Methionine synthase (MS) catalyzes methylation of homocysteine, the last step in the biosynthesis of methionine, which is essential for the regeneration of tetrahydrofolate and biosynthesis of S-adenosylmethionine. Here, we report that MS is localized to the nucleus of Pichia pastoris and Candida albicans but is cytoplasmic in Saccharomyces cerevisiae. The P. pastoris strain carrying a deletion of the MET6 gene encoding MS (Ppmet6) exhibits methionine as well as adenine auxotrophy indicating that MS is required for methionine as well as adenine biosynthesis. Nuclear localization of P. pastoris MS (PpMS) was abrogated by the deletion of 107 C-terminal amino acids or the R742A mutation. In silico analysis of the PpMS structure indicated that PpMS may exist in a dimer-like configuration in which Arg-742 of a monomer forms a salt bridge with Asp-113 of another monomer. Biochemical studies indicate that R742A as well as D113R mutations abrogate nuclear localization of PpMS and its ability to reverse methionine auxotrophy of Ppmet6. Thus, association of two PpMS monomers through the interaction of Arg-742 and Asp-113 is essential for catalytic activity and nuclear localization. When PpMS is targeted to the cytoplasm employing a heterologous nuclear export signal, it is expressed at very low levels and is unable to reverse methionine and adenine auxotrophy of Ppmet6. Thus, nuclear localization is essential for the stability and function of MS in P. pastoris. We conclude that nuclear localization of MS is a unique feature of respiratory yeasts such as P. pastoris and C. albicans, and it may have novel moonlighting functions in the nucleus.

Methionine synthase (MS) catalyzes the terminal step of de novo biosynthesis of methionine. It transfers a methyl group from 5-methyltetrahydrofolate to L-homocysteine to form L-methionine and tetrahydrofolate (THF) (1). Two functionally and structurally distinct types of MS have been identified: cobalamin-dependent and cobalamin-independent enzymes of ∼140,000 and ∼86 kDa size, respectively (2). Although bacteria such as Escherichia coli contain both types of MS, mammals, including humans, have only the cobalamin-dependent enzyme. Fungi, plants, and some bacteria lack the ability to obtain cobalamin or to synthesize cobalamin de novo and therefore possess only the cobalamin-independent enzyme (2). Despite catalyzing similar reactions, the cobalamin-dependent and -independent enzymes exhibit significant differences in their structure as well as mechanism of action, and therefore, the latter are attractive targets for the development of antifungals against pathogenic fungi such as Candida albicans and Cryptococcus neoformans (3, 4). The crystal structures of cobalamin-independent MS of E. coli, Thermogota maritima, Arabidopsis thaliana, and C. albicans are known (5–9). In these species, MS was shown to fold into two (βα)₈ barrels with the N- and C-terminal barrels joined by an inter-domain linker. C. albicans MS (CaMS) consists of two (βα)₈ barrels, and the active site is located between the two domains (8, 9). Binding sites for a zinc ion and the substrates L-homocysteine and 5-methyltetrahydrofolate glutamate have also been mapped. Binding of L-homocysteine or methionine was shown to result in conformational rearrangements at the amino acid binding pocket, moving the catalytic zinc into position to activate the thiol group (8, 9).

Yeast strains carrying a deletion in the gene encoding MS exhibit multiple phenotypes. In Saccharomyces cerevisiae and Aspergillus nidulans, methionine auxotrophy caused by MS dysfunction can be fully rescued by methionine supplementation (3, 10). However, C. albicans carrying deletion of both the alleles encoding MS (met6/met6) cannot grow on media supplemented with exogenous methionine (3). Similarly, the met6 strain of C. neoformans grows at a slow rate in minimal media supplemented with methionine (4), whereas the met6 strain of Fusarium graminearum is defective in aerial hyphal growth when cultured on methionine-supplemented media (11). In the case of Schizosaccharomyces pombe, disruption of met26 encoding MS results in a pleiotropic phenotype with defects in cell growth and development.

This work was supported in part by Research Grant EMR/2015/000567 and J. C. Bose Fellowship Grant SB/S2/JCB-025/2015 from the Science and Engineering Research Board, Department of Science and Technology, New Delhi, India (to P. N. R.), and by the Department of Biotechnology–Indian Institute of Science partnership program. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Fig. S1.

1 Recipient of post-doctoral fellowship of Department of Biotechnology, New Delhi, India.
2 To whom correspondence should be addressed: Dept. of Biochemistry, FE15, Biology Bldg., Indian Institute of Science, Bangalore 560012, India. Tel./Fax: 91-80-23601492; E-mail: pnr@biochem.iisc.ernet.in.
3 The abbreviations used are: MS, methionine synthase; PpMS, MS encoded by P. pastoris genome; ScMS, MS encoded by S. cerevisiae genome; CaMS, MS encoded by C. albicans genome; MAT, methionine adenosyltransferase; THF, tetrahydrofolate; NES, nuclear export signal; MAS, membrane anchor signal; NLS, nuclear localization signal; TRITC, tetramethylrhodamine isothiocyanate; PDB, Protein Data Bank; F, forward; R, reverse.
methionine as well as adenine auxotrophy due to homocysteine accumulation and defective purine biosynthesis, MS may have other unknown functions in certain yeast species.

The genome of *P. pastoris*, a methylo trophic yeast, encodes a cobalamin-independent MS (PpMS), which shares 77 and 79% amino acid sequence identity with the MS of *S. cerevisiae* (ScMS) and CaMS, respectively (13). Here, we report that deletion of *MET6* results in methionine as well as adenine auxotrophy in *P. pastoris* but only methionine auxotrophy in *S. cerevisiae*. MS is uniquely localized to the nucleus of *P. pastoris* and *C. albicans*, although it localizes to the cytosol in *S. cerevisiae*. Arg-742 and Asp-113 located in the C- and N-terminal regions of PpMS interact with each other and facilitate association between two monomers in a dimer-like configuration. Interaction between Arg-742 and Asp-113 is essential for the stability, catalytic activity, and nuclear localization of MS. Taken together, our results suggest that MS is present in the nucleus of respiratory yeasts such as *P. pastoris* and *C. albicans*, and it possesses unique biochemical properties that have not been reported in any other species.

**Results**

**Deletion of *P. pastoris MET6* results in methionine as well as adenine auxotrophy**

Our laboratory has been studying key transcription factors involved in the regulation of carbon metabolism in the respiratory yeast, *P. pastoris* (14–21). While studying methionine metabolism, a methionine auxotroph (*Ppmet6*) was generated by replacing the coding region of *MET6* encoding MS with a Zeocin expression cassette in the *P. pastoris* GS115 strain (Fig. 1, A and B). MS deficiency resulted in methionine auxotrophy, and *Ppmet6* was unable to grow in a methionine-deficient medium, as expected (Fig. 1C). However, methionine supplementation did not restore the growth of *Ppmet6* (Fig. 1D) under conditions in which it readily restored the growth of *S. cerevisiae met6* strain (*Scmet6*) (Fig. 1, E and F). Of the various components tested, adenine when added to YNBD + methionine medium restored growth of *Ppmet6* (Fig. 1, G and H) indicating that MS deficiency results in methionine as well as adenine auxotrophy in *P. pastoris*.

**MS localizes to the nucleus of *P. pastoris* and *C. albicans***

To further characterize *P. pastoris* MS (PpMS), we first examined its subcellular localization. PpMS was expressed as a histidine-tagged protein in *E. coli*, purified, and injected into rabbits to generate anti-PpMS antibodies that reacted with a protein of ~86 kDa in the cell lysates of GS115 but not *Ppmet6* (Fig. 2, A and B). Immunofluorescence studies revealed that PpMS is present in the nucleus of *P. pastoris* cells cultured in YPD, YPG, or YPM media (Fig. 2C). To further validate these results, *p. pastoris* strain expressing chromosomally tagged PpMS-GFP (*Pp-PpMSGFP*) was constructed, and the localization of GFP fusion protein was visualized by direct examination of GFP fluorescence as well as immunofluorescence of DAPI-stained cells using anti-GFP and anti-PpMS antibodies. The results indicate that PpMSGFP localizes to the nucleus (Fig. 2, D and E). To further confirm nuclear localization of PpMS, *P. pastoris* GS115 strain was transformed with pGAP-PpMS<sub>Myc</sub> plasmid expressing c-Myc epitope-tagged PpMS (*PpMS<sub>Myc</sub>*) from the GAPDH promoter, and immunofluorescence studies were carried out with anti-c-Myc antibodies. The results indicate that PpMS<sub>Myc</sub> localizes to the nucleus (Fig. 2F). PpMS<sub>Myc</sub> expression was confirmed by SDS-PAGE followed by Western blotting of whole-cell extracts using anti-c-Myc as well as anti-PpMS antibodies (Fig. 2G).

Because nuclear localization of MS has not been reported in any species thus far and to study the generality of this phenomenon, MS localization was examined in *C. albicans*, another...
respiratory yeast, which also exhibits methionine as well as adenine auxotrophy when MET6 is deleted (3). We confirmed that CaMS is immunoreactive to anti-PpMS antibodies by Western blotting (Fig. 2H). Immunoﬂuorescence studies indicate that CaMS localizes to the nucleus in C. albicans as well (Fig. 2I). Because the S. cerevisiae met6 strain does not exhibit adenine auxotrophy, we examined MS localization in this yeast species. ScMS was expressed as a GFP fusion protein (ScMSGFP) in S. cerevisiae met6 strain to generate Sc-ScMSGFP strain. Direct visualization of GFP fluorescence (Fig. 3A) as well as immunoﬂuorescence using anti-GFP antibodies (Fig. 3B) indicates that ScMSGFP is localized to the cytoplasm. Interestingly, when ScMSGFP was expressed in the Ppmet6 strain (Pp-ScMSGFP), it localized to the nucleus as evident from the direct visualization of GFP ﬂuorescence (Fig. 3C) and by immunoﬂuorescence using anti-GFP antibodies (Fig. 3D). When PpMSGFP was expressed in S. cerevisiae (Sc-PpMSGFP), it localized to the cytoplasm as evident from the direct visualization of GFP ﬂuorescence (Fig. 3E) and by immunoﬂuorescence using anti-GFP antibodies (Fig. 3F). Differential subcellular localization of MS in P. pastoris and S. cerevisiae was further conﬁrmed by confocal microscopy (Fig. 3G). Western blot analysis revealed that PpMS is present in the cytoplasm as well as nuclear fractions of P. pastoris, although it is present only in the cytoplasm of S. cerevisiae (Fig. 3H). Taken together, these results indicate that ScMS is cytosolic in S. cerevisiae but nuclear in P. pastoris, and PpMS is nuclear in P. pastoris but cytoplasmic in S. cerevisiae.

**Identification of nuclear localization signal of PpMS**

To understand the mechanism of nuclear localization of PpMS, we examined whether catalytic activity is required for its nuclear localization. In CaMS, the Asp-614 residue in the active site is essential for binding to homocysteine, and consequently, CaMSD612A mutant enzyme possesses only 2% of the native enzyme activity (22). Because this aspartate residue is conserved in PpMS (Fig. 4A), we introduced the D612A mutation into PpMSGFP. The Ppmet6 strain expressing PpMSD612AGFP was catalytically inactive as evident from its inability to grow in YNBD medium (Fig. 4B). Direct ﬂuorescence as well as immunoﬂuorescence using anti-GFP antibodies revealed that PpMSD612AGFP localizes to the nucleus (Fig. 4C–E) indicating that catalytic activity is not required for nuclear localization of PpMS.

Analysis of PpMS amino acid sequence using PSORT II, PredictNLS, and NetNTS databases indicated that PpMS does not contain a nuclear localization signal. Methanol-inducible alcohol oxidase (AOX), a peroxisomal protein was visualized with mouse anti-AOX antibodies and TRITC-conjugated goat anti-mouse antibodies. Direct examination of GFP ﬂuorescence in live P. pastoris cells expressing PpMSGFP, E, immunoﬂuorescence of PpMSGFP with rabbit anti-PpMS antibodies and FITC-conjugated goat anti-rabbit antibodies as well as mouse anti-GFP antibodies and TRITC-conjugated goat anti-mouse antibodies, F, immunoﬂuorescence of PpMSMyc with anti-c-Myc antibodies. G, Western blot analysis of c-Myc epitope-tagged PpMS (PpMSMyc) in whole-cell extracts using anti-c-Myc and anti-PpMS antibodies. H, Western blot analysis of whole-cell extracts of P. pastoris (GS115), Ppmet6, and C. albicans with anti-PpMS antibodies. PpMS is indicated by an asterisk. Protein molecular mass markers (kDa) are indicated. I, immunoﬂuorescence of CaMS in C. albicans using anti-PpMS antibodies. In all the experiments, nuclei were stained with DAPI.

**Figure 2. Subcellular localization of MS in P. pastoris and C. albicans.** A, protein proﬁle of whole-cell lysates of GS115 and Ppmet6 strains as analyzed by SDS-PAGE. B, Western blot analysis of whole-cell lysates using anti-PpMS antibodies. Protein molecular mass markers (kDa) are indicated. C, immunoﬂuorescence of PpMS in P. pastoris cells cultured in YPD, YPG, or YPM using rabbit anti-PpMS antibodies and FITC-conjugated goat anti-rabbit antibodies. Methanol-inducible alcohol oxidase (AOX), a peroxisomal protein was visualized with mouse anti-AOX antibodies and TRITC-conjugated, goat anti-mouse antibodies. D, direct examination of GFP fluorescence in live P. pastoris cells expressing PpMSGFP. E, immunoﬂuorescence of PpMSGFP with rabbit anti-PpMS antibodies and FITC-conjugated goat anti-rabbit antibodies as well as mouse anti-GFP antibodies and TRITC-conjugated goat anti-mouse antibodies, F, immunoﬂuorescence of PpMSMyc with anti-c-Myc antibodies. G, Western blot analysis of c-Myc epitope-tagged PpMS (PpMSMyc) in whole-cell extracts using anti-c-Myc and anti-PpMS antibodies. H, Western blot analysis of whole-cell extracts of P. pastoris (GS115), Ppmet6, and C. albicans with anti-PpMS antibodies. PpMS is indicated by an asterisk. Protein molecular mass markers (kDa) are indicated. I, immunoﬂuorescence of CaMS in C. albicans using anti-PpMS antibodies. In all the experiments, nuclei were stained with DAPI.
mutant carrying a deletion of 107 C-terminal amino acids, and when expressed in the *P. pastoris* GS115 strain, it remained in the cytosol (Fig. 4, C–E). Thus, the C-terminal region is essential for nuclear localization of PpMS.

Methionine is converted to S-adenosylmethionine by methionine adenosyltransferase (MAT) also known as S-adenosylmethionine synthetase. In mammalian cells, MATI/III was reported to be present in the nucleus, and basic amino acid residues in the C-terminal region of MATI/III are important for nuclear localization (23). The C-terminal region of PpMS also contains basic amino acid residues, many of which are conserved in ScMS as well as CaMS (Fig. 4F). To examine their role in nuclear localization, we substituted specific arginine/lysine residues present in the C-terminal region of PpMS by alanine. *P. pastoris* strains expressing PpMS<sup>K740A</sup>GFP, PpMS<sup>R742A</sup>GFP, PpMS<sup>K759A</sup>GFP, or PpMS<sup>R762A</sup>GFP were generated, and their expression was confirmed by Western blotting with anti-GFP antibodies (Fig. 4G). Analysis of MS-GFP localization by fluorescence microscopy indicates that all the mutants localize to the nucleus except PpMS<sup>R742A</sup>GFP, which remained in the cytosol (Fig. 4H). Expression of PpMS<sup>R742A</sup>GFP in *Ppmet6* did not result in the restoration of the growth of cells cultured in methionine- and adenine-deficient medium (Fig. 5, A and B). To understand the effect of R742A mutation on enzyme function, the same mutation was introduced into ScMS to generate ScMS<sup>R742A</sup>GFP. ScMS<sup>R742A</sup>GFP as well as PpMS<sup>R742A</sup>GFP were expressed in the *S. cerevisiae* strain, and their expression was confirmed by Western blotting using anti-GFP antibodies (Fig. 5C). ScMS<sup>R742A</sup>GFP and PpMS<sup>R742A</sup>GFP failed to reverse the methionine auxotrophy of *Smet6* (Fig. 5, D and E) indicating that the R742A mutant is catalytically inactive.

To examine the effect of extranuclear localization on enzyme stability and function, we generated *Ppmet6* strains expressing PpMSGFP-NES and PpMSGFP-MAS in which a nuclear export signal (NES) or a membrane anchor signal was fused to the C terminus of PpMS, respectively (Fig. 6A). We also generated PpMSGFP-NLS, in which a nuclear localization signal was fused to the C terminus of PpMSGFP (Fig. 6A). As expected, PpMSGFP-NES and PpMSGFP-NLS were targeted to the cytoplasm and nucleus, respectively (Fig. 6B). Although PpMSGFP-MAS was targeted to the plasma membrane, nuclear localization was not completely abrogated (Fig. 6B, B and C). Although expression of PpMSGFP-NLS and PpMSGFP-MAS was observed in almost all cells, expression of PpMSGFP-NES was restricted to a small subset of cells (Fig. 6B). Western blot analysis indicated that expression of PpMSGFP-NES was the lowest among the three proteins (Fig. 6D), and as a result, PpMSGFP-NES expression does not result in the reversal of methionine and adenine auxotrophy of *Ppmet6*. Pp-PPMSGFP-NES strain exhibited severe growth retardation even in medium containing methionine and adenine (Fig. 6, E and F).

**Interaction between Arg-742 and Asp-113 is essential for catalytic activity and nuclear localization**

To understand the mechanism by which the R742A mutation affects enzyme function, structural modeling exercise was carried out to examine the role and significance of Arg-742 at the C-terminal end on the enzyme activity. BLAST search
Nuclear localization of yeast methionine synthase

A

CaMS 600 VNDLEGAGITVIQVDEPAIREGLPLRAGKERS 631
PpMS 598 VNELEPPSVEVIQVDEPAIREGLPLRSQERS 630
ScMS 598 VNDLEAAGIKVQVDEPALREGLRREGTERS 630

B

Pp-PpMSGFP
Ppmet6
Pp-PpMS<sup>D612A</sup>GFP

C

PpMSGFP

1 768

GFP

PpMS<sup>D612A</sup>GFP

1 768
D612A

PpMSA<sup>99</sup>GFP

1 100 768

PpMSA<sup>661-768</sup>GFP

1 661 768

D

E

Immunoflorescence

DAPI  PpMSGFP  MERGE

F

PpMS 737 CGLKTRGWEERASLTNMEAAKTREKLYAQN 768
CaMS 739 CGLKTRGPEVKEESTLNMVEAAKEFRAYK 767
ScMS 736 CGLKTRGWEETRLSSTMVEAAKFREQYKNN 768

G

H

PpMS<sup>K740A</sup>GFP

PpMS<sup>R742A</sup>GFP

PpMS<sup>K759A</sup>GFP

PpMS<sup>R762A</sup>GFP

I

Immunoflorescence

DAPI  Anti-GFP  MERGE
revealed that PpMS shares the highest similarity with CaMS (PDB codes 3PPC, 3PPF, and 3PPH) with a query coverage of 99%, e-value of −0, and amino acid sequence identity of 78% (Fig. 7A). 3PPC was used as the base template, and the short stretches of 8 and 6 residues that were missing in the N and C termini, respectively, were modeled based on corresponding regions in 3PPH (Fig. 7A). Superposition of all the structural templates revealed that the substrate-binding site for homocysteine, zinc ion, and the methyl donor have distinct pockets and are seen in between the N- and C-terminal barrel domains (6–8). In our structural model, Arg-742 is located at a distance of 8 Å from the nearest atom in the substrate-binding pocket and clearly far away from the substrate-binding pocket. Hence, it cannot have any direct effect on the binding of any of the substrates, nor can it be critically involved in stabilizing the pocket. Therefore, we focused our attention on the quaternary structure of cobalamin-independent MS of C. albicans (3PPC) and T. maritima (PDB code 1XPG). The biological assemblies obtained from the PDB database for these two proteins consisted of two subunits of the same polypeptide chain that form a "dimer-like" assembly. With this information, a corresponding dimer-like association was modeled for the PpMS as well, by superposing the whole-length polypeptide chain of our protein with the A and B chains of the structural templates as shown in Fig. 7B. A subsequent energy minimization of the interface of the dimer-like association was also carried out. It was observed that Arg-742 of one subunit makes extensive interactions, mainly hydrogen bonding and ionic nature, with the residues of the other subunit. Of the many interactions, ionic interaction of Arg-742 with Asp-113 was identified, whose side chains were positioned appropriately for forming a salt bridge (Fig. 6, C and D, and supplemental Fig. 1). Apart from the Arg-742–Asp-113 salt bridge, hydrogen-bonding interactions of Thr-106–Ser-527, Gln-449–Lys-85, Ser-711–Lys-681, Arg-103–Glu-23, Glu-745–Lys-179, Glu-745–Arg-82, and Arg-748–Asp-180 could also be deciphered from the model. The full-length model of the protein in the PDB format is given as supplemental material. The extensive nature of interactions between the two subunits was suggestive of a biological association between the two subunits rather than as an artifact of crystallization. Thus, structural modeling indicated that Arg-742 could play an important role in the stabilization of a dimer-like assembly through an ionic interaction with Asp-113 of a neighboring subunit.

To validate these in silico studies, detailed biochemical investigations were carried out. Because R742A mutation results in an inactive enzyme, we examined the effect of the D113R/D113A mutation on enzyme function. PpMS D113AF and PpMS D113RGFP were expressed in Scm66 as well as PpMS D113RGFP.

**Nuclear localization of yeast methionine synthase**

Western blot analysis of multiple clones of each mutant indicated that PpMS D113AF and PpMS D113RGFP are expressed at lower levels than PpMS GFP (Fig. 8, A–C) suggesting that Asp-113 is essential for the stability of the protein. D113A mutation abrogates enzyme function as evident from the inability of PpMS D113AF/PpMS D113RGFP to restore the growth of Scm66 and Ppmet6 in methionine-deficient media (Fig. 8, D and E). Subcellular localization studies indicate that PpMS D113RGFP is localized to the cytoplasm of *P. pastoris* cells (Fig. 8, F and G). Thus, mutation of either Arg-742 or Asp-113 results in catalytically inactive, cytosolic PpMS.

**Discussion**

Recruitment of metabolic enzymes to the nuclear compartment for alternative functions is well-documented. For example, enzymes of carbon metabolism such as phosphoglycerate kinase, aldolase, enolase, glyceraldehyde-3-phosphate dehydrogenase, fumarase etc., known conventionally as cytoplasmic enzymes with housekeeping functions, localize to the nucleus and perform moonlighting functions in gene transcription, DNA replication, DNA repair, DNA methylation, nuclear RNA export, etc. (24–28). Similarly, the mitochondrial pyruvate dehydrogenase complex translocates from the mitochondria to the nucleus to provide acetyl-CoA necessary for histone acetylation (29). Among the enzymes of methionine metabolism, MATII localizes to the nucleus of mammalian cells and serves as a transcriptional corepressor of Maf oncoprotein (30). Thus far, there is no report on nuclear localization of MS in any species. In this study, we demonstrate that MS is present in the nucleus as well as the cytoplasm of *P. pastoris* and *C. albicans* but only in the cytosol of *S. cerevisiae*. As a first step toward understanding the function of PpMS in the nucleus, we first focused our attention on the mechanism of nuclear translocation of PpMS. Analysis of various mutants indicated that deletion of 107 C-terminal amino acids or more specifically, mutation of a C-terminal amino acid (R762A) abrogates nuclear localization of PpMS. When expressed in Ppmet6 and Scm66 strains, the cytosolic PpMS R762A mutant was unable to reverse methionine auxotrophy. Thus, R742A mutation abrogates catalytic activity as well as nuclear localization of PpMS. On the contrary, D612A mutation within the active site abrogates catalytic activity but not nuclear localization of PpMS. Although cobalamin-independent MS has been studied in a number of bacterial and yeast species, the importance of Arg-742 has not been examined thus far, and therefore a detailed investigation was undertaken. PpMS shares a high degree of structural similarity with the CaMS and *T. maritima* MS, and an in silico analysis indicated that these enzymes may exist in a dimer-like configuration in which Arg-742 of a monomer can

---

**Figure 4. Identification of amino acid residues required for nuclear localization of PpMS.** A, partial amino acid sequence of CaMS, PpMS, and ScMS. The aspartate residue (Asp-614 in CaMS and Asp-612 in PpMS and ScMS) essential for catalytic activity is shown in red. B, analysis of methionine auxotrophy of Ppmet6 strain expressing PpMS D113AF. C, schematic representation of PpMS GFP and mutant PpMS GFP proteins. D, direct examination of GFP fluorescence in live *P. pastoris* cells expressing PpMS GFP and mutant PpMS GFP proteins. E, immunofluorescence of *P. pastoris* expressing PpMS GFP and mutant PpMS GFP proteins using mouse anti-GFP antibodies and TRITC-conjugated goat anti-mouse antibodies. DAPI was used for staining the nucleus. F, amino acid sequence of the C-terminal region of PpMS, CaMS, and ScMS. Arginine and lysine residues that are conserved in all three proteins are shown in red. G, Western blot analysis of whole-cell extracts of *P. pastoris* strains expressing PpMS GFP, PpMS D113AF, PpMS D113RGFP, PpMS K740AF, and PpMS K740RGFP. Protein molecular mass markers (kDa) are indicated. H, direct examination of GFP fluorescence in live *P. pastoris* cells expressing PpMS D113AF and PpMS D113RGFP, PpMS D113RF, PpMS D113RGFP, PpMS K740AF, and PpMS K740RGFP. I, immunofluorescence of *P. pastoris* expressing PpMS D113AF, PpMS D113RGFP, PpMS K740AF, and PpMS K740RGFP, using mouse anti-GFP antibodies and TRITC-conjugated goat anti-mouse antibodies. DAPI was used for staining the nucleus.
form a salt bridge with Asp-113 of another monomer. To confirm this, biochemical studies were carried out, and the results indicate that the D113R/D113A mutation abrogates catalytic activity as well as nuclear localization of PpMS. Furthermore, PpMS\textsuperscript{D113R}/PpMS\textsuperscript{D113A} are expressed at much lower levels than PpMS in \textit{S. cerevisiae} and \textit{P. pastoris} indicating that this mutation may affect the stability of the protein as well. In the case of PpMS\textsuperscript{R742A}GFP and PpMS\textsuperscript{D113R}GFP, the inability to associate in a dimer-like configuration may lead to their cytoplasmic retention, poor stability, and loss of function. Attempts to demonstrate the existence of a PpMS dimer by native PAGE and gel filtration chromatography of recombinant PpMS or chemical cross-linking and communoprecipitation of epitope-tagged PpMS expressed in \textit{P. pastoris} cell extracts were not successful. Thus, we were unable to provide direct evidence for dimer formation. It is possible that the association between PpMS monomers is not sufficiently strong or is transient thus obscuring the detection through conventional biochemical techniques (6).

It is interesting to note that cytosolic localization of PpMSGFP in \textit{Scmet6} results in reversal of methionine auxotrophy. However, PpMSGFP-NES is unable to reverse methionine and adenine auxotrophy of \textit{Ppmet6}. This may be due to very low levels of the protein. It is possible that forceful targeting of the protein to cytoplasm renders it unstable and susceptible for proteolytic degradation. Thus, nuclear association is essential for the stability and function PpMS in \textit{P. pastoris} but not \textit{S. cerevisiae}. The factor(s) that contributes to the stability of PpMS in \textit{P. pastoris} remains to be investigated. It is also pertinent to note that methionine and adenine fail to completely rescue the growth of \textit{Ppmet6}. Thus, in addition to its role in methionine and adenine metabolism, PpMS may have other moonlighting functions in the nucleus in \textit{P. pastoris}.

Homocysteine is a substrate not only for MS but also for cystathionine \(\beta\)-synthase. The latter catalyzes the first step of transsulfuration pathway involving the synthesis of cystathionine from serine and homocysteine (Fig. 9). In \textit{S. pombe}, which lacks the enzymes of transsulfuration pathway, MS deficiency leads to accumulation of homocysteine resulting in defective purine biosynthesis and adenine auxotrophy (12). Expression of enzymes of the transsulfuration pathway in the MS-deficient strain of \textit{S. pombe}, restores growth in the absence of adenine (12). The genome of \textit{P. pastoris} encodes cystathionine \(\beta\)-synthase (PAS\_chr2-2\_0137) as well as cystathionine \(\gamma\)-lyase (PAS\_chr1-4\_0489), and thus, adenine auxotrophy of \textit{Ppmet6} is unlikely to be due to homocysteine toxicity. Methionine biosynthesis is intimately linked to purine biosynthesis as THF generated by MS-catalyzed homocysteine remethylation reaction is essential for \textit{de novo} purine biosynthesis. It is possible that a decrease in the intracellular pool of THF in \textit{Ppmet6} may lead to defective purine biosynthesis and impaired growth. Because the accumulation of purine biosynthetic intermediates

Figure 5. Comparison of growth of different \textit{P. pastoris} and \textit{S. cerevisiae} strains in methionine-deficient and methionine-sufficient YNBD media. 

\textbf{A}, Western blot analysis of extracts of \textit{P. pastoris} strains cultured in methionine-sufficient YNBD medium using anti-GFP antibodies. Phosphoglycerate kinase (PGK) served as loading control. Protein molecular mass markers (kDa) are indicated. \textbf{B}, growth of various strains in different media as indicated. \textbf{Error bars} in growth curves indicate S.D. \textit{n} = \textit{3}. \textbf{C}, Western blot analysis of extracts of \textit{S. cerevisiae} strains cultured in methionine-sufficient YNBD medium. Protein molecular mass markers (kDa) are indicated. \textbf{D}, growth of various \textit{S. cerevisiae} strains in methionine-deficient and -sufficient YNBD liquid medium. \textbf{Error bars} in growth curves indicate S.D. \textit{n} = \textit{3}. \textbf{E}, growth of various \textit{S. cerevisiae} strains in methionine-deficient and -sufficient YNBD agar plates.
such as 5′-phosphoribosyl-5-aminimidazole-4-carboxamide (AICAR) interferes with methionine biosynthesis (31), it is possible that accumulation of 5-methyl-THF in Ppmet6 results in THF deficiency leading to defective purine biosynthesis. These aspects are schematically represented in Fig. 9.

Respiratory yeasts such as P. pastoris, Hansenula polymorpha, and C. albicans favor respiratory growth, which is characterized by low glucose uptake and high biomass yield. S. cerevisiae follows a respiro-fermentative growth characterized by high glucose uptake and low biomass yield. Key differences
Figure 7. In silico analysis of the PpMS structure. A, sequence alignment that was used for model building of the query MS sequence with two templates. B, structural superposition of the dimer-like association of the model with the two structural templates, 3PPC (CaMS) and 1XPG (T. maritima MS). The A chain of the assembly is shown in blue for all the three structures, and the B chain is shown in red. The ionic interaction involving Arg-742–Asp-113 is shown in a space-filling representation. C, dimer-like assembly of PpMS in the model is shown in a schematic representation. The interface of the two chains is shown in surface representation with the residues involved in interactions in line representation. D, surface representation of the interface of two chains are shown in red and pink colors, in which Arg-742–Asp-113 are shown in ball and stick representation.
have been reported between the respiro-fermentative and respiratory yeasts. For example, RNA interference has not been demonstrated in \textit{S. cerevisiae} but is effective in \textit{C. albicans} (32). Although respiratory yeasts such as \textit{C. albicans} possess a mitochondrial dihydrololate dehydrogenase, \textit{S. cerevisiae} possesses a cytosolic enzyme whose activity is independent of ubiquinone.
Nuclear localization of yeast methionine synthase

![Figure 9. Cross-coupling of purine and methionine biosynthetic pathways.](image)

**Figure 9. Cross-coupling of purine and methionine biosynthetic pathways.** Step 1, serine hydroxymethyltransferase; step 2, thymidylate synthase; step 3, dihydrofolate reductase; step 4, methylene tetrahydrofolate dehydrogenase; step 5, methylene tetrahydrofolate reductase; step 6, methionine adenosyltransferase/SAM synthetase; step 7, methyl transferases; step 8, S-adenosylhomocysteine hydrolase; step 9, cystathionine β-synthase; step 10, cystathionine γ-lyase. DHF, dihydrofolate. The transsulfuration pathway is indicated by a dotted circle.

and the presence of oxygen, enabling the latter to grow under anaerobic conditions (33–34). Cyanide-insensitive alternative oxidase involved in the oxidation of ubiquinone by molecular oxygen is present in aerobic yeasts but not in *S. cerevisiae* (35).

The fact that MS, a key enzyme required for multiple metabolic pathways, is cytosolic in *S. cerevisiae* but nuclear in *P. pastoris* and *C. albicans* suggests that differential localization of MS is another key feature that distinguishes respiratory yeasts from respiro-fermentative yeasts. We speculate that nuclear localization of MS may be a common feature of many respiratory yeasts, and MS may have novel moonlighting functions in the nucleus of these yeasts. Furthermore, methylotrophic yeasts may harbor novel sulfur metabolic pathways as was shown recently in *H. polymorpha* in which methionine is synthesized only through cysteine and cystathionine, and the sulfur metabolism is centered on cysteine rather than methionine (36).

Thus, understanding sulfur metabolism of methylotrophic yeasts may lead to better exploitation of these yeasts for the production of high-value sulfur-containing amino acids and metabolites.

**Experimental procedures**

**Yeast strains, media, and transformation**

*P. pastoris* strain GS115 (*his4*) was a generous gift of James Cregg (37). *S. cerevisiae* BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from Euroscarf, Germany. *C. albicans* SC5314 strain was a generous gift of Parag Sadhale and has been described previously (38). *P. pastoris* was cultured at 30 °C in shake flasks in YP medium (1% yeast extract, 2% peptone) containing 2% glucose (YPD), 2% glycerol (YPG), or 2% methanol (YPM) as the sole source of carbon. *P. pastoris* GS115 strain (*his*) was cultured in minimal synthetic medium containing 0.67% yeast nitrogen base (YNB), histidine (50 μg/ml), and 2% glucose (YNBD). *P. pastoris* was transformed with DNA by electroporation (Gene Pulser Xcell Electroporation System, Bio-Rad) as per the manufacturer’s instructions. Zeocin-resistant colonies were selected on YPD plates containing Zeocin (100 μg/ml). Transformants expressing *HIS4* gene were plated on YNB plates lacking histidine. *S. cerevisiae* and *C. albicans* were grown in YPD medium or YNB medium containing 2% glucose with appropriate auxotrophic supplements. List of yeast strains used in this study are given in Table 1.

**Measurement of growth rate of yeast cells**

A single colony of the indicated yeast strain was inoculated into 5 ml of YPD and grown overnight at 30 °C in a shaker incubator. Absorbance was measured at 600 nm, and cells corresponding to 0.1 A₆₀₀ units were inoculated into flasks containing 25 ml of YNBD medium containing (Met⁺) or lacking (Met⁻) methionine and cultured at 30 °C. Aliquots were taken at 2–6-h intervals, and A₆₀₀ was recorded. Growth rate experiments were performed in triplicate. In some experiments, adenine was added at a final concentration of 20 mg/liter. For spot tests, cells were pre-grown on YPD medium overnight, spun down, washed twice in sterile distilled water, resuspended to a density of 2.7 × 10⁷ cells/ml (A₆₀₀ 1.0), and serially diluted (1:10 dilutions). Five μl of each dilution was spotted onto YNBD + agar plates and incubated at 30 °C for 72 h.

**Overexpression of PpMS in E. coli and generation of anti-PpMS antibodies**

The primer pair 5’-CCGCTCGAGATGGTTCAATCATGGTC-3’ and 5’-CCCAAGCTTTAATTCTGAGC-3’ was designed based on the *P. pastoris MET6* gene sequence...
Table 1 Yeast strains used in this study

| Strain          | Genotype                  | Source/Ref. |
|-----------------|---------------------------|-------------|
| P. pastoris (GS115) | his4                      | 32          |
| S. cerevisiae (BY4741) | MATa his4ΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ | Euroscarf    |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |
| Ppmet6         | his4 met6ΔΔ A::Zeo         | This study   |
| Scmet7115      | MATa hisΔΔ les2ΔΔ met15ΔΔ ura3ΔΔ A::Zeo | This study   |

(GenBankTM accession number Y601648) and used in a PCR to amplify the MET6 gene from P. pastoris genomic DNA. The Xhol and HindIII restriction sites are underlined. cDNA was digested with Xhol with HindIII and cloned into the E. coli PRSETA vector (Invitrogen) in-frame with the vector-encoded histidine tag, and PpMS was expressed as histidine-tagged protein in E. coli (BL21) cells (39). The E. coli cell lysate expressing PpMS was subjected to SDS-PAGE, and the band corresponding to PpMS was visualized

Preparation of yeast nuclear extract and cytosol

Nuclear extract was isolated as described (41) with modifications. Briefly, yeast cells were isolated in YPD at 30 °C to A600

between 4 and 5. A 2-liter culture yielded ~10 g of cells (wet weight). The cell pellet was resuspended in 1 volume (10 ml) of zymolyase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1.2 m sorbitol, 30 mM DTT) and incubated at room temperature for 15 min. Cells were pelleted at 4000 rpm (Beckman JA-20) for 5 min at 4 °C, and the pellet, resuspended in 3 volumes (30 ml) of Zymolyase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1.2 m sorbitol, 1 mM DTT) containing 1 ml of long life zymolyase (1.5 units/ml from G-Biosciences) and incubated at 37 °C for 3–5 h with gentle stirring until a 10-fold decrease in A600 (in presence of 1% SDS), was observed. Spheroplasts were pelleted by centrifugation at 4000 rpm for 5 min and at 4 °C. The pellet was resuspended in 0.1 ml of 18% Ficoll 400 (which was dissolved in 20 mM KH2PO4 at pH 7.5 and 0.5 mM CaCl2) per g of cells using a glass rod. The cells were then diluted with 3 ml of

18% Ficoll and homogenized in a Dounce homogenizer with a Teflon pestle (~10 strokes). The mixture was then diluted with equal volume of 0% Ficoll (1 m sorbitol and 0.5 mM CaCl2), and 10 ml of it was carefully loaded on preformed Percoll gradient. A 32.5% Percoll gradient was formed by mixing 0 and 100% (1M sorbitol and 0.5 mM CaCl2 at pH 7.5) of Percoll in an appropriate ratio (Percoll was purchased from Sigma). Percoll mixture (34 ml) was centrifuged at 27,000 × g for 50 min in an SW28 rotor in an ultracentrifuge (Beckmann Coulter). Nuclei thus obtained were loaded onto the Percoll gradient and centrifuged at 9500 × g for 4 h in a swinging bucket rotor. Among the three bands formed, the top band containing nuclei was isolated, mixed with 2 volumes of 0% Percoll, and then centrifuged to obtain a nuclear pellet. This pellet was resuspended in an equal volume of nuclear lysis buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgSO4, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 1 × protease inhibitor mix), and the purity was examined in a fluorescence microscope after staining with DAPI. After careful examination under a microscope and DAPI staining, we found that the top layer had the least contamination of unbroken cells and cellular debris. Purity of the nuclear fraction was assayed by Western blotting using appropriate nuclear markers.

For the preparation of cytosol, yeast cells were cultured overnight in YPD, and 5 ml of cells were pelleted and resuspended in 2 volumes of zymolyase buffer containing 20 μl of long life zymolyase (1.5 units/μl from G-Biosciences) and incubated at 37 °C for 30–60 min. Spheroplasts were washed twice with zymolyase buffer and resuspended in 2 volumes of yeast lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM MgCl2, 10 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 7 mM β-mercaptoethanol, 1 × protease inhibitor mix). Cells were lysed by pipetting up and down using a pipetman and incubated on ice for 20 min. Following centrifugation at 10,000 × g for 30 min at 4 °C, the supernatant (cytosol) was removed, stored at ~80 °C in aliquots, and subjected to SDS-PAGE and Western blot analysis.
Nuclear localization of yeast methionine synthase

Fluorescence microscopy

Late log phase cultures of *P. pastoris* were treated with 3.7% formaldehyde for 1 h, and spheroplasts were prepared by treating the cells with Zymolase (1.5 units/μl) in a Zymolase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 1 mM sorbitol for 1 h at 30 °C. Cells were centrifuged at 1600 rpm for 10 min at room temperature in a microcentrifuge (Eppendorf) and resuspended in PBST (phosphate-buffered saline containing 0.05% Tween 20). The cells were spread onto glass coverslips. Following treatment with chilled methanol and acetone for 7 min and 30 s, respectively, the coverslips were air-dried. They were incubated sequentially with blocking buffer (PBS containing 20 mg/ml BSA), blocking buffer containing appropriate primary and secondary antibodies. Finally, the smears were treated with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min for staining the DNA. The coverslips were washed in PBS and air-dried, and cells were visualized by either a fluorescent microscope (Leica) or a confocal microscope (Confocal Zeiss LSM880-Airyscan).

Generation of *P. pastoris* strain expressing chromosomally GFP-tagged *PpMS* (*Pp-PpMSGFP*)

The chromosomal *MET6* gene of *P. pastoris* was tagged in-frame with the DNA sequence encoding green fluorescent protein (GFP) so that the *PpMET6-GFP* gene is expressed under the control of the *MET6* promoter. The *PpMET6-GFP* expression cassette consisting of 1000 bp of 3'-flanking region of *PpMET6* followed by GFP ORF and Zeocin expression cassette was generated by four different PCRs. First, 1000 bp of *MET6* gene encompassing +1305 to +2304 bp were amplified from *P. pastoris* genomic DNA by PCR using the primer pair 5'-CTTGGCTTTTGTTCCCAACCAACACC-3' (1F, nucleotides from position +1305 to +1330 bp of *MET6*) and 5'-CTCCTTTAATATGCGGATCCGCTATAGT-3' (1R, +25 to +1 bp of GFP (uppercase)) and +2305 to +2281 bp of *MET6* (lowercase)). In another PCR, a 714-bp region from the pREP41GFP vector (42) was amplified using the primer pair 5'-ctacgctagaagagctgtggggt-3' and 5'-AGCTATGTTGTTGCGGATCCGCTATTAGTAAATTTG-3' (2F, position from +2279 to +2304 bp of *MET6*) (lowercase) and position +1 to +25 bp of GFP (uppercase)) and 5'-AGCTATGTTGTTGCGGATCCGCTATTAGTAAATTTG-3' (2R, 962–990 bp of *pGAPZA* vector (uppercase)) and +741 to +713 bp of GFP (lowercase). In the third PCR, the Zeocin expression cassette was amplified using the primer pair 5'-ctacgctagaagagctgtggggt-3' and 5'-AGCTATGTTGTTGCGGATCCGCTATTAGTAAATTTG-3' (2R, 962–989 bp of *pGAPZA* vector (lowercase)) and 5'-AGCTATGTTGTTGCGGATCCGCTATTAGTAAATTTG-3' (2R, 2161 to 2136 bp of *pGAPZA* vector). Final PCR was carried out using all the above three PCR products (50 ng each) together with 1F and 3R primers to generate the *PpMET6-GFP* expression cassette (2941 bp), which was transformed into *P. pastoris* by electroporation. Zeocin-resistant colonies in which the *PpMET6-GFP* expression cassette was integrated into the chromosomal *PpMET6* locus by homologous recombination were selected by plating the transformants on YPD plates containing Zeocin (100 μg/ml). *P. pastoris* strain expressing chromosomally GFP-tagged MS (PpMSGFP) was designated as *Pp-PpMSGFP*.

Construction of *P. pastoris* strain expressing *ScMSGFP* (*Pp-ScMSGFP*)

The expression cassette encoding *ScMSGFP* consisting of −1001 bp of *P. pastoris* *MET6* promoter fused to *S. cerevisiae* *MET6* ORF and GFP ORF was generated by four different PCRs. First, 1000 bp of *P. pastoris* *MET6* promoter encompassing −1000 bp was amplified from *P. pastoris* genomic DNA by PCR using the F1 and R1 primer pair (F1, 5'-CGGTCTCAGCAGGGACGT-TGATGCCGGCCTTT-3' → −1000 to −968 bp of *P. pastoris* genomic DNA using the primer pair 5'-CACGCTGGAGACCACATGTCGAGCAACTGAAATTTCTGGTTT-3' (3F, nucleotides 2136–2160 of *pGAPZA* (uppercase); nucleotides 2308–2333 of *MET6* (lowercase)) and 5'-CGGTCTCAGCAGGGACGT-TGATGCCGGCCTTT-3' (3R, nucleotides 3207–3182 of *MET6*). Finally, the three PCR products containing overlapping regions were pooled (50 ng each) and used as templates in a final PCR along with primers 1F and 3R. The final 3.0-kb PCR product thus obtained was used to transform the *P. pastoris* GS115 strain to generate Zeocin-resistant *Ppmet6* strain in which *MET6*-coding region was replaced by Zeocin expression cassette.
**Nuclear localization of yeast methionine synthase**

BY7471 strain, and G418-resistant colonies were selected to obtain Scmet6met15 strain.

Because MET15 was already deleted in BY4741, in order to study methionine auxotrophy of Scmet6met15 caused by the deletion of MET6 alone, it became necessary to express MET15 in Scmet6met15. MET15 was amplified from S. cerevisiae genomic DNA using the primer pair 5′−CGCGATCCAGTC−CATCTTCTAGCTCTTTGTTCTAGTGGTTTTTGCGCCAGGACAC−3′ and 5′−CCGTCGAAGCTACGACTTTTGTTCTAGTGCTAGGAG−3′. The BamHI and XhoI restriction sites in the primers are underlined. DNA encoding Myc tag in the reverse primer is shown in italics. The PCR product was cloned into pRS416TEF vector (51) and transformed into Scmet6met15 strain, and expression of MET15 gene product (O-acetylmethionine−O-acetylseryln sulfhydrylase) was confirmed by Western blotting using anti-Myc antibodies (details will be provided on request). This strain in which ScMET6 is deleted but ScMET15 is functional is designated as Scmet6.

The gene encoding ScMSGFP was amplified by PCR using the primer pair 5′−AAAACCTCGAGATGTTTCTAGCTCTTTGTTCTAGTGGTTTTTGCGCCAGGACAC−3′ and 5′−CCGTCGAAGCTACGACTTTTGTTCTAGTGCTAGGAG−3′. The BamHI and XhoI restriction sites in the primers are underlined. DNA encoding Myc tag in the reverse primer is shown in italics. The PCR product was cloned into pRS416TEF vector (52) to generate the pRS415−ScMSGFP plasmid that was transformed into the electrocompetent Scmet6 strain. Transformants were selected on LEU− plates. S. cerevisiae expressing ScMSGFP was designated as Sc-ScMSGFP.

**Construction of P. pastoris strain expressing c-Myc epitope-tagged PpMS (Pp-PpMS<sub>Mye</sub>)**

To express PpMS as c-Myc epitope-tagged protein in P. pastoris, the cDNA encoding PpMS was amplified by PCR from pPSETA−PpMS plasmid using the primer pair 5′−CGCGTCGAGTCTGCTACTTTTTGAGGAG−3′ and 5′−ATAACATACGCGGCCGCAATCTGAGGTTTTCTACAC−3′. The XhoI and NotI sites are underlined. Following digestion with XhoI and NotI, the PCR product was cloned into pGAPZA vector, in-frame with vector-encoded c-Myc epitope to generate pGAPZA−PpMS vector, which was linearized with AvrII and transformed into electrocompetent P. pastoris GS115 strain to generate the Pp-PpMS<sub>Mye</sub> strain expressing c-Myc-tagged PpMS (PpMS<sub>Mye</sub>).

**Generation of P. pastoris strains expressing PpMSGFP, PpMSΔ99GF, and PpMSΔ661−768GFp**

Genes encoding PpMSGFP and PpMSΔ99GF were generated by PCR amplification of genomic DNA isolated from the Pp-PpMSGFP strain expressing chromosomally GFP-tagged PpMS as a template, a common reverse primer (5′−ATAAGAATCGGGCAGCTAGGTTTCTAGCTGCTACTTTCTACAC−3′) and the following forward primers: PpMSGFP, 5′−CCGTCGAAGCTACGACTTTTGTTCTAGTGCTAGGAG−3′. NotI and XhoI restriction sites in the reverse and forward primers, respectively, are underlined. Following restriction digestion, the PCR products were cloned into pGAPZA vector.

**Construction of Scmet6−1, Scmet6, and Sc-ScMSGFP strains**

MET6 was deleted from the BY7471 strain to generate the Scmet6met15 strain as described (51). Briefly, KanMX4 cassette was amplified using primers 5′−AGGTTGCTGTTGCTTTTCTACAC−3′ (Sc MET6 from +1 to +50 bp are shown in lowercase) and 5′−GGCAATTCTTTTTCTACAC−3′ (Sc MET6 from +1260 to +1310 bp are shown in lowercase). The PCR product was transformed to the pREP41GFP vector, in-frame with vector-encoded c-Myc epitope to generate pGAPZA−PpMS vector, which was linearized with AvrII and transformed into electrocompetent P. pastoris GS115 strain to generate the Pp-PpMS<sub>Mye</sub> strain expressing c-Myc-tagged PpMS (PpMS<sub>Mye</sub>).

**Analysis of PpMS structure**

The query PpMS sequence (Uniprot accession number C4QZU2, 768 amino acids long) was searched against the Protein Data Bank (43) using protein BLAST (44) (basic local alignment tool) and BLOSUM62 substitution matrix to find the structural templates that were homologous to PpMS. A homology model was obtained using Modeler (version 9) (45), and the structural model was verified to have acceptable geometrical and stereo-chemical parameters using PROCHECK (46). A loop refinement process using an inbuilt modeler script was also carried out to correct the loop regions. A structural similarity search was carried out using DALI (47), and the identified homologs were aligned using Mustang (48). Energy minimization of the modeled protein was carried out using Chiron (49). Calculation of the interface contacts was carried out using Protein Interactions Calculator server (50). PyMOL (57) was used for structure visualization and generation of images.

**Construction of Scmet6−1, Scmet6, and Sc-ScMSGFP strains**

MET6 was deleted from the BY7471 strain to generate the Scmet6met15 strain as described (51). Briefly, KanMX4 cassette was amplified using primers 5′−AGGTTGCTGTTGCTTTTCTACAC−3′ (Sc MET6 from +1 to +50 bp are shown in lowercase) and 5′−GGCAATTCTTTTTCTACAC−3′ (Sc MET6 from +1260 to +1310 bp are shown in lowercase). The PCR product was transformed to the pREP41GFP vector, in-frame with vector-encoded c-Myc epitope to generate pGAPZA−PpMS vector, which was linearized with AvrII and transformed into electrocompetent P. pastoris GS115 strain to generate the Pp-PpMS<sub>Mye</sub> strain expressing c-Myc-tagged PpMS (PpMS<sub>Mye</sub>).
Nuclear localization of yeast methionine synthase

To generate pGAPZA expressing PpMSΔ661–768GFP, we carried out three different PCRs. First, PpME6 gene (1983 bp) was amplified by PCR using the primer pair 5’-CGGGTTCGAGATGTTCACTGTCG-3’ (PpMSGFP-FP, Xhol site is underlined, and +1 to +18 bp of ME6 gene are shown in lowercase), and 5’-ctcattgatctctactgagt-3’ (PpMSGFP-RP, +1 to +23 bp of GFP gene are shown in uppercase, and +1960 to +1983 bp of ME6 gene are shown in uppercase). In the second PCR, the gene encoding GFP was amplified using the primer pair 5’-ctcattgatctctactgagt-3’ (PpMSGFP-RP, +1 to +23 bp of GFP gene are shown in uppercase) and 5’-ATAAGATTGCCGCGCGCtaggtctactgagt-3’ (GFP-RP, NotI site is underlined, and +744 to +723 bp of GFP gene are shown in lowercase). In the third and final PCR, the products of the first two PCRs were used as templates, and PCR was carried out with PpMSGFP-FP and GFP-RP primers. The final PCR products were restricted with Xhol and NotI and cloned into pIB vector (Invitrogen). All the recombinant pIB-PpMSGFP plasmids were linearized with AvrII and transformed into P. pastoris GS115 strain. Zeocin-resistant colonies were screened for GFP expression.

Construction of PpPpMSGFP-NLS, PpPpMSGFP-MAS, and PpPpMSGFP-NES strains expressing MSGFP fused to NLS, MAS, and NES, respectively

The strain Pp-PpMSGFP-NLS was constructed by transformation of the plasmid pIB-PpMSGFP-NLS into the P. pastoris met6 strain. To generate pIB-PpMSGFP-NLS, PCR was carried out with the primer pair 5’-CGGGTTCGAGATGTTCACTGTCG-3’ (KpnI site is underlined) and 5’-CCCCTCGAAGCTCACTTCGTTTTTATAAGGTGGTCGCTGGTCTTGATAG-3’ (Xhol site is underlined, and DNA encoding NLS is italicized) using plasmid pIB-PpMSGFP as a template.

The strain Pp-PpMSGFP-MAS was constructed by transformation of the plasmid pIB-PpMSGFP-MAS into the P. pastoris met6 strain. To generate pIB-PpMSGFP-MAS, PCR was carried out with primer pair 5’-CGGGTTCGAGATGTTCACTGTCG-3’ (KpnI site is underlined) and 5’-CCCCTCGAAGCTCACTTCGTTTTTATAAGGTGGTCGCTGGTCTTGATAG-3’ (Xhol site is underlined, and DNA encoding NLS is italicized) using plasmid pIB-PpMSGFP as a template.

The strain Pp-PpMSGFP-NES was constructed by transformation of the plasmid pIB-PpMSGFP-NES into the P. pastoris met6 strain. To generate pIB-PpMSGFP-NES, PCR was carried out with primer pair 5’-CGGGTTCGAGATGTTCACTGTCG-3’ (KpnI site is underlined) and 5’-CCCCTCGAAGCTCACTTCGTTTTTATAAGGTGGTCGCTGGTCTTGATAG-3’ (Xhol site is underlined, and NES is italicized) using plasmid pIB-PpMSGFP as a template. This NES (IESALSDALAALEL) is present in P. pastoris ribosome-associated molecular chaperone (PAS_chr3_0731, GenBank™ accession number XP_002492959.1), and a similar sequence (IEAALSDALAALQI) is present in the S. cerevisiae homolog Hsp70 Ssb1p (55, 56).

All the PCR products were cloned into pIB3 vector, and the recombinant plasmids were linearized with Sall and transformed into Ppm6et.

Expression of PpMSD612AGFP in Ppmet6 strain

An expression cassette consisting of 1.0 kb of MET6 promoter and MET6GFP-coding region amplified by PCR from genomic DNA isolated from Pp-PpMSGFP strain, expressing PpMSGFP using the primer pair 5’-CGGGTTCGAGATGTTCACTGTCG-3’ (GFP-RP, NotI site is underlined, and +744 to +723 bp of GFP gene are shown in lowercase) and 5’-ATAAGATTGCCGCGCGCtaggtctactgagt-3’ (KpnI and HindIII restriction sites are underlined. The PCR product was digested with KpnI and HindIII and cloned into pIB3 vector (Addgene, catalog no. 25452) to obtain pIB3-PpMSGFP. D612A mutation was introduced into PpMSGFP by site-directed mutagenesis using the QuickChange method (Stratagene, CA), essentially as described by the manufacturer. Briefly, PCR was carried out with pIB3-PpMSGFP as template, PfuTurbo DNA polymerase, and the following mutagenic primers: 5’-CTCGAGATATCGTAGTCTGGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. The mutant codon is underlined. Following confirmation of the mutation by DNA sequencing, recombinant pIB3-PpMSD612AGFP was linearized with Sall and transformed into Ppm6et strain by electroporation. Transformants were plated on YNBD His− agar and screened for GFP expression.

Generation of P. pastoris strains expressing chromosomally GFP-tagged PpMS5740A, PpMS742A, PpMS759A, and PpMS762A

K740A, R742A, K759A, and R762A mutations were introduced into PpMSGFP encoded by pIB3-PpMSGFP by site-directed mutagenesis using the QuickChange method (Stratagene, CA), essentially as described by the manufacturer. Briefly, PCR was carried out with pIB3PpMSGFP as template, PfuTurbo DNA polymerase, and the following mutagenic primers: 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. The PCR product was digested with KpnI and HindIII and cloned into pIB3-PpMS5740A GFP was generated using the primer pair, 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. pIB3-PpMS742A GFP was generated using the primer pair, 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. pIB3-PpMS759A GFP was generated using the primer pair, 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. pIB3-PpMS762A GFP was generated using the primer pair, 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’. Point mutations were confirmed by sequencing the recombinant plasmids. These plasmids were used to transform PpMSGFP by PCR using the primer pair, 5’-CTGGACGTGCTGGTCTTTGCGACTGAC-3’ and 5’-CTGCCATGCTGCTGGCGGAAGGACATGAG-3’.
of pGAPZA vector are shown in uppercase, and +741 to +713 bp of GFP are shown in lowercase). In another PCR, the Zeocin expression cassette was amplified from pGAPZA vector using the primer pair 5’-ctataaagacgtccacacacTAACTGCGGTATCCCC- CACACACCATAGCT-3’ (3’F, +717 to +714 bp of GFP are shown in lowercase, and 962–989 bp of pGAPZA vector are shown in lowercase) and 5’-TGCTTACATGGTGTCCACGGT- G-3’ (3’R, 2161–2136 bp of pGAPZA vector). Final PCR was carried out using the two PCR products (50 ng each) as templates together with 1’F and 3’R primers to generate mutant PpMSGFP-Zeocin expression cassettes that were transformed into P. pastoris GS115 strain by electroporation. Zeocin-resistant colonies in which genes encoding mutant PpMSGFP fusion proteins were integrated into the chromosomal MET6 locus were selected by plating the transformants on YPD plates containing Zeocin (100 µg/ml) and screened for GFP expression.

**Generation of S. cerevisiae strains expressing PpMSR742AGFP, PpMSD612AGFP, and ScMSD612AGFP**

The PpMSR742AGFP and PpMSD612AGFP were amplified from pIB3-PpMSR742AGFP and pIB3-PpMSD612AGFP plasmids, respectively, by PCR and cloned into pRS415TEF vector. The recombinant plasmids were transformed into Scmet6 strain, and transformants were selected on Leu− plates. pScMSD612AGFP plasmid was generated by introducing D612A mutation into pScMSGFP vector by site-directed mutagenesis using the QuickChange method (Stratagene, CA). Following confirmation of the mutation by DNA sequencing, recombinant pScMSD612AGFP was transformed into Scmet6 strain by electroporation. Transformants were plated on YNBD Leu− agar and screened for GFP expression.

**Author contributions**—U. S. and P. N. R. planned the experiments and organized the data; U. S., H. R. V. K. and S. S. K. performed the experiments; R. B. and N. C. carried out the structural analysis. U. S. and P. N. R. take the responsibility for integrity of the data and accuracy of data analysis. P. N. R. wrote the paper.

**Acknowledgment**—The confocal microscopy facility at the Indian Institute of Science is acknowledged.

**Note added in proof**—In the version of this article that was published as a Paper in Press on July 12, 2017, the wrong images were used in Fig. 2F. This error has now been corrected and does not affect the results or conclusions of this work.

**References**

1. González, J. C., Banerjee, R. V., Huang, S., Sumner, J. S., and Matthews, R. G. (1992) Comparison of cobalamin-independent and cobalamin-dependent methane synthases from Escherichia coli: two solutions to the same chemical problem. *Biochemistry* 31, 6045–6056
2. Matthews, R. G., Smith, A. E., Zhou, Z. S., Taurog, R. E., Bandarian, V., Evans, J. C., and Ludwig, M. (2003) Cobalamin-dependent and cobalamin-independent methane synthases: are there two solutions to the same chemical problem? *Helv. Chim. Acta* 86, 3939–3954
3. Suliman, H. S., Appling, D. R., and Robertus, J. D. (2007) The gene for cobalamin-independent methane synthase is essential in *Candida albicans*: a potential antifungal target. *Arch. Biochem. Biophys.* 467, 218–226
4. Pascon, R. C., Ganous, T. M., Kingsbury, J. M., Cox, G. M., and McCusker, J. H. (2004) *Cryptococcus neoformans* methionine synthase: expression analysis and requirement for virulence. *Microbiology* 150, 3013–3023
5. González, J. C., Pearso, K., Penner-Hahn, J. E., and Matthews, R. G. (1996) Cobalamin-independent methionine synthase from *Escherichia coli*: a zinc metalloenzyme. *Biochemistry* 35, 12228–12234
6. Pechal, R., and Ludwig, M. L. (2005) Cobalamin-independent methionine synthase (MetE): a face-to-face double barrel that evolved by gene duplication. *PLoS Biol.* 3, e31
7. Ferrer, J. L., Ravanel, S., Robert, M., and Dumas, R. (2004) Crystal structures of cobalamin-independent methionine synthase complexed with zinc, homocysteine, and methylenetetrahydrofolate. *J. Biol. Chem.* 279, 44235–44238
8. Ubbi, D., Kavanagh, K. L., Monzongo, A. F., and Robertus, J. D. (2011) Structure of *Candida albicans* methionine synthase determined by employing surface residue mutagenesis. *Arch. Biochem. Biophys.* 513, 19–26
9. Ubbi, D., Kago, G., Monzongo, A. F., and Robertus, J. D. (2014) Structural analysis of a fungal methionine synthase with substrates and inhibitors. *J. Mol. Biol.* 426, 1839–1847
10. Kaczprzak, M. M., Lewadowska, I., Matthews, R. G., and Paszewski, A. (2003) Transcriptional regulation of methionine synthase by homocysteine and choline in *Aspergillus nidulans*. *Biochem. J.* 376, 517–524
11. Seong, K., Hou, Z., Tracy, M., Kistler, H. C., and Xu, J. R. (2005) Random insertion mutagenesis identifies genes associated with virulence in the wheat scab fungus *Fusarium graminearum*. *Phytopathology* 95, 744–750
12. Fujita, Y., Ukena, E., Iefuji, H., Giga-Hama, Y., and Takegawa, K. (2006) Homocysteine accumulation causes a defect in purine biosynthesis: further characterization of Schizosaccharomyces pombe methionine auxotrophs. *Microbiology* 152, 397–404
13. Huang, L., Li, D. Y., Wang, S. X., Zhang, S. M., Chen, J. H., and Wu, X. F. (2005) Cloning and identification of methionine synthase gene from *Pichia pastoris*. *Acta Biochim. Biophys. Sin.* 37, 371–378
14. Kranthi, B. V., Balasubramaniam, N., and Rangarajan, P. N. (2006) Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, *Pichia pastoris* and its identification as ζ crystallin. *Nucleic Acids Res.* 34, 4060–4068
15. Kranthi, B. V., Kumar, R., Kumar, N. V., Rao, D. N., and Rangarajan, P. N. (2009) Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in *Pichia pastoris*. *Biochim. Biophys. Acta* 1789, 460–468
16. Kranthi, B. V., Kumar, H. R., and Rangarajan, P. N. (2010) Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast *Pichia pastoris*. *Yeast* 27, 705–711
17. Kumar, N. V., and Rangarajan, P. N. (2011) Catabolite repression of phosphoenolpyruvate carboxykinase by a zinc finger protein under biotin- and pyruvate carboxylase-deficient conditions in *Pichia pastoris*. *Microbiology* 157, 3361–3369
18. Kumar, N. V., and Rangarajan, P. N. (2012) The zinc finger proteins Mxr1p and repressor of phosphoenolpyruvate carboxykinase (ROP) have the same DNA binding specificity but regulate methanol metabolism antagonistically in *Pichia pastoris*. *J. Biol. Chem.* 287, 34465–34473
19. Sahu, U., Krishna Rao, K., and Rangarajan, P. N. (2014) Trm1p, a Zn(II)(2)(Cys(6))−type transcription factor, is essential for the transcriptional activation of genes of methanol utilization pathway, in *Pichia pastoris*. *Biochem. Biophys. Res. Commun.* 451, 158–164
20. Sahu, U., and Rangarajan, P. N. (2016) Regulation of acetate metabolism and acetyl-CoA synthetase 1 (ACS1) expression by methanol expression regulator 1 (Mxr1p) in the methylotrophic yeast *Pichia pastoris*. *J. Biol. Chem.* 291, 3648–3657
21. Sahu, U., and Rangarajan, P. N. (2016) Methanol expression regulator 1 (Mxr1p) is essential for the utilization of amino acids as the sole source of carbon by the methylotrophic yeast, *Pichia pastoris*. *J. Biol. Chem.* 291, 20588–20601
22. Prasannan, P., Suliman, H. S., and Robertus, J. D. (2009) Kinetic analysis of site-directed mutants of methionine synthase from *Candida albicans*. *Biochem. Biophys. Res. Commun.* 382, 730–734
Nuclear localization of yeast methionine synthase

23. Reytor, E., Pérez-Miguelsanz, J., Alvarez, L., Pérez-Sala, D., and Pajares, M. A. (2009) Conformational signals in the C-terminal domain of methionine adenosyltransferase I/I1 determine its nucleocytoplasmic distribution. FASEB J. 23, 3347–3360
24. Jeffery, C. J. (1999) Moonlighting proteins. Trends Biochem. Sci. 24, 8–11
25. Jeffery, C. J. (2003) Multifunctional proteins: examples of gene sharing. Ann. Med. 35, 28–35
26. Jeffery, C. J. (2003) Moonlighting proteins: old proteins learning new tricks. Trends Genet. 19, 415–417
27. Kim, I. W., and Dang, C. V. (2005) Multifaceted roles of glycolytic enzymes. Trends Biochem. Sci. 30, 142–150
28. Yogev, O., Yogev, O., Singer, E., Shaulian, E., Goldberg, M., Fox, T. D., and Pines, O. (2010) Furmarase: a mitochondrial metabolic enzyme and a cytosolic/nuclear component of the DNA damage response. PLoS Biol. 8, e1000328
29. Sutendra, G., Kinnaird, A., Dromparis, P., Paulin, R., Stenson, T. H., Haromy, A., Hashimoto, K., Zhang, N., Flaim, E., and Michelakis, E. D. (2014) A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation. Cell 158, 84–97
30. Katoh, Y., Ikura, T., Hoshikawa, Y., Tashiro, S., Ito, T., Ohma, M., Kera, Y., Noda, T., and Igarashi, K. (2011) Methionine adenosyltransferase II serves as a transcriptional coressor of Maf oncprotein. Mol. Cell. 41, 554–566
31. Holmes, W. B., and Appling, D. R. (2002) Cloning and characterization of methenyltetrahydrofolate synthetase from Saccharomyces cerevisiae. J. Biol. Chem. 277, 20205–20213
32. Drinnenberg, I. A., Weinberg, D. E., Xie, K. T., Mower, J. P., Wolfe, K. H., Fink, G. R., and Bartel, D. P. (2009) RNAi in budding yeast. Science 326, 544–550
33. Nagy, M., Lacroute, F., and Thomas, D. (1992) Divergent evolution of pyrimidine biosynthesis between anaerobic and aerobic yeasts. Proc. Natl. Acad. Sci. U.S.A. 89, 8966–8970
34. Zameitat, E., Gojkovic´, Z., Knecht, W., Piskur, J., and Löffler, M. (2006) Biochemical characterization of recombinant dihydroorotate dehydrogenase from the opportunistic pathogenic yeast Candida albicans. FEBS J. 273, 3183–3191
35. Vanlerbergh, G. C., Vanlerbergh, A. E., and McIntosh, L. (1997) Molecular genetic evidence of the ability of alternative oxidase to support respiration carbon metabolism. Plant Physiol. 113, 657–661
36. Sohn, M. J., Yoo, S. J., Oh, D. B., Kwon, O., Lee, S. Y., Sibirny, A. A., and Kang, H. A. (2014) Novel cysteine-centered sulfur metabolic pathway in the thermostolerant methylotrophic yeast Hansenula polymorpha. PLoS ONE 9, e100725
37. Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985) Pichia pastoris as a host system for transformations. Mol. Cell. Biol. 5, 3376–3385
38. Gillum, A. M., Tsay, E. Y., and Kirsch, D. R. (1984) Isolation of the Candida albicans gene for orotidine-5’-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol. Gen. Genet. 198, 179–182
39. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
40. Lee, C., Levin, A., and Branton, D. (1987) Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 166, 308–312
41. Ide, G. J., and Saunders, C. A. (1981) Rapid isolation of yeast nuclei. Curr. Genet. 4, 85–90
42. Craven, R. A., Griffiths, D. J., Sheldrick, K. S., Randall, R. E., Hagan, I. M., and Carr, A. M. (1998) Vectors for the expression of tagged proteins in Schizosaccharomyces pombe. Gene 221, 59–68
43. Daskowski, R. A., Macarthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst. 26, 283–291
44. Hasegawa, H., and Holm, L. (2009) Advances and pitfalls of protein structural alignment. Curr. Opin. Struct. Biol. 19, 341–348
45. Konagurthu, A. S., Whisstock, J. C., Stuckey, P. J., and Lesk, A. M. (2006) MUSTANG: a multiple structural alignment algorithm. Proteins 64, 559–574
46. Ramachandran, S., Kota, P., Ding, F., and Dokholyan, N. V. (2011) Automated minimization of steric clashes in protein structures. Proteins 79, 261–270
47. Tina, K. G., Bhadra, R., and Srinivasan, N. (2007) PIC: protein interactions calculator. Nucleic Acids Res. 35, W473–W476
48. Pines, O. (2010) Fumarase: a mitochondrial metabolic enzyme and a cytosolic/nuclear component of the DNA damage response. Curr. Protoc. Bioinformatics Chapter 5, Unit 5.6
49. Schrödinger, LLC (2012) The PyMOL Molecular Graphics System, Version 1.5.0, Schrödinger, LLC, New York Harbor, NY