

Supplementary Materials

Communication

***DPH1* gene mutations identify a candidate SAM pocket in radical enzyme Dph1•Dph2 for diphthamide synthesis on EF2**

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1. Supplementary Tables

Table S1. Yeast strains used and generated in this study.

Strain	Genotype	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf *
Y02262	BY4741 but <i>dph1Δ::kanMX4</i>	Euroscarf
KU18	BY4741 but <i>DPH1-(HA)₆::HIS3MX6</i>	This study
KU129	BY4741 but <i>dph1G238A; KILEU2</i>	This study
KU328	BY4741 but <i>dph1G238A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU285	BY4741 but <i>dph1H261A; KILEU2</i>	[1]
KU329	BY4741 but <i>dph1H261A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU287	BY4741 but <i>dph1Q321A; KILEU2</i>	This study
KU330	BY4741 but <i>dph1Q321A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU289	BY4741 but <i>dph1V349A; KILEU2</i>	This study
KU331	BY4741 but <i>dph1V349A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU291	BY4741 but <i>dph1R370A; KILEU2</i>	This study
KU332	BY4741 but <i>dph1R370A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU293	BY4741 but <i>dph1D374A; KILEU2</i>	This study
KU333	BY4741 but <i>dph1D374A-(HA)₆::HIS3MX6; KILEU2</i>	This study
KU25	BY4741 but <i>dph1C368S-(HA)₆::HIS3MX6</i>	This study
KU123	BY4741 but <i>dph1Q321A/V349A; ::loxP::</i>	This study

* <http://www.euroscarf.de/index.php?name=News>

Table S2. Primers used for PCR-based gene engineering and genomic verification.

Name	Sequence (5' → 3')	Usage *
DPH1KOURAF	CTCATGAACTATCTGCTGCGAATTTTAAGGATAATCGGATA GCCAGCTGAAGCTTCGTACGC	ko
DPH13'UTRLEUF	CGTTTTTGACGGCTTGCAGGCGAACTAAATTGTCTAAAAT TCAAAACCAGCTGAAGCTTCGTACG	smi
DPH13'UTRLEUR	GAATAAAAATAGGCTTGACCAGCAGTGATATCAAGTTAGA AGGCATTGCATAGGCCACTAGTGGATCTG	ko/smi
DPH15'UTRF	GTGATGGTAGATTATAGCAAG	ko-ver
DPH13'UTRR	GGAAATATGCTTGGCAAATC	ko-ver
DPH1G238AFW	GGGGTGAAGTATTGGCGTGTACTTCTG	sdm
DPH1G238ARV	CTTTCAGAAGTACACGCCAATACTTCAC	sdm
DPH1H261AFW	CGGTGATGGTAGATTGCTTTGGAGTC	sdm
DPH1H261ARV	TATCATTCGAGACTCCAAAGCAAATCTACC	sdm
DPH1Q321AFW	GGTGCATTAGGTAGAGCAGGTAATTTAAAC	sdm
DPH1Q321ARV	CAGTGTTTAAATTACCTGCTCTACCTAATG	sdm
DPH1V349AFW	GAAAATTATTCTAAGTGAAGCTTTTCCCC	sdm
DPH1V349ARV	GCTTTTGGGGAAAAGCTTCACTTAG	sdm
DPH1R370AFW	CAGGTCGCATGTCCTGCACTGTCCATC	sdm
DPH1R370ARV	CCCAATCGATGGACAGCGTAGGACATGC	sdm
DPH1D374AFW	CCTAGACTGTCCATCGCTTGGGGTTATG	sdm
DPH1D374ARV	GAAGGCATAACCCCAAGCGATGGACAG	sdm
DPH1C368SFW	CAAATTGATGTTTTTGTTCAGGTCGCATCTCCTAGACTGTCC	sdm
DPH1C368SRV	GAAGGCATAACCCCAATCGATGGACAGTCTAGGAGATGCG ACCTG	sdm
DPH1S2	CATATGTAACAGGAAGACAAGTGACAACAAAACTATTTA AACTAATCGATGAATTCGAGCTCG	tag
DPH1S3	CGAAGCTAAAGGATACGGGCGTGGGGAACTCCGAAACA TGCGATTGAACGTACGCTGCAGGTCGAC	tag

* Abbreviations used: (i) ko – gene knock-out

(ii) smi – selection marker insertion

(iii) ko-ver – verification of gene knockout

(iv) sdm – site-directed mutagenesis

(v) tag – epitope tagging



2. Supplementary Figures

Figure S1 (for legend, see next page)

CmaDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
ScDph1	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
AtDph1	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
DmdDph1	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
NmdDph1	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
HaDph1	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
ScDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
AtDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
DmdDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
NmdDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
HaDph2	1	1	MSGESTESKKQRRRTGRKSGSHSHDKITTVAEENHEIIHQKRSRATGRSVHNVVEDILMDKELMEAKILLESNHEIKTMIRK--HAKRIALQPEGLIISLIISDILE--QFCGVE--VLVNGDVSTGACCIIDFTARALDCDFVHYAHSLV 157
CmaDph2	84	EYEN	-----VVFIEARSNIDIIIPAVKTA--LMLKA--NRIGLITTVQVHVKLE-----EACKVIKYGKE--CVTIGKGDPRATYEQVIG--NFTAAV--DCEEFYIYG--SGIHPGLVAIA--TKKRVI 195
ScDph1	158	PIDVT	-----KIKVAVFVTINIQEDHIIKTLQ--KHFPGK--SRITAGTIOFNPVH--SVRKLIDHEHMLXIITPQI--KPLSRGEVIG--TSERLDK--EYDAMVFIQ--DGRHLESAMH--NPEIPAFKY 277
AtDph1	130	PIDST	-----KIPCLVFEVQIDVCKLNTIM--LHLSADVKHIIAGTIQTSAIR--AVRPELEKQGN--VLIPQS--KPLSRGEVIG--TSERLDK--EYDAMVFIQ--DGRHLESAMH--NPEIPAFKY 277
DmdDph1	142	PVDQC	-----GIVKLVFVQIDVCKLNTIM--LHLSADVKHIIAGTIQTSAIR--AVRPELEKQGN--VLIPQS--KPLSRGEVIG--TSERLDK--EYDAMVFIQ--DGRHLESAMH--NPEIPAFKY 277
NmdDph1	135	PMDTS	-----VQVQVRLVXFVQIDVCKLNTIM--LHLSADVKHIIAGTIQTSAIR--AVRPELEKQGN--VLIPQS--KPLSRGEVIG--TSERLDK--EYDAMVFIQ--DGRHLESAMH--NPEIPAFKY 277
HaDph1	140	PMDTS	-----VQVQVRLVXFVQIDVCKLNTIM--LHLSADVKHIIAGTIQTSAIR--AVRPELEKQGN--VLIPQS--KPLSRGEVIG--TSERLDK--EYDAMVFIQ--DGRHLESAMH--NPEIPAFKY 277
ScDph2	131	AIQH	-----LPVVSFGTFPFDLALVVEHQ--RAFPDLSKICIMANAFSKHLSQLYHLKGLDHYTHIIYSQVHTSAVEKEFT--LIDTFHVEDVDQVGT--KHSVIFGQ--HDKADNISPEDIYHLFIITPQ--DPRL--LYLSTVF--QSVHIF 274
AtDph2	95	PTSV	-----IPAFVFGKASINVSQVKNLI--DYAKSDKPIMILIGLEYAVHVPQIREELGELKSDQLSVANVLCFSRSPKIDREHSHPRYESSDSDLSRSRISGLGLWDLPGESKIDYILFWIGSD--SSAN--NVLVITF--N--GCDIVRY 244
DmdDph2	121	RAAS	-----LPVLYLPELPDVLHVLVSKLQGLRAESIDRQVCVYIDIGYQH--PYGEKIKQISE--LIEPKELIEFPFIEFESTTRET--NLERICIFIGAD--HQRT--NHLISLTA--PAVQVMIY 237
NmdDph2	113	PPAS	-----QLPITFVLGQRPVLELCAKAFE--AQHPDPTAPVLLSEPAACAHLE--PLANLILPKYQDL--LISRPALPLVGSFSSQFESLERFGR--CFPLMPGRLEEGYAFYVGSQASDSDSDPDLISRLILGWTPGRPFSC 250
HaDph2	113	PPAR	-----PLPVAEVLQRQSVALELCWKAFF--AQHPDPAKPVLLSEPAACAHLE--ALATILRPYIDL--LVSSPAFPQVGSLSPEF--NLERFGR--REPLMPGRLEEGYAFYVGSQASDSDSDPDLISRLILGWTPGRPFSC 250
CmaDph2	196	DPTFHQ	AVESPE--RFLRKGGYIAKATGAKIFGIIVSTKSG--RMKLAQKLEIADKHGKIGYIIHDIH--VTEQQLAFAK--ADAVVNTACHETIT--AERFNAPVLTPQEFELVIGE-----310
ScDph1	278	DYNRKFT	REGYDQQLVEVRAEAIEVARQKQVGLILGALGQ--SHLVTKNLEKHLIANGKTVKVIKILSH--VFPQKLNHFQ--IDVFVQVACHETIT--GYAFHFKPLITPYEASVLLKQVDFSEKY-----YPMHY-----408
AtDph1	251	DYVGLK	LEEXDHKGHRETRAIARAREAKTWGIVLGLTGQ--HPKILERLEKMEKIGDSTVLLSH--LSPTRVAFEDSVDAMVQIACHETIT--GEAFKPLITTFEAEIATGLD--WEHDS--380
DmdDph1	260	DYKFKFT	QYDHSNQHLRLDAVQAKARRIGIILGLTGQ--SVRHFLEKHLIANGKIGETITLISH--PQKALFAD--IDAFVQIACHETIT--GSASFQPLIHPYELSVLGD--VEMTPHSSPRUNAYPMDF-----396
NmdDph1	252	DYVGLK	SREYDQHQATQEAATAARSASWGLIIGTGQ--SPKILEHLESQLRHGILPFRVLLSH--PQKALFAD--IDAFVQIACHETIT--GSASFQPLIHPYELSVLGD--VEMTPHSSPRUNAYPMDF-----396
HaDph1	252	DYVGLK	SREYDQHQATQEAATAARSASWGLIIGTGQ--SPKILEHLESQLRHGILPFRVLLSH--PQKALFAD--IDAFVQIACHETIT--GSASFQPLIHPYELSVLGD--VEMTPHSSPRUNAYPMDF-----396
ScDph2	275	DALP	GHVTPFPF--LHRYKTHVARTAGCIGILVNTLSR--TRETINEHLEKTRKSKHYLVVGH--VAKLANHFE--IDWCLIGCSQGIIVDQVNEFYK--IITPYEALHLSREVTWIG--WVDFRDAIDEIFQNLGGQDTSAST--425
AtDph2	245	DAEDS	LVTFEYQORILKRYLYLVEKADANIIGILVGLVANGYLMHMHQALISANGKSYILMNGHP--VAKLANHFE--CDVYIYISCAQTALID--SKEFSPVITPTEFANLNRSGSEWIGA--YLAHFQDVHNSKSESEANIG-----390
DmdDph2	238	D6SDS	LSKHPITAGFTRRYFHEKCKDAQTLGLIVATLS--GLDVTVRLQTHAKRGKIKQLISVGH--IPAKLANHFE--IDCFVLIGCF--NMH--SKEYKPVSVFTEAHALNP--NMHR-----YPAVYDFKQLPEGRSFLFDAD--386
NmdDph2	251	CPDTG	QDQAGKAGIRARLXILIERADARVVGLLAGTIGV--AQRHLEALHRLKITEANGKRSYVLAHVP--PAKLANHFE--MDVFLILLACE--LALAP--SGGFFRPVITPCELEAACHP--AMPPP-----GLAPHILHYAELLPGSPFNVPFPPPESE 403
HaDph2	251	CPDTG	QDQAGKAGIRARLXILIERADARVVGLLAGTIGV--AQRHLEALHRLKITEANGKRSYVLAHVP--PAKLANHFE--MDVFLILLACE--LALAP--SGGFFRPVITPCELEAACHP--AMPPP-----GLAPHILHYAELLPGSPFNVPFPPPESE 403
CmaDph2	311		-----RWEHRE--HDEHIQ-----323
ScDph1	409		-----YKANG--RETPKHAE-----425
AtDph1	381		-----RVHSSGGCKEDKETSACKNEHVDDK--KMDGALDGDYPMHYAQEG--EMWSYIKKSSRP--TRRPLFSV--453
DmdDph1	397		-----YASGSLGPTWPF--KPAIGECENKPSADCC--GRCTRAELEKDDSGKAAALDILL--FKKG-----454
NmdDph1	381		-----YSGSLGPTWPF--KPAIGECENKPSADCC--GRCTRAELEKDDSGKAAALDILL--FKKG-----454
HaDph1	386		-----YSGSLGPTWPF--KPAIGECENKPSADCC--GRCTRAELEKDDSGKAAALDILL--FKKG-----454
ScDph2	426	SDEFF	VDVGRYTSRPTALHILEAAD--DDDSKQTRTASGAVGTSTASALQHRSMKGLSDVSTSDVNTGADIEGISCVARGYSGDFREDANKHEK-----EPIYVGRSGKASGYKHE-----491
AtDph2	381	SEEP	SFTQGGYVEDKINDQKNGEEDTGTMTLVQAAEKALQLRGNDHSLTKQTAA--KSGPEFLNRYVGRGLEINSENTLP--LSFEDRTWQGLINSENTLP--AKLQOGLSGIP--INYSHQ-----469
DmdDph2	387	IPED	SVLSGR--LRGAVHDSLETGG--DPASALATQAKALMTDTG--LSFEDRTWQGLINSENTLP--AKLQOGLSGIP--INYSHQ-----469
NmdDph2	404	IMDTD	SVLSGR--LRGAVHDSLETGG--DPASALATQAKALMTDTG--LSFEDRTWQGLINSENTLP--AKLQOGLSGIP--INYSHQ-----469
HaDph2	404	IMDTD	SVLSGR--LRGAVHDSLETGG--DPASALATQAKALMTDTG--LSFEDRTWQGLINSENTLP--AKLQOGLSGIP--INYSHQ-----469

Figure S1. Alignment between archaeal *CmnDph2* and eukaryal Dph1 and Dph2 sequences. Amino acid sequences of *CmnDph2* were aligned to sequences of Dph1 and Dph2 from *S. cerevisiae*, *A. thaliana*, *D. melanogaster*, *M. musculus* and *H. sapiens*. Eukaryal Dph1 residues conserved (dark green) to the SAM pocket residues (G158, H180, Q237, V265, R285 and D289) of *CmnDph2* (red) are shown boxed. The SAM pocket residue in position 290 from *CmnDph2* (D290) is less conserved and occupied in any of the eukaryal Dph1 query sequences with a conserved tryptophan residue. Note that eukaryal Dph2 sequences (light green and boxed) are not that conserved at the sites invariant between Dph1 or *CmnDph2* - except for counterpart analogues to *CmnV265* and *CmnD290* in Dph2 from *A. thaliana* (D332) and *D. melanogaster* (I309, D335) (see Fig. S2 in bold light green).

Figure S2

<i>CmnDph2</i>	G158	H180	Q237	V265	R285	D289	D290
<i>ScDph1</i>	G238	H261	Q321	V349	R370	D374	W375
<i>AtDph1</i>	G208	H234	Q294	L322	R344	D348	W349
<i>DmDph1</i>	G221	H243	Q303	I331	R352	D356	W357
<i>MmDph1</i>	G213	H235	Q295	I323	R344	D348	W359
<i>HsDph1</i>	G218	H240	Q300	I328	R349	D353	W354
<i>ScDph2</i>	E228	-	R315	P343	Q364	I368	V369
<i>AtDph2</i>	S195	-	A288	P316	Q337	L348	D342
<i>DmDph2</i>	S197	-	E281	I309	F330	Y334	D335
<i>MmDph2</i>	P193	D231	A294	P322	L343	A347	P348
<i>HsDph2</i>	P193	D231	A294	P322	L343	A347	P348

Figure S2. The SAM pocket from archaeal *CmnDph2* is conserved in eukaryal Dph1, not Dph2. Excerpt of the amino acid sequence alignment (see Fig. S1 above for full details) between eukaryal Dph1 and Dph2 from *S. cerevisiae*, *A. thaliana*, *D. melanogaster*, *M. musculus* and *H. sapiens* and *CmnDph2* (see Fig. S1 for full alignment). Dph1 residues conserved to *CmnDph2* (dark green) and a few scattered analogous Dph2 residues (light green) are highlighted in bold.

Figure S3

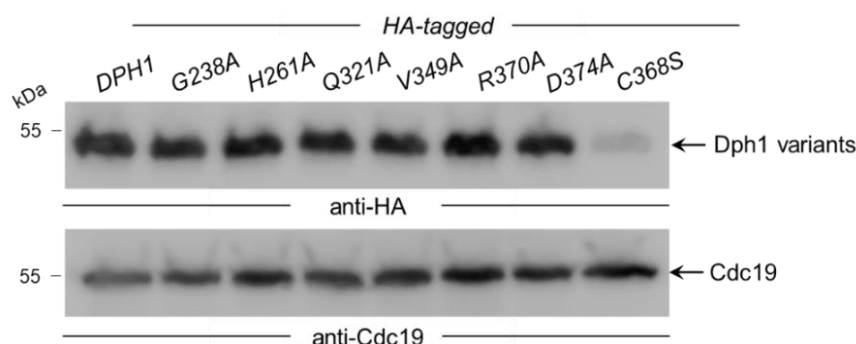


Figure S3. Dph1 protein expression analysis from wild-type and mutant strain backgrounds. Total extracts of yeast strains expressing HA-tagged Dph1 variants as indicated were subjected to Western blot analyses using *anti-HA* and *anti-Cdc19* antibodies to investigate cellular levels of Dph1, and pyruvate kinase Cdc19 as control. As a negative control, HA-tagged C388S mutant was included, which produces a highly labile catalytic Dph1 mutant protein.

Figure S4

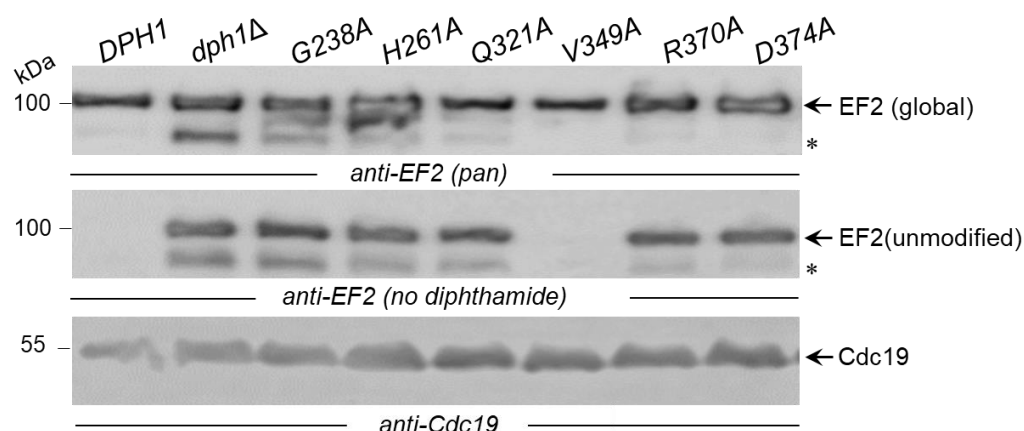


Figure S4. Detection of unmodified EF2 pools using Western blot analysis. Protein extracts of indicated yeast backgrounds were subjected to *anti-EF2(no diphthamide)* Western blots to recognize pools of EF2 not modified with diphthamide [2]. Detection of global EF2 amounts with *anti-EF2(pan)* or Cdc19 with *anti-Cdc19* antibodies is shown in parallel Western blots. EF2 degradation products are marked with an asterisk.

3. Supplementary References

1. Ütkür, K.; Mayer, K.; Khan, M.; Manivannan, T.; Schaffrath, R.; Brinkmann, U. DPH1 and DPH2 variants that confer susceptibility to diphthamide deficiency syndrome in human cells and yeast models. *Dis. Model. Mech.* **2023**, *16*, dmm050207, doi: 10.1242/dmm.050207.
2. Stahl, S.; da Silva Mateus Seidl, A.R.; Ducret, A.; Kux van Geijtenbeek, S.; Michel, S.; Racek, T.; Birzele, F.; Haas, A.K.; Rueger, R.; Gerg, M.; Niederfellner, G.; Pastan, I.; Brinkmann, U. Loss of diphthamide pre-activates NF-kappaB and death receptor pathways and renders MCF7 cells hypersensitive to tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 10732–10737. doi: 10.1073/pnas.1512863112.