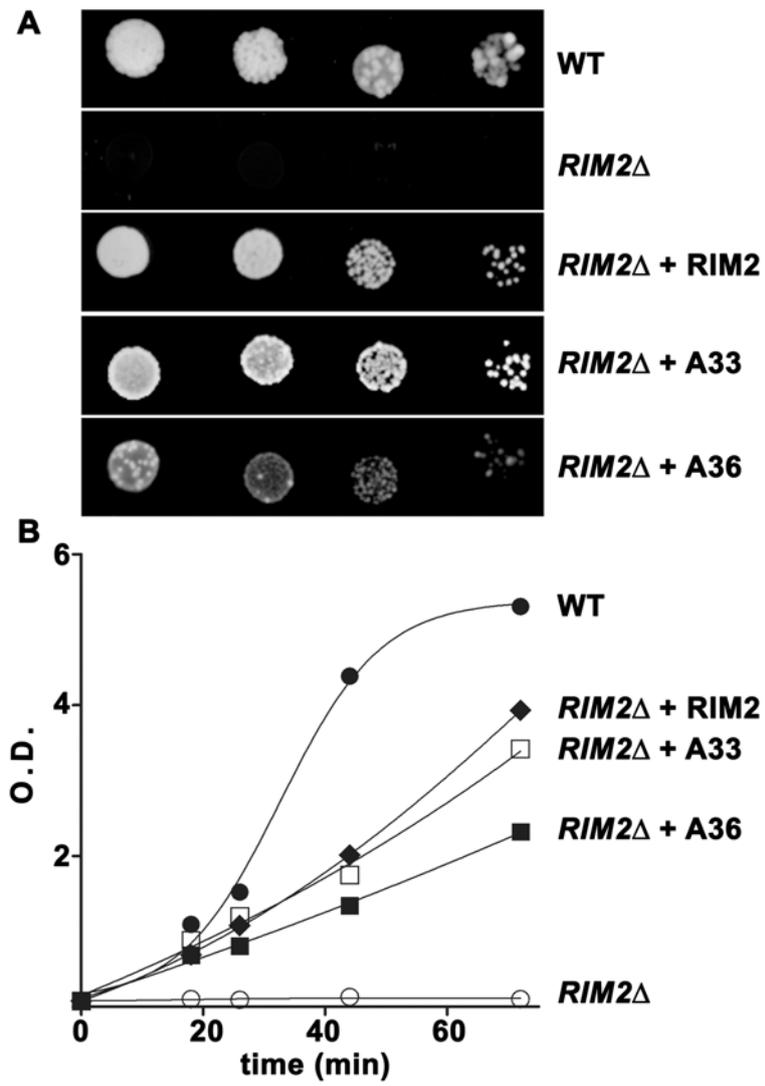


Figure 9



**The human SLC25A33 and SLC25A36 genes of solute carrier family 25 encode two mitochondrial pyrimidine nucleotide transporters**

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*J. Biol. Chem.* published online October 15, 2014

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Access the most updated version of this article at doi: [10.1074/jbc.M114.610808](https://doi.org/10.1074/jbc.M114.610808)

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