microbial cell

Research Report

www.microbialcell.com

Yeast gene *KTI13* (alias *DPH8*) operates in the initiation step of diphthamide synthesis on elongation factor 2

Meike Arend[1], Koray Ütkür[1], Harmen Hawer[1], Klaus Mayer[2], Namit Ranjan[3], Lorenz Adrian[4], Ulrich Brinkmann[2] and Raffael Schaffrath[1,*]

[1] Institute of Biology, Division of Microbiology, University of Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany.
[2] Roche Pharma Research & Early Development, Large Molecule Research, Roche Innovation Center München, Nonnenwald 2, 82377 Penzberg, Germany.
[3] Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany.
[4] Environmental Biotechnology, Helmholtz Centre for Environmental Research - UFZ, 04318 Leipzig, Germany.
* Corresponding Author:
Raffael Schaffrath, Institute of Biology, Division of Microbiology, University of Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany; Phone: +49-561-804-4175; Fax: +49-561-804-4337; E-mail: schaffrath@uni-kassel.de

ABSTRACT **In yeast, Elongator-dependent tRNA modifications are regulated by the Kti11•Kti13 dimer and hijacked for cell killing by zymocin, a tRNase ribotoxin. Kti11 (alias Dph3) also controls modification of elongation factor 2 (EF2) with diphthamide, the target for lethal ADP-ribosylation by diphtheria toxin (DT). Diphthamide formation on EF2 involves four biosynthetic steps encoded by the *DPH1-DPH7* network and an ill-defined *KTI13* function. On further examining the latter gene in yeast, we found that *kti13*Δ null-mutants maintain unmodified EF2 able to escape ADP-ribosylation by DT and to survive EF2 inhibition by sordarin, a diphthamide-dependent antifungal. Consistently, mass spectrometry shows *kti13*Δ cells are blocked in proper formation of amino-carboxyl-propyl-EF2, the first diphthamide pathway intermediate. Thus, apart from their common function in tRNA modification, both Kti11/Dph3 and Kti13 share roles in the initiation step of EF2 modification. We suggest an alias *KTI13/DPH8* nomenclature indicating dual-functionality analogous to *KTI11/DPH3*.**

doi: xxx
Received *originally: 16.05.2023;*
In revised form: 31.07.2023,
Accepted *07.08.2023,*
Published *08.08.2023*

Keywords*: budding yeast; EF2 diphthamide modification; diphtheria toxin; tRNA modification; elongator; tRNase zymocin.*

Abbreviations:
KTI *– Kluyveromyes lactis toxin insensitive,* ***FeS*** *– iron-sulfur,* ***mcm^{5}s^{2}U34*** *– methoxy-carbonyl-methyl-thio-uridine,* ***EF2*** *– elongation factor 2,* ***DT*** *– diphtheria toxin,* ***nLC-MS/MS*** *– nano-liquid chromatography tandem mass spectrometry,* ***YPD*** *– yeast peptone dextrose.*

INTRODUCTION

Zymocin is a trimeric (αβγ) chitinase and tRNase toxin complex from *Kluyveromyes lactis* that kills *Saccharomyces cerevisiae* cells [1,2]. Expression in *S. cerevisiae* of its tRNase subunit γ alone (aka γ-toxin) is lethal [3] suggesting subunits α and β mediate zymocin contact with sensitive cells for γ-toxin uptake [2,3]. Accordingly, screens for zymocin survivors identified mutations in non-target (class I) and toxin-target (class II) genes termed *KTI* (*K. lactis* toxin insensitive) [4]. While class I loci encode cell wall and membrane components (chitin, sphingolipids, H^+ pump Pma1) for zymocin docking [5-7], class II genes identified the γ-toxin effector role of the tRNA modifier complex Elongator (Elp1-Elp6) [2,8,9]. Its tRNA acetylase subunit (Elp3) uses iron-sulfur (FeS) and radical SAM (RS) cofactors to modify wobble uridines (U34) in tRNA anticodons [10-12]. This includes methoxy-carbonyl-methyl-thio-uridine (mcm^{5}s^{2}U34) groups, which are hijacked for anticodon cleavage by γ-toxin. Hence, Elongator mutants lacking the mcm^{5}s^{2}U34 groups resist the tRNase attack [13,14].

Among *KTI* loci not coding for Elongator subunits are regulatory genes: *KTI11* (aka *DPH3*), *KTI12*, *KTI13* and *KTI14* (aka *HRR25*) [2,4,15]. Kti12 binds tRNA and supports Elongator phosphorylation by kinase Kti14 [16,17]. Together with Sit4, a phosphatase antagonistic to Kti14, the tRNA modification activity of Elongator likely is phosphoregulated [9,15,18]. Kti11 is a rubredoxin-like electron carrier and dimerizes with Kti13 to effect Elongator-dependent tRNA modifications [18,19]. *KTI11* is also allelic with *DPH3* [20] and acts in diphthamide decoration of translation elongation factor 2 (EF2), a protein essential for life [21-23]. Diphthamide synthesis involves four steps encoded by a network (*DPH1-DPH7*) that is conserved in eukaryotes [24,25]. The EF2 décor is important for reading frame maintenance during mRNA translation and accurate protein biosynthesis [26,27]. Imbalanced proteostasis as a result of diphthamide deficiency has been attributed to neuropathies and various types of cancer in humans [28]. Of note, diphthamide underlies the human diphtheria disease since it is targeted by corynebacterial diphtheria toxin (DT) for ADP-ribosylation and EF2 inactivation [29] (**Fig. 1A**).

In line with dual roles for tRNA and EF2 modification, Kti11/Dph3 co-purifies with Elongator, EF2 and Dph1•Dph2 [30]. The latter enzyme uses (similar to Elp3) FeS and SAM

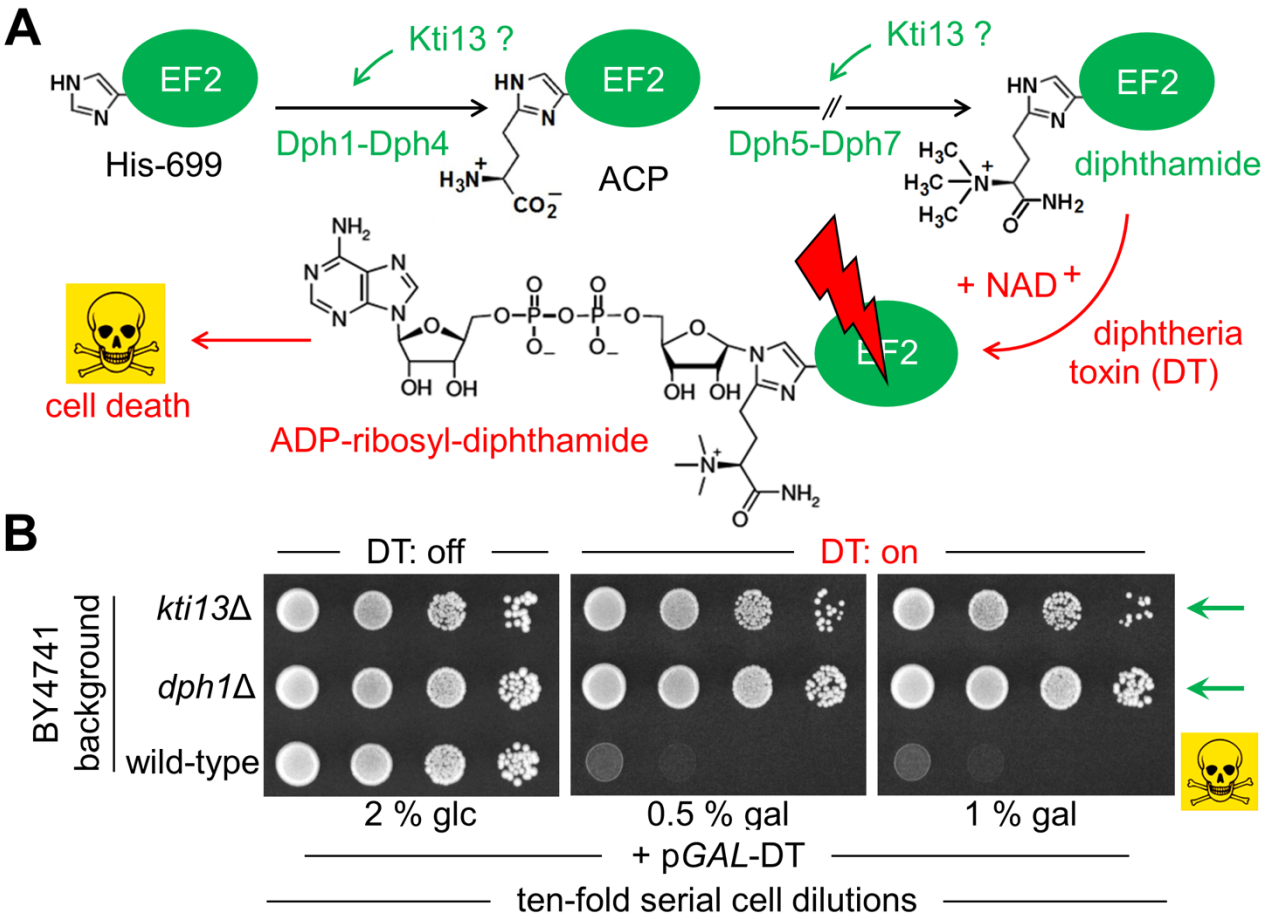

FIGURE 1: Potential role of yeast *KTI13* in diphthamide modification. (A) Simplified pathway overview [24,25]. Diphthamide synthesis initiates with modifcation of EF2 at His-699 by ACP involving proteins Dph1-Dph4. Subsequential reactions to convert ACP into end product diphthamide entail Dph5-Dph7. Potential Kti13 involvement in the synthesis steps is indicated ('?'). Diphthamide can be hijacked by diphtheria toxin (DT) for ADP-ribosylation in an NAD^+ fashion and induces cell death by EF2 inactivation (skull-crossbones). **(B)** *KTI13* and *DPH1* gene deletion strains resist against DT cytotoxicity. Yeast strains carrying p*GAL*-DT [39], a plasmid for galactose-inducible expression of the lethal ADP-ribosylase domain from DT (see A) were spotted onto medium containing 0.5-1% (w/v) galactose (gal) or 2% (w/v) glucose (glc). Following DT induction, growth inhibition of diphthamide-proficient wild-type is distinguishable from DT resistance of diphthamide-deficient *dph1*Δ and *kti13*Δ mutants (green arrows).

cofactors for RS chemistry and formation of 3-amino-3-carboxyl-propyl-EF2) (ACP: **Fig. 1A**), the first diphthamide pathway intermediate [31,32]. Dph3/Kti11 in a dimer with Kti13, donates electrons to the FeS clusters in Dph1•Dph2 and possibly, Elp3 [33-36], which likely enables proper FeS redox states for RS-based modification chemistry. Whether the dimer feeds into both RS enzymes [18] or limits electron flow to Elongator as suggested [19] is moot. That *KTI11/DPH3* and *KTI13* gene functions may be related to each modification pathway, is supported by reports showing that both loci genetically interact with the Elongator and EF2 networks [18,22,37,38]. In relation to Kti11/Dph3, however, the precise role of Kti13 and its position within the diphthamide pathway have been less clear. On further studying *KTI13* gene function, we found that *kti13*Δ mutants survive EF2 inhibition by sordarin, a diphthamide-dependent antifungal, and evade ADP-ribosylation of EF2 by DT. Consistently, *kti13*Δ cells are drastically reduced in ACP formation indicating that proper initiation of the EF2 décor depends on Kti13. This is similar to Kti11/Dph3, which is why we suggest an alias nomenclature: *KTI13/DPH8*.

RESULTS AND DISCUSSION

*kti13*Δ phenotypes diagnostic for a *bona fide* diphthamide defect

To study Kti13 in more detail (**Fig. 1A**) we subjected a *kti13*Δ null-mutant raised in strain BY4741 to DT expression under *GAL*-promoter control [22,39]. In presence of galactose, DT expression was lethal to wild-type, while *kti13*Δ cells survived the toxin attack on EF2 (**Fig. 1B**). The resistance phenotype is robust and similar to the *dph1*Δ mutant (**Fig. 1B**), which is blocked in the first step of the diphthamide pathway [21]. Similar to other *kti* strains or mutants (*kti11/dph3*Δ, *kti12*Δ, *kti14/hrr25*, *sit4*Δ) lacking Elongator regulators crucial for tRNA modification [2,15,9,40], *kti13*Δ cells also copied zymocin resistance (**Fig. 2A**). When we compared growth of this mutant set in the presence of

sordarin, a diphthamide-dependent EF2 inhibitor other than DT [22,41], solely *kti11/dph3*Δ and *kti13*Δ cells would protect against the antifungal (**Fig. 2A**). As shown previously, sordarin resistance is a trait diagnostic for failure to initiate or complete EF2 modification with diphthamide [39,41]. Thus, two out of five Elongator regulators tested, apparently share dual-functional roles in tRNA and EF2 modification pathways: Kti11/Dph3 and Kti13.

An EF2 pool not modified with diphthamide accumulates in *kti13*Δ cells

Next, we analyzed protein extracts from the above set of mutants (*kti11/dph3*Δ, *kti12*Δ, *kti13*Δ, *kti14/hrr25*, *sit4*Δ) by Western blots (**Fig. 2B**). We used anti-EF2(pan), an antibody against EF2 regardless of modification, and anti-EF2(no diphthamide) shown to be specific for unmodified EF2 [29,42,43] (Fig. S1). *kti13*Δ cell extracts produced strong anti-EF2(no diphthamide) Western signals indicative for EF2 species not modified with diphthamide in absence of Kti13 (**Fig. 2B**). This is a read-out very similar to unmodified EF2 pools from *kti11/dph3*Δ cells (**Fig. 2B**), which like other step one mutants (*dph1*Δ, *dph2*Δ, *dph4*Δ) fail to initiate diphthamide synthesis (**Fig. 1A**) [20,21]. Previously, step one mutants were shown to raise EF2 protein levels, possibly to compensate for diminished EF2 function in absence of diphthamide [25,26]. Here, anti-EF2(pan) Western blots on *kti13*Δ and *kti11/dph3*Δ extracts also revealed upregulated EF2 levels (**Fig. 2B**). Thus, Kti13 and Kti11/Dph3 are diphthamide-related but differ from Kti12, Kti14 and Sit4, which are dispensable for making diphthamide based on anti-EF2(no diphthamide) blots (**Fig. 2B**). Nonetheless, we observed similar anti-EF2(pan) signals between *kti12*Δ and *kti13*Δ cells (**Fig. 2B**), suggesting an unheard EF2 upregulation under conditions that disturb

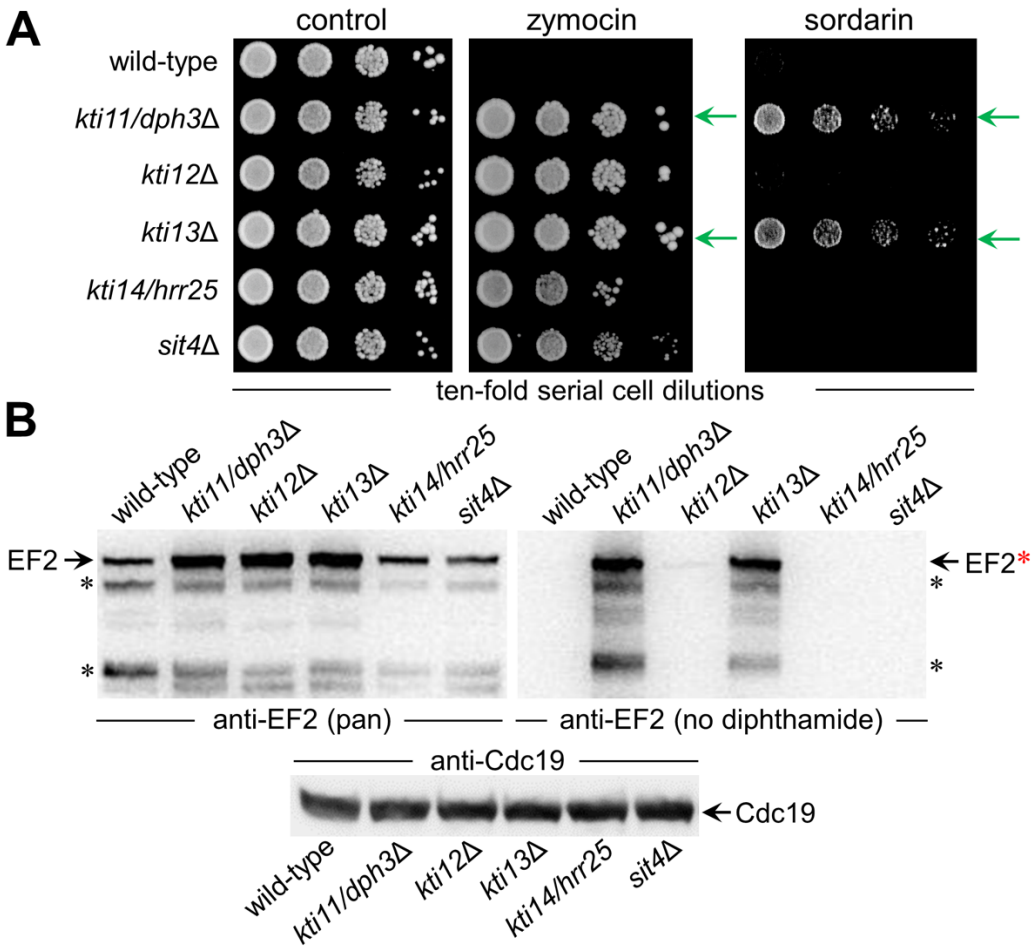

FIGURE 2: Among genes involved in Elongator regulation and tRNA modification, *KTI11* and *KTI13* also function in EF2 modification. (A) Growth assays in response to zymocin (0.02% [v/v]) or sordarin (9 µg/mL) and diagnostic for tRNA or diphthamide modfciation defects, respectively. Dilutions of cells with indicated genotypes were incubated at 30°C for 3 days. Note, that while all *kti*Δ and *sit4*Δ mutants resist growth inhibition by Elongator-dependent tRNase zymocin, only *kti11/dph3*Δ and *kti13*Δ cells are protected (green arrows) against diphthamide-dependent EF inhibitor sordarin. **(B)** Western blot analysis of total cell extracts from strains with genotypes as in A in order to profile their amounts of total EF2 and unmodified EF2 using anti-EF2(pan) (left panel) and anti-EF2(no diphthamide) antibodies (right panel), respectively. Black asterisks (left & right panels) denote EF2 degradation products, the red asterisk indicates full-length unmodified EF2 (right panel). The anti-Cdc19 antibody (bottom panel) was used as loading control. Note the anti-EF2(no diphthamide) Western blot (right panel) detects unmodified EF2 pools for *kti11/dph3*Δ and *kti13*Δ cells indicative for diphthamide defects.

tRNA (*kti12*Δ) but not diphthamide modification. In sum, among five known Elongator and tRNA modification regulators, two also contribute to diphthamide modification: Kti11/Dph3 and Kti13. Hence, to go with *KTI11/DPH3*, we suggest to SGD an alias nomenclature indicating bifunctional nature: *KTI13/DPH8*.

*kti13*Δ cells block proper initiation of EF2 modification with diphtamide

To further examine the position of Kti13 in the diphthamide pathway, we purified His-tagged EF2 from strain TKY675 [44]. Other than the full EF2 gene (*EFT1 EFT2*) complement of BY4741, TKY675 harbors a double knockout (*eft1*Δ *eft2*Δ) with a single-copy plasmid carrying *EFT2-[His]$_6$* [44]. To diagnose diphthamide status in TKY675 prior to EF2 purification, we used DT assays (as above for BY4741). *dph1*Δ and *kti13/dph8*Δ mutants survived DT, yet their phenotype was weaker compared to BY4741 counterparts (**Fig. 1B**) and diminished by increasing DT loads (**Fig. 3A**). This suggests strain-specific variation due to EF2 copy number effects, a notion supported by Western blots showing significantly reduced EF2 pools and lower (than BY4741) levels of unmodified EF2 in *dph1*Δ from TKY675 (Fig. S1).

Next, we purified His-tagged EF2 from TKY675 in a two-step process coupling immobilized (IMAC: Fig. S2) with size exclusion chromatography (SEC: Fig. S3) for profiling diphthamide modification by nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS) [43]. Previously, yeast, plant and human EF2 were found predominantly diphthamide modified [26,42,43]. In line with this scenario, we hardly detected any unmodified EF2 from total cell extracts of yeast strains BY4741 and TKY675 in anti-EF2(no diphthamide) Western blots (**Fig. 2B**, Fig. S1). However, upon purification of His-tagged EF2, nLC-MS/MS detected similar amounts of modified and unmodified peptides from TKY675 (wild-type: **Fig. 3B**). So, in contrast to normal *EFT1 EFT2* gene dosage and EF2 levels in BY4741, EF2 purified from TKY675 with single-copy *EFT2-[His]$_6$* apparently is not fully modified (Fig. S4). Whether this suggests the affinity-tag on EF2 or gene copy number reduction in TKY675 compromise the modification efficiency of the pathway is unclear. The observed imbalance, however, seems not to be accounted to the His-tag alone based on similar EF2 protein patterns in anti-His versus anti-EF2(pan) Western blots (Fig. S5).

Nonetheless, nLC-MS/MS on EF2 purified from the *dph1*Δ mutant reliably identified an unmodified tryptic

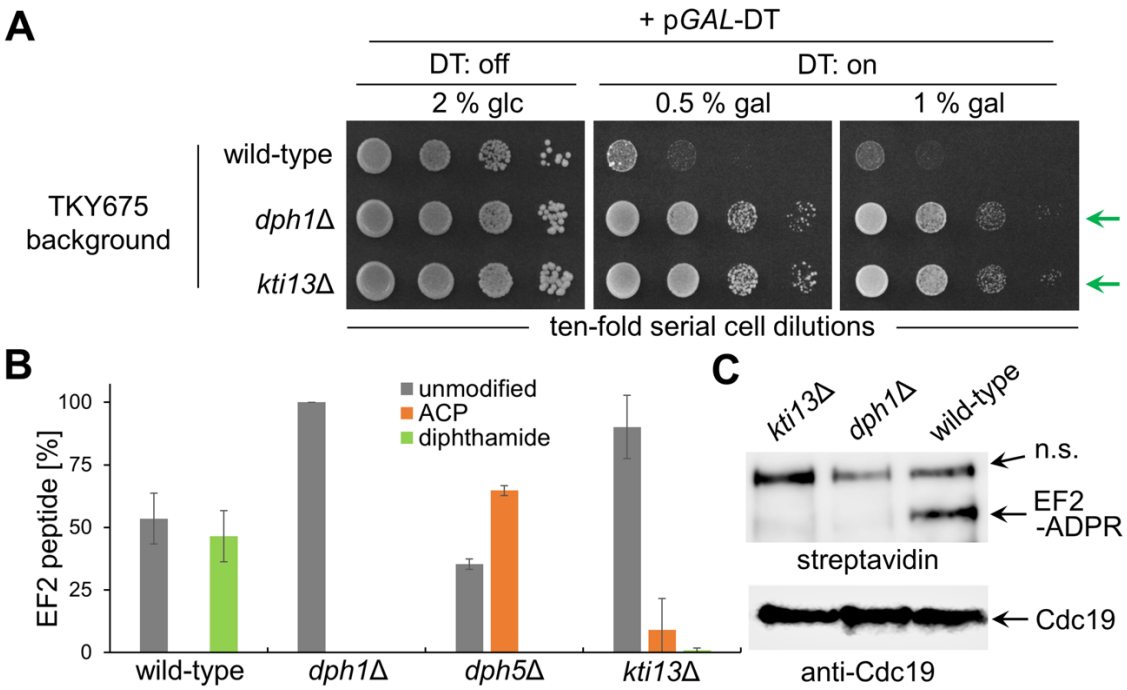

FIGURE 3: *KTI13* is required for proper initiation of diphthamide synthesis on EF2. (A) *kti13*Δ and *dph1*Δ mutants in strain TKY675 resist against DT cytotoxicity. The assay was essentially performed as for BY4741 (Fig. 1B). Following galactose-inducible DT expression, wild-type growth inhibition is distinct from DT resistance (green arrows) of diphthamide-deficient mutants (*kti13*Δ, *dph1*Δ). **(B)** Profiling diphthamide modification states on EF2 purified from wild-type, *dph1*Δ, *dph5*Δ and *kti13*Δ cells via nLC-MS/MS. Amounts of modification states were normalized to amounts of unmodified EF2 in *dph1*Δ (EF2 peptide [%]). *kti13*Δ contains pools of unmodified EF2 comparable to *dph1*Δ and drastically reduced ACP levels (~9%) in relation to *dph5*Δ (~65%). **(C)** ADP-ribosylation (ADPR) assay. Cell extracts from indicated genotypes were incubated with 200 ng DT and biotin-NAD [5 µM] at 25 °C for 1 h. The transfer to EF2 of biotin-ADP-ribose (EF2-ADPR) was detected by Western blot (top panel) using an HRP-streptavidin conjugate recognizing the biotin moiety of the reaction product [26,42]. An anti-Cdc19 Western blot (bottom panel) served as control for sample loading. Note that solely diphthamide-modified EF2 from wild-type cells undergoes detectable ADPR. As has been previously detected in similar assays [29,39], there is an unspecific (n.s.) reaction product of high molecular weight.

peptide with no intermediate or modified variant detectable in TKY675 (**Fig. 3B**). This is consistent with earlier studies that exclusively identified unmodified EF2 in plant and human *dph1*Δ cell lines [42,43] and a yeast *dph2*Δ mutant lacking the Dph1 partner to initiate diphthamide synthesis on EF2 [39,45]. In control purifications from a *dph5*Δ mutant, which fails to use ACP (**Fig. 1A**) for formation of methyl-diphthine [39,46], we detected unmodified EF2 (~35%) and ACP (~65%) supporting previous data that ACP accumulates when step two of diphthamide pathway is blocked in the absence of Dph5 (**Fig. 1A**) [46,47]. Importantly, His-tagged EF2 purified from *kti13/dph8*Δ cells mostly appeared unmodified with minor ACP (~9%) and low diphthamide (~1%) amounts (**Fig. 3B**). Thus, nLC-MS/MS reveals similar profiles among *dph1*Δ and *kti13/dph8*Δ mutants strongly suggesting the latter has a step one defect and fails in proper formation of ACP-modified EF2, the first pathway intermediate (**Fig. 1A**).

Unmodified EF2 from step one *kti13*Δ mutant escapes ADP-ribosylation by DT

In further support that *KTI13/DPH8* deletion copies diphthamide step one mutants are assays using biotinylated NAD^+ as ADP-ribosyl donor [26,39] for ADP-ribosylation (ADPR) of EF2 by DT *in vitro*. Using an HRP-streptavidin conjugate to detect biotin in the ADPR reaction product [42], wild-type EF2 was found to yield robust bio-ADPR-EF2 signals (**Fig. 3C**). EF2 from *dph1*Δ or *kti13/dph8*Δ cells, however, lacked diphthamide-dependent ADPR acceptor activity indicating loss of diphthamide on EF2 evades ADPR by DT (**Fig. 3C**). These data fully agree with our anti-EF2(no diphthamide) blots showing unmodified EF2 from *kti13/dph8*Δ and *dph1*Δ (**Fig. 2B**; Fig. S1) cells and their DT resistance *in vivo* (**Fig. 1B**; **Fig. 3A**). These are features similar to *kti11/dph3*Δ cells lacking the electron donor that Kti13/Dph8 dimerizes with to drive Elongator-dependent tRNA modification [18,37]. Whether in analogy, the diphthamide function of Kti11/Dph3 also requires Kti13/Dph8 in the dimer for electron transfer and ACP synthesis by RS enzyme Dph1•Dph2 is plausible given drastically reduced ACP formation in *kti13*Δ cells (**Fig. 3B**) and *in vivo* traits typical of tRNA and EF2 modification loss caused by dimer interface mutations [18,22,37]. However, while electron transfer is essential for Dph1•Dph2 to form ACP *in vitro*, Kti13/Dph8 is dispensable in theses reconstitution assays [34,37].

CONCLUSION

We show here that apart from its effector role for Elongator-dependent tRNA modification in yeast, Kti13 alias Dph8 also operates in step one of the diphthamide modification pathway (**Fig. 4**). Although Kti13/Dph8 is important *in vivo* for EF2 modification by diphthamide, low ACP levels (~9%) detectable in *kti13/dph8*Δ cells by MS suggest its presence for the diphthamide pathway to operate is not as catalytically critical as its partner protein Kti11/Dph3 [21,39]. In line with this, previous surveys on the tRNA modification pathway revealed low levels of Elongator activity (~15%) in *kti13*Δ but none at all in *kti11*Δ mutants [9,40]. So residual EF2 and tRNA modification activity in the absence of *KTI13/DPH8* suggests an accessory role for gene product Kti13/Dph8. Perhaps it mediates proper electron flow from Kti11/Dph3 to either RS client (**Fig. 4**) for physiological modification reactions by Dph1•Dph2 and Elongator and thus, helps avoid inappropriate, harmful ones. Alternatively, in the dimer, Kti13/Dph8 may protect its RS clients

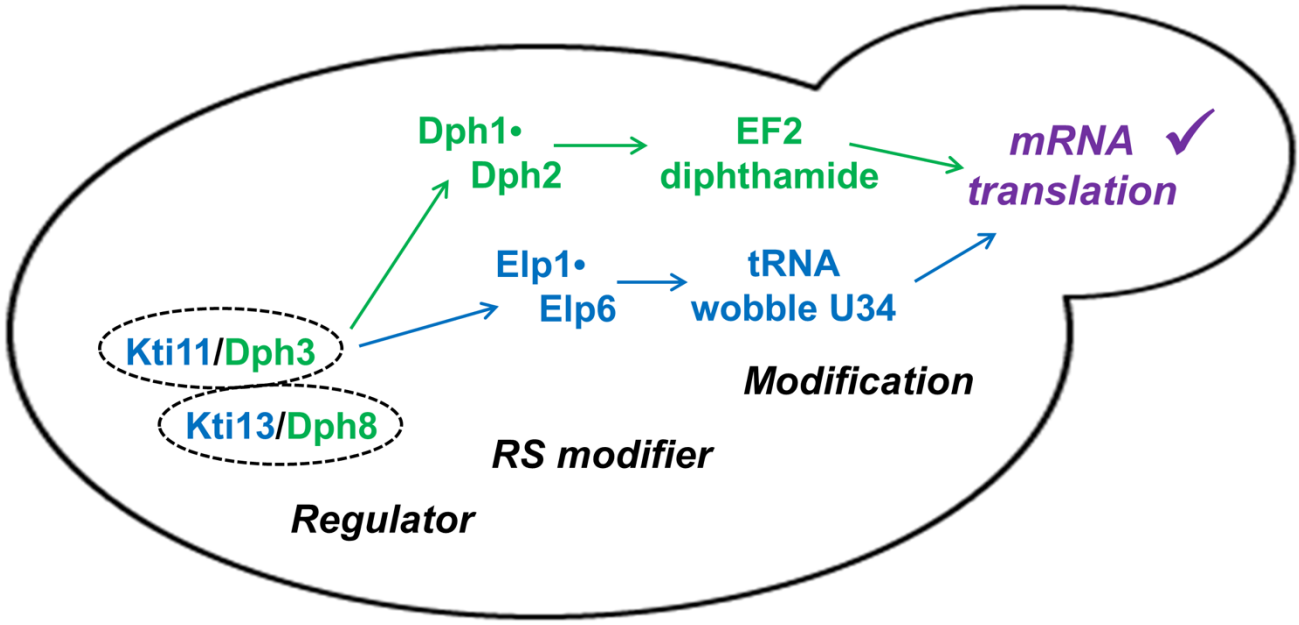

FIGURE 4: Kti11•Kti13 dimer (alias Dph3•Dph8), dual modification regulator. The dimer is located upstream of two radical SAM (RS) enzyme complexes (Dph1•Dph2; Elongator: Elp1•Elp6). Its dual regulator roles ensure proper synthesis of diphthamide on EF2 and modification of tRNA anticodon wobble uridine (U34) bases in order to support accurate mRNA translation and *de novo* protein synthesis [27,50].

against oxygen toxicity and FeS cluster damage as recently suggested for yeast and human Dph1•Dph2 [48,49]. Being located upstream of two RS modifiers (**Fig. 4**) that impact on the accuracy of tRNA decoding and EF2 translocation, a better understanding of how the Kti11•Kti13 (alias Dph3•Dph8) dimer affects mRNA translation is in need [27,50], particularly, in the light of clinically relevant roles for tRNA and EF2 modifications that have recently been shown to emerge in human disease syndromes [51,52].

MATERIALS AND METHODS

Strains, media, growth conditions and assays

S. cerevisiae and *K. lactis* yeast strains used throughout this study are listed in Table S1. Yeast gene deletion strains were generated based on PCR-mediated protocols using oligonucleotides and gene specific primers (Table S2) with pUG6 plasmid templates [26,39,53]. Strains were grown in complete yeast peptone dextrose (YPD) or minimal synthetic defined (SD) media [54] at 30˚C unless otherwise stated. For zymocin response analyses, ten-fold serial cell dilutions of *S. cerevisiae* tester strains (starting OD_{600}: 1.5) were spotted onto YPD plates lacking or containing 0.02-0.5% (v/v) zymocin. The latter tRNase complex was partially purified from *K. lactis* killer strain AWJ137 (Table S1) by ultrafiltration [55]. Incubation was for 2-4 days at 30˚C. For sordarin assays, yeast cells were cultivated at 30 °C on YPD supplemented with 5-10 µg/mL sordarin produced from *Sordaria araneosa* (Sigma-Aldrich). DT growth assays involved galactose-inducible expression of the cytotoxic ADP-ribosylase fragment A [29] from DT, using vector pSU9 [39].

Analysis of EF2 diphthamide modification status

Diagnosis of EF2 diphthamide modification states *in vivo* involved Western blots on total yeast cell extracts and antibodies that detect global EF2 pools irrespective of diphthamide modification (anti-EF2[pan]) or specifically recognize unmodified forms of EF2 (anti-EF2[no diphthamide]) [42]. Both antibodies were previously shown to detect human EF2 [42]. As the diphthamide target sequences between human (708-TLHADAIHRGGGQIIPT-724) [42] and yeast (692-TLHADAIHRGGGQIIPT-708) cells are identical [26], anti-EF2(no diphthamide) is suited to differentiate diphthamide modification states of EF2 from *S. cerevisiae* [26]. Total yeast cell extracts were generated as previously described [56] and protein concentrations determined by the Bradford assay [57]. 8 µl Lämmli samples were subjected to SDS-PAGE (12% [w/v] polyacrylamide) and blotted onto PVDF membranes (Merck/Millipore). These were probed overnight at 4˚C with the anti-EF2(pan) anti-EF2(no diphthamide) antibodies [26] and developed with anti-rabbit secondary antibody HRP-conjugate (Dako; working concentration: 1:2000) and Lumi-Light Western blotting substrate (Roche) as previously described [26,42]. Protein loading was controlled in parallel Western blots with anti-Cdc19 antibodies recognizing pyruvate kinase. Diphthamide-dependent ADPR acceptor activity of EF2 in the presence of DT was tested *in vitro* [58]. The assays used total yeast extracts and biotinylated NAD^+ as ADP-ribosyl donor for DT essentially as previously described with human and yeast EF2 resources [58,59].

Two-step purification of His-affinity tagged EF2 by IMAC and SEC

His-tagged EF2 from strain TKY675 carrying *EFT2-[His]$_6$* on pTKB612 (Table S1) was detected with anti-$(His)_6$ antibodies (Santa Cruz Biotechnology, USA) in Western blots. Purification by IMAC and SEC used 5 ml HisTrap columns (GE Healthcare, Chicago, USA) (Fig. S2, S3). For detailed IMAC and SEC protocols including modifications from the one originally described [44], see Supplemental Material.

Detection of EF2 diphthamide modification states by mass spectrometry

Isolated EF2 proteins from the various TKY675 backgrounds (wild-type, *dph1Δ*, *dph5Δ* and *kti13/dph8Δ*) were analyzed via nLC-MS/MS to determine their diphthamide modification states in accordance with an earlier description for EF2 modification analysis from *Arabidopsis thaliana* [43]. Yeast proteins were separated by SDS-PAGE (7.5% [w/v] polyacrylamide), stained with Coomassie Blue, and excised as bands from the gel. Disulfides were reduced with dithionite and cysteine residues were alkylated with iodoacetamide, followed by trypsin digestion of proteins overnight, all within the gel piece as described [60]. Trypsin-digested fragments were eluted from the gel pieces and desalted using ZipTips [61] before analysis by nLC-MS/MS on a Thermo Orbitrap Fusion mass spectrometer (ThermoFisher) with injection via an electrospray ion source (Tri-Versa NanoMate, Advion). Acquisition of mass spectra was done at a resolution of 120,000 for MS1 scans and 60,000 for MS2 scans with operation parameters described in detail elsewhere [61]. Diphthamide-modified ($C_{81}H_{137}N_{25}O_{23}$), ACP-modified ($C_{78}H_{130}N_{24}O_{24}$) and diphthamide-unmodified ($C_{74}H_{123}N_{23}O_{22}$) precursor masses of 1829,03, 1786,97, and 1685,92, respectively, of target peptide 686-VNILDVTLHADAIHR-700 (with diphthamide target residue, His-699, underlined) were identified with ProteomeDiscoverer Version 2.4 (ThermoFisher) using SequestHT as the search engine and yeast EF2 (Eft1, Eft2) sequence as a database. Parameters included carbamidomethylation of cysteine as a fixed and the diphthamide modification of histidine ($+C_7H_{14}N_2O$, m = 142.11 g) as a variable modification. We allowed no missed cleavage, a precursor charge state of +2 to +7, a precursor m/z tolerance of ±3 ppm, and a fragment mass tolerance of 0.1 Da. The false discovery rate was set to 1% at the peptide identification level using the Target Decoy PSM Validator node. Precursor abundance was estimated with the Minora node in ProteomeExplorer.

ACKNOWLEDGEMENTS

We thank Tessa Hübner (MPI, Göttingen, Germany) and Benjamin Scheer (UFZ, Leipzig, Germany) for assistance with EF2 purification and nLC-MS/MS, Dr Roland Klassen (Kassel University, Germany) for critical reading of the manuscript and Prof Jeremy Thorner (University of California, Berkeley, USA) for kindly donating anti-Cdc19 antibod-

ies. The work was supported by DFG (Bonn, Germany) Priority Program 1927 *Iron-Sulfur for Life* to LA (AD178/7-1) and RS (SCHA750/21-1) and by a *Diphthamide* Pilotgrant to RS (#2887) from ZFF (Kassel University, Germany).

SUPPLEMENTAL MATERIAL

All supplemental data for this article are available online at www.microbialcell.com.

CONFLICT OF INTEREST

KM and UB are employed by and members of Roche Pharma Research & Early Development, and are co-inventors on patent applications that cover assays to detect presence or absence of diphthamide. Roche is interested in targeted therapies and diagnostics. All other authors declare no conflict of interest.

COPYRIGHT

Please cite this article as: Meike Arend, Koray Ütkür, Harmen Hawer, Klaus Mayer, Namit Ranjan, Lorenz Adrian, Ulrich Brinkmann and Raffael Schaffrath (**2023**). Yeast gene *KTI13* (alias *DPH8*) operates in the initiation step of diphthamide synthesis on elongation factor 2. **Microbial Cell**: *in press*.

REFERENCES

1. Butler AR, O'Donnell RW, Martin VJ, Gooday GW, and Stark MJ (**1991**). *Kluyveromyces lactis* toxin has an essential chitinase activity. **Eur J Biochem** 199: 483–488. doi: 10.1111/j.1432-1033.1991.tb16147.x

2. Jablonowski D, and Schaffrath R (**2007**). Zymocin, a composite chitinase and tRNase killer toxin from yeast. **Biochem Soc Trans** 35: 1533–1537. doi: 10.1042/BST0351533

3. Stark MJ, Boyd, A, Mileham AJ, and Romanos, MA (**1990**). The plasmid-encoded killer system of *Kluyveromyces lactis*: a review. **Yeast** 6: 1–29. doi: 10.1002/yea.320060102

4. Butler AR, White JH, Folawiyo Y, Edlin A, Gardiner D, and Stark MJ (**1994**). Two *Saccharomyces cerevisiae* genes which control sensitivity to G1 arrest induced by *Kluyveromyces lactis* toxin. **Mol Cell Biol** 14: 6306–6316. doi: 10.1128/mcb.14.9.6306-6316.1994

5. Jablonowski D, Fichtner L, Martin VJ, Klassen R, Meinhardt F, Stark MJ, and Schaffrath, R (**2001**). *Saccharomyces cerevisiae* cell wall chitin, the *Kluyveromyces lactis* zymocin receptor. **Yeast** 18: 1285–1299. doi: 10.1002/yea.776

6. Mehlgarten C, and Schaffrath R (**2004**). After chitin-docking, toxicity of *Kluyveromyces lactis* zymocin requires *Saccharomyces cerevisiae* plasma membrane H^+-ATPase. **Cell Microbiol** 6: 569–580. doi: 10.1111/j.1462-5822.2004.00383.x

7. Zink S, Mehlgarten C, Kitamoto HK, Nagase J, Jablonowski D, Dickson RC, Stark MJ, and Schaffrath, R (**2005**). $M(IP)_2C$, the major yeast plasma membrane sphingolipid, governs toxicity of *Kluyveromyces lactis* zymocin. **Eukaryot Cell** 4: 879–889. doi: 10.1128/EC.45.879-889.2005

8. Frohloff F, Fichtner L, Jablonowski D, Breunig KD, and Schaffrath, R (**2001**). *Saccharomyces cerevisiae* Elongator mutations confer resistance to the *Kluyveromyces lactis* zymocin. **EMBO J** 20: 1993–2003. doi: 10.1093/emboj/20.8.1993

9. Huang B, Johansson MJ, and Bystrom AS (**2005**). An early step in wobble uridine tRNA modification requires the Elongator complex. ***RNA*** 11: 424–436. doi: 10.1261/rna.7247705

10. Paraskevopoulou C, Fairhurst SA, Lowe DJ, Brick P, and Onesti S (**2006**). The Elongator subunit Elp3 contains a Fe4S4 cluster and binds S-adenosylmethionine. **Mol Microbiol** 59: 795–806. doi: 10.1111/j.1365-2958.2005.04989.x

11. Lin TY, Abbassi NEH, Zakrzewski K, Chramiec-Głąbik A, Jemioła-Rzemińska M, Różycki J, and Glatt S (**2019**). The Elongator subunit Elp3 is a non-canonical tRNA acetyltransferase. **Nat Commun** 10: 625. doi: 10.1038/s41467-019-08579-2

12. Abbassi NE, Biela A, Glatt S, and Lin TY (**2020**). How Elongator acetylates tRNA bases. **Int J Mol Sci** 21: 8209. doi: 10.3390/ijms21218209

13. Lu J, Huang B, Esberg A, Johansson MJ, and Byström AS (**2005**). The *Kluyveromyces lactis* gamma-toxin targets tRNA anticodons. **RNA** 11: 1648–1654. doi: 10.1261/rna.2172105

14. Jablonowski D, Zink S, Mehlgarten C, Daum G, and Schaffrath R (**2006**). tRNAGlu wobble uridine methylation by Trm9 identifies Elongator's key role for zymocin-induced cell death in yeast. **Mol Microbiol** 59: 677–688. doi: 10.1111/j.1365-2958.2005.04972.x

15. Schaffrath R, and Leidel SA (**2017**). Wobble uridine modifications – a reason to live, a reason to die?! **RNA Biol** 14: 1209–1222. doi: 10.1080/15476286.2017.1295204

16. Krutyhołowa R, Hammermeister A, Zabel R, Abdel-Fattah W, Reinhardt-Tews A, Helm M, Stark MJ, Breunig KD, Schaffrath R, and Glatt S. (**2019**) Kti12, a PSTK-like tRNA dependent ATPase essential for tRNA modification by Elongator. **Nucleic Acids Res**47: 4814–4830. doi: 10.1093/nar/gkz190

17. Abdel-Fattah W, Jablonowski D, Di Santo R, Scheidt V, Hammermeister A, ten Have SM, Thüring KL, Helm M, Schaffrath, R, and Stark MJ (**2015**). Phosphorylation of Elp1 by Hrr25 is required for Elongator-dependent tRNA modification in yeast. **PLoS Genet** 11: e1004931. doi: 10.1371/journal.pgen.1004931

18. Glatt S, Zabel R, Vonkova I, Kumar A, Netz DJ, Pierik AJ, Rybin V, Lill R, Gavin AC, Balbach J, Breuing KD, and Müller CW (**2015**). Structure of the Kti11/Kti13 heterodimer and its double role in modification of tRNA and eukaryotic elongation factor 2. **Structure** 23: 149–160. doi: 10.1016/j.str.2014.11.008

19. Kolaj-Robin O, McEwen AG, Cavarelli J, and Séraphin B (**2015**). Structure of the Elongator cofactor complex Kti11/Kti13 provides insight into the role of Kti13 in Elongator-dependent tRNA modification. **FEBS J** 282: 819–833. doi: 10.1111/febs.13199

20. Liu S, and Leppla SH (**2003**). Retroviral insertional mutagenesis identifies a small protein required for synthesis of diphthamide, the target of bacterial ADP-ribosylating toxins. **Mol Cell** 12: 603–613. doi: 10.1016/j.molcel.2003.08.003

21. Liu S, Milne GT, Kuremsky JG, Fink GR, and Leppla SH (**2004**). Identification of the proteins required for biosynthesis of diphthamide, the target of bacterial ADP-ribosylating toxins on translation elongation factor 2. **Mol Cell Biol** 24: 9487–9497. doi: 10.1128/MCB.24.21.9487-9497.2004

22. Bär C, Zabel R, Liu S, Stark MJ, and Schaffrath R (**2008**). A versatile partner of eukaryotic protein complexes that is involved in multiple

biological processes: Kti11/Dph3. **Mol Microbiol** 69: 1221–1233. doi: 10.1111/j.1365-2958.2008.06350.x

23. Jørgensen R, Merrill AR, and Andersen GR (**2006**). The life and death of translation elongation factor 2. **Biochem Soc Trans** 34: 1–6. doi: 10.1042/BST20060001

24. Su X, Lin Z, and Lin H (**2013**). The biosynthesis and biological function of diphthamide. **Crit Rev Biochem Mol Biol** 48: 515–521. doi: 10.3109/10409238.2013.831023

25. Schaffrath R, Abdel-Fattah W, Klassen, R, and Stark MJ (**2014**). The diphthamide modification pathway from *Saccharomyces cerevisiae* – Revisited. **Mol Microbiol** 94: 1213–1226. doi: 10.1111/mmi.12845

26. Hawer H, Ütkür K, Arend M, Mayer K, Adrian L, Brinkmann U, Schaffrath R (2018). Importance of diphthamide modified EF2 for translational accuracy and competitive cell growth in yeast. **PLoS One** 13: e0205870. doi: 10.1371/journal.pone.0205870.

27. Djumagulov M, Demeshkina N, Jenner L, Rozov A, Yusupov M, Yusupova G (2021). Accuracy mechanism of eukaryotic ribosome translocation. **Nature** 600: 543–546. doi: 10.1038/s41586-021-04131-9

28. Hawer H, Hammermeister A, Ravichandran KE, Glatt S, Schaffrath R, Klassen R (2019). Roles of Elongator dependent tRNA modification pathways in neurodegeneration and cancer. **Genes** 10: 19. doi: 10.3390/genes10010019.

29. Uthman S, Liu S, Giorgini F, Stark MJ, Costanzo M, and Schaffrath R (**2012**). Diphtheria disease and genes involved in formation of diphthamide, key effector of the diphtheria toxin. In**: Insight and Control of Infectious Disease in Global Scenario**, Kumar R (ed), INTECH OAP, pp 333–356. doi: 10.5772/31680

30. Fichtner L, Jablonowski D, Schierhorn A, Kitamoto HK, Stark MJ, and Schaffrath R (**2003**). Elongator's toxin-target (TOT) function is NLS dependent and suppressed by post-translational modification. **Mol Microbiol** 49: 1297–1307. doi: 10.1046/j.1365-2958.2003.03632.x

31. Zhang Y, Zhu X, Torelli AT, Lee M, Dzikovski B, Koralewski RM, Wang E, Freed J, Krebs C, Ealick SE, and Lin H (**2010**). Diphthamide biosynthesis requires an organic radical generated by an iron-sulphur enzyme. **Nature** 465: 891–896. doi: 10.1038/nature09138

32. Dong M, Dando EE, Kotliar I, Su X, Dzikovski B, Freed JH, and Lin H (**2019**). The asymmetric function of Dph1-Dph2 heterodimer in diphthamide biosynthesis. **J Biol Inorg Chem** 24: 777–782. doi: 10.1007/s00775-019-01702-0

33. Sun J, Zhang J, Wu F, Xu C, Li S, Zhao W, Wu Z, Wu J, Zhou CZ, and Shi Y (**2005**). Solution structure of Kti11p from *Saccharomyces cerevisiae* reveals a novel zinc-binding module. **Biochemistry** 44: 8801–8809. doi: 10.1021/bi0504714

34. Dong M, Su X, Dzikovski B, Dando EE, Zhu X, Du J, Freed JH, and Lin H (**2014**). Dph3 is an electron donor for Dph1-Dph2 in the first step of eukaryotic diphthamide biosynthesis. **J Am Chem Soc** 136: 1754–1757. doi: 10.1021/ja4118957

35. Zhang Y, Su D, Dzikovski B, Majer SH, Coleman R, Chandrasekaran S, Fenwick MK, Crane BR, Lancaster KM, Freed JH, and Lin H (**2021**). Dph3 enables aerobic diphthamide biosynthesis by donating one iron atom to transform a [3Fe-4S] to a [4Fe-4S] cluster in Dph1-Dph2. **J Am Chem Soc** 143: 9314–9319. doi: 10.1021/jacs.1c03956

36. Lin Z, Dong M, Zhang Y, Lee EA, and Lin H (**2016**). Cbr1 is a Dph3 reductase required for the tRNA wobble uridine modification. **Nat Chem Biol** 12: 995–997. doi: 10.1038/nchembio.2190

37. Zabel R, Bär C, Mehlgarten C, and Schaffrath, R (**2008**). Yeast -tubulin suppressor Ats1/Kti13 relates to the Elongator complex and interacts with Elongator partner protein Kti11. **Mol Microbiol** 69: 175–187. doi: 10.1111/j.1365-2958.2008.06273.x

38. Fichtner L, and Schaffrath R (**2002**). *KTI11* and *KTI13*, *Saccharomyces cerevisiae* genes controlling sensitivity to G1 arrest induced by *Kluyveromyces lactis* zymocin. **Mol Microbiol** 44: 865–875. doi: 10.1046/j.1365-2958.2002.02928.x

39. Uthman S, Bär C, Scheidt V, Liu S, ten Have S, Giorgini F, Stark MJ, and Schaffrath R (**2013**). The amidation step of diphthamide biosynthesis in yeast requires *DPH6*, a gene identified through mining the *DPH1–DPH5* interaction network. **PLoS Genet** 9: e1003334. doi: 10.1371/journal.pgen.1003334

40. Huang B, Lu J, and Byström AS (**2008**). A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*. **RNA** 14: 2183–2194. doi: 10.1261/rna.1184108

41. Shao Y, Molestak E, Su W, Stankevič M, Tchórzewski M (**2022**). Sordarin – an anti-fungal antibiotic with a unique modus operandi. **Br J Pharmacol** 179: 1125–1145. doi: 10.1111/bph.15724

42. Stahl S, da Silva Mateus Seidl AR, Ducret A, Kux van Geijtenbeek S, Michel S, Racek T, Birzele F, Haas AK, Rueger R, Gerg M, Niederfellner G, Pastan I, and Brinkmann U (**2015**). 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** 112: 10732–10737. doi: 10.1073/pnas.1512863112

43. Zhang H, Quintana J, Ütkür K, Adrian L, Hawer H, Mayer K, Gong X, Castanedo L, Schulten A, Janina N, Peters M, Wirtz M, Brinkmann U, Schaffrath R, and Krämer U (**2022**). Translational fidelity and growth of *Arabidopsis* require stress-sensitive diphthamide biosynthesis. **Nat Comm** 13: 4009. doi: 10.1038/s41467-022-31712-7

44. Jørgensen R, Carr-Schmid A, Ortiz PA, Kinzy TG, and Andersen, GR (**2002**). Purification and crystallization of the yeast elongation factor eEF2. **Acta Crystallogr D Biol Crystallogr** 58: 712–715. doi: 10.1107/s0907444902003001

45. Ortiz PA, Ulloque R, Kihara GK, Zheng H, and Kinzy TG (**2006**). Translation elongation factor 2 anticodon mimicry domain mutants affect fidelity and diphtheria toxin resistance. **J Biol Chem** 281: 32639–32648. doi:10.1074/jbc.M607076200

46. Mattheakis LC, Shen WH, and Collier RJ (**1992**). *DPH5*, a methyltransferase gene required for diphthamide biosynthesis in *Saccharomyces cerevisiae*. **Mol Cell Biol 12**: 4026–4037. doi: 10.1128/mcb.12.9.4026-4037.1992.

47. Lin Z, Su X, Chen W, Ci B, Zhang S, and Lin H (**2014**). Dph7 catalyzes a previously unknown demethylation step in diphthamide biosynthesis. **J Am Chem Soc** 136: 6179–6182. doi: 10.1021/ja5009272

48. Zhang Y, Su D, Dzikovski B, Majer SH, Coleman R, Chandrasekaran S, Fenwick MK, Crane BR, Lancaster KM, Freed JH, and Lin H (**2021**). Dph3 enables aerobic diphthamide biosynthesis by donating one iron atom to transform a [3Fe-4S] to a [4Fe-4S] cluster in Dph1-Dph2. **J Am Chem Soc** 143: 9314–9319. doi: 10.1021/jacs.1c03956

49. Baik AH, Haribowo AG, Chen X, Queliconi BB, Barrios AM, Garg A, Maishan M, Campos AR, Matthay MA, and Jain IH (**2023**). Oxygen toxicity causes cyclic damage by destabilizing specific Fe-S cluster-containing protein complexes. **Mol Cell** 83: 942–960.e9. doi: 10.1016/j.molcel.2023.02.013

50. Nedialkova DD, and Leidel SA (**2015**). Optimization of codon translation rates via tRNA modifications maintains proteome integrity. **Cell** 161: 1606–1618. doi: 10.1016/j.cell.2015.05.022

51. Hawer H, Mendelsohn BA, Mayer K, Kung A, Malhotra A, Tuupanen S, Schleit J, Brinkmann U, and Schaffrath R (**2020**). Diphthamide-deficiency syndrome: a novel human developmental disorder and ribosomopathy. **Eur J Hum Genet** 28: 1497–1508. doi: 10.1038/s41431-020-0668-y

52. Gaik M, Kojic M, Wainwright BJ, and Glatt S (**2023**). Elongator and the role of its subcomplexes in human diseases. **EMBO Mol Med** 15: e16418. doi: 10.15252/emmm.202216418

53. Gueldener U, Heinisch J, Koehler GJ, Voss D, and Hegemann JH (**2002**). A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. **Nucleic Acids Res** 30: e23. doi: 10.1093/nar/30.6.e23

54. Sherman F (**2002**). Getting started with yeast. **Methods Enzymol** 350: 3–41. doi: 10.1016/s0076-6879(02)50954-x

55. Klassen R, Wemhoff S, Krause J, and Meinhardt F (**2011**). DNA repair defects sensitize cells to anticodon nuclease yeast killer toxins. **Mol Genet Genomics** 285: 185–195. doi: 10.1007/s00438-010-0597-5

56. Zachariae W, Shin TH, Galova M, Obermaier B, abd Nasmyth K (**1996**). Identification of subunits of the anaphase-promoting complex of *Saccharomyces cerevisiae*. **Science** 274: 201–1204. doi: 10.1126/science.274.5290.1201

57. Bradford MM (**1976**). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Chem** 72: 248–254. doi: 10.1006/abio.1976.9999

58. Mayer K, Schröder A, Schnitger J, Stahl S, and Brinkmann U (**2017**). Influence of DPH1 and DPH5 protein variants on the synthesis of diphthamide, the target of ADPRibosylating toxins. **Toxins** 9: 78. doi: 10.3390/toxins9030078

59. Shankar SP, Grimsrud K, Lanoue L, Egense A, Willis B, Hörberg J, Alabdi L, Mayer K, Ütkür K, Monaghan KG, Krier J, Stoler J, Alnemer M, Shankar PR, Schaffrath R, Alkuraya FS, Brinkmann U, Eriksson LA, Lloyd K, Rauen KA, and Undiagnosed Diseases Network (**2022**). A novel *DPH5*-related diphthamide-deficiency syndrome causing embryonic lethality or profound neurodevelopmental disorder. **Genet Med** 24: 2207. doi: 10.1016/j.gim.2022.07.021

60. Kublik A, Deobald D, Hartwig S, Schiffmann CL, Andrades A, von Bergen M, Sawers RG, and Adrian L (**2016**). Identification of a multiprotein reductive dehalogenase complex in *Dehalococcoides mccartyi* strain CBDB1 suggests a protein dependent respiratory electron transport chain obviating quinone involvement. **Environ Microbiol** 18: 3044–3056. doi: 10.1111/1462-2920.13200

61. Seidel K, Kuhnert J, and Adrian L (**2018**). The complexome of *Dehalococcoides mccartyi* reveals its organohalide respiration-complex is modular. **Front Microbiol** 9: 1130. doi: 10.3389/fmicb.2018.01130