**Supporting Information**

# Allosteric inhibition of MTHFR prevents futile SAM cycling and maintains nucleotide pools in one carbon metabolism

Muskan Bhatia[1], Jyotika Thakur[2], Shradha Suyal[1], Ruchika Oniel[3], Rahul Chakraborty[4], Shalini Pradhan[4], Monika Sharma[5], Shantanu Sengupta[4], Sunil Laxman[3], Shyam Kumar Masakapalli[2], Anand Kumar Bachhawat[1*]

[1]Department of Biological Sciences, Indian Institute of Science Education and Research (IISER Mohali), S.A.S. Nagar, Punjab 140306, India

[2]BioX Center, School of Basic Sciences, Indian Institute of Technology Mandi, Kamand, Himachal Pradesh 175005, India

[3]Institute for Stem Cell Biology and Regenerative Medicine (inStem), NCBS-TIFR campus, Bangalore 560065, India

[4]Council of Scientific and Industrial Research—Institute of Genomics and Integrative Biology, New Delhi 110020, India

[5]Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER Mohali), S.A.S. Nagar, Punjab 140306, India

## List of Material Included

Supplementary Figures: S1-S9

Supplementary Tables: S1, S2, S3, S5

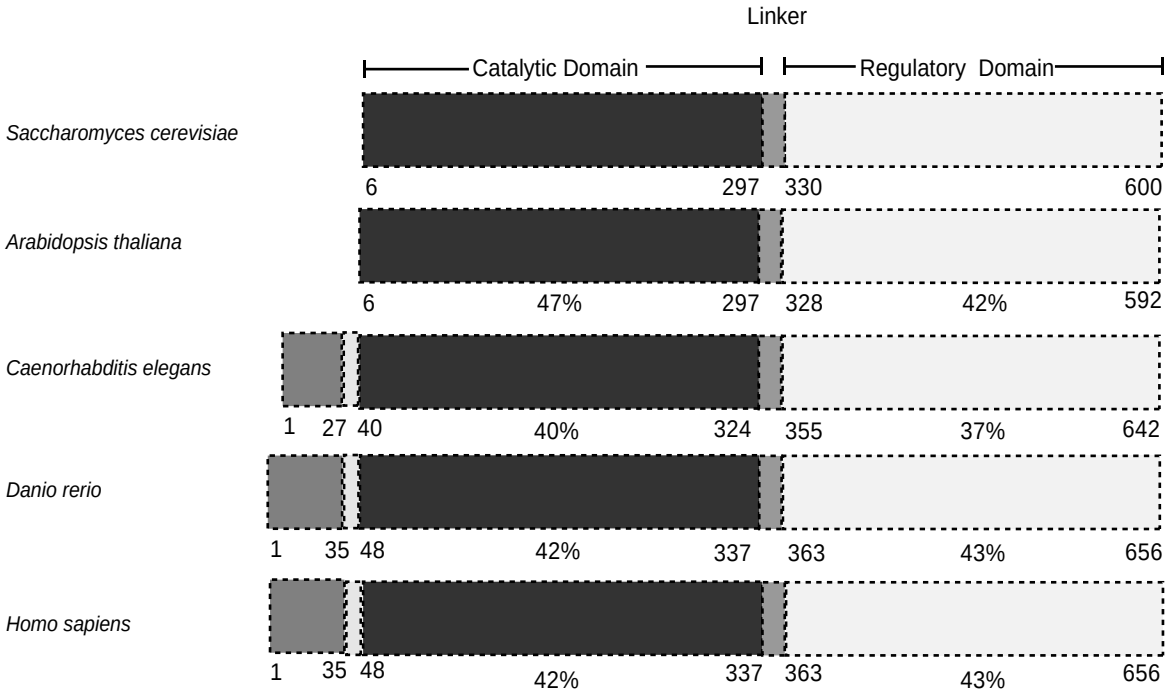



**Figure S1 Schematic representation of eukaryotic MTHFR domain organization.**
Domain organization of MTHFR orthologs across evolution. Numbers represent approximate amino acid numbers in each organisms. Percentage represents the identity of amino acids within each domain of *Saccharomyces cerevisiae* across different organisms.

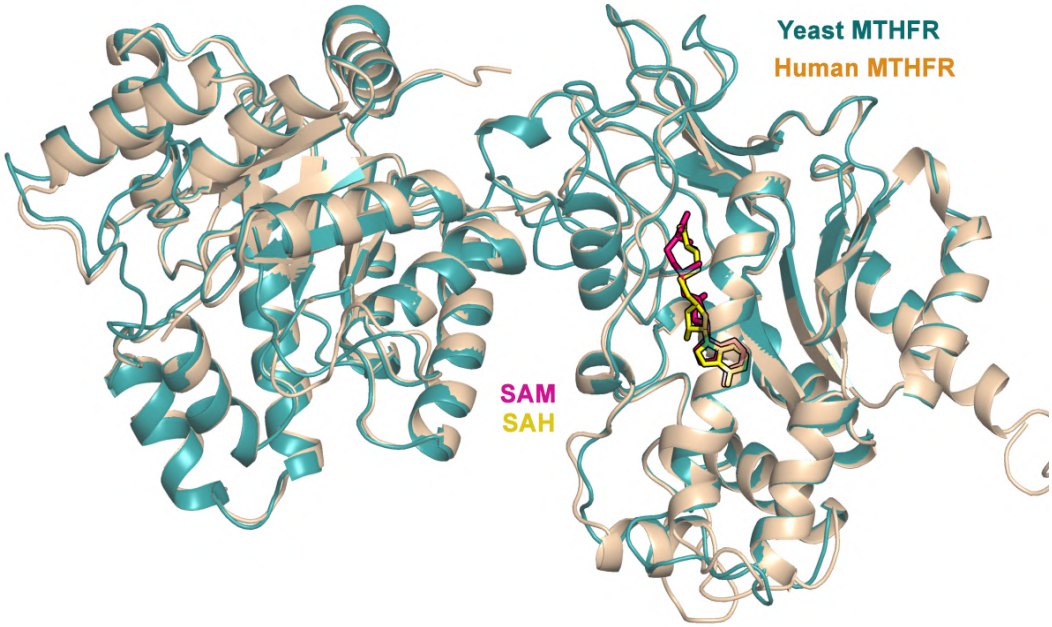



**Figure S2 Structural alignment of modeled yeast MTHFR with human MTHFR.**
Cartoon representation of modeled yeast MTHFR structure (cyan colored) aligned with respect to human MTHFR structure (wheat colored). The respective bound ligands are shown in sticks with SAH in yellow and SAM in magenta. The yeast modeled structure showed rmsd value of 0.48 Å with respect to the human crystal structure.

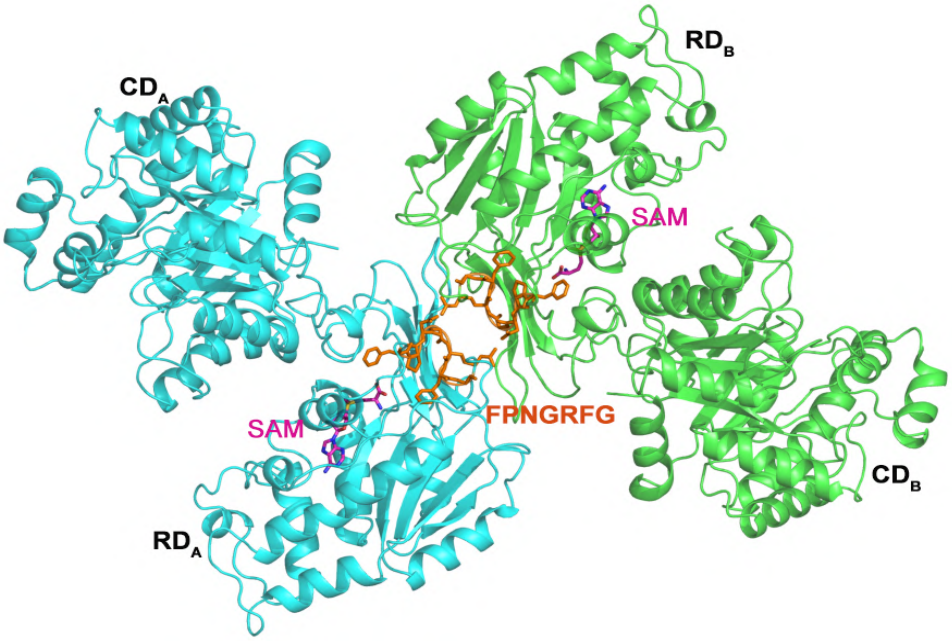



**Figure S3 Cartoon representation of modeled yeast MTHFR structure.**
Homology modeling studies using Modeler 9v20 indicates yeast MTHFR, Met13p, exists as a dimer where each monomer is composed of a catalytic domain and a regulatory domain. The two monomers are shown in cyan and green color. The two monomers interact with each other via the regulatory domain. $CD_A$ and $CD_B$- Catalytic domain of the two monomers (A and B); $RD_A$ and $RD_B$- Regulatory domain of the two monomers (A and B); bound SAM is represented by sticks in pink.

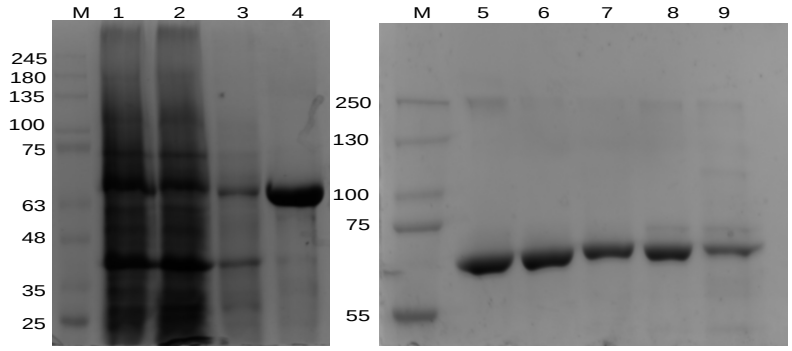



**Figure S4 Protein purification of yeast MTHFR proteins.**
Yeast MTHFR WT and mutant proteins were recombinantly expressed and purified from bacterial expression system using NiNTA affinity chromatography. The purified protein was run on 12% SDS PAGE and band of corresponding to molecular weight of WT MET13p (69 kDa) was observed. Lane 1 Crude extract, Lane 2 Flow through, Lane 3 Wash, Lane 4 MET13_M2, Lane 5 MET13_WT, Lane 6 MET13_P354A, Lane 7 MET13_R357A, Lane 8 MET13_Y404A, and Lane 9 MET13_E422A.

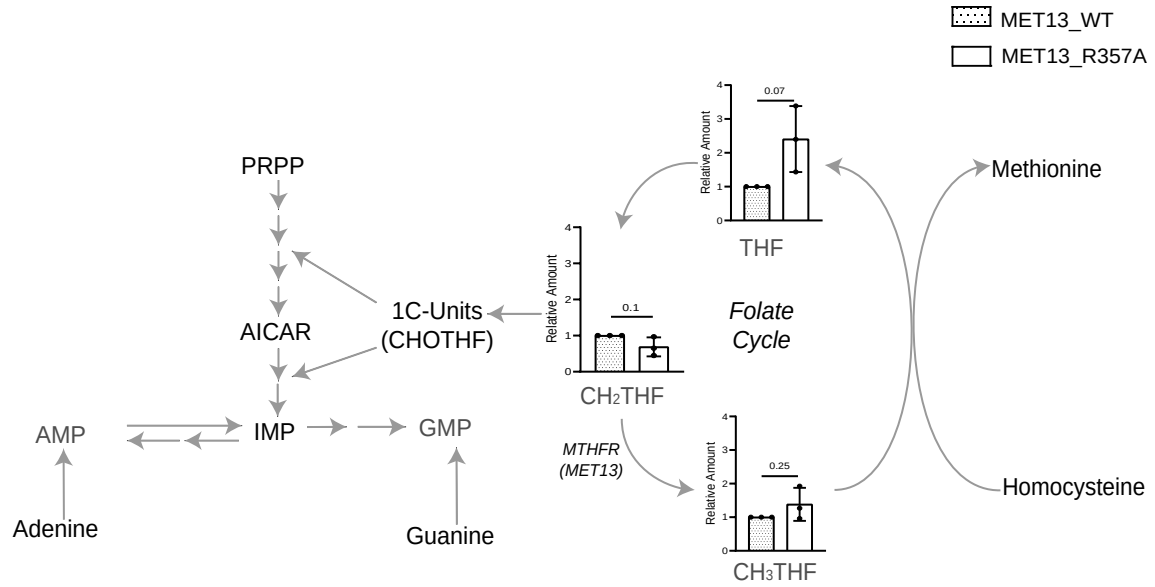



**Figure S5 MTHFR deregulation disrupts cellular folate pools.**
**T**he relative abundance of folate pools in transformants bearing the WT MET13 or deregulated mutant of yeast MTHFR, MET13_R357A. Transformants were grown overnight in SD minimal media along with GSH (200µM) as a sulfur source, and re-inoculated to medium containing methionine (200µM) and different types of folate intermediates were determined by LC-MS/MS. Error bars indicate SD. n = 4.

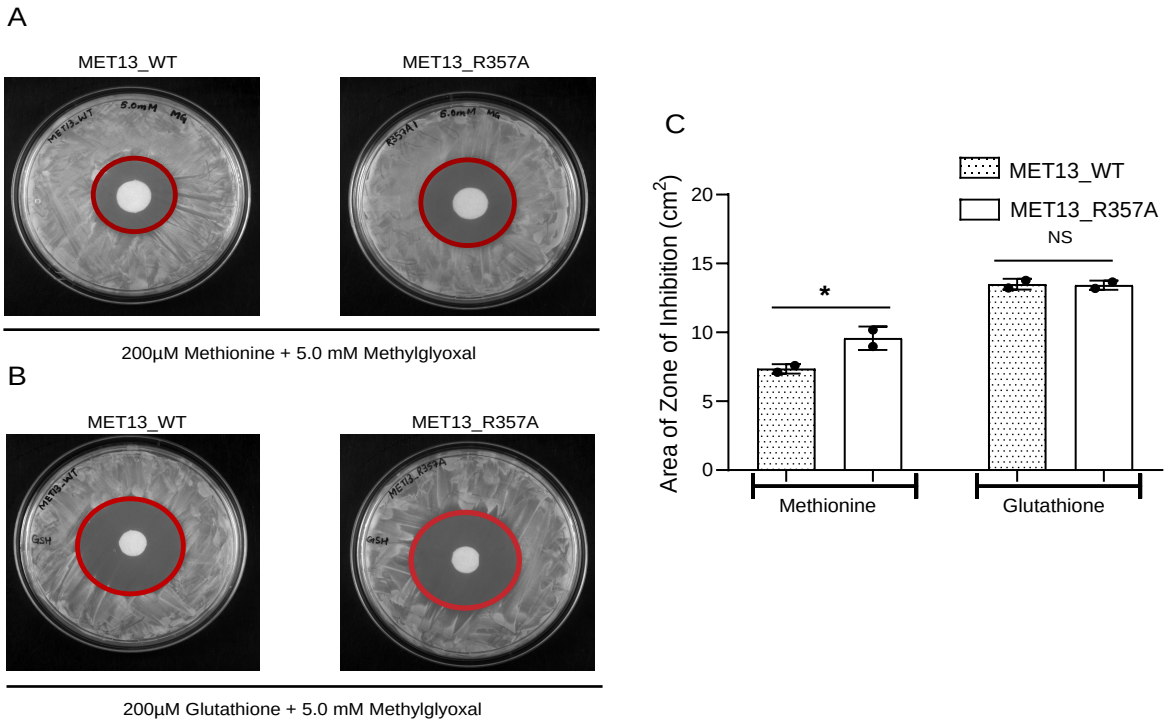



**Figure S6 Increased sensitivity of deregulated MET13_R357A mutant towards methylglyoxal (MG), GSH specific oxidizing agent.**
Transformant bearing the deregulated mutant, MET13_R357A, exhibits sensitivity to MG when grown overnight in SD minimal media along with GSH (200µM) as sulfur source and re-inoculated to medium containing methionine (200µM). At the exponential phase (1.0-1.5 OD), 5 OD of cells were plated onto SD plates supplemented with either (A) methionine or (B) Glutathione. Filter disc containing 5.0mM MG was placed onto the lawn of cells, which were allowed to grow at 30°C for 48 h. The experiment was repeated twice, and a representative data set of two biological replicates is shown in the above figure. (C) The graph corresponds to the zone of inhibitions calculated using the average of two independent biological replicates. Error bars indicate SD. n = 2. *p < 0.05, NS is not significant

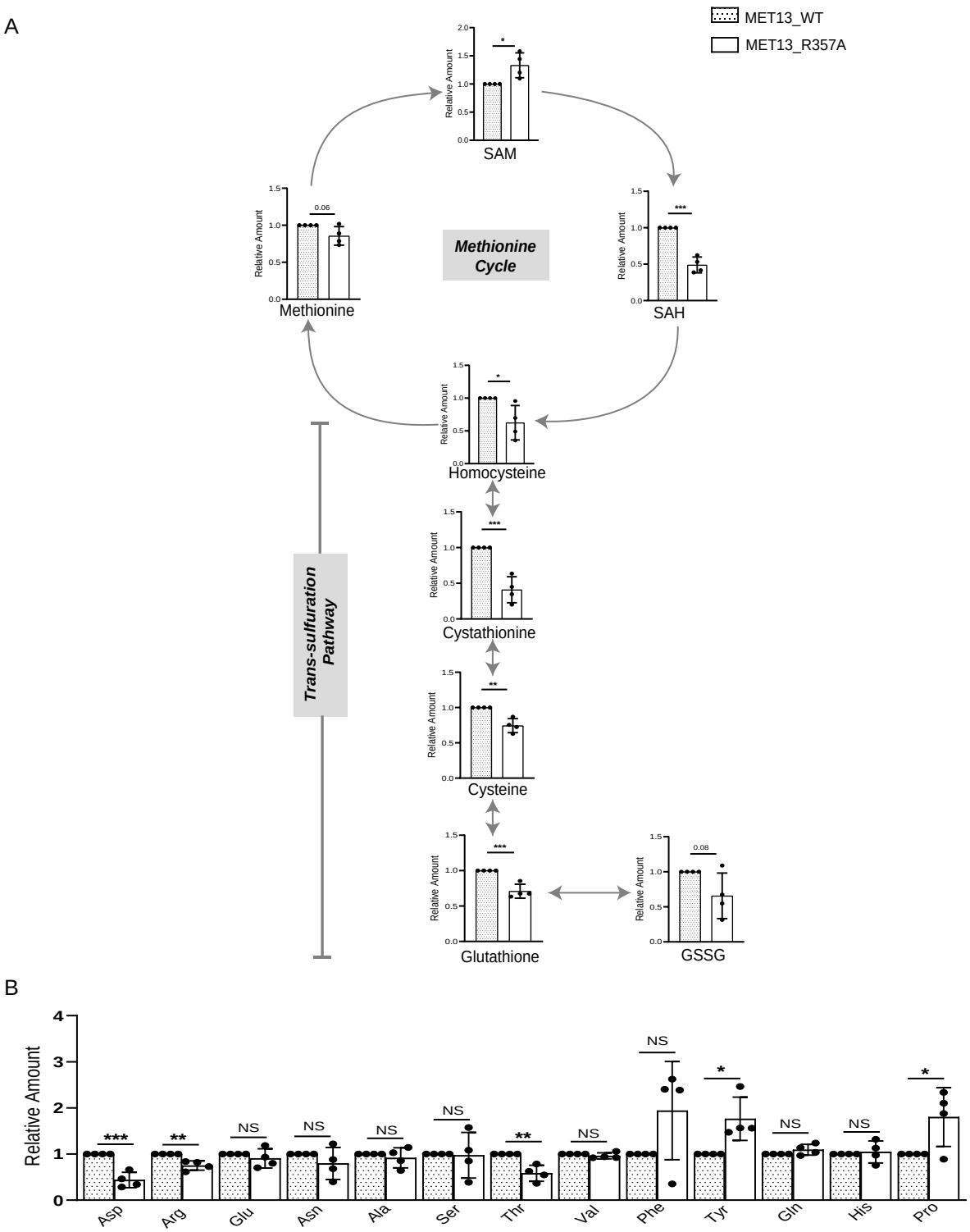
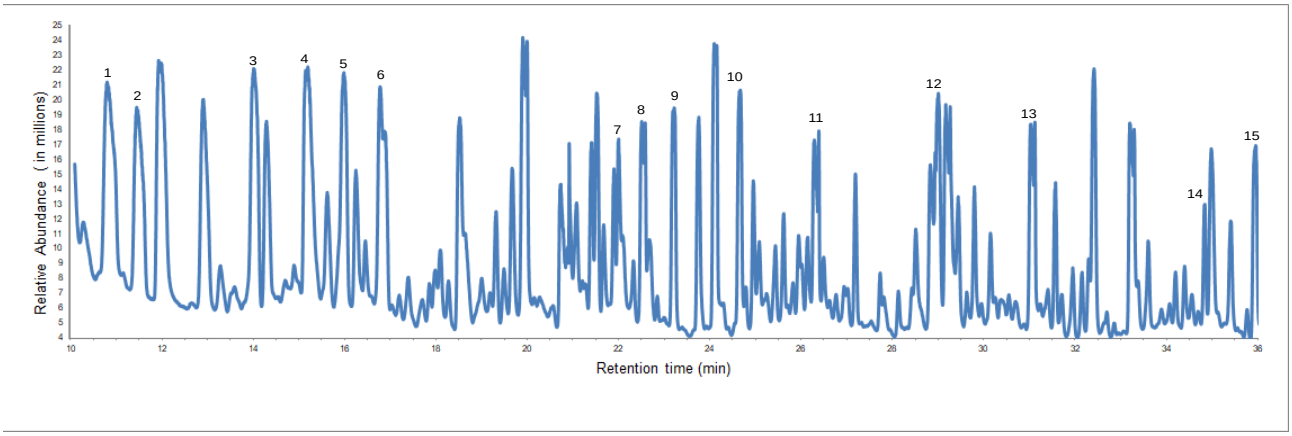



**Figure S7 The impact of the deregulated MTHFR on amino acids, SAM and glutathione.**
Relative intracellular levels of the amino acid pools estimated from yeast transformants of MET13_WT or MET13_R357A in *met13Δmet15Δ* (ABC2613) that were grown overnight in minimal media with amino acid supplements and GSH. These were then re-inoculated at 0.15 OD600 in fresh SD media without any sulfur source. Methionine was added to the transformants after 3 hours of secondary inoculation, and samples were collected in triplicates at 0.5 or 1.0 OD. The graph here corresponds to the representative data set of 0.5 OD cells plotted using the average of three biological replicates and three technical replicates of each of these biological replicates along with ±S.D values. Error bars indicate SD. n = 3. *p < 0.05, **p < 0.01, p***< 0.001 and NS is not significant. (A) Sulphur containing amino acids and intermediates of reverse-trans-sulfuration (B) Non-sulphur amino acids.

A




B

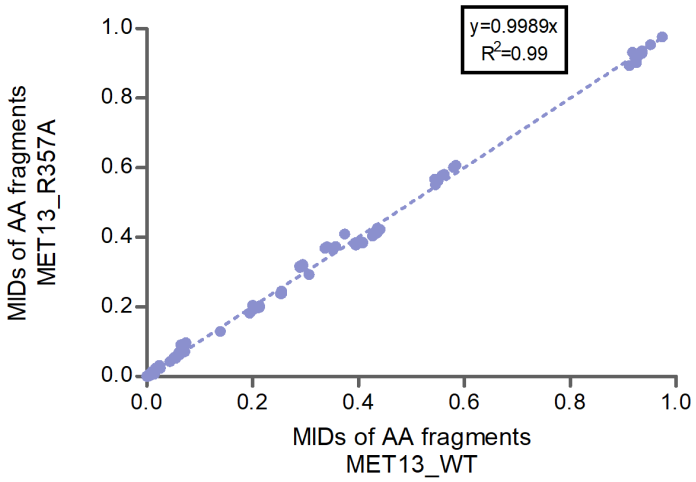



**Figure S8 Relative flux analysis of wild-type and feedback insensitive, deregulated MTHFR mutant.**
(A) GC-MS spectra of amino acids derived from the yeast cells. 15 TBDMS derivatized amino acids were detected from the cell hydrolysates of MET13_WT and MET13_R357A. The m/z fragments of each amino acids resulted in Mass isotopomer distributions. The peaks corresponding to the detected amino acids, with their respective elution times (in minutes), are indicated in Supplementary Table S2. (B) Mass isotopomer distributions of amino acid pools observed between the MET13_WT and MET13_R357A obtained from [1-$^{13}$C] glucose feeding. The TBDMS derivatized protein hydrolysate from these cells was subjected to GC-MS for accurate analysis of mass isotopomer distribution in amino acids. Due to ionization in mass spectroscopy, the TBDMS derivatized amino acids formed different fragments such as [M-0]+, [M-15]+, [M-57]+, [M-85]+, [M-159]+, and [M-R]+ (where, R denotes the side chain of an amino acid often resulting in fragment [f302]+), where [M-57]+, [M-85]+ fragments of each amino acid were plotted using linear regression.

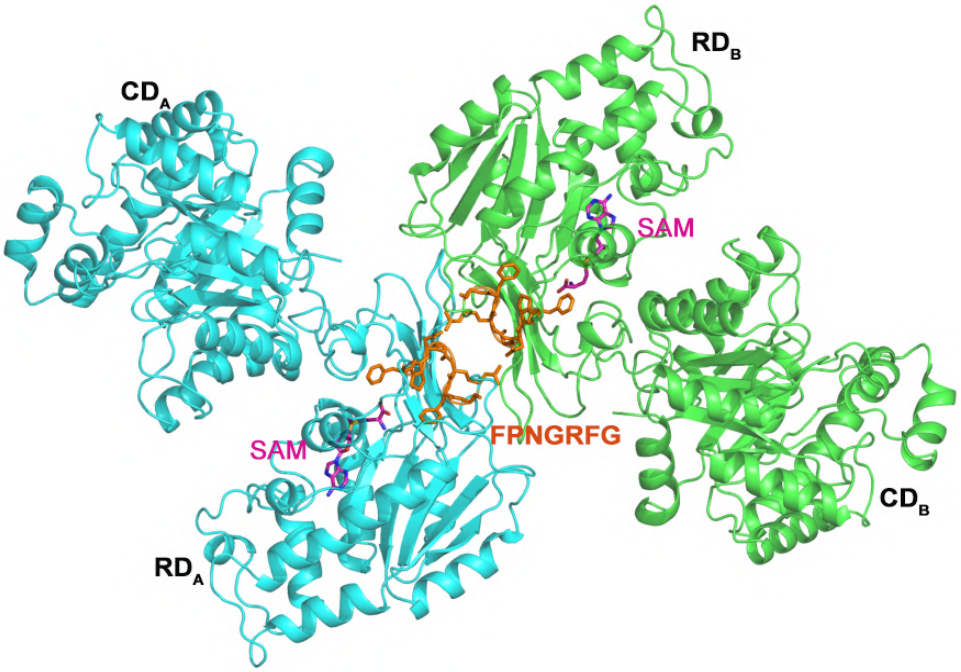



**Figure S9 Cartoon representation of modeled yeast MTHFR structure highlighting residues critical for allosteric inhibition by SAM.**
Investigation towards identifying residues critical for regulation of MTHFR by SAM, revealed a 7 amino acid stretch, 353-F-P-N-G-R-F-G-359, within the CR1 region of the regulatory domain. The 'FPNGRFG' loops for both the monomers at the interface region is shown in orange color. The two monomers are shown in cyan and green color. $CD_A$ and $CD_B$- Catalytic domain of the two monomers (A and B); $RD_A$ and $RD_B$- Regulatory domain of the two monomers (A and B); bound SAM is represented by sticks in magenta.

**Table S1 Regulatory domain mutants of CR1, CR2, and CR3 of the Met13p**

| Mutant Name | Residues Mutated | Region of Regulatory Domain |
|---|---|---|
| M1 | R344A.T345A | CR1 |
| M2 | R357A.S361A | CR1 |
| M4 | T440A.N442A.S443A.Q444A.P445A | CR2 |
| M5 | T519A.W520A | CR3 |
| M6 | F523A.P524A.E527A | CR3 |

**Table S2 List of TBDMS derivatized amino acids detected using GC-MS from the yeast cell hydrolysates**

| S. No. | Retention Time (RT) | Amino acids identified | Derivatives |
|---|---|---|---|
| **1** | 10.787 | Alanine | 2TBDMS |
| **2** | 11.438 | Glycine | 2TBDMS |
| **3** | 14.009 | Valine | 2TBDMS |
| **4** | 15.151 | Leucine | 2TBDMS |
| **5** | 15.967 | Isoleucine | 2TBDMS |
| **6** | 16.762 | Proline | 2TBDMS |
| **7** | 21.894 | Methionine | 2TBDMS |
| **8** | 22.504 | Serine | 3TBDMS |
| **9** | 23.2 | Threonine | 3TBDMS |
| **10** | 24.657 | Phenylalanine | 2TBDMS |
| **11** | 26.381 | Aspartic acid | 3TBDMS |
| **12** | 28.928 | Glutamic acid | 3TBDMS |
| **13** | 31.033 | Lysine | 3TBDMS |
| **14** | 34.986 | Histidine | TBDMS |
| **15** | 35.942 | Tyrosine | 3TBDMS |

**Table S4 Yeast strains**

| Strain | Relevant Genotype | Source |
|---|---|---|
| BY4742 | MATα *his3Δ leu2Δ 0 lys2Δ0 ura3Δ0* | Euroscarf |
| *met13Δ* (BY4742) | BY4742, *ygl125wΔ*::*KanMX* | Euroscarf |
| BY4741 | MATa *his3Δ leu2Δ 0 met15Δ0 ura3Δ0* | Euroscarf |
| *met13Δ* (BY4741) | BY4741, *ygl125wΔ*::*KanMX* | Euroscarf |
| *met6Δ. met13Δ* (BY4742) | BY4742, *yer091cΔ*::*KanMX, ygl125wΔ*:: *LEU2Δ* | This study |
| *mup1Δ.met13Δ* (BY4742) | BY4742, *ygr055wΔ*::*KanMX, ygl125wΔ*:: *LEU2Δ* | This study |

**Table S5 Primer List**

| Primer Name | Sequence (5' 3') |
|---|---|
| MET13_XbaI F | TGACGATCTAGAATGAAGATCACAGAAAAATTAGAGCAACATAG |
| MET13-His Cla I R | GATCCTATCGATTTAATGGTGGTGATGGTGATGTAGGCTTAGTAGGA TGGAATGG |
| MET13_Seq (600bp) F | TGGTGTTCCCAAGTTAGAGCTGCG |

| | |
|---|---|
| MET13_SacI F | TGACGAGAGCTCATGAAGATCACAGAAAAATTAGAGCAACATAG |
| MET13_Y404A F | TTGGTCATCAACGCCTTGAATGGAAAC |
| MET13_Y404A R | GTTTCCATTCAAGGCGTTGATGACCAAGAAGG |
| MET13_E422A F | CCCATCAATGATGCAATAAATCCAATC |
| MET13_E422A R | GATTGGATTTATTGCATCATTGATGGG |
| MET13_M1 F | TGGAAGAGAAGACCTTACGCCTATGTCGCAGCAGCCTCTCAATGGGCCGTGGACG |
| MET13_M1 R | GTCCACGGCCCATTGAGAGGCTGCTGCGACATAGGCGTAAGGTCTTCTCTTCCAG |
| MET13_M2 F | GGGCCGTGGACGAATTCCCCAACGGTGCATTCGGTGATGCGTCTTCTCCTGCGTTCGG |
| MET13_M2 R | CCGAACGCAGGAGAAGACGCATCACCGAATGCACCGTTGGGGAATTCGTC |
| MET13_M4 F | AACCAGCATTCTATCATCGCTATAGCCGCTGCAGCTCAAGTCAACGGCATTAGGTC |
| MET13_M4 R | CCTAATGCCGTTGACTTGAGCTGCAGCGGCTATAGCGATGATAGAATGCTGGTTCAGC |
| MET13_M5 F | TCCAAGTCCAACGCTGTGGCTGCGGGTATTTTCCCCGGCAGAG |
| MET13_M5 R | TCTGCCGGGGAAAATACCCGCAGCCACAGCGTTGGACTTGGAG |
| MET13_M6 F | GCTGTGACTTGGGGTATTGCCGCCGGCAGAGCAATTCTTCAACCTACCATTGTCG |
| MET13_M6 R | AATGGTAGGTTGAAGAATTGCTCTGCCGGCGGCAATACCCCAAGTCACAGCG |
| MET13_S340A F | TGGAAGAGAAGACCTTACGCCTATGTCGCAAGAACC |
| MET13_S340A R | GGTTCTTGCGACATAGGCGTAAGGTCTTCTCTTCC |
| MET13_Y341A F | GAGAAGACCTTACTCCGCTGTCGCAAGAACCTCTCAATGG |
| MET13_Y341A R | TGAGAGGTTCTTGCGACAGCGGAGTAAGGTCTTCTCTTCC |
| MET13_R344A F | TTACTCCTATGTCGCAGCAACCTCTCAATGGGCCG |
| MET13_R344A R | ACGGCCCATTGAGAGGTTGCTGCGACATAGGAGTAAGG |
| MET13_T345A F | ACTCCTATGTCGCAAGAGCCTCTCAATGGGCCGTGG |
| MET13_T345A R | CCACGGCCCATTGAGAGGCTCTTGCGACATAGGAGTAAGG |
| MET13_W348A F | CGCAAGAACCTCTCAAGCGGCCGTGGACGAATTCCC |
| MET13_W348A R | GGGAATTCGTCCACGGCCGCTTGAGAGGTTCTTGCG |
| MET13_F353A F | ATGGGCCGTGGACGAAGCCCCCAACGGTAGATTCGG |
| MET13_F353A R | CCGAATCTACCGTTGGGGGCTTCGTCCACGGCCCATTGAGAGG |
| MET13_P354A F | GGGCCGTGGACGAATTCGCCAACGGTAGATTCGGTG |
| MET13_P354A R | CACCGAATCTACCGTTGGCGAATTCGTCCACGGCCC |
| MET13_G356A F | GGACGAATTCCCCAACGCTAGATTCGGTGATTCG |
| MET13_G356A R | CGAATCACCGAATCTAGCGTTGGGGAATTCGTCC |
| MET13_R357A F | CGAATTCCCCAACGGTGCATTCGGTGATTCGTCTTCTCCTGCG |
| MET13_R357A R | GAGAAGACGAATCACCGAATGCACCGTTGGGGAATTCGTCC |
| MET13_G359A F | CCCCAACGGTAGATTCGCTGATTCGTCTTCTCCTGC |
| MET13_G359A R | GCAGGAGAAGACGAATCAGCGAATCTACCGTTGGGG |
| MET13_S361A F | CCAACGGTAGATTCGGTGATGCGTCTTCTCCTGCGTTCGG |
| MET13_S361A R | CCGAACGCAGGAGAAGACGCATCACCGAATCTACCGTTGG |
| MET13_E22A F | ACTTACTCATTCGCGTACTTCGTCCCG |
| MET13_E22A R | CGGGACGAAGTACGCGAATGAGTAAGT |
| LEU2-MET13-DEL F | ATGAAGATCACAGAAAAATTAGAGCAACATAGACAGACCTAACTGTGGGAATACTCAGGT |
| LEU2 MET13 DEL R | TAGGCTTAGTAGGATGGAATGGATTTGATCATCTGGAGAATTAAGCAAGGATTTTCTTAA |
| MET13 prom F | TCATTCTATCCCTCGGATTATAGACTGTG |