The Amidation Step of Diphthamide Biosynthesis in Yeast Requires DPH6, a Gene Identified through Mining the DPH1-DPH5 Interaction Network

Shanow Uthman1, Christian Bär1, Viktor Scheidt2, Shihui Liu3, Sara ten Have4, Flaviano Giorgini1, Michael J. R. Stark4, Raffael Schaffrath1,2

1 Department of Genetics, University of Leicester, Leicester, United Kingdom, 2 Institut für Biologie, FG Mikrobiologie, Universität Kassel, Kassel, Germany, 3 Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, 4 Centre for Gene Regulation and Expression, College of Life Sciences, MSI/WTB Complex, University of Dundee, Dundee, Scotland

Abstract

Diphthamide is a highly modified histidine residue in eukaryal translation elongation factor 2 (eEF2) that is the target for irreversible ADP ribosylation by diphtheria toxin (DT). In Saccharomyces cerevisiae, the initial steps of diphthamide biosynthesis are well characterized and require the DPH1-DPH5 genes. However, the last pathway step—amidation of the intermediate diphthine to diphthamide—is ill-defined. Here we mine the genetic interaction landscapes of DPH1-DPH5 to identify a candidate gene for the elusive amidase (YLR143w/DPH6) and confirm involvement of a second gene (YBR246w/DPH7) in the amidation step. Like dph1-dph5, dph6 and dph7 mutants maintain eEF2 forms that evade inhibition by DT and sordarin, a diphthamide-dependent antifungal. Moreover, mass spectrometry shows that dph6 and dph7 mutants specifically accumulate diphthine-modified eEF2, demonstrating failure to complete the final amidation step. Consistent with an expected requirement for ATP in diphthine amidation, Dph6 is therefore a candidate for the elusive amidase, while Dph7 apparently couples diphthine synthase (Dph5) to diphthamide amidation. The latter conclusion is based on our observation that dph7 mutants show drastically upregulated interaction between Dph5 and eEF2, indicating that their association is kept in check by Dph7. Physiologically, completion of diphthamide synthesis is required for optimal translational accuracy and cell growth, as indicated by shared traits among the dph mutants including increased ribosomal –1 frameshifting and altered responses to translation inhibitors. Through identification of Dph6 and Dph7 as components required for the amidation step of the diphthamide pathway, our work paves the way for a detailed mechanistic understanding of diphthamide formation.

Introduction

Regulation of biological processes by posttranslational modification can involve the function, distribution and interaction capabilities of the modified protein [1–3]. Though most modification pathways such as phosphorylation and ubiquitin conjugation target many different proteins, some exceptional ones uniquely target just a single polypeptide [4]. One prominent example is diphthamide formation on eukaryal translation elongation factor 2 (eEF2) [5]. Strikingly, this modification is pathobiologically important because it is hijacked for eEF2 inhibition by sordarin fungicides and by diphtheria toxin (DT) produced by pathovarieties of Corynebacterium diphtheriae that cause the severe human disease syndrome diphtheria [6–8]. Both agents efficiently block protein synthesis by inactivating the essential function of the modified translation factor, via stalling the diphthamide modified form of eEF2 on ribosomes and irreversible ADP ribosylation of eEF2, respectively [9–12]. Diphthamide itself is a highly modified histidine residue on eEF2 – 2-[3-carboxyamido-3-(trimethylamino)-propyl]-histidine – which is conserved from yeast (H699) to man (H715) (Figure 1) [5,8,13]. Intriguingly, it is absent from the bacterial eEF2 analog, EF-G, thus conferring immunity on the DT producer.

Among the archaea and eukarya, diphthamide formation involves a conserved biosynthetic pathway, which has been extensively dissected in Saccharomyces cerevisiae via isolation of
**Author Summary**

Diphthamide is an unusual modified amino acid found uniquely in a single protein, eEF2, which is required for cells to synthesize new proteins. The name refers to its target function for eEF2 inactivation by diphtheria toxin, the disease-inducing agent produced by the pathogen Corynebacterium diphtheriae. Why cells require eEF2 to contain diphthamide is unclear, although mice unable to make it fail to complete embryogenesis. Cells generate diphthamide by modifying a specific histidine residue in eEF2 using a three-step biosynthetic pathway, the first two steps of which are well defined. However, the enzyme(s) involved in the final amidation step are unknown. Here we integrate genomic and molecular approaches to identify a candidate for the elusive amidase (Dph6) and confirm involvement of a second protein (Dph7) in the amidation step, showing that failure to synthesize diphthamide affects the accuracy of protein synthesis. In contrast to Dph6, however, Dph7 may be regulatory. Our data strongly suggest that it promotes dissociation of eEF2 from diphthine synthase (Dph5), which carries out the second step of diphthamide synthesis, and that Dph5 has a novel role as an eEF2 inhibitor when diphthamide synthesis is incomplete.

Diphthamide Biosynthesis Requires Dph6 and Dph7

![Figure 1. The biosynthetic pathway for modification of eEF2 by diphthamide. For roles played by the bona fide diphthamide genes DPH1–DPH5 in steps 1 and 2 of the pathway, see main text. The ill-defined step 3, conversion of diphthine to diphthamide by amidation, is highlighted (red label). It likely involves ATP and ammonium cofactors in a reaction catalyzed by unidentified DPH gene product(s). Step 4 indicates diphthamide can be hijacked for eEF2 inactivation and cell death induction by antifungals, i.e. sordarin and bacterial ADP ribosylase toxins (ADPRtox); alternatively, it has been reported to undergo cell growth related physiological ADP ribosylation (ADPRphys?) by elusive cellular modifier(s). ACP, 2-(3-amino-carboxyl-propyl)-histidine; SAM: S-adenosylmethionine. doi:10.1371/journal.pgen.1003334.g001](image-url)
ciently, by ADP ribosylation [29,31]. Thus amidase-deficient mutants may display DT sensitivity in vivo and thereby escape identification in screens for DT resistant yeast mutants.

Indication that additional proteins are involved in diphthamide biosynthesis has come from recent work on WDR85 and its potential yeast ortholog YBR246w [41,42], while our preliminary investigation of the yeast DPH1 genetic interaction network [13] implicated both YBR246w and YLR143w as novel proteins potentially involved in the diphthamide pathway. Here we further exploit yeast genome-wide genetic interaction and chemical genomics databases [43,44] to demonstrate that YLR143w (DPH6) and YBR246w (DPH7) cluster tightly with all known members of the diphthamide gene network. We find that dph6 and dph7 mutants phenocopy sordarin and DT traits typical of the bona fide dph1-dph5 mutants, which are defective in the first two steps of diphthamide synthesis. Importantly, we show that DPH6 and DPH7 deletions block the final amidation step of the diphthamide pathway, cause diphthine modified forms of eEF2 to accumulate and consequently abolish ADP ribose acceptor activity upon DT treatment. Thus conversion of diphthine to diphthamide depends on Dph6 and Dph7.

Results

Yeast Gene Interaction Databases Predict Diphthamide Functions for YLR143w (DPH6) and YBR246w (DPH7)

To identify factors involved in the terminal amidation step of the diphthamide modification pathway (Figure 1), we took advantage of synthetic genetic array (SGA) screens, which previously enabled systematic mapping of genetic interactions among yeast deletion collections using high-density arrays of double mutants [45,46]. SGA analysis provides the set of genetic interactions for a given gene – the genetic interaction profile – and thereby the phenotypic signatures indicative of functions of both known genes and unassigned ORFs [47]. For example, genes with similar interaction profiles are often functionally related in shared biochemical pathways and/or protein complexes [48,49]. Consistent with this, SGA analysis revealed that the diphthamide gene network members have highly correlated interaction profiles and tightly cluster in the global genetic interaction landscape from yeast [45].

Since our preliminary examination of the yeast genetic interaction landscape placed two uncharacterized yeast ORFs, YLR143w and YBR246w, within the diphthamide gene network [13], we next examined this network in more detail by mining the SGA DRYGIN database for quantitative S. cerevisiae genetic interactions [44,50]. We compared DPH1, DPH2, DPH4, DPH5, YLR143w and YBR246w gene interactions with every array ORF represented in the SGA network and deposited at DRYGIN, ranking the similarity between all possible pairwise profiles according to their Pearson correlation coefficient (PCC; see Table S1 for full details). As expected, the other known DPH genes scored significantly highly among the correlation profiles generated for each specific DPH query gene, consistently being ranked among the top ten genetic interactors (Figure 2A). Strikingly, YLR143w and YBR246w were among the top interactors of DPH1, DPH2, DPH4 and DPH5, while the most correlated interactors for YLR143w and YBR246w included each other and several bone fide DPH genes (Figure 2A). Such highly correlated interaction patterns suggest that YLR143w and YBR246w are both functionally interrelated and qualify as candidate ORFs of the pathway for eEF2 modification by diphthamide. In line with this notion, the two eEF2 encoding gene copies, EFT1 and EFT2, also ranked among the top ten interactors of DPH1, DPH2 and DPH5 (Figure 2A).

For independent validation of these correlations, we searched the FitDB yeast fitness database [51], which contains genome-scale phenotypic profiles for diploid yeast deletion collections in response to more than 1100 different growth conditions [45,52]. Here, scoring gene interaction profiles by homologous co-sensitivity revealed that among the top loci to phenocluster with YBR246w are DPH2, DPH4 and DPH5, while top interactors of YLR143w include DPH4, DPH5, YBR246w and DPH2 (Figure 2B). A similar pattern of interaction is shown by DPH5 (Figure 2B), DPH2 and DPH4 (data not shown). Based on correlated interaction profiles, FitDB ascribes GO terms enriched for processes concerning peptide-diphthamide biosynthesis from peptidyl-histidine to YLR143w and YBR246w with p-values of 2×10\(^{-3}\) and 9×10\(^{-3}\) respectively (Figure 2C). Collectively, the FitDB and DRYGIN profiles thus provide robust phenotypic signatures suggesting novel roles in the diphthamide pathway for YBR246w and YLR143w, which are tightly clustered within the DPH gene network (Figure 2C). This notion is consistent with a recent report that YBR246w and its mammalian homolog, WDR85, have a diphthamide related function [41,42]. Since YLR143w is as yet unassigned in the Saccharomyces genome database (SGD), based on the evidence below that YLR143w and YBR246w are indeed diphthamide synthesis genes we have named them DPH6 (YLR143w) and DPH7 (YBR246w).

**DPH6 and DPH7 Deletions Cause Phenotypes Typical of Bonafide Diphthamide Mutants**

To verify the predicted roles for DPH6 and DPH7 in the diphthamide pathway, we next examined strains deleted for these ORFs for phenotypes specifically linked to defects in diphthamide formation on eEF2, namely sordarin resistance and response to DT [6,7]. Sordarin traps eEF2 on the 80S ribosome [53], blocking mRNA translation elongation and yeast cell growth [54] in a fashion that depends on diphthamide synthesis [6,7]. As a result, diphthamide mutants dph1-dph5 efficiently protect against sordarin inhibition [6,7]. Like dph1-dph5, dph6 and dph7 mutants showed robust resistance towards sordarin at 10 μg/ml, a concentration inhibitory to the wild-type (Figure 3A). This resistance was comparable to that shown by eEF2 substitution mutants eEF2H699I and eEF2H699N (Figure 3A), which lack the His699 acceptor residue for diphthamide modification [37]. Thus DPH6 and DPH7 are novel sordarin effectors, a feature they share with the diphthamide synthesis genes DPH1-DPH5 [6,7].

Diphthamide modification plays a key effector role for inhibitory ADP ribosylation of eEF2 by DT, hence dph1-dph5 mutants in both yeast and mammalian cells confer resistance towards DT [14,16]. We therefore compared DT-dependent ADP ribosylation of eEF2 in vitro between wild-type cells and dph1, dph5, dph6 and dph7 mutants. While the translation factor from wild-type cells was efficiently modified by the toxic ADP ribosylase (Figure 3B), eEF2 extracted from dph1, dph5, dph6 and dph7 mutants could not be ADP ribosylated by exogenously added DT under the conditions used (Figure 3B). Such lack of ADP ribose acceptor activity in vitro strongly suggests defects in the diphthamide pathway and that DPH6 and DPH7 encode novel functions required for diphthamide formation. To further address this experimentally, we assayed the response of dph6 and dph7 mutants to intracellular expression of the ADP ribosylase domain of DT (DTA) using GAL5, a truncated variant of the GAL1 promoter [55]. When DTA expression was induced by 0.1% galactose, dph6 and dph7 mutants were indeed found to show some protection against DTA in contrast to wild-type cells (Figure 3C).

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consistent with defects in diphthamide formation. However, at a higher level of expression on 2% galactose, they showed wild-type like sensitivity to DTA whereas \(dph1\) and \(dph5\) mutants remained fully resistant (Figure 3C). This suggests that eEF2 forms from \(dph6\) or \(dph7\) mutants, although not substrates in vitro (Figure 3B), can nonetheless be ADP ribosylated in vivo if DTA is expressed at a sufficiently high level [30]. While our work was in progress, eEF2 from a \(ybr246w/dph7\) mutant was shown to be a very weak substrate for ADP ribosylation when treated with 10 mM DT [42], a 500-fold increase in concentration over that used in our in vitro ADP ribosylation assays (Figure 3B). Thus eEF2 from the \(dph6\) or \(dph7\) mutants is resistant to sordarin and shows a vastly reduced ability to be ADP-ribosylated by DT, strongly suggesting that the diphthamide pathway is defective. Since the intermediate diphthine can serve as a sub-optimal substrate for ADP ribosylation using excess levels of DT or upon overexpressing its toxic ADP ribosylase domain from inside cells [29,31], the properties of eEF2 from \(dph6\) and \(dph7\) mutants are consistent with a defect in the final step of the pathway that converts diphthine to diphthamide. Our analysis is therefore entirely consistent with the above database predictions and indicates \(DPH6\) and \(DPH7\) constitute novel candidate loci for diphthamide biosynthesis.

Mass Spectrometry Reveals Diphthine Accumulation in \(dph6\) and \(dph7\) Mutants Due to a Block in the Terminal Amidation Step of the Diphthamide Pathway

Given the above evidence, we next examined whether eEF2 prepared from cells deleted for either \(DPH6\) or \(DPH7\) carried any modification on His699, the eEF2 residue that is modified to
generate diphthamide. eEF2 preparations made from wild-type and gene deletion strains expressing His6-tagged eEF2 were digested with trypsin and examined by mass spectrometry. The His6-tagged form was chosen as the source of eEF2 since expression rescued the inviability of an eft1 eft2 double mutant lacking eEF2 function, and it is thus considered to be biologically active [56]. Strains lacking either DPH1, in which the first step of diphthamide biosynthesis is blocked, or lacking DPH5 (encoding diphthine synthase), were used respectively as controls for complete lack of modification and presence of ACP, the first intermediate in the diphthamide pathway [14,16,30,32]. All strains expressed similar levels of His6-tagged eEF2 (data not shown).

The modified histidine in eEF2 (His699) is located in the tryptic peptide 686-VNILDVTLHADAIHR-700 and, as expected, unmodified versions of this peptide were readily detected in eEF2 prepared from the dph1 mutant (Figure S1C). Unmodified peptide was also found in eEF2 prepared from dph5, dph6 and dph7 deletion strains as well as from wild-type cells (Figures S1 and S2). Thus even in wild-type cells not all of the eEF2 is modified by diphthamide. In addition to the unmodified peptide, we readily detected diphthamide-modified peptide in eEF2 prepared from the wild-type strain (Figure 4A), but failed to detect this in any of the mutants. Instead, ACP-modified peptide was found in eEF2 prepared from the dph5 mutant (Figure 4B), as expected given its known role in generating diphthine [32] from the ACP intermediate in the pathway.

In contrast, eEF2 from the dph7 mutant generated spectra consistent with the presence of diphthine on His699, in which the m/z values for both the parent ions and the daughter ions in the MS/MS spectra were higher in a manner consistent with the 0.984 Da extra mass associated with presence of a carboxyl group in diphthine rather than the amide group in diphthamide (Figure 4C). Thus each of the doubly-charged daughter ions in Figure 4C is larger by an m/z of 0.5 than the corresponding ion in the wild-type spectrum (Figure 4A). Furthermore, the quite

Figure 3. DPH6 and DPH7 deletion strains copy traits typically related to the bona fide diphthamide mutants dph1-dph5. (A) Sordarin resistance. Ten-fold serial cell dilutions of the indicated yeast strains, BY4741 wild-type (wt) background and its dph1-dph7 gene deletion derivatives (upper panels) as well as an MKK-derived eft1 eft2 double deletion background maintaining plasmid pEFT2 wild-type or H699 substitution (H699 N and H699I) alleles of EFT2 (lower panels), were grown on YPD plates in the absence (control) or presence (+sor) of 10 μg ml⁻¹ sordarin. Growth was assayed for 3 d at 30°C. Sordarin resistant (R) and sensitive (S) responses are indicated. (B) Lack of in vitro ADP ribose acceptor activity of eEF2. Cell extracts obtained from dph1, dph5, dph6 and dph7 mutant and wild-type (wt) strains were incubated with (+DT) or without (−DT) 20 nM diphtheria toxin in the presence of biotin-NAD (10 μM) at 37°C for 1 hour. The transfer of biotin-ADP-ribose to eEF2 was detected by Western blotting using a streptavidin-conjugate. Two unknown non-specific bands (indicated by *) served as internal controls for even sample loading. (C) DT phenotype. As indicated, yeast dph mutants and wild-type control (wt) were tested for sensitivity to intracellular expression of DTA, the cytotoxic ADP ribosylase fragment of DT. This in vivo assay involved galactose-inducible expression from vector pSU8 (see Materials and Methods). Serial cell dilutions were replica spotted onto raffinose (2% raf) and galactose-inducing conditions using concentrations (2, 0.2 and 0.1% gal) suited to achieve gradual down-regulation of DTA toxicity. Growth was for 3 days at 30°C. DTA sensitive (S) resistant (R), partially resistant (PR) and reduced sensitive (RS) phenotypes are indicated.

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different elution times of the diphthine-modified and diphthamide-modified peptide that are evident from the extracted ion chromatograms (Figure S3) are consistent with differently modified forms of eEF2. As noted in previous studies [32,33,36], some of the ions in our MS/MS spectra had undergone neutral loss of the trimethylamino group during MS/MS, as indicated by loss of 59,110 mass units.

Two types of spectra corresponding to the peptide with modified His699 were seen when eEF2 from the dph6 mutant was analyzed. In some spectra (Figure 4D), the parent ion m/z and MS/MS data indicated the presence of diphthine as in the dph7 mutant, with some daughter ions again showing neutral loss of the trimethylamino group during MS/MS as noted above. However, we also detected peptide forms in which elimination of the trimethylamino group occurred prior to MS/MS. As indicated by the lower parent ion m/z (Figure 4E) and an MS/MS spectrum in which all assignable peaks corresponded to ions lacking the trimethylamino group. Such trimethylamino elimination prior to mass spectrometry was observed previously when diphthine-modified Pyrococcus horikoshii EF2 was generated in an in vitro reaction [32], indicating that this modification might be unstable. However, we failed to detect any pre-mass spectrometry loss of the trimethylamino group when eEF2 from the dph7 mutant was analyzed. Thus while eEF2 from both mutants carries diphthine, the modification appears to be more labile in the dph6 mutant and may be protected from trimethylamino elimination by the absence of Dph7.

Figure S3 shows extracted ion chromatograms for ions with m/z values corresponding to the His699-containing peptide modified with diphthamide, diphthine or with ACP, indicating that the ACP-modified peptide was only present in the dph5 mutant, the diphthine-modified peptide was only present in the dph6 and dph7 mutants, and diphthamide-modified peptide was only seen in wild-type cells. Our mass spectrometry analysis therefore shows that in yeast strains lacking either DPH5 or DPH7, modification of His699 progresses only as far as diphthine. Thus both loci indeed qualify as novel diphthamide synthesis genes with likely roles in conversion of diphthine to diphthamide.

Protein–Protein Interactions Between Dph6, Dph7, Dph5, and EF2

Although Dph6 and Dph7 appear to function within the same step of the diphthamide synthesis pathway, using co-immune precipitation they were not found to interact either with one another or with Dph2 and Dph3, players involved in the two earlier pathway steps (Figure S4; Figure S5 and data not shown). However, in support of our evidence that Dph6 is a diphthamide biosynthetic factor, we observed co-immune precipitation that Dph6-HA bound to a fraction of His6-tagged eEF2 (Figure 5A). Intriguingly, this interaction was independent of Dph7 (Figure 5A), suggesting Dph7 may not mediate interaction between Dph6 and the translation factor. Dph7 is also unlikely to play an indirect role through regulation of DPH6 gene expression because Dph6 protein levels were unaffected in the DPH7 deletion strain (Figure 5A).

Inactivation of WDR85, the mammalian homolog of Dph7, was recently shown to dramatically enhance association of diphthine synthase Dph5 with eEF2 [41]. We therefore examined whether Dph7 impacts on the interaction between Dph5 and eEF2 in budding yeast. We found that a much higher level of affinity tagged eEF2 could be co-immune precipitated with HA-tagged Dph5 from extracts of the dph7 mutant in comparison to wild-type extracts (Figure 5B). A smaller increase was also seen with the dph6

Figure 4. MS/MS spectra of diphthamide-, ACP-, and diphthine-modified EF2 peptide 686-VNILDVTLHADAIHR-700 from wild-type and mutant yeast strains. Spectra are shown for (A) diphthamide-modified peptide from the wild-type yeast strain; (B) ACP-modified peptide from the dph5Δ mutant; (C) diphthine-modified peptide in the dph7Δ strain; (D) diphthine-modified peptide in the dph6Δ strain; (E) diphthamide-modified peptide in the dph6Δ strain with loss of the trimethylamino group before analysis in the mass spectrometer indicated by the parent ion m/z. In each case the parent ion m/z and charge state is indicated. In (A, C) and (D), * indicates neutral loss of trimethylamino during MS/MS. The inset in (C) shows greater detail for the more crowded part of the MS/MS spectrum. Figure S2A indicates how the B and Y ions are derived from the peptide sequence.

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Figure 5. Co-immune precipitations reveal eEF2 interactions with Dph6 and Dph5. (A) eEF2 interacts with Dph6 in a fashion that is independent of Dph7. (B) eEF2 interaction with Dph6 is dramatically enhanced by elimination of Dph7 or Dph1. Yeast strains co-expressing (His)6-tagged eEF2 with Dph6-HA (A) or Dph5-HA (B) in the background of wild-type (A: Dph7 and B: wt) and dph mutant strains (A: dph7; B: dph1, dph6 and dph7) were subjected to immune precipitations (IP) using the anti-HA antibody. Strains expressing (His)6-tagged eEF2 on their own served as IP controls (A and B: no HA-tag). Subsequently, the precipitates were probed with anti-HA (A: top left panel; B: first panel) and anti-(His)6 antibodies (A: bottom left panel) to check for the content of Dph6-HA (A) and Dph5-HA (B), respectively (all indicated by arrows). The content of HA-tagged Dph6 (A) and Dph5 (B) as well as (His)6-marked eEF2 (A and B) in the protein extracts prior to IP (pre-IP) was examined on individual Western blots using anti-HA (A: top right panel; B: fourth panel) and anti-(His)6 antibodies (A: bottom right panel; B: third panel), respectively. While absence of Dph7 hardly affected the Dph6:eEF2 interaction (A), Dph5:eEF2 interaction was strongly enhanced by inactivating DPH7 or DPH1 (B).

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Physiological Implications of the Diphthamide Modification on eEF2

Although the precise biological function of diphthamide is unclear, its location at the tip of the eEF2 anticodon mimicry domain IV predicts a potentially important role in translation. Consistent with this, structure-function studies have shown that domain IV is sufficiently proximal for interaction with tRNA in the decoding P-site of the ribosome [57] and alterations of invariant tip residues, including H699 substitutions that cannot be diphthamide modified, confer biologically significant traits including thermosensitive growth defects [37,58]. Nonetheless, when compared to their wild-type parental strain, we found no significant changes in the growth performance of dph1 mutant or dph3 and dph7 cells under conditions of wild-type DPH5 copy number and normal Dph5 expression levels (Figure 5B). Taken together, our results suggest that binding of Dph5 to incompletely modified eEF2 may be inhibitory to the function of the translation factor. Our data also indicate that both unmodified eEF2, and diphthine-modified eEF2 in the absence of Dph7, show strongly enhanced binding to Dph5. Furthermore, since we failed to detect association between Dph3 and Dph6 despite demonstrating interaction of each with eEF2, it is likely that Dph5 and Dph6 do not bind concurrently to eEF2 and that their binding may therefore be mutually exclusive.

Diph6 

Dph6 contains three conserved domains consistent with its functioning as an enzyme (Figure S8). The amino-terminal 225 residues constitute an Alpha_ANH_like_IV domain (cd1994) in the NCBI Conserved Domain Database [62], also known as DUF71, a member of the adenine nucleotide alpha hydrolyase superfamily that is predicted to bind ATP. Many DUF71 proteins from archaea to mammals contain the highly conserved motif – E215GG/D/EXE230– (Dph6 numbering), which has been proposed to be involved in substrate binding and catalysis and which is replaced by –ENGE/F/Y/E– in a group of related DUF71 proteins implicated in biotin synthesis [60]. Based on this we generated a dph6 allele encoding two substitutions in this region (G216N, E220A) and tested its functionality by monitoring complementation of sordarin resistance in a yeast dph6 knockout strain. Figure 7 clearly shows that this small change completely inactivates the function of Dph6, demonstrating that the Alpha_ANH_like_IV domain is critical for the conversion of diphthine to diphthamide. The C-terminal portion of Dph6 contains two domains related to the YjgF-YER057c-UK114 protein family (eu_ANAH_C1: cd06155 and eu_ANAH_C2: cd06166) that may promote homotrimerisation and formation of an inter-subunit cleft that has been proposed to bind small molecule ligands [63–65]. Several key residues in human UK114 required for homotrimerisation and ligand binding [66] are present in Dph6 (Figure S8) including arg-107, which in E. coli TdcF forms a bidentate salt bridge with the carboxylic acid group of bound ligands [63]. Deletion of residues 335–415 encompassing much of the YjgF-YER057c-UK114 region abolished the function of Dph6 as monitored by sordarin resistance (Figure 7), while truncation of Dph6 at the first of the two conserved domains by insertion of a mscS tag also eliminated Dph6 function (Figure 7) despite detectable expression of the truncated polypeptide (data not shown), indicating that the YjgF-YER057c-UK114 domains are also important for Dph6 function and that the Alpha_ANH-like_IV domain is nonfunctional on its own. Since Salmonella enterica YigF has an enamine/imine deaminase activity that is conserved in human UK114 [67] it is possible that the YigF-YER057c-UK114 domains in Dph6 are used to generate ammonia for diphthamide formation.
Figure 6. dph mutants show sensitivity to elevated diphthine synthase levels and confer reduced translational accuracy. (A) DPH5 overexpression in dph1-dph4 and dph7 mutants causes cytotoxicity and a severe cell growth defect. Cells of yeast strains with the indicated genetic backgrounds and maintaining plasmid pGAL-DPH5 for galactose inducible overexpression of diphthine synthase Dph5 were serially diluted and replica spotted onto glucose (2% glc) and galactose (2% gal) media to assay their response to DPH5 overexpression. Growth was for 3 days at 30°C. Unaltered (T), slightly weakened tolerance (~T) and sensitive (S) responses are indicated. Note that dph1-dph4 and dph7 mutants are extremely sensitive to DPH5 overexpression. (B) Ribosomal frameshift analysis reveals erroneous translation in dph1-dph7 mutants. Strains with the indicated genetic backgrounds were transformed with control (pJD240.0) or lacZ21 frameshift (pJD240.21) plasmids [59] to monitor lacZ expression through β-galactosidase (β-Gal) production using O-nitrophenol-D- galactopyranoside assays and to score translation efficiency (pJD240.0) and fidelity (pJD240.21). Ribosomal −1 frameshifts are expressed relative to the level of overall translation efficiency with statistical significance determined by one-way ANOVA followed by Dunnett’s multiple comparison. With the exception of dph4 and dph7, post-hoc comparison found that all other mutant backgrounds showed a significant increase in ribosomal −1 frameshifting relative to wild-type (wt) yeast cells (* = P < 0.05; *** = P < 0.001; ns. = not significant).

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Figure 7. Both the Alpha_ANH_like_IV and YjgF-YER057c-UK114 domains in Dph6 are essential for its functionality. (A) Diagram showing the DPH6 wild-type and mutant constructs tested in (B), indicating the Alpha_ANH_like_IV (ANH) and YjgF-YER057c-UK114 (UK114) domains and the position of point mutations, an in-frame deletion (- - - - -) and triple myc epitope tag (myc3) as appropriate. (B) Ten-fold serial cell dilutions of a dph6 deletion strain carrying the constructs shown in (A) or the corresponding empty vector (top panel, pMS61/62; lower panel, pSU7 [wt DPH6]; lower panel, pSU7 [wt DPH6]; Table S3) were spotted onto SCD-Leu plates with or without 10 μg/ml sordarin and grown at 30°C for 3 days.

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Taken together, these properties suggest a direct, ATP-dependent role for Dph6 in diphthamine amidation proceeding via an adenylated intermediate and with ammonia acting as the source of the amide group. Such a direct role has now been demonstrated by Su et al., who have used an in vitro assay system to show that Dph6 has diphthamine synthetase activity [61]. Although proteins showing Dph6-like domain organization are readily identified in fungi, plants, amphibians and insects (Figure S8), they are largely absent from archaebacterial and mammalian proteomes. However, mammals and archaebacteria have separate proteins showing strong similarity to either the adenine nucleotide alpha hydrolase domain or to the YjgF-YER057c-UK114 related regions (Figure S8 and data not shown), suggesting Dph6 functionality may be split between different polypeptides in these cases. It is therefore surprising that expression of the human DPH6 ortholog in a yeast dph6 mutant can restore diphthamide biosynthesis [61] despite lacking the YjgF-YER057c-UK114 domains that are essential in the yeast protein (Figure 7; [61]). Thus while the core function of the enzyme must therefore reside in the Alpha_ANH_like_IV domain, it will be interesting to determine the role of the YjgF-YER057c-UK114 domains in Dph6 from lower eukaryotes.

Dph7

Dph7 has four well-defined WD40 repeats (Figure S9) and its predicted structure consists exclusively of β-sheet elements [41,68]. Although its human homolog WDR85 has been implicated in the first step of diphthamide biosynthesis [41], our work and that of Su et al. [42] show that the pathway can proceed as far as diphthine in the absence of DPH7 and that the block is therefore in conversion of diphthine to diphthamide. Furthermore, this block cannot be bypassed simply by introducing DPH6 on a multicopy plasmid to increase the level of diphthamide synthetase (data not shown). How then might Dph7 contribute to diphthamine amidation? Its domain structure suggests it could act as an adaptor molecule for diphthamine amidation [42], but this notion is at odds with our failure to detect interaction between Dph7 and Dph6 (see above).

Our intriguing finding that eEF2 binds much more Dph5 in the absence of Dph7 suggests an alternative role, namely that Dph7 is needed to displace Dph5 from diphthine-modified eEF2 to allow the amidation reaction to occur. Similar findings in mammalian cells upon inactivation of WDR85 support this notion [41]. Together with our data showing that viability of dph1-dph4 and dph7 cells is extremely sensitive to excess Dph5 in comparison to wild-type or dph6 cells, it appears that binding of Dph5 to eEF2 is inhibitory to the function of the translation factor and negatively interferes with cell growth unless eEF2 carries the completed diphthamide modification. Perhaps in addition to catalyzing methylation of ACP-modified eEF2, Dph5 binds to newly-synthesised eEF2 to exclude it from functioning in translation until the diphthine amidation step takes place (Figure 8). Consistent with this proposal is our observation that the level of Dph5 associated with eEF2 in the dph1 mutant, in which modification of His191 cannot be initiated, is drastically increased and virtually indistinguishable from the enhanced Dph5-eEF2 interaction seen when Dph7 is absent. Dph7 may be needed to displace Dph5 once diphthine has been generated so that Dph6 can carry out the diphthine to diphthamide conversion (Figure 8), a notion consistent with the sensitivity of the dph7 mutant to DPH5 overexpression. In contrast, the dph6 mutant may tolerate Dph5 overexpression because Dph7 is present to displace it.

Two other seemingly unrelated functions have been previously proposed for DPH7. Firstly, it emerged from a genetic screen as a potential negative regulator of RNA polymerase I (Rrt2), although no other DPH genes were similarly identified [69]. Secondly, DPH7 has been implicated in retromer mediated endosomal recycling and named ERE1 [68]. The connection between endosomal recycling and diphthamide biosynthesis is currently unclear and it remains to be determined whether Dph7 is multifunctional or if these other roles are linked to its eEF2 modification function.

Diphthamide on eEF2 is the target for bacterial ADP-ribosylase toxins and also affects toxicity of sordarin and ricin, a ribosome inhibiting protein toxin from plants [70]. Although this emphasizes its pathological relevance, the physiological significance of diphthamide remains enigmatic and elusive. Nonetheless, the evolutionary conservation of the diphthamide pathway among eukaryotes and the embryonic lethality of mice that cannot synthesize diphthamide [38] strongly suggest that it is important in translation related processes. In support of this notion, evidence presented here and by others shows that diphthamide mutants cause increased translational framesshifting, a defect also observed in mammalian cells [6,36,71]. Diphthamide modification may have particular importance in multicellular organisms or when cells are stressed [4]. Mutation of mammalian diphthamide synthesis genes affects cell proliferation and development: inactivation of DPH1/3/ERE1/KTI11 is associated with tRNA modification defects and neurodegeneration and mutations in DPH1/OVCA1 revealed a tumor suppressor role for this diphthamide synthesis gene in ovarian cancer [27,38-40,72]. Regardless of its physiological functions, our data indicate that the diphthamide pathway is more complex than originally anticipated and comprises, in
addition to Dph1-Dph5, two further components, Dph6 and Dph7, which operate in the terminal amidation step (Figure 8). While it is now clear that Dph6 is diphthamide synthetase [61], in the future it will be important to understand why the archael and mammalian orthologs can dispense with the otherwise conserved YigF-YER057c-UK114 domains and to define the precise role of Dph7. It will also be crucial to explore the potential role of diphthine synthase (Dph5) as a potential regulator of the entire pathway and the reasons for apparent lability of diphthine in the dph6 mutant that is suggested by our data (Figure 8).

Materials and Methods

Strains, Media, Growth Conditions, and Growth Assays
Yeast strains used in this study are listed in Table S2 and plasmids in Table S3. Cultures were grown in complete (YPD) or minimal (SD) media [73] at 30°C unless otherwise stated. For phenotypic assays, YPD was supplemented with 10 µg/ml sordarin sodium salt from Sordaria araneosa (Sigma-Aldrich). Yeast transformations with plasmid DNAs were performed following the lithium acetate protocol [74]. Diphtheria toxin (DT) growth assays were performed using one-way ANOVA followed by Dunnett’s multiple comparison post test and was performed with Graphpad Prism 5.0 software essentially as previously described [75]. Gene deletions were confirmed via diagnostic PCR on genomic DNA preparations using target ORF-specific primer pairs (Table S4) as well as sordarin response assays. C-terminal tagging of DPH1, DPH2, DPH5, DPH6/1LR143a and DPH7/1BR246 was performed according to previously published in vivo PCR-based epitope tagging protocols [78] using appropriate S3/S2 primer pairs (Table S4). Tagged genes were confirmed by Western blot detection with anti-HA or anti-c-Myc antibodies (Santa Cruz Biotechnology A-14 and F7, respectively). Detection of HA- or c-Myc-tagged Dph1, Dph2, Dph5, Dph6 and Dph7 as well as Dph3 and Elp2 in co-immune precipitation (Co-IP) assays were performed as previously described [6,77,79].

DPH6 Constructs
pSU6 was generated by insertion into YCplac111 [80] of a genomic PCR fragment including DPH6 together with 829 bp of upstream and 59 bp of downstream sequence flanked by Evori and BomHI sites incorporated using PCR primers (Table S4). The insert was verified by sequencing and shown to complement a dph6 knockout strain. pSU7 was made by cloning the DPH6 insert from pSU6 into YEplac181 [80]. To generate a G216N E220A dph6 mutant, pSU6 was digested with AgeI and BomBI and the small DPH6 fragment replaced by an identical synthetic fragment (Integrated DNA Technologies) carrying the G216N E220A mutations, generating independent clones pMS61 and pMS62. The replaced region was verified by DNA sequencing. pMS67 and pMS68 were generated from pSU6 by replacing the BomBI-SalI fragment carrying the C-terminal region of DPH6 and downstream sequence with a synthetic BomBI-SalI fragment in which codons 335–685 were replaced by sequence encoding the linker and triple myc tag from pYM23 [81]. To generate pMS72, the smaller NheI-SpeI fragment of pSU7 was excised and the large fragment ligated to generate an in-frame fusion that removed DPH6 codons 347–471, checking the resulting fusion by DNA sequencing.

In Vitro ADP Ribosylation Assay
Yeast cell extracts were prepared as described previously [15]. ADP ribosylation reactions were performed at 37°C for 1 hour in a volume of 40 µl ADP ribosylation buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM DTT) containing 30 µg of yeast extract, 50 ng fully-nicked DT and 10 µM 6-biotin-17-NAD (Trevigen). Samples were then mixed with SDS sample buffer, boiled for 5 min and run on 4–25% SDS-PAGE gradient gels (Invitrogen). The proteins were transferred to nitrocellulose.
membranes and Western blotting was performed using streptavidin-IR conjugate (Rockland Immunocchemicals, Gilbertsville, PA) and scanned on an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

Expression and Purification of Affinity-Tagged eEF2-(His)₆
BY4741 wild-type yeast cells as well as dph1, dph5, ylr142w/dph6 and ybr244w/dph7 mutants thereof carrying an ef2 null-allele were transformed with plasmid pTKB612 (a kind gift from A. R. Merrill, University of Guelph, Ontario, Canada), which expresses a (His)₆-tagged form of translation elongation factor eEF2 (Table S3) that is fully functional and able to complement an ef1 ef2 double mutant [36]. In order to express and purify (His)₆-tagged eEF2 for MS/MS analysis, 750 ml of yeast culture were grown in YPD to an OD₆₀₀ 2.0 and harvested by centrifugation. The pellet was resuspended in 3 ml B60 buffer (50 mM HEPES-KOH pH 7.3, 60 mM KOAc, 5 mM Mg(OAc)₂, 0.1% Triton X100, 10% (v/v) glycerol, 1 mM NaF, 20 mM glycercophosphate, complete protease inhibitor [Roche]) without DTT and cells were lysed in a bead beater. The lysate was centrifuged twice at 13,500 rpm for 30 min, and the protein concentration measured with a NanoDrop spectrophotometer. Five mg total protein was applied to 2 mg anti-(His)₆-tag Dynabeads (Invitrogen, Lincoln, NE). The beads were washed three times with lysis buffer and resuspended in 3 ml B60 buffer. The lysate was centrifuged at 13,500 rpm for 30 min. and the protein concentration measured with a NanoDrop spectrophotometer. Five mg total protein was applied to 2 mg anti-(His)₆-tag Dynabeads (Invitrogen, #101-05D) and purified according to manufacturer’s instructions. The identity of purified eEF2 fraction was confirmed by SDS-PAGE and Western blot analysis using an anti-(His)₆ antibody (Abcam, #ab18184).

Analysis of Diphthamide Pathway Modifications on eEF2 by Mass Spectrometry
Crude yeast eEF2 preparations from wild-type and dph mutants strains were separated by SDS-PAGE, using 4–12% Bis-Tris precast gels (Invitrogen, Carlshad, USA) and the area of the gel containing eEF2 was excised after staining with Instant Blue Coomassie (Expedeon, Cambridge, UK). In-gel digests were performed with trypsin, subsequent to reduction and alkylation with diithiothreitol and iodoacetamide, with the resulting peptides cleaned over C₁₈ columns. Peptides were then analyzed via HPLC-MS/MS using a Dionex U300 HPLC (Dionex California) with a 15 cm PepMap C₁₈ column coupled to a Thermo Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptides were eluted from the C₁₈ column at 300 nL/min over 120 min using a linear 5–90% (v/v) acetonitrile gradient. The Orbitrap Velos was operated in positive ion mode, with an ion source voltage of 1.2 kV and capillary temperature 200°C, using a lock mass of 445.120924. The initial survey scan was performed at 60000 resolution, FTMS scanning from 335–1800 Da. The top 15 most intense ions were selected for MS/MS sequencing, using collision-induced dissociation (CID; MS/MS charge state 1+ rejected, >2+ accepted). Protein identification was performed using MaxQuant 1.2.2.5 [82] against a proteome database generated from the *Saccharomyces* Genome database [83]. Manual annotation of the modified peptide spectra corresponding to the modified EF2 peptide and generation of extracted ion chromatograms were done using the Thermo Xcalibur software for spectra visualization.

Supporting Information
Figure S1 MS/MS spectra of unmodified eEF2 peptide 686-VNILDVTHLDAIHHR-700 from wild-type and mutant yeast strains. (A) Cartoon showing how the B and Y ions seen in the MS/MS spectra map onto the tryptic peptide containing His-699. Y1 to Y13 and B14 ions contain His-699 and their m/z values are therefore informative regarding the modification state of His-699. (B–F) MS/MS spectra of unmodified peptide in eEF2 obtained from the indicated yeast strains: the parent ion m/z and charge state is indicated in each case.

Figure S2 Extracted ion chromatograms of unmodified EF2 peptide 686-VNILDVTHLDAIHHR-700. In (A), peaks corresponding to doubly-charged ions (m/z unmodified peptide 843.97, extracted mass range 843.8–844.0) are shown while triply-charged ions (m/z unmodified peptide 562.98, extracted mass range 562.5–563.2) are shown in (B). The yeast strain to which each chromatogram pertains is indicated. Note that in (B) an intensity of 580,000 corresponding to unmodified peptide with m/z 562.98 was not resolved from a different, more abundant ion with m/z 563.02 in the wt sample. Peak annotations are as follows: RT, retention time; AA, peak area; BP, parent ion m/z.

Figure S3 Extracted ion chromatograms of modified eEF2 peptide 686-VNILDVTHLDAIHHR-700. (A) Peaks corresponding to triply-charged ions (m/z diphthamine-modified peptide 610.68, m/z diphthamide-modified peptide 610.35, extracted masses 610.2–610.9). (B) Triply-charged ions (m/z AC-modified peptide 596.66, extracted masses 596.2–596.8). Peak annotations are as follows: RT, retention time; AA, peak area; BP, parent ion m/z.

Figure S4 Failure to detect interaction by TAP-based co-immune precipitation between Dph6 or Dph7 and either Dph2 or diphthine synthase Dph5, factors integral to the first two steps of diphthamide synthesis. Co-immune precipitations were performed using magnetic beads (Dynabeads, Invitrogen) coated to anti-CBP antibodies (Santa Cruz Biotechnology) specific for the calmodulin binding peptide (CBP) of the TAP-tag. The indicated strains expressed DPH6-TAP or DPH7-TAP in conjunction with HA-tagged versions of either DPH2 or DPH5. A strain co-expressing respectively, HA- and TAP-tagged variants of Dph1 and Dph3, step 1 pathway players previously shown to associate with one another [6,20] served as a positive internal control for interaction. The presence of the respective proteins within the immune precipitates (IP) was assessed using anti-HA and anti-CBP Western blots (A) or anti-HA immune blots on total protein extracts obtained prior to the IP protocol (preIP, B). Asterisks indicate breakdown products of Dph2-HA, Dph3-TAP and Dph6-TAP.

Figure S5 Failure to detect Dph6-Dph7 interaction by co-immune precipitation. Co-immune precipitations using the anti-HA-antibody were performed with the indicated strains expressing DPH6-c-myc or DPH7-c-myc on their own or in parallel with HA-tagged versions of DPH2 or DPH5, respectively. A strain co-producing c-Myc- and HA- and tagged versions of the Elp2 subunit (ELP2-c-myc) of the elongator complex, and Kit12 (KTI12-HA), a protein known to interact with ELP2 [84], was used as internal positive control. The presence of the respective proteins was assessed in individual anti-c-Myc and anti-HA immune blots (top two panels) and crude extracts (pre IP; bottom two panels). The asterisk denotes an unspecific band that originates from the anti-HA-antibody present in the IPs.

Figure S6 Lack of effect of dph1-dph7 gene knockouts on growth performance and viability. (A) The wild-type parental strain and diphthamide deficient mutants dph1, dph6 and dph7 were grown in YNB minimal media supplemented with His, Met, Ura, Leu to
cover the auxotrophic markers (Table S2) under standard laboratory conditions over a period of 50 h. OD_{600} was monitored at 2 h intervals. (B) To address a potential temperature sensitive phenotype, ten-fold serial cell dilutions of the indicated strains were spotted on YPD plates and grown at 30°C or 39°C. Note that only the dph4/dph5 ORF, which affects additional biosynthetic pathways [8,15] apart from diphthamide biosynthesis [13] shows temperature sensitivity (S) while the other dph mutants tolerate high temperatures (T).

**Figure S7** Altered growth performance of dph1-dph7 mutants in response to translation elongation indicator drugs under standard or elevated cultivation temperatures. Ten-fold serial cell dilutions of wild-type parental strain as well as diphthamide mutants dph1-dph7 were replica spotted on YPD plates without (control) and supplemented with hygromycin (20 µg/ml), anisomycin (20 µg/ml) or paromomycin (1.5 mg/ml) and grown at 30°C (A) or 37°C (B). Reduced or improved performance of the dph mutants relative to wild-type behavior reflects respectively, enhanced sensitivity or improved tolerance towards the drug in question respectively.

**Figure S8** Conservation of the DPH6 gene product, Dph6. (A) Representation of Dph6 indicating the conserved adenine nucleotide alpha hydrolase (cd1994) and YigF-YER057c-UK114 related (cd06155, cd06166) domains discussed in the main text. (B) The Dph6 amino acid sequence was aligned using Clustal with representative examples of putative orthologs from other organisms (identified by PSI-BLAST). Sequences are as follows (database accession numbers in parentheses): DPH6, S. cerevisiae Dph6/Ylr143w; Sp_mug71, Schizaosachromyces pombe (NP 595310); At_AAH_H_4, Arabidopsis thaliana endoribonuclease (NP 187098); DI_AAH_H_4, Dictyostelium fasciculatum endoribonuclease L-PSP domain-containing protein (EGG21287); XI_AAH_H_4, Xenopus laevis ATP binding domain 4 (NP 001085655); Hs_AAH_H_4, Human ATP binding domain containing protein 4 (NP 543281); Mm_AAH_H_4, mouse ATP binding domain containing protein 4 (NP 079951); Hs UK114, human ribonuclease UK114/p14.5/L-PSP (NP 005827); Mm UK114, mouse UK114/p14.5/L-PSP (NP 0052313). Note that the last two sequences appear twice in the alignment so that the sequence relationships to each of the YigF-YER057c-UK114 related domains in the non-mammalian proteins can be shown. *, conserved residues shown to be important for trimerisation and ligand binding [63,66].

**Figure S9** Conservation of the DPH7 gene product, Dph7. (A) Representation of Dph7 showing the location of the conserved WD40 domains. (B) The Dph7 amino acid sequence was aligned using Clustal with representative examples of putative orthologs from other organisms (identified by PSI-BLAST). Sequences are as follows (database accession numbers in parentheses): DPH7, S. cerevisiae Dph7/Ybr246w Sp_WD85, Schizaosachromyces pombe WD repeat protein (CAA21429); At_WD85, Arabidopsis thaliana WD40 domain-containing protein (NP 211016); Dd_WD85, Dictyostelium discoideum WD40 repeat-containing protein (XP 646601); Xr_WD85, Xenorhopus tropicalis WD repeat-containing protein 85-like (XP 002942023); Hs_WD85, Human WD repeat-containing protein 85 (NP 620133); Mm_WD85, mouse unnamed protein (BAE 92074).

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**Author Contributions**

Conceived and designed the experiments: SU CB VS SL StH MJRS RS. Performed the experiments: SU CB VS SL StH. Analyzed the data: SU CB VS SL StH FG MJRS RS. Contributed reagents/materials/analysis tools: FG MJRS RS. Wrote the paper: FG MJRS RS. Contributed analysis tools: FG MJRS RS. Contributed advice and assistance in SGA data analysis. In addition we thank Dr. Natascha Mumday and Dr. Wael Abdel-Fattah for technical assistance, and Jonas Teunen for help with Figure 8.

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