Supporting Information

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SI Materials and Methods

Expression and Purification of Yeast eEF-2. The plasmid p423 MET25-eEF-2 (hosted by strain HL610E) was transformed into *S. cerevisiae* strains with BY4741 background (OpenBiosystems). The list of strains used in this study can be found in Table S1. The expression and purification of eEF-2 was the same as previously reported (1).

Rh-NAD Labeling of the Endogenous eEF-2. The yeast cell lysates were prepared as described previously (2). The concentrations of the lysates were determined using absorbance at 280 nm. For each labeling reaction, the lysate was diluted to 10 μL with the final A_{280} of 0.16. Then the lysate and Rh-NAD (25 μM) were incubated with DT at 30 °C in 25 mM Tris·HCl (pH 8.0), 50 mM NaCl, 30 mM DTT, and 2 mM EDTA. The DT concentration was 100 nM and the reaction time was 15 min if not specified otherwise. The reaction mixture was resolved by SDS/PAGE. Rhodamine fluorescence signal from protein gel was visualized on a Fisher Scientific UV transilluminator.

Design and Development of an in Vivo Assay to Detect Yeast Mutants That Are Defective in the Last Step of Diphthamide Biosynthesis. To develop such an assay, we need a diphtheria toxin (DT) mutant that can differentiate diphthamide from diphthine. The wild-type DT can ADP-ribosylate both diphthamide and diphthine and thus cannot be used in such an assay. In the reported structure of Exotoxin A (ETA), Asp461 forms hydrogen bonds with diphthamide amide (3, 4). This is presumably the residue discriminating diphthamide and diphthine, as the negatively charged diphthine would be repelled by the negatively charged Asp461. In DT, this residue is replaced by an asparagine residue (Asn45), which may not differentiate diphthamide from diphthine. Therefore, we thought an Asn45Asp (N45D) mutant of DT may be able to differentiate diphthamide from diphthine. The DT catalytic domain was cloned from pLMY101into p426GAL1 or p415GALS plasmid using primers XS182 (5′-agtcagGGATCCatgagcagaaaactgtttgcg-3′) and XS183 (5′-agtcagCTCGAGTTAcggagaatacgcgggacga-3′). The DT mutants were made by overlap extension PCR (5). The primers for N45D were XS180 (5′-gccaaaatctggtacacaaggaGATtatgacgatgattggaaaggg-3′) and XS181 (5′-tccttgtgtaccagattttggc-3′). The primers for E148S were XS178 (5′-ctgaggggagttctagcgttTCAtatattaataactgggaacaggc-3′) and XS179 (5′-aacgctagaactcccctcag-3′). The cells are transformed with plasmids containing DT mutant or wild type. The results showed that, when strong promoter GAL1 was used, DT N55D was still too toxic (Fig. S3). But when a weaker promoter GALS was used, *Dybr246w* strain can grow but the WT strain cannot (Fig. S3). Therefore, we have established a system using GALS-driven DT N45D mutant to discriminate diphthamide and diphthine-containing eEF-2.

Yeast Growth Assay. Cells were transformed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). Transformed yeast cells were grown on synthetic complete medium with uracil dropout. The carbon sources were used as indicated. Colony formation was recorded 3 d after plating.

Cloning, Expression, and Purification of YLR143W. Yeast YLR143W was amplified from yeast genomic DNA, which was extracted from BY4741 using a Pierce Yeast DNA Extraction Kit. The primers used were XS198 (5′-agtcagGGATCCATGAAGTTTATAGCATTAATATCAGG-3′) and XS199 (5′-agtcagGTCGACttaGGAACGAATATGCAACCCAAA-3′). The amplified gene was inserted into pET28a vector for protein production. The pET28a YLR143W plasmid was transformed into BL21 pRARE2 strain. Cells were grown in 2 L LB medium at 37 °C and 200 rpm (MaxQ 5000 Shaker, Thermo Scientific, Asheville, NC). It took 4–5 h for the OD600 to reach 0.5 after inoculation with the overnight culture. Then the culturing temperature was changed to 15 °C, and the protein expression was induced by 0.1 mM isopropyl-D-thiogalactoside. Cells were harvested after incubation at 15 °C for 20 h. The protein was purified using a HisTrap column (GE Healthcare). Protein concentrations were determined by Bradford assay.

[α-^{32}P]-ATP Autoradiography Detecting Reaction Products. To detect the product formed from ATP, the diphthine amidation reaction was performed in 10 μL solutions with 2 μCi [α-^{32}P]-ATP (PerkinElmer, 800 Ci/mmol, 2 mCi/mL), 80 mM Tris·HCl (pH 8.0), 15 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10 mM NH_4Cl. The eEF-2 concentration was 0.5 μM and the purified YLR143W concentration was 0.75 μM. The reactions were incubated at room temperature for 5 min. The ADP standard spot was generated with L-glutamine synthetase (Sigma, G3144). The reaction contained 2 μCi [α-^{32}P]-ATP (PerkinElmer, 800 Ci/mmol, 2 mCi/mL), 80 mM Tris·HCl (pH 8.0), 15 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 mM NH_4Cl, and 1 mM L-glutamate. The AMP spot was generated with acyl-CoA synthetase (Sigma, A3352). The reaction contained 2 μCi [α-^{32}P]-ATP (PerkinElmer, 800 Ci/mmol, 2 mCi/mL), 80 mM Tris·HCl (pH 8.0), 15 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM CoA, and 1 mM sodium myristate. The reactions were incubated at room temperature for 45 min. A total of 0.5 μL of each reaction were spotted onto cellulose PEI-F TLC plates (Baker-flex) and developed with 2 M lithium chloride in water. After development, the plates were air-dried and exposed to a Phosphor Imaging screen (GE Healthcare). The signal was detected using a STORM860 phosphorimager (GE Healthcare).

In-gel Trypsin Digestion of eEF-2 and Nano Liquid Chromatography/MS/MS Analysis. The in-gel digestion of eEF-2, the nano liquid chromatography/MS/MS analysis, and the associated data analysis were the same as previously used (1).

1. Su X, et al. (2012) YBR246W is required for the third step of diphthamide biosynthesis. *J Am Chem Soc* 134(2):773–776.
2. Du J, Jiang H, Lin H (2009) Investigating the ADP-ribosyltransferase activity of sirtuins with NAD analogues and 32P-NAD. *Biochemistry* 48(13):2878–2890.
3. Jørgensen R, Wang Y, Visschedyk D, Merrill AR (2008) The nature and character of the transition state for the ADP-ribosyltransferase reaction. *EMBO Rep* 9(8):802–809.
4. Carroll SF, Collier RJ (1988) Amino acid sequence homology between the enzymic domains of diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A. *Mol Microbiol* 2(2):293–296.
5. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77(1):51–59.

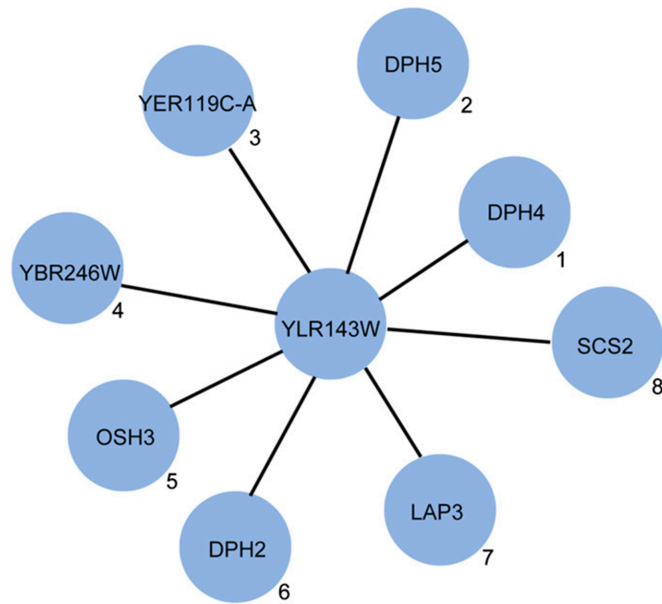

Fig. S1. The top eight cofitness correlations to YLR143W deletion strain. The rank of each correlation is labeled to the bottom right of each circle.

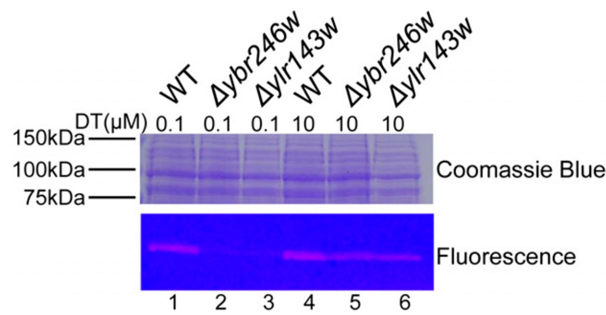

Fig. S2. Rh-NAD labeling of total lysates from various parental strains. The parental strains are specified at the top. (*Upper*) eEF2 band on Coomassie Blue–stained SDS/PAGE gel. (*Lower*) Fluorescence labeling. In lanes 1–3, low DT concentration (0.1 μM) was used. In lanes 4–6, high DT concentration (10 μM) was used.

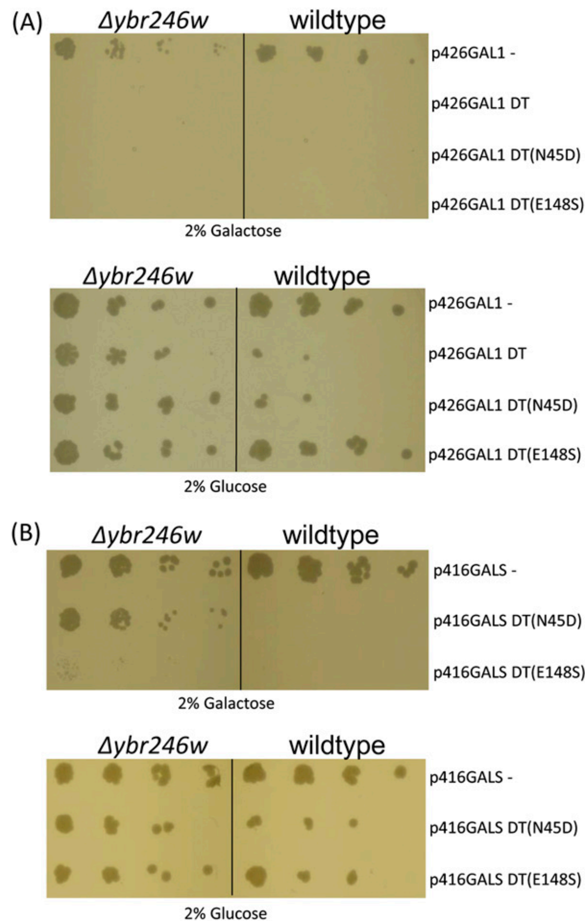

Fig. S3. Growth assay using DT mutants. (*A*) DT (N45D) in p426GAL1 gave no phenotypic difference between the wild type (WT) (BY4741) and *Δybr246w*. (*B*) DT (N45D) in p416GALS gave phenotypic difference between the WT and *Δybr246w*. The parental strains used are specified at the top. The control and toxin-encoding plasmids used are specified to the right. The cells were grown on 2% galactose (*Upper*) or 2% glucose (*Lower*).

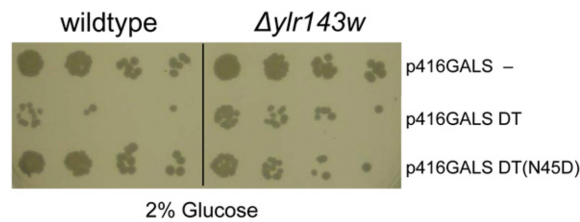

Fig. S4. Control plates of diphtheria toxin sensitivity assay. The parental strains used are specified at the top. The control and toxin-encoding plasmids used are specified to the right. The cells were grown on 2% glucose.

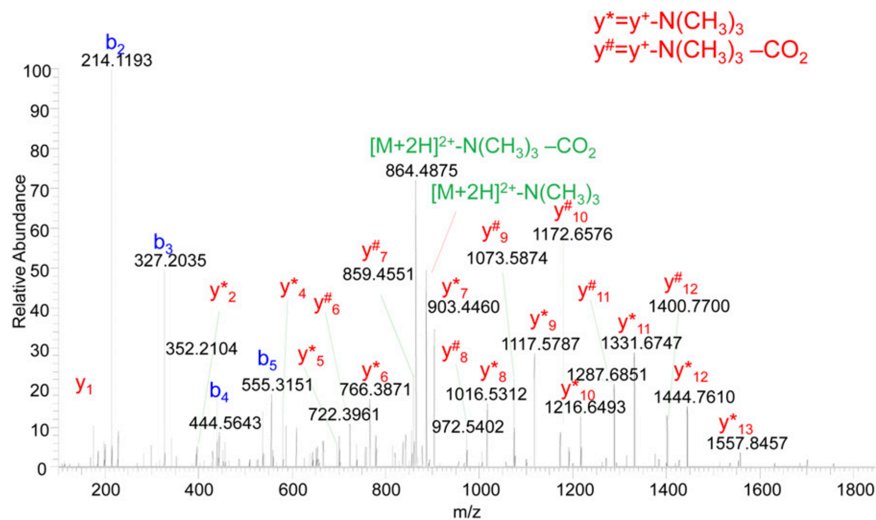

Fig. S5. MS/MS spectrum of the diphthine-containing peptide derived from the eEF2 purified from *Δylr143w*. Two neutral loss patterns were observed in the spectrum and are labeled y* and $y^{\#}$.

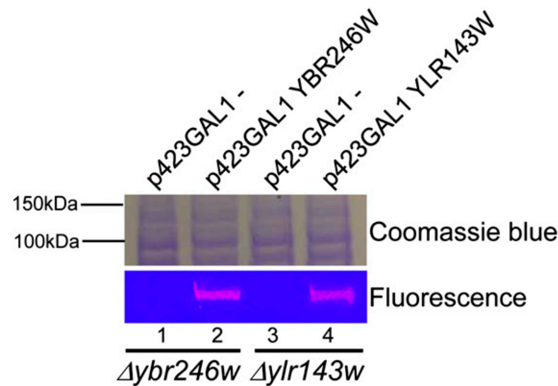

Fig. S6. YBR246W and YLR143W restore diphthamide biosynthesis in their deletion strains. The deletion strains used are specified at the bottom. The plasmids used are specified at the top. (*Upper*) Lysates on Coomassie Blue–stained SDS/PAGE gel. (*Lower*) Fluorescence labeling. In all lanes, low DT concentration (0.1 μM) was used.

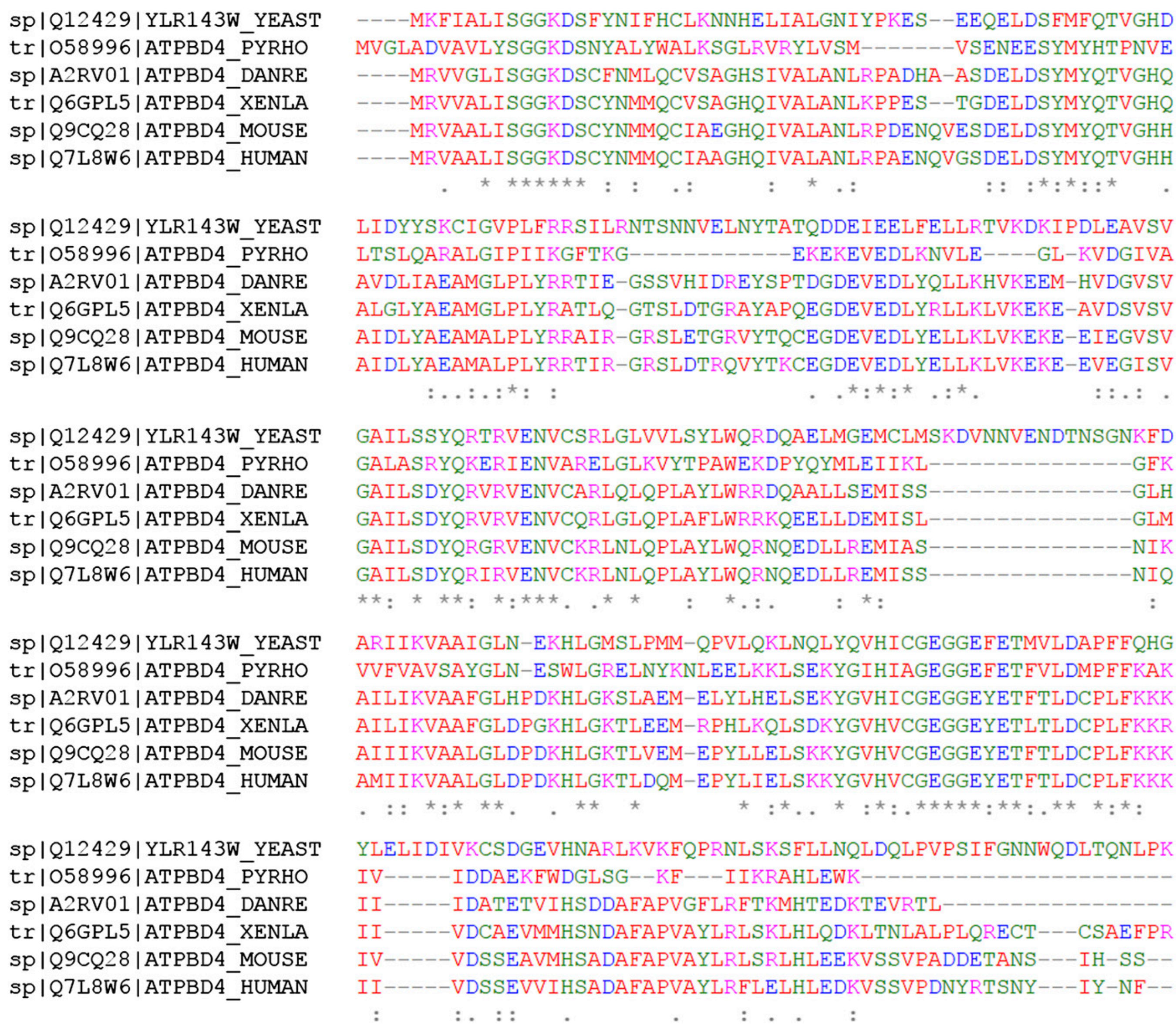

Fig. S7. Clustal Omega (http://www.clustal.org/omega) sequence alignment result. The compared sequences are from *S. cerevisiae*, *P. horikoshii*, *Danio rerio*, *Xenopus laevis*, *Mus musculus,* and *Homo sapiens*.

Table S1. Yeast strains used

Strain	Genotype	Source
HL813Y	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems (YSC1048)
HL814Y	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ybr246wΔ*	Open Biosystems (YSC1021-552106)
HL904Y	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems (YSC1049)
HL940Y	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ybr246wΔ*	Open Biosystems (YSC1021-547231)
HL1025Y	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ylr143wΔ*	Open Biosystems (YSC1021-552695)
HL823Y	HL813Y [p423MET25 EFT1-Histag]	(1)
HL824Y	HL814Y [p423MET25 EFT1-Histag]	(1)
HL825Y	HL815Y [p423MET25 EFT1-Histag]	(1)
HL968Y	HL941Y [p423MET25 EFT1-Histag]	(1)
HL1026Y	HL1025Y [p423MET25 EFT1-Histag]	This study
HL958Y	HL904Y [p426GAL1 DT-F2]	This study
HL959Y	HL904Y [p426GAL1 DT-F2 (N45D)]	This study
HL960Y	HL904Y [p426GAL1 DT-F2 (E148S)]	This study
HL962Y	HL904Y [p426GAL1 -]	This study
HL963Y	HL940Y [p426GAL1 DT-F2]	This study
HL964Y	HL940Y [p426GAL1 DT-F2 (N45D)]	This study
HL965Y	HL940Y [p426GAL1 DT-F2 (E148S)]	This study
HL967Y	HL940Y [p426GAL1 -]	This study
HL1008Y	HL813Y [p416GALS DT-F2]	This study
HL1013Y	HL814Y [p416GALS DT-F2]	This study
HL1058Y	HL813Y [p416GALS DT-F2 (N45D)]	This study
HL1059Y	HL813Y [p416GALS DT-F2 (E148S)]	This study
HL1060Y	HL813Y [p416GALS -]	This study
HL1061Y	HL814Y [p416GALS DT-F2 (N45D)]	This study
HL1062Y	HL814Y [p416GALS DT-F2 (E148S)]	This study
HL1063Y	HL814Y [p416GALS -]	This study
HL1064Y	HL1025Y [p416GALS DT-F2]	This study
HL1065Y	HL1025Y [p416GALS DT-F2 (N45D)]	This study
HL1066Y	HL1025Y [p416GALS DT-F2 (E148S)]	This study
HL1067Y	HL1025Y [p416GALS -]	This study
HL1039Y	HL814Y [p423GAL1 YBR246W]	This study
HL1040Y	HL1025Y [p423GAL1 YLR143W]	This study
HL1068Y	HL814Y [p423GAL1 -]	This study
HL1069Y	HL1025Y [p423GAL1 -]	This study
HL1054Y	HL1025Y [p423GAL1 YLR143W-Histag]	This study
HL1055Y	HL1025Y [p423GAL1 YLR143W(1-264)-Histag]	This study
HL1056Y	HL1025Y [p423GAL1 ph ATPBD4-Histag]	This study
HL1057Y	HL1025Y [p423GAL1 Human ATPBD4-Histag]	This study

1. Su X, et al. (2012) YBR246W is required for the third step of diphthamide biosynthesis. *J Am Chem Soc* 134(2):773–776.

Other Supporting Information Files

Dataset S1 (XLSX)