Supporting Information

Dph7 catalyzes a previously unknown demethylation step in diphthamide biosynthesis

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Method and materials

Expression and purification of yeast eEF-2

The eEF-2 proteins from *Δdph5, Δdph6* and *Δdph7* strains were expressed and purified as previously described[1], with a minor modification on the method for lysing cells. Cells were lysed using a beadbeater (BioSpec Products, Inc., USA) instead of the Emulsi Flex-C3 cell disruptor.

Cloning and expression of Dph7

Yeast YBR246W (Dph7) was amplified from yeast genomic DNA, which was extracted from BY4741 using Pierce Yeast DNA Extraction Kit. The primers used were ZL001 (5'- agtcagACTAGTATGcatcatcatcatcatcatGACTCTATTCAAGAATCAG ATG -3') and ZL002 (5'- agtcagGTCGACCTAAACTATCCATGTTTGCAAG -3'). The amplified gene was inserted into the p423GAL1 vector for protein production. The p423GAL1-Dph7 plasmid was transformed into BY4741 strain. Cells were grown in 2 liters of synthetic complete galactose media lacking histidine at 30°C and 200 rpm for 24 hours or till the OD600 reached 2.0. Subsequent cell harvesting and purification were the same as those for eEF-2 protein purification described above. Protein concentrations were determined by Bradford assay.

Cloning of eEF-2 with $(His)_6$ and flag tags

DNA encoding eEF-2 with C-terminal $(His)_6$ and flag tags was amplified from plasmid p423MET25-EFT1His (from strain HL610E). The primers used were ZL010 (5'-agtcagGGATCCATGGTTGCTTTCACTGTTGACCA-3') and ZL011 (5'-agtcagCTCGAGTTActtgtcatcgtcgtccttgtagtcgggatgatgatgatgatgatgCAATTTGTCG TAATATTCTTGCC -3'). The amplified fragment was inserted into the p423 MET25 vector. The plasmid p423MET25-ETF1His&Flag was transformed into *Δdph7* strain. Expression and purification of the double tagged eEF-2 were the same as the procedures described above for purification of yeast eEF-2.

In vitro diphthine amidation and ADP-ribosylation using Rh-NAD

The reconstitution of the amidation reaction on *Δdph6* eEF-2 was same as previously described.[2] Dph7 (50 nM) was used for the reconstitution of diphthamide formation on

Δdph7 eEF-2. Detection of diphthamide formation was done by ADP-ribosylation reaction using Rh-NAD labeling as described.[2] The fluorescence signals were visualized using a Fisher Scientific Ultraviolet Transilluminators (Figure 2) or a Typhoon 9400 Imager (Figure 3).

Dph7 treatment of Δdph7 eEF-2 and repurification of treated eEF-2

Flag-tagged *Δdph7* eEF2 (2 μM) was incubated with 50 nM of Dph7 at 30°C for 1 hour. The reaction mixture was then incubated with the anti-flag M2 affinity gel (SIGMA-ALDRICH, USA) for 1 hour at 4°C with gentle shaking on a platform shaker. The anti-flag resins were collected by centrifugation at 1000xg for 5 minutes and washed with TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) three times. The flag tagged *Δdph7* eEF-2 was eluted by 100 μg/mL of FLAG peptides (SIGMA-ALDRICH, USA) in TBS.

Non-enzymatic hydrolysis of methylated diphthine to diphthine

Δdph7 eEF-2 and *Δdph6* eEF-2 were buffer exchanged into a buffer containing 25 mM Tris-HCl pH 9.0 and 150 mM NaCl using Amicon Ultra Centrifugal Filters (EMD Millipore, USA). Buffer exchanged eEF-2 proteins were incubated at 30°C for various time intervals. *In vitro* diphthine amidation and detection of diphthamide formation were carried out as described above using DT and Rh-NAD.

Dph7-catalyzed hydrolysis of methylated diphthine

Δdph7 eEF2 (2 μM) was incubated with Dph7 (50 nM) in buffer containing Tris-HCl pH 8.0 and 150 mM NaCl at 30°C for 1 hour. As a control, *Δdph7* eEF2 (2 μM) in buffer containing Tris-HCl pH 8.0 and 150 mM NaCl was incubated at 30°C for 1 hour without Dph7. Then 10 μg of both Dph7-treated and untreated *Δdph7* eEF2 samples were used for subsequent in-solution trypsin digestion and MS analysis.

Cloning and purification of Dph5

Yeast YLR172C (Dph5) was amplified from yeast genomic DNA, which was extracted from BY4741 using Pierce Yeast DNA Extraction Kit. The primers used were XS220 (5'-agtcagGTCGACTTACTCGTCGCTGTCGTCTTCT-3') and XS221 (5'-agtcagG GATCCATGCTTTATTTGATCGGACTTG-3'). The amplified gene was inserted into pET28a vector for protein production. The pET28a YLR172C plasmid was transformed into BL21 pRARE2 strain. Cells were grown in 2 liters of LB medium at 37°C and 200 rpm. It took 4-5 hours for the OD600 to reach 0.8 after inoculation with the overnight culture. Then the culturing temperature was changed to 16°C, and the protein expression was induced by 0.4 mM isopropyl-D-thiogalactoside (IPTG). Cells were harvested after incubation at 16°C for 18 hours. The purification using HisTrap column (GE Healthcare) was the same as eEF-2 protein described above. Protein concentrations were determined by Bradford assay.

In vitro reconstitution of Dph5 activity

Δdph5 eEF2 (2μM) was incubated with Dph5 (50 nM) and SAM (100 μM) in buffer containing Tris-HCl pH 8.0 and 150 mM NaCl at 30°C for 1 hour. As a control, *Δdph5* eEF2 (2μM) was incubated with SAM (100μM) in the same buffer at 30°C for 1 hour without Dph5. Then 10 μg of both Dph5-treated or untreated *Δdph5* eEF2 samples were used for subsequent in-solution trypsin digestion and MS analysis.

In-solution trypsin digestion of eEF-2

The eEF2 samples (10 μg each) were denatured and reduced using 6 M urea and 10 mM dithiothreitol (DTT) in 100 μL of Tris-HCl pH 8.0 buffer at room temperature (RT) for 1 hour. Iodoacetamide (IDA, final concentration 40 mM) was added to the mixture and left at RT for 1 hour. Extra IDA was quenched by addition of 4 μL of 1 M DTT and left at RT for 1 hour. The mixture was then diluted six times by adding a solution containing 50 mM Tris-HCl pH 8.0 and 1 mM $CaCl_2$. Then 0.5 μg of trypsin (Promega, reconstituted at 100 μg/mL) was added and the digestion was allowed to proceed at 37°C for 18 hours. The resulting solution was acidified with 10% trifluoroacetic acid to pH 2-3 and then desalted using a Sep-Pak C18 1 cc Vac Cartridge (Waters, USA). The eluted solution was lyophilized.

Protein Identification with nano LC/MS/MS Analysis

The nano LC/MS/MS analysis was the same as previously described.[1] Acquired MS and MS/MS raw spectra were processed using Mascot 2.3 against Swissprot database with a taxonomy filter of Saccharomyces Cerevisiae and one missed cleavage site by trypsin was allowed. Mass tolerances for precursor ions were set at 10 ppm and for MS/MS were set at 100 mmu. A fixed carbamidomethyl modification on cysteine, variable modifications on methionine oxidation, deamindation of asparagine and glutamine, and variable substitutions on histidine including unmodified, ACP-modified, methylated diphthine, diphthine and diphthamide as well as the possible elimination products were specified. All MS/MS spectra of identified peptides were manually inspected and verified using Xcalibur 2.2 software.

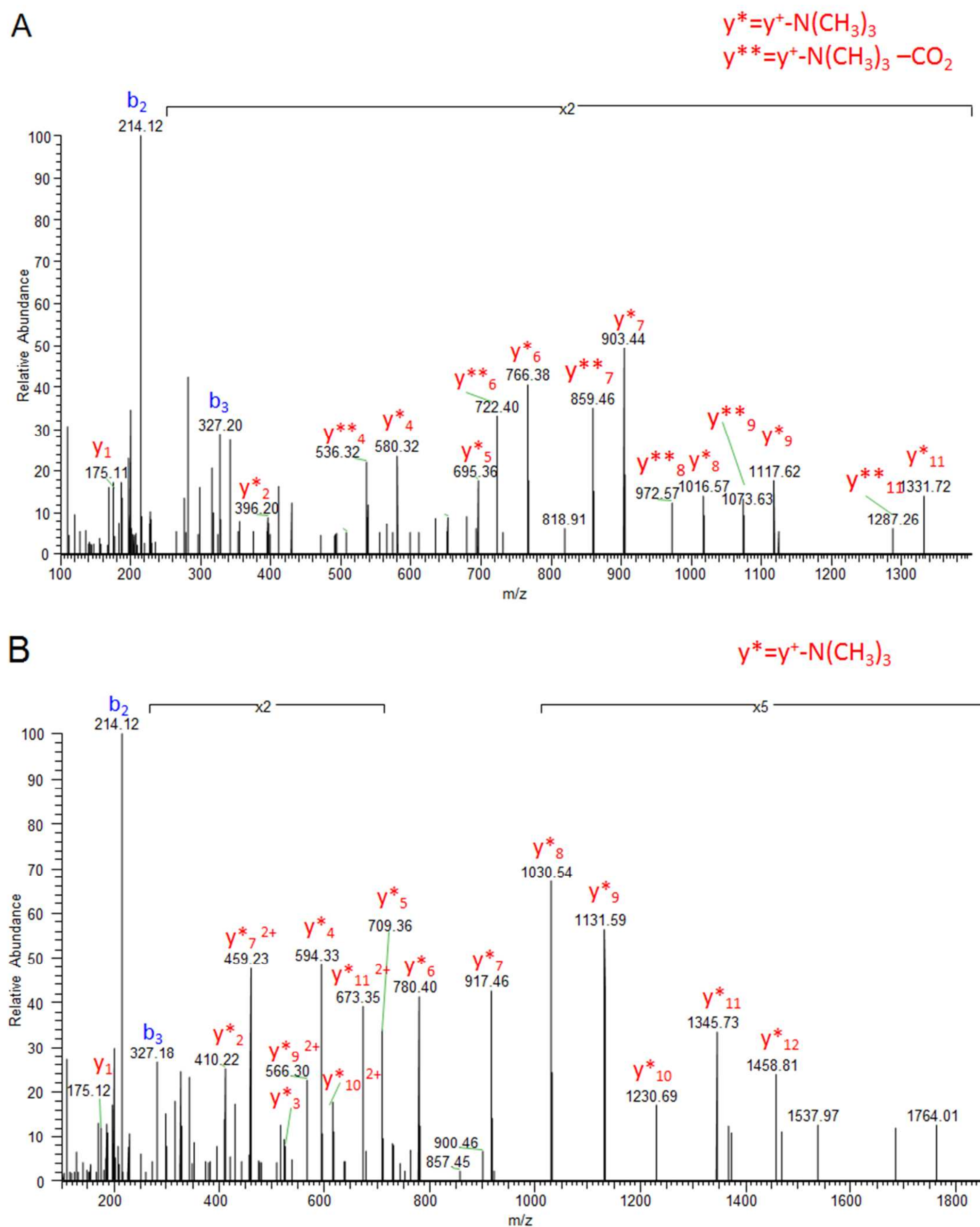

Figure S1. MS/MS spectra of diphthine (A, observed parental ion *m/z* 610.6793, Calculated *m/z* 610.6792) and methylated diphthine (B, observed parental ion *m/z* 615.3514, Calculated *m/z* 615.3518) containing peptides from *Δdph7* eEF-2. A neutral loss of the trimethyl amino group on each of y ion series was observed and labeled as y*. Neutral loss of both the trimethyl amino group and carbon dioxide was observed and labeled as y**.

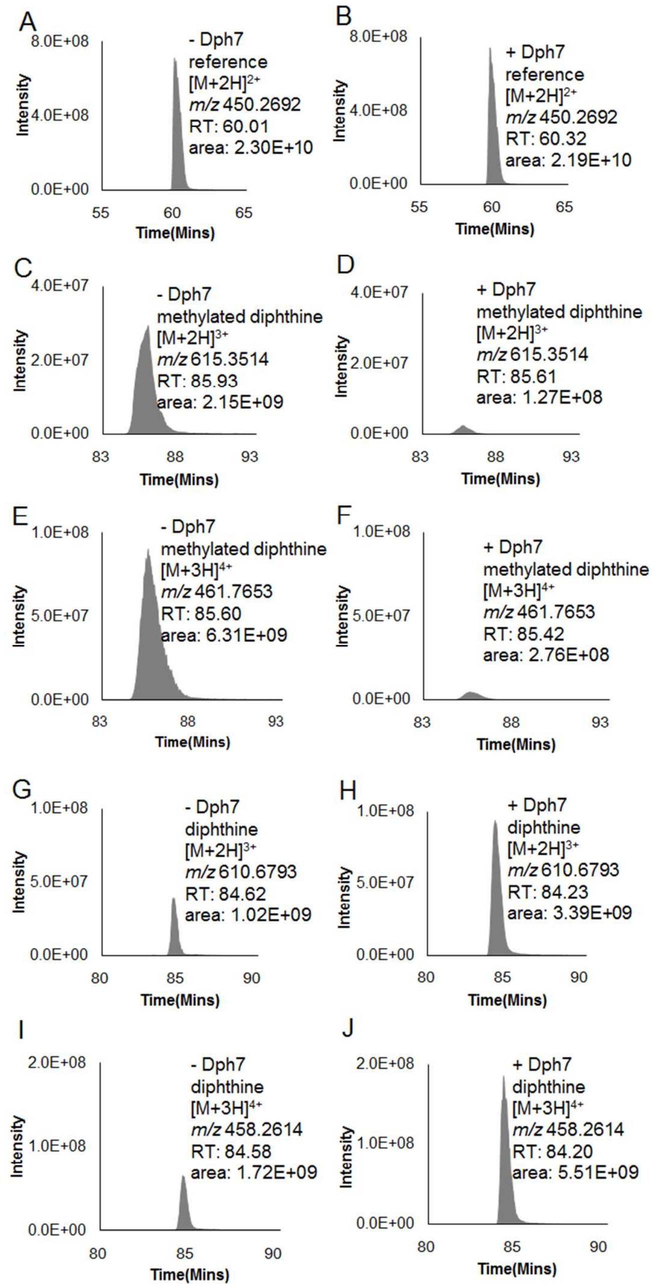

Figure S2. Dph7 hydrolyzes methylated diphthine to form diphthine. Relative quantification of tryptic peptides from Δdph7 eEF-2 with or without Dph7 treatment is shown by extracted ion chromatograms of the target ions. Peaks correspond to reference peptide^{2+} (A and B, observed *m/z* 450.2692, calculated *m/z* 450.2691); methylated diphthine^{3+} (C and D, observed *m/z* 615.3514, calculated *m/z* 615.3518); methylated diphthine^{4+} (E and F, observed *m/z* 461.7653, calculated *m/z* 461.7658); diphthine^{3+} (G and H, observed *m/z* 610.6793, calculated *m/z* 610.6792); diphthine^{4+} (I and J, observed *m/z* 458.2614, calculated *m/z* 458.2619). "-Dph7" indicates peptides from Δdph7 eEF-2 without Dph7 treatment. "+Dph7" indicates peptides from Δdph7 eEF-2 with Dph7 treatment.

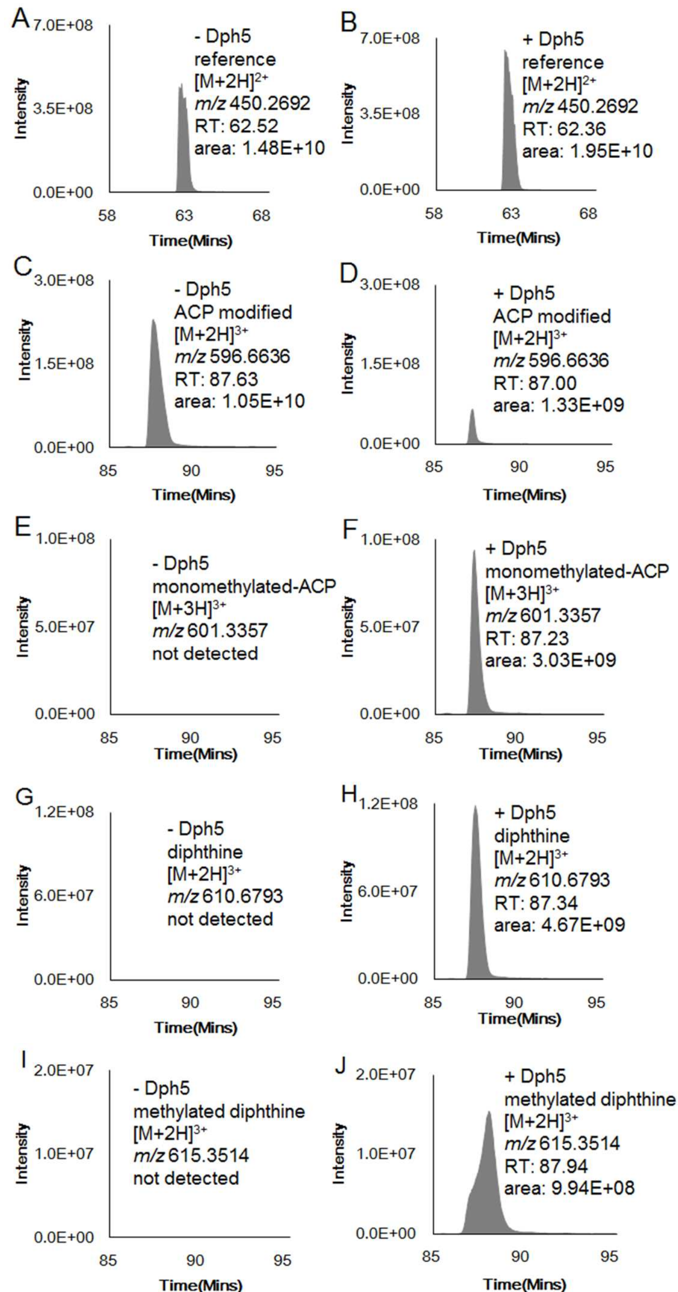

Figure S3. Dph5 generates methylated diphthine. Relative quantification of tryptic peptides from Δdph5 eEF-2 with or without Dph5 treatment is shown by extracted ion chromatograms of the target ions. Peaks correspond to reference peptide^{2+} (A and B, observed *m/z* 450.2692, calculated *m/z* 450.2691); ACP-modified^{3+} (C and D, observed *m/z* 596.6636, calculated *m/z* 596.6643); monomethylated-ACP^{3+} (E and F, observed *m/z* 601.3357, calculated *m/z* 601.3361); diphthine^{3+} (G and H, observed *m/z* 610.6793, calculated *m/z* 610.6792); methylated diphthine^{3+} (I and J, observed *m/z* 615.3514, calculated *m/z* 615.3518). "–Dph5" indicates peptides from Δdph5 eEF-2 without Dph5 treat-ment. "+Dph5" indicates peptides from Δdph5 eEF-2 with Dph5 treatment.

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Dph7_HoSa       LHLLMVNETRPRLQKVASWQAHQFEAWIAAFNYWH--PEIVYSGGDDGLLRGWDTRVPG- 229
Dph7_MuMu       LHLLMVNEGTAELQLVASWPAHHFEAWIAAFNYWQ--TELVYSGGDDCLLRGWDTRMLG- 228
Dph7_DaNo       LHLLAVSETGPRLQAVATWPAHRFEAWIAAFNYWQ--TEIVYSGGDDGLLKGWDTRMAPD 144
Dph7_AnPl       LNLFSIDESAPSVHVLNQWKAHKFEAWIAAFNYWN--TDVVYSGGDDNLLKGWDTRCSPE 173
Dph7_GaGa       LNLFSIDESAPSVHVLNQWEAHKFEAWIAAFNYWN--IDIVYSGGDDSLLKGWDTRCNPE 100
Dph7_MaZe       ISVLSLAEG--ALTTLSQWKAHDFEAWISAFSYWD--TQLVYSGGDDCKLKGWDLRIGPS 219
Dph7_BoMo       VTILTVNGN--GIEKRSSWRAHGFEAWIGAFNYWN--TNLLYSGGDDCLFKCFDVRIQD- 212
Dph7_ArTh       ASVVSFTDS--NLETVQEWKGHDFELWTASFDLNN--PNLVYTGSDDCKFSCWDIRDSPA 198
Dph7_SaCe       YEVQGATEKVIHVESGQFLKPHELECWTAEFGSLQPFQDVVFTGGDDSRIMAHDLRSKEF 234
                                     *  * *   *            * **      * *

Dph7_HoSa       KFLFTS--KRHTMGVCSIQSSPH-----REHILATGSYDEHILLWDTRNMKQ-------- 274
Dph7_MuMu       TPVFTS--KRHCMGVCSIQSSPH-----QEHILATGSYDEHVLLWDTRNIRQ-------- 273
Dph7_DaNo       MPLFTS--ERHTMGVCSIHSSPH-----QEHVLATGSYDEHVLLWDTRHMQQ-------- 189
Dph7_AnPl       TPVFTS--KRHSMGVCSIQCSPH-----RENLLATGSYDEHVLLWDTRNMKQ-------- 218
Dph7_GaGa       TPVFTS--RRHSMGVCSIQCSPH-----RENLLATGSYDEHVLLWDTRNMKQ-------- 145
Dph7_MaZe       SPTFIS--KRHSMGVCSIHSNPH-----REHILATGSYDEQVLLWDGRNMRQ-------- 264
Dph7_BoMo       GPVAVN--KSHEAGVTSIRSHVD-----VEHQLLTGSYDEKVRLWDARKMKS-------- 257
Dph7_ArTh       DNRVFQNSKVHTMGVCCISSNPS-----DPYSIFTGSYDETLRVWDTRSVSR-------- 245
Dph7_SaCe       IWSNNR---IHDAGVVSIKCSQPNFRNNKPTSIITGSYDDNIRSLDLRMMGESIFPGANV 291
                          *  **  *                *****      * *

Dph7_HoSa       -PLADTPVQGG--VWRIKWHPFHH--------HLLLAACMHSGFKILNCQKAMEE-RQEA 322
Dph7_MuMu       -PLADVPVQGG--VWRLKWHPVHH--------HLLLAACMHNGFKILNCQKAIEE-KQDI 321
Dph7_DaNo       -PFADAHVQGG--VWRLKWHPFHR--------HLLLAACMHNGFKILSCHKSE---KQEV 235
Dph7_AnPl       -PLADTHVEGG--VWRLKWHPTCD--------FVLLAACMQSGFKILDCRGSMAENMEEC 267
Dph7_GaGa       -PLADTHVEGG--VWRLKWHPTCD--------FVLLAACMQSGFKILDCRGSMAENTEEC 194
Dph7_MaZe       -PLSETPLGGG--VWRLKWHPSHQ--------HLLLAACMHNDFHILHCQQALEGSAGAC 313
Dph7_BoMo       -CITETCVNGG--VWRLKWHPITP--------NVVLAACMYGGFRILHIDDGVN------ 300
Dph7_ArTh       -PLNETSVSLGGGVWRIKHHPSLS--------GVVLAACMHNGFALAKVSDGKGE----- 291
Dph7_SaCe       PTVNKLACDLGGGVWRFVESPIDQEQSHHNGSDRLLVCCMYNGAKVVTMNDNSDE--YFQ 349
                          *  ***    *              *  **

Dph7_HoSa       TVLTSHTLPDSLVYGADWSWLLFR--------------SLQRAPSWSFPSNLG--TKTAD 366
Dph7_MuMu       TVLTSHEMPNSLVYGADWSWLFHS--------------MKPTPTWFFDQNDMG--VKAAD 365
Dph7_DaNo       NIVSSFMWHNSLAYGADWSWFSLR--------------PLQAQQPASFTSSLHSDTGVSN 281
Dph7_AnPl       IILSSYVLHNSLAYGADWSRLCPRDSLSAAQDSAATYQSLGELVARPEEGDERLNLQVRN 327
Dph7_GaGa       VILSSYVLHNSLAYGADWSRLCPRDSLAALQDSAAVCQPLEQPVARSEEGDERLNLQVRN 254
Dph7_MaZe       PIVTSYILHSSLAYGADWSRLSLED--------------HTACSPPATEPKESPAENRGH 359
Dph7_BoMo       -VVSEYLEHCSIAYGADWC----------------------------------------- 318
Dph7_ArTh       -VLESYNKHHSLAYGADWYRGKDQ------------------------------------ 314
Dph7_SaCe       IQHYLKKGHDSMCYGGDWS----------------------------------------- 368
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Dph7_HoSa       ----------------QATAATTRDCGVNPEEADSAFSLLATCSFYDHALHLWEWEGN-- 452
Dph7_MuMu       ----------------SSSSVKTRDLSHCSGGQSFDNSLLATCSFYDHVLHLWKWETNQA 461
Dph7_DaNo       ----------------LAPGSKIFDHDLHVDGANFENCVLATCSFYDHVLHLWKWEMS-- 376
Dph7_AnPl       --PESVGSSDPGVKRPNRMSLDRSDD----SASPKEMSIVATCSFYDNILHVWKWEMSLA 439
Dph7_GaGa       DSSKSASSCDLGVKRSNGIGQDGSGDSVRSSDSPKATSIVATCSFYDNILHIWKWEMNLA 374
Dph7_MaZe       -----------------------------DEDAPSLSCLLASCSFYDHMLHVWRWDWMPD 433
Dph7_BoMo       ----------------------------------HGRDLVATCSFYDRSLHLSEIQLNLD 344
Dph7_ArTh       -----------------------------------KQSVVATCSFYDRLLRVWMPITDFS 339
Dph7_SaCe       ------------------------------------NSLIATCSFYDNSLQTWIV----- 387
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Figure S4. Representative sequences from multiple alignments of Dph7 orthologs using CLUSTAL W[3] showing conserved residues. The protein sequences were obtained from the NCBI protein database: Dph7_HoSa, Homo sapiens Dph7 (GI:24308452); Dph7_MuMu, Mus musculus Dph7 (GI:21313066); Dph7_DaNo, Dasypus novemcinctus Dph7 (GI:488589513); Dph7_AnPl, Anas platyrhynchos Dph7 (GI:514780369); Dph7_GaGa, Gallus gallus Dph7 (GI:513212019); Dph7_MaZe, Maylandia zebra Dph7 (GI:499047390); Dph7_BoMo, Bombyx mori Dph7 (GI:512887870); Dph7_ArTh, Arabidopsis thaliana Dph7 (GI:15242588); Dph7_SaCe, Saccharomyces cerevisiae Dph7 (GI:6319723).

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)
HL824Y	HL814Y [p423MET25-EFT1-Histag]	[1]
HL968Y	HL941Y [p423MET25-EFT1-Histag]	[1]
HL1026Y	HL1025Y [p423MET25-EFT1-Histag]	[2]
HL1105Y	HL813Y [p423GAL1-Dph7-Histag]	This study
HL1215Y	HL814Y [p423MET25-EFT1-His&Flagtag]	This study

References

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