SUPPLEMENTARY INFORMATION

Cbr1 is a Dph3 reductase required for the tRNA wobble uridine modification

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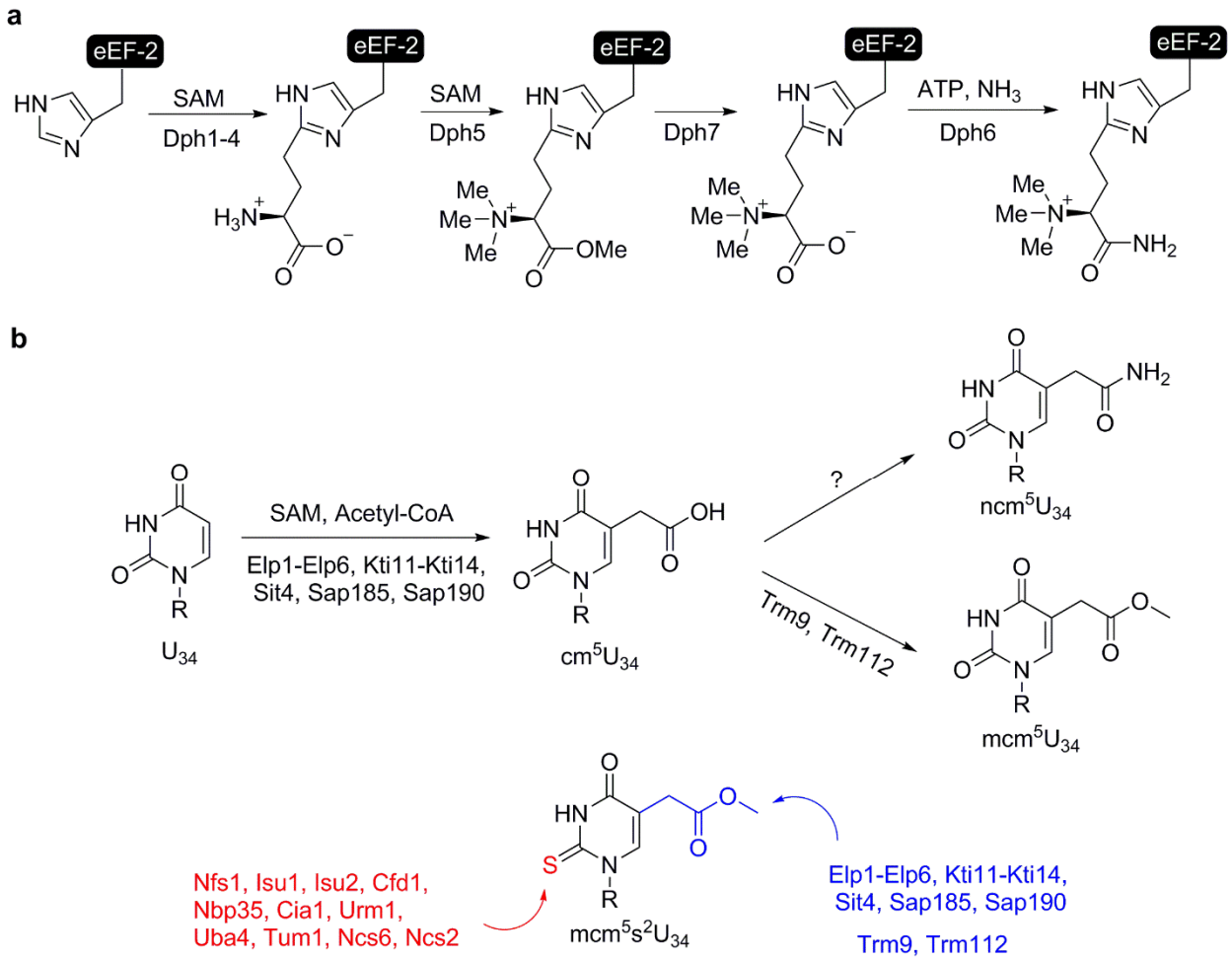
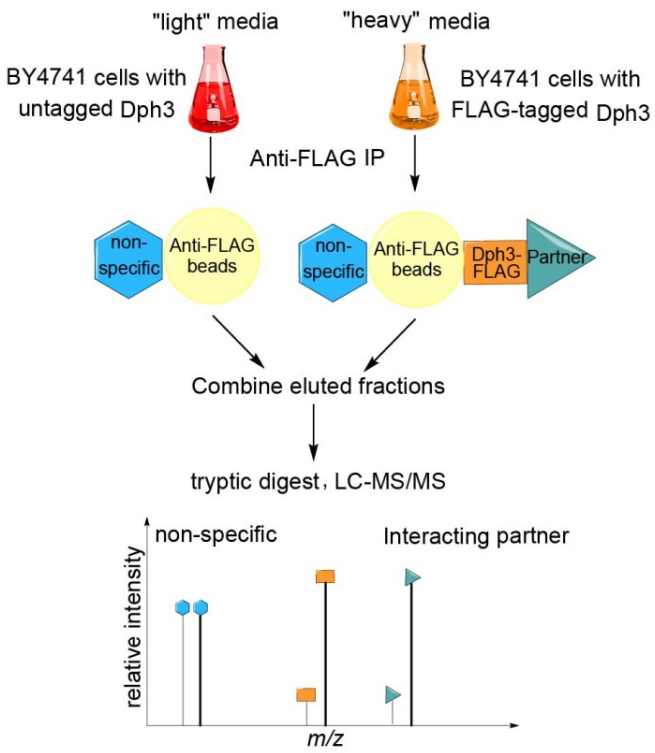
SUPPLEMENTARY RESULTS



Supplementary Figure 1. Reaction schemes for diphthamide and tRNA wobble uridine modification. (A) Diphthamide biosynthesis pathway in eukaryotes. (B) tRNA wobble uridine modifications in eukaryotes.

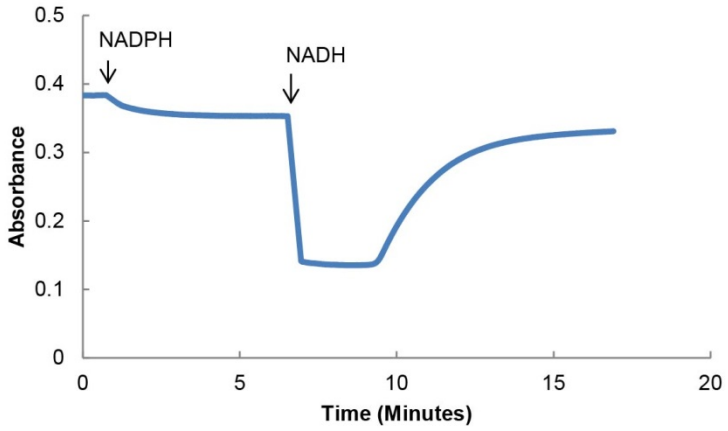
a



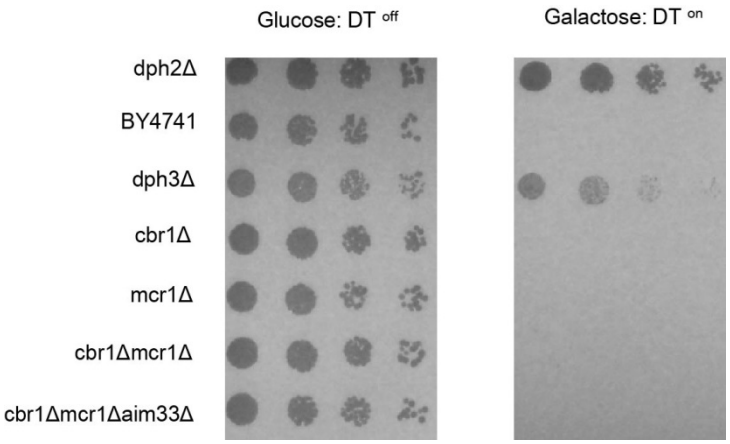
b

Protein	Heavy to light ratio
Elongator subunit Elp1	500.00
Dph2	500.00
Dph1	500.00
Elongator subunit Elp2	500.00
Elongator subunit Elp3	500.00
Serine/threonine protein kinase Hrr25	500.00
Deoxyhypusine hydroxylase	500.00
Cytochrome b_5 reductase Cbr1	500.00
F1F0 ATP synthase subunit 4	452.37
H(+)-transporting V1 sector ATPase subunit H	32.00
Mitochondrial import receptor subunit TOM40	25.06
Kti13	13.75
Regulator of ribosome biosynthesis Rrs1	11.20

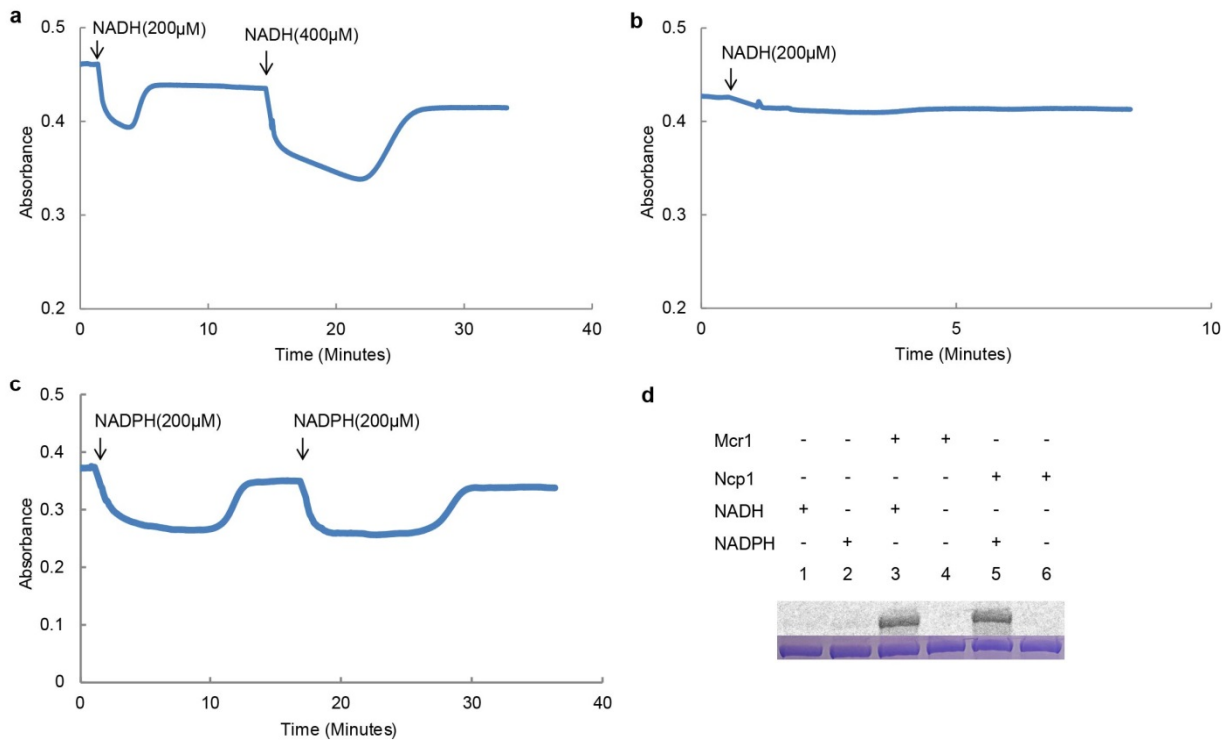
Supplementary Figure 2. Dph3 interactome study identifies Cbr1 as a potential Dph3 reductase. (A) Schematic workflow of the Dph3 SILAC interactome study. (B) A list of proteins with high H/L ratios from Dph3 SILAC interactome study. 500 was set as the maximum H/L ratio to make it mathematically meaningful for peptides not detected in the light sample. The table lists protein with H/L ratio greater than 10.



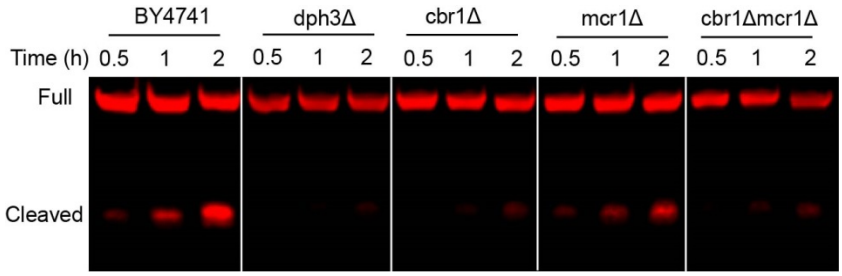
Supplementary Figure 3. Cbr1 uses NADH but not NADPH for Dph3 reduction. Reduction of Dph3 by Cbr1 was monitored using the 488 nm absorbance of oxidized Dph3. Figure is representative of three experimental repeats.



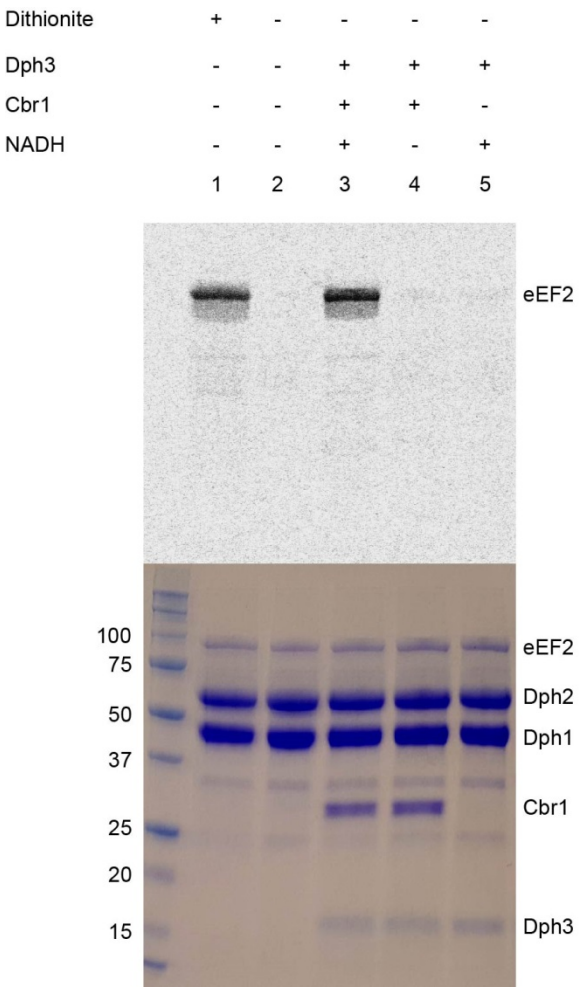
Supplementary Figure 4. DT sensitivity assay showing that diphthamide formation is not affected in Cbr1 or Mcr1 deletion strains. The strains used are specified on the left. Each row represents a serial dilution from left to the right. Figure is representative of three biological triplicates.



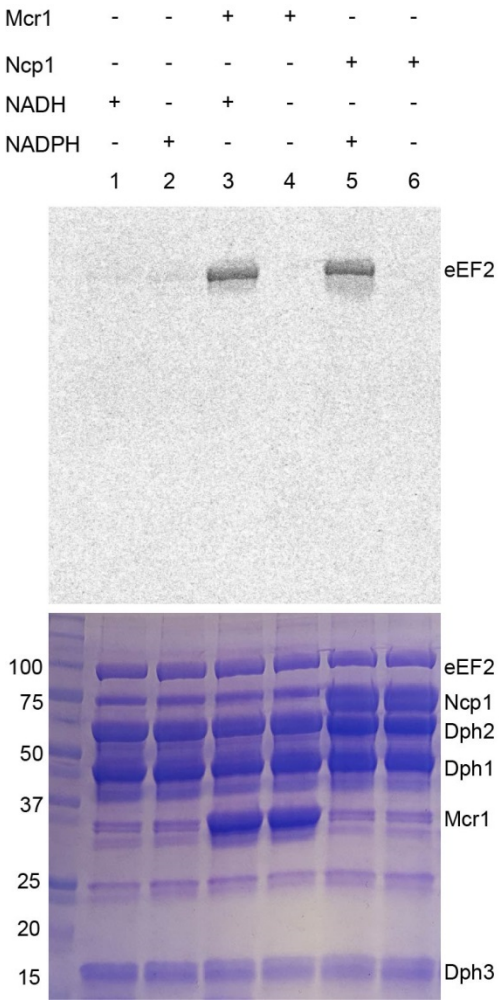
Supplementary Figure 5. Mcr1 and Ncp1 reduce Dph3 *in vitro*. (A) Reduction of Dph3 by Mcr1 monitored using the 488 nm absorbance of oxidized Dph3. (B) Reduction of Dph3 by Pga3 monitored using the 488 nm absorbance of oxidized Dph3. (C) Reduction of Dph3 by Ncp1 monitored using the 488 nm absorbance of oxidized Dph3. (D) *In vitro* reconstitution of the first step of diphthamide biosynthesis on eEF2 using Dph1-2, carboxy-^{14}C-SAM in the presence of either Mcr1/NADH or Ncp1/NADPH. Autoradiography shows labeled eEF2 substrate. Bottom panel shows eEF2 stained with Coomassie blue. Supplementary Figure 5a, 5b and 5c are representative of three experimental repeats. Supplementary Figure 5d shows representative image from two experimental repeats. Full gel images for Supplementary Figure 5d are shown in Supplementary Figure 9.



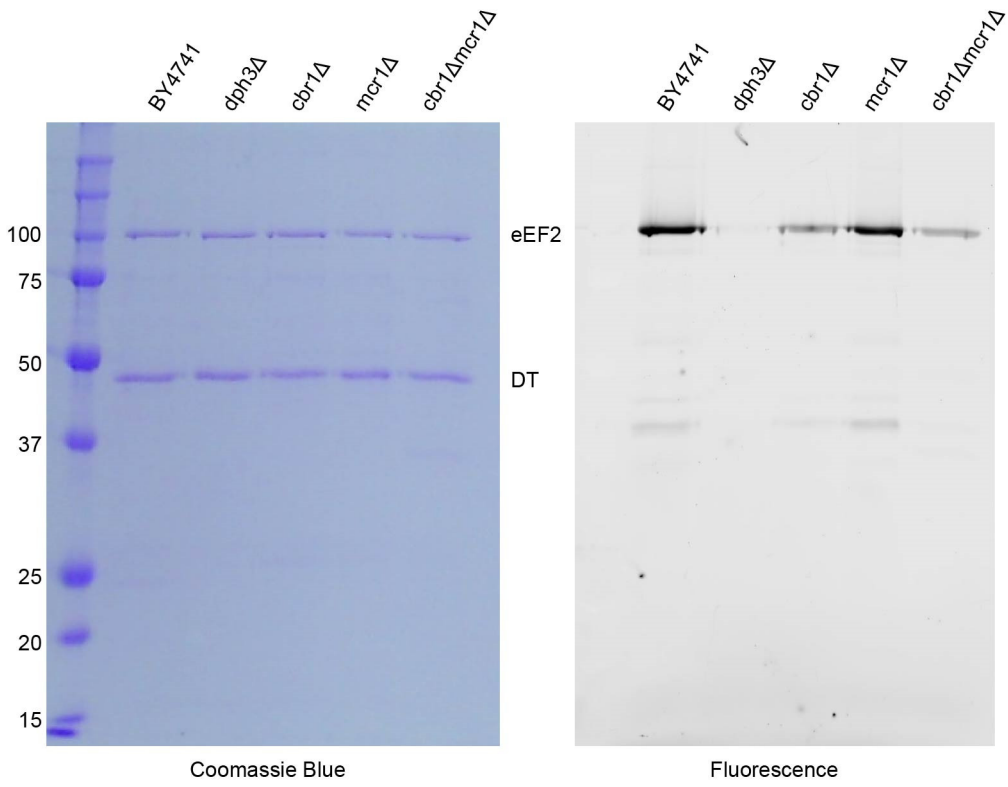
Supplementary Figure 6. Time course for *In vitro* γ-toxin treatment of isolated tRNA from cells. Samples were analyzed by northern blot with glu-tRNA 5' probe. The positions of the full length or cleaved glu-tRNA are labeled on the left. Figure shows representative image from two experimental repeats.



Supplementary Figure 7. Full gel images for Figure 1c.



Supplementary Figure 8. Full gel images for Supplementary Figure 5d.



Supplementary Figure 9. Full gel images for Figure 2d.

Supplementary Table1. Yeast strains used

Strain	Genotype	Source
HL813Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems (YSC1048)
HL1352Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dph3-6xGLY-3xFLAG::HIS3MX6*	This study
HL815Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dph2Δ::KANMX*	Open Biosystems (YSC1021-553846)
HL1429Y	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dph3Δ::KANMX*	Open Biosystems (YSC6273-201938235)
HL1355Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cbr1Δ::KANMX*	Open Biosystems (YSC6273-201920517)
HL1396Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mcr1Δ::KANMX*	Open Biosystems (YSC6273-201936518)
HL1439Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cbr1Δ::KANMX mcr1Δ::NATMX6*	This study
HL1400Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cbr1Δ::KANMX mcr1Δ::NATMX6 aim33Δ::HIS3MX6*	This study
HL1433Y	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 elp3Δ::KANMX*	Open Biosystems (YSC6273-201929585)
HL1401Y	HL813Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1402Y	HL815Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1403Y	HL1429Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1405Y	HL1355Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1406Y	HL1396Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1407Y	HL1439Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1442Y	HL1433Y [pLF16, *CEN LEU2* UASGAL-γ-toxin]	This study
HL1416Y	HL813Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study
HL1417Y	HL815Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study
HL1418Y	HL1429Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study

Supplementary Table1. Continued

Strain	Genotype	Source
HL1419Y	HL1355Y [pHL1025, p416 GALS DT-F2 (N45D)][a]	This study
HL1420Y	HL1396Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study
HL1440Y	HL1439Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study
HL1441Y	HL1400Y [pHL1025, p416 GALS DT-F2 (N45D)]	This study
HL1443Y	HL813Y [pHL610E, p423 met25 eEF2 C-His][b]	This Study
HL1444Y	HL1429Y [pHL610E, p423 met25 eEF2 C-His]	This Study
HL1445Y	HL1355Y [pHL610E, p423 met25 eEF2 C-His]	This Study
HL1446Y	HL1396Y [pHL610E, p423 met25 eEF2 C-His]	This Study
HL1447Y	HL1439Y [pHL610E, p423 met25 eEF2 C-His]	This Study
HL1448Y	HL813Y [pHL610E, p423 met25 eEF2 C-His; pHL1025, p416 GALS DT-F2 (N45D)]	This Study
HL1449Y	HL1429Y [pHL610E, p423 met25 eEF2 C-His; pHL1025, p416 GALS DT-F2 (N45D)]	This Study
HL1450Y	HL1355Y [pHL610E, p423 met25 eEF2 C-His; pHL1025, p416 GALS DT-F2 (N45D)]	This Study
HL1451Y	HL1396Y [pHL610E, p423 met25 eEF2 C-His; pHL1025, p416 GALS DT-F2 (N45D)]	This Study
HL1452Y	HL1439Y [pHL610E, p423 met25 eEF2 C-His; pHL1025, p416 GALS DT-F2 (N45D)]	This Study

[a] Source reference for p416 GALS DT-F2 (N45D): Su, X. et al. *Proc Natl Acad Sci USA* 109, 19983-19987 (2012).

[b] Source reference for p423 met25 eEF2 C-His: Su, X. et al. *J Am Chem Soc.* 134, 773–776 (2012).

Supplementary Table 2. List of primers used

Primers for constructing endogenous Dph3 FLAG tag

ZL210 GCAGGCATCCACCCCCCTGAGCCTATTGCCGCTGCTGCCcggatccccgggttaattaa

ZL211 CTTTATTTCTATTTGTATTCTCGATCTAGCCTCTCATCTgaattcgagctcgtttaaac

Primers for deletion of *mcr1* gene

ZL244 ATAACGTATATAGGTTAAAATAATATTCCAAGTCAAAAACcggatccccgggttaattaa

ZL245 ATCCGAAATTAAAAAAAATATCAATTACTTTCCTCCATGCgaattcgagctcgtttaaac

Primers for verification of *mcr1* deletion

ZL354 ATAACGTATATAGGTTAAAATAATATTCC

ZL307 CAATTACTTTCCTCCATGC

Primers for deletion of *aim33* gene

ZL242 TATCACATTTTTTCTTTGTAAAAAGCAACCATTCGCAACAcggatccccgggttaattaa

ZL243 TGCTTATTTACATGAAAAATCATCAATCGTAAACAGTTGAgaattcgagctcgtttaaac

Primers for verification of *aim33* deletion

ZL250 GTATGTTTAGTATTAACTCATATCAC

ZL251 AAATACGAATATATATCTAAATATAATTAATGC

Primers for the *cbr1* gene

ZL224 CAGAGTGAATTCAAGACCAAGCCTGTGCT

ZL222 AGTCAGCTCGAGTTAAAACACAAACACCTGGT

Primers for the *pga3* gene

ZL234 AGTCAGGGATCCAAAAGAAGAAGATCACTGTA

ZL235 AGTCAGCTCGAGTTAAAAGACGAAGACTTGAT

Primers for the *mcr1* gene

ZL240 CAGAGTGAATTCAACCGTAACCAACATTCC

ZL241 AGTCAGCTCGAGTTAAAATTTGAAAACTTGGT

Primers for the *ncp1* gene

ZL316 AGTCAGCCATGGGCCATCATCATCATCATCATATGTCCGATGACGGAGATAT

ZL318 AGTCAGCTCGAGTTACCAGACATCTTCTTGGTAT

Primers for the γ-toxin

ZL436 AGTCAGCCATGGGCCATCATCATCATCATCATCATGCAGCTACTACTGCGAGA

ZL437 AGTCAGCTCGAGTTATACACATTTTCCATTCTGTAG