New Genes Involved in the Synthesis of Diphthamide, a Modification of Eukaryotic Translation Elongation Factor 2 with Roles in Diphtheria Disease and Ovarian Cancer Formation

A thesis submitted for the degree of Doctor of Philosophy

by

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Department of Genetics
University of Leicester
November 2012
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ABSTRACT

Diphthamide, the target of Diphtheria toxin, is a unique post-translational modification on His699 (S. cerevisiae) of translation elongation factor 2 (eEF2) found in eukarya and archaea. It serves as the unique target for bacterial ADP-ribosylating toxins such as Diphtheria Toxin, Exotoxin A and Cholix toxin. So far six genes have been known to be involved in the complex three-step biosynthesis pathway: bona fide diphthamide genes DPH1-DPH5 and the recently identified YBR246w. While the latter was shown to be involved in the final step of the pathway, its exact role remains unclear. Dph1-Dph4 facilitate the initial step of the pathway and the methytransferase, Dph5, the second step. Surprisingly, after almost four decades of intensive research the enzyme catalyzing the final step, the conversion of the intermediate diphthine into the final product diphthamide, has remained elusive. We sought to exploit yeast genetic interaction and chemical genomic databases in order to identify novel diphthamide biosynthesis genes. A novel candidate gene YLR143w was identified and we here present genetic, phenotypic and biochemical analyses that clearly identify YLR143w as a novel diphthamide biosynthesis gene. Our observations implicate that YLR143w is the main catalytic enzyme necessary for the third step of the pathway, while YBR246w has a regulatory role involving Dph5-EF2 interaction.

Furthermore, we demonstrate that Dph1 is likely the primary catalytic enzyme which generates the initial modification on the His699 residue.

In addition to the implications in bacterial pathogenesis, diphthamide and the biosynthesis genes DPH1, DPH3 and DPH4 are associated with cancer formation as well as defects in embryonic development and cell proliferation control. We here demonstrate that diphthamide deficient yeast cells display a significant increase in -1 frameshifting during translation and propose that this is the underlying cause of the phenotypes seen in mammalian organisms.
New Genes Involved in the Synthesis of Diphthamide, a Modification of Eukaryotic Translation Elongation Factor 2 with Roles in Diphtheria Disease and Ovarian Cancer Formation

Shanow Uthman

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Publications


Scientific Meetings


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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ART</td>
<td>ADP-Ribosylating Toxins</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>co-IP</td>
<td>co-immunoprecipitation</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPT</td>
<td>Diphtheria–Pertussis–Tetanus</td>
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<td>DT</td>
<td>Diphtheria toxin</td>
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<td>DTT</td>
<td>Dithiotreitol</td>
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<tr>
<td>ECL</td>
<td>Electrognerated chemiluminescence</td>
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<tr>
<td>HCl</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>His</td>
<td>Histidine</td>
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<td>kilobases</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>LB</td>
<td>Luria Bertani Medium</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
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<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
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<tr>
<td>ONPG</td>
<td>Ortho-Nitrophenyl-β-galatoside</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<td>PAGE</td>
<td>Polyacrylamide gelelectrophoresis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PEG</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylfulfonylfluoride</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SD</td>
<td>Synthetic dropout</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
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<td>Tris Borate EDTA</td>
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<tr>
<td>UV</td>
<td>Ultraviolet light</td>
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<tr>
<td>YEPD</td>
<td>Yeast extract peptone dextrose medium</td>
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CHAPTER 1

INTRODUCTION
1 Introduction

1.1 Post-translational modifications - diversifying the proteome

One of the fundamental laws of genetics postulates that DNA is transcribed into RNA and RNA in turn is translated into protein. It is the basis of molecular biology and a beautifully simple principle, but soon becomes very complex when looked upon in detail. The end product of this cycle is the formation of proteins, which carry out most of the work in a cell. Proteins determine cell structure, function and regulation and therefore the synthesis of proteins is a fundamental process to every living organism.

The regulation of gene expression is highly sophisticated and allows the translation of genes into their gene products. Every single step from DNA to RNA and the final protein product is meticulously regulated in order to ensure proper cell function. First, transcription initiation at alternative promoters allows the tissue specific expression of genes and together with differential regulation of transcription termination represents important mechanisms of gene expression at the DNA level (Ayoubi and Van De Ven 1996). Next, alternative mRNA splicing delivers several isoforms of proteins translated from the same template RNA and finally alterations of the freshly synthesized protein, which undergoes so called post-translational modifications (PTMs). The regulation of these three main stages of translation enables the increase of complexity from the genome with ~25,000 human genes to the transcriptome (~100,000 transcripts) and finally an estimated one million proteins in the proteome (Jensen 2004). Here we
focus on post-translational modifications (PTM) which in contrast to the first two steps of gene expression regulation, present a much faster way for the cell to respond to intracellular and extracellular signals. PTMs are chemical changes to the structure of the newly synthesized protein thereby regulating its function. The importance of PTMs is reflected in the number of genes that are assigned to them. The human genome includes more than 500 hundred protein kinases, more than 500 protein phosphatases and numerous methyltransferases, ubiquitinyl ligases, acetylases and deacetylases (reviewed by (Lin H. 2008; Lin and Begley 2011). In general, PTMs are additions of functional groups to side chains of amino acids in a protein, which in turn determines its activity, localization and interaction with other components of the cell. PTMs are generated by enzymatic activity and can occur at any point – right after translation the chemical modification of the newly synthesized peptide ensures the proper folding and localization of the protein. Kinases and proteases on the other hand act as an ON/OFF switch and shift the protein from an active into an inactive state. These are reversible forms of PTM whereas the irreversible addition of tags, such as during ubiquitination, is irreversible and leads to the degradation of the tagged protein. Errors in the vast network of PTM can impair protein function and even lead to disease, such as the deregulated phosphorylation cascade in the MAPK/ERK pathway, which is implicated in the formation of many cancers. Therefore investigating and targeting PTM provide us with invaluable therapeutic approaches for heart diseases, cancer and neurodegenerative diseases. Inhibitors of kinases and proteases for example have been used as a treatment for cancer, inflammation as well as infection by viruses such as HIV (reviewed by (Lin and Begley 2011).
PTMs can be classified according to their underlying chemistry (phosphorylation, acetylation, methylation, etc.), the target residue (Ser/Thr, Lys, Arg, etc.) or the biological function they regulate. The latter includes changes that act as an ON/OFF switch for enzymatic activity, the addition of protein tags like ubiquitin, creation or masking of recognition sites and the cleavage of proteins into smaller fragments (proteolysis). The list of PTM and the resulting amino acid variations is very long, some of which are more common and others are unique to a single protein (reviewed by (Lin H. 2008).

1.2 Diphthamide, a peculiar amino acid variation

Translation elongation factor 2 (EF2) from both archaeb and eukayotes (eEF2) carries a unique PTM on His\(^{699}\) in yeast and His\(^{715}\) in mammalian cells, called diphthamide (Figure 1.1) (Van Ness et al. 1980b; Moehring et al. 1984; Chen et al. 1985b). It is an unusual amino acid variation, not only because it is exclusively found on eEF2, but also because it is generated in a complex pathway involving enzymes encoded by more than five diphthamide biosynthesis genes, DPH1-DPH5 (Pappenheimer 1977; Chen et al. 1985b; Liu and Leppla 2003a). In 1974 Robinson et al. first reported the discovery of this histidine derivate as the unique target for diphtheria toxin, which hence was named diphthamide (Robinson et al. 1974; Van Ness et al. 1980b). Even though diphthamide was identified almost 4 decades ago, its role in cell physiology remains elusive. However, it is associated with several distinct phenotypes which will be discussed here. Adding to its peculiar nature is that diphthamide is subject to a secondary PTM: it can be mono-ADP-ribosylated by
endogenous and exogenous ADP-ribosyltransferases (ARTs) (Figure 1.3) (Honjo et al. 1968; Van Ness et al. 1980a; Sitikov et al. 1984; Fendrick and Iglewski 1989a). In contrast to the unique diphthamide, mono-ADP-ribosylated proteins can be found in every major compartment of the cell, which highlights its diverse application as a PTM. In the case of eEF2, the tandem of PTMs (diphthamide formation and its consequent ADP-ribosylation) renders the translation factor inactive, which in turn arrests de novo protein synthesis and ultimately leads to cell death (Pappenheimer 1977; Oppenheimer and Bodley 1981).
Figure 1.1. Ribbon diagram of ADP-ribosylated eukaryotic translation elongation factor 2 (eEF2) from *S. cerevisiae* (Jorgensen et al. 2006). eEF2 contains 6 domains (I-V and G’), which move relative to each other in order to perform conformational changes essential for eEF2 function. The diphthamide modification is located at the tip of domain IV, whereas the binding site for the antifungal sordarin (black) is at the interface between domains III, IV and V. Normal eEF2 function requires binding of GDP (yellow) to its binding site in domain I. ADPR-DIPH, ADP-ribosylated diphthamide.
1.3 Mono-ADP-ribosylation of diphthamide - friend or foe?

If ADP-ribosylation (ADP-R) of diphthamide causes translation inhibition and results in cell death, why would the cell put so much effort into the formation of a complex modification like diphthamide in the first place? The answer lies in the difference of the nature of the ADP-R catalyzed by cellular ATRs (ADP-ribosyl transferases) versus exogenous ARTs. While the endogenous process is reversible, ADP-R via exogenous transferases like diphtheria toxin is an irreversible PTM (Pappenheimer 1977; Corda and Di Girolamo 2003). It has been proposed that the reversible manner of cellular diphthamide ADP-R could act as an ON/OFF switch for eEF2 function, which could serve as a regulatory mechanism during translation. This putative regulatory role for diphthamide is supported by the discovery of endogenous ADP-ribosylation of eEF2 in the absence of bacterial toxins (Fendrick and Iglewski 1989b; Fendrick et al. 1992). Recently, Jaeger et al. reported that inteleukin-1β (IL-1β) ADP-ribosylates diphthamide in cardiomyocytes in a similar way to toxin mediated ADP-ribosylation and thereby might regulate eEF2 function (Jager et al. 2011). However, cellular mono-ADP-ribosylation of diphthamide is still poorly understood and deserves further attention before we can draw conclusions regarding the diphthamide modification. Nevertheless, mimicking this process allows bacterial toxins to target a vital cellular function and blocking it in an irreversible manner. What we know about diphthamide to date mainly stems from studies on bacterial protein toxins, therefore it seems compelling to take a closer look at microbial pathology.
1.4 Microbial Competition

Microorganisms have developed various mechanisms to secure survival in competitive environments. Amongst them is the well-known strategy of bacterial toxin secretion, which allows the toxin producing organism to compete and flourish in the microbial jungle. They are classified as endotoxins, which act in the close proximity of the bacterial cell wall, and exotoxins, which are secreted in order to attack cellular components of competitors at a remote site. Based on the molecular mechanism of action, the latter can be allocated into specific categories, such as the rather large group of ADP-ribosyltransferase (ART) family of bacterial toxins. ARTs act by blocking specific components of the host’s translational machinery such as mRNA synthesis at the initiation, elongation and termination step or the general ribosome machinery including rRNAs and tRNAs. Diphtheria Toxin (DT) from *Corynebacterium diphtheriae*, Exotoxin A (ETA) from *Pseudomonas aeruginosa* and Cholix Toxin from *Vibrio cholerae* make up the DT group of ADP-Ribosylating toxins, which shares a specific target protein: translation elongation factor 2 (eEF2) (Deng and Barbieri 2008; Uthman S. 2011). These DT toxins belong to the two-component A-B family of pathogenic bacterial toxins, where initially the B domain facilitates binding to surface receptors of the host cell and upon uptake is followed by the enzymatic action of the A domain. This enzymatic activity involves the above mentioned covalent transfer of a single ADP-Ribose moiety from NAD$^+$ to the diphthamide residue on eEF2 (Figure 1.1). The mechanistic action of DT, ETA and cholix toxin involves three main steps: receptor-mediated endocytosis, release of the catalytic fragment into the cytoplasm by cleavage of the disulfide bridge between the A and B components and finally the ADP-
ribosylation of the target eEF2 protein. In order to generate the ADP-R, the enzyme first binds and cleaves NAD\(^+\) between the nicotinamide and the nicotinamide-ribose to sever an ADP-Ribose group and consequently transfer it to diphthamide on eEF2 (Yates et al. 2006). By covalent modification of a key player of the translational machinery, these microorganisms have developed a highly efficient way of eliminating their competitors. In fact, the uptake of a single molecule of DT is sufficient to kill a cell (Yamaizumi et al. 1978). Interestingly, eubacteria do not carry the diphthamide modification on their eEF2 homologue (EF-G), hence the toxin attacks the target cell without affecting the protein synthesis of toxin producing bacteria (Collier 2001). The very similar mode of action between DT, ETA and cholix toxin that share ART activity and the same target protein, suggests that they share functional domains. However, sequence analysis of DT and ETA reveals low similarity (20% sequence identity) within their catalytic domains and therefore BLAST search for cellular ARTs does not generate any putative hits (Yates et al. 2006).

The prototype and best characterized member of bacterial ADP-ribosylating toxins is diphtheria toxin (DT), the causative agent of diphtheria disease in humans. DT is secreted by the gram-positive bacterium, Corynebacterium diphtheriae, and causes respiratory and cutaneous lesions that in severe cases can cause life-threatening complications such as loss of motor function, myocarditis and peripheral neuropathy. In particular, human skin, throat and pharynx can be colonized by C. diphtheriae biotypes gravis, intermedius and mitis which differ in colony morphology, growth and virulence properties. The infectious nature of the pathogen caused epidemics prior to the routine use of the Diphtheria–Pertussis–Tetanus (DPT) vaccine, which now has nearly
eradicated new diphtheria disease incidences (reviewed by (Pappenheimer 1977; Murphy 1996).

Another prominent member of the bacterial A-B toxin family is Exotoxin A (ETA) secreted by the gram-negative bacillus *P. aeruginosa*. This prevalent pathogen is associated with multi-drug-resistant infections including urinary tract infections, pneumonia and sepsis in immuno-compromised patients. In fact it is the primary cause of death in individuals with cystic fibrosis. In contrast to diphtheria disease, there is no preventive vaccine available for *P. aeruginosa* infections which together with its resistance towards many antibiotics makes it a potent human pathogen ((Deng and Barbieri 2008). The prevalent and opportunistic nature of ETA stresses the significance of current research centered around the diphthamide modification on eEF2.

1.5 eEF2 - key player in the translation machinery

Eukaryotic translation elongation comprises the formation of the newly synthesised polypeptide on the ribosome with the help of two highly conserved GTPases - translation elongation factors eEF1A and eEF2. While eEF1A is responsible for the selection of the aminoacyl-tRNA and its delivery to the acceptor site (A-site) of the ribosome, eEF2 facilitates the translocation of the peptidyl-tRNA from the A-site to the P-site (peptidyl site) as well as the simultaneous translocation of the deacylated tRNA from the P-site to the E-site on the 80S ribosome thereby exposing the A-site for a consecutive round of polypeptide elongation (Gomez-Lorenzo et al. 2000; Taylor et al. 2007). In detail, once translation is initiated, the 80S ribosome carries an mRNA with its start codon and the corresponding methionine-tRNA at the P site of the
ribosome. The GTPase, eEF1A binds an aa-tRNA and transfers it to the A site of the ribosome. To do so the anticodon of the aa-tRNA binds the codon sequence of the mRNA. Next, ribosomal RNA in the peptidyl transferase center (PTC) facilitate peptide bond formation between the newly incorporated amino acid and the methionine, which is bound to the tRNA in the P site. Upon GTP hydrolysis eEF1A in its GDP bound state is released to covalently bind another aa-tRNA, which is dependent on a guanine nucleotide exchange factor to transform GDP-eEF1A to its GTP bound form (Dever and Green 2012). In order to translocate the bound tRNAs, the 40S and 60S subunits of the ribosome perform a rapid rotation motion relative to each other. This motion is dependant on the action of eEF2, which reaches into the decoding centre of the ribosome and upon GTP hydrolysis undergoes conformational changes that unlock the ribosome and allow the ratcheting movement of the subunits (Dever and Green 2012). Once the tRNAs are moved from the A to the P site and from the P to the E site (exit site), eEF2 locks the ribosomal subunits again for the next elongation cycle. At the end of the translocation step, the deacylated-tRNA occupies the E site, the peptidyl-tRNA the P site and the A site is free for the next round of the peptide elongation. Interestingly, yeast and fungi have a third translation elongation factor, eEF3, an ATPase with 2 ATP binding domains. eEF3 has been reported to bind to the 80S ribosome in its post-translocation stage (Andersen et al. 2006). The exact role of eEF3 is unclear, however it was shown to bind the 60S as well as the 40S subunit (where exactly) as well as the E site and therefore has been suggested to aid the dissociation of the de-acylated tRNA from the ribosome (Triana-Alonso et al. 1995; Andersen et al. 2006). To date, no homolog of eEF3 has been found in other species and though yeast
eEF1a and eEF2 can complement their mammalian counterparts, they in return cannot facilitate translation elongation in yeast in the absence of eEF3 (Skogerson and Engelhardt 1977). Translation elongation is a highly conserved process from bacteria to eukaryotes, however it is unclear why fungal protein synthesis selectively is dependant on the action of a third elongation factor, eEF3 (Rodnina and Wintemeyer 2009).

The accuracy and efficiency of the eEF2 mediated translocation of tRNAs within the ribosome is fundamental to the translation machinery. eEF2 is composed of six domains, I-V and G’ that can move relative to each other, a property central to the enzymatic activity of eEF2 (Figure 1.1). Upon GTP hydrolysis eEF2 as well as the small and large ribosome subunits undergo large-scale conformational changes in order to facilitate the translocation of the mRNA and tRNAs relative to the ribosome (Aevarsson et al. 1994; Jorgensen et al. 2003). The diphthamide modification on eEF2 is located at the tip of domain IV, which reaches into the ribosomal decoding center. In fact, cryo-electron microscopy reconstruction of ribosome bound eEF2 demonstrated that the diphthamide containing loop of yeast eEF2 (His694-Ile698) is close enough to interact with the codon-anticodon interface between the P-site bound tRNA and mRNA (Figure 1.2) (Agrawal et al. 1999; Stark et al. 2000; Spahn et al. 2004). It has been proposed that the movement of diphthamide on eEF2 from the GTP- to GDP-bound state would disrupt the interaction between the decoding center and the mRNA-tRNA duplex during the translocation cycle, a property that contributes to the maintenance of the correct reading frame (Taylor et al. 2007). In line with this, data from Ortiz et al. and our own group showed that amino acid substitutions (D696A, I698A, H699N) as
well as the loss of the diphthamide modification on His699 alone showed an increase in -1 frameshifting (Ortiz et al. 2006a; Bar et al. 2008).

![Figure 1.2. Cryo-EM reconstruction of ADP-ribosylated eEF2 in complex with the ribosome](image)

Cryo-EM derived densities are displayed in computationally separated structures of the 60S (grey) and 40S (yellow) ribosomal subunits, P/E site tRNA (green), ADP-ribosylated eEF2 (red) and the ADP-Ribose moiety is circled. The images visualize how close the tip of domain IV of eEF2 (with the diphthamide modification) reaches to the tRNA in the ribosomal decoding centre.

### 1.6 The multi-step pathway of diphthamide biosynthesis

The unique posttranslationally modified histidine residue, diphthamide or 2-[3-carboxyamido-3-(trimethylammonio)-propyl] histidine, is highly conserved from lower archaea to humans (Figure 1.3). It is found on His600 in the archaeon *Pyrococcus*
*horikoshii*, His\(^699\) in yeast and His\(^715\) in mammalian eEF2 (Moehring et al. 1984; Chen et al. 1985b; Liu et al. 2004b). The biosynthesis of diphthamide is a multi-step process of addition of chemical groups to the histidine precursor in eEF2. Initially a 3-amino-3-carboxypropyl group (ACP) from S-adenosylmethionine (SAM) is transferred to the C-2 position of the imidazole ring of the histidine residue resulting in the first product, the ACP-intermediate. The SAM-dependant tri-methylation of the amino-group follows and produces the second intermediate, diphthine (Chen and Bodley 1988; Mattheakis et al. 1992). In the 3\(^{rd}\) and final step, the carboxyl group of the diphthine intermediate is amidated in an ATP-dependant manner, which completes the diphthamide modification (Liu et al. 2004b). The first 2 steps of the PTM are dependent upon SAM as a donor for functional groups: the ACP group and the methyl group. The genes involved in diphthamide synthesis were first identified in genome-wide screens for diphtheria toxin (DT) and *Pseudomonas* Exotoxin A (ETA) resistant mutants in yeast and CHO (Chinese hamster ovary) cells (Moehring et al. 1984; Chen et al. 1985a). The loss of diphthamide as a recognition motif makes them resistant to DT and ETA, and therefore resistant mutants can be considered to be involved in the synthesis of the PTM. DT resistant mutants were classified into 5 complementation groups, *dph1-dph5*. These diphthamide biosynthesis genes were assigned to the first 2 steps of the pathway, with *dph1-dph4* mutants lacking any modification on His\(^699\) and *dph5* blocking the modification after the formation of the ACP-intermediate. However, no mutants were found with the diphthine intermediate, most likely because diphthine can still be ADP-ribosylated by DT though it is a poor substrate compared to the final diphthamide modification (Moehring et al. 1984). Therefore the amidase at the end of the pathway
has repeatedly escaped DT and ETA screening. As for the other *dph* genes and their protein products, Dph1-Dph4 facilitate the initial transfer of the ACP group followed by the trimethylation by Dph5.

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Figure 1.3. The proposed biosynthesis pathway and ADP-ribosylation of diphthamide (Zhang et al. 2010). The posttranslational modification of the histidine residue (*S. cerevisiae* His<sup>699</sup>) on eEF2 is a 3 step process starting with the initial transfer of a 3-amino-3-carboxypropyl (ACP) group to the C2 position of the imidazole ring. Four protein products, Dph1-Dph4, are involved in the formation of the resulting ACP-intermediate, which further undergoes trimethylation by the methyltransferase, Dph5, and results in the second intermediate, diphthine. A yet unknown amidase generates the final diphthamide product by amidating the carboxyl group of diphthine. Diphthamide
is subject to a tandem posttranslational modification: ADP-ribosylation by bacterial toxins (diphtheria toxin, Pseudomonas exotoxin A and cholix toxin) and endogenous ADP-ribosyltransferases. eEF2, translation elongation factor 2.

1.7 **Archael diphthamide synthesis**

In an effort to further dissect the pathway, the archaeon *Pyrococcus horikoshii* (*P. horikoshii*) was used as a model organism for *in vitro* reconstruction of diphthamide formation. Two homologues of the yeast *Dph* genes can be found in *P. horikoshii*, termed *PhDph2* and *PhDph5*. PhDph2 forms a homodimer with 3 conserved cysteine residues (Cys59, Cys163 and Cys287), which are grouped together in the center and serve as binding sites for a [4Fe-4S] cluster (Figure 1.4). The cleavage of the ACP group from SAM is catalyzed by such a [4Fe-4S] cluster in the reduced state. Each PhDph2 can bind one [4Fe-4S] cluster, however only one such cluster is sufficient for the reaction (Zhang et al. 2010; Zhu et al. 2011). Interestingly, PhDph2 is more similar to *S. cerevisiae* Dph1, however Dph1 and Dph2 are homologous to each other. Therefore it was proposed that the archael PhDph2 homodimer evolved into a Dph1-Dph2 heterodimer in eukaryotes. The cleavage of an ACP radical from SAM and the formation of a C-C bond with the imidazole ring of His	extsuperscript{600} is followed by the activity of the archael diphthine synthase, PhDph5 (Zhu et al. 2010). This methyltransferase was shown to be sufficient to catalyze the mono-, di- and trimethylation in *P. horikoshii* with SAM as methyl donor. Interestingly, even though the reconstitution of archael diphthine synthesis was successful, Zhu et al. (2010) reported that the resulting trimethylamino group is readily eliminated *in vitro*. It will be interesting to see if this holds true for eukaryotic diphthine.
Figure 1.4. Ribbon diagram of PhDph2 homodimer and stereoview of the electron density assigned to a bound [4Fe-4S] cluster (Taylor et al. 2007). A. *Pyrococcus horikoshii* Dph2 forms a homodimer with 3 conserved cysteine residues (Cys59, Cys163 and Cys287) clustered in the center. B. Structural representation of a [4Fe-4S] cluster bound to the 3 cysteine residues of a single PhDph2 peptide. Fe: orange, S: yellow.
1.8 Dph3 and Dph4 – two versatile little proteins

Work from our own group and others has established that yeast Dph1, Dph2 and Dph3 interact with each other and form a complex in order to catalyze the formation of the ACP-intermediate (Fichtner et al. 2003b; Liu et al. 2004b; Baer et al. 2008; Zhang et al. 2010). Dph4, a J-domain protein, has been proposed to chaperone the correct assembly of the complex and its individual components. Type III J-proteins are a class of heat shock proteins that act as co-chaperones for Hsp70. In this role, Dph4 stimulates the ATPase activity of Hsp70 and is therefore involved in the chaperone regulation cycle (Thakur et al. 2012). The C-terminus of Dph4 contains a CSL-domain similar to full-length Dph3, allowing them to bind zinc and iron. Both Fe-Dph3 and Fe-Dph4 are redox active, which in light of the very recent findings in archael in vitro studies, might suggest that they act as electron reservoir for the [4Fe-4S] cluster thereby transforming it into the reduced state necessary for the cleavage of the ACP radical (Sun et al. 2005; Wu et al. 2008; Thakur et al. 2012). Moreover, their iron binding property indicates a putative iron donor role for the formation of the [4Fe-4S] cluster. Furthermore, Dph3 has been implicated in multiple biological processes. DPH3 is allelic with KTI11 (Kluyveromyces lactis killer toxin zymocin insensitive gene 11) and is a partner protein of the Elongator complex in yeast (Fichtner et al. 2003b). This Elongator complex has a diverse set of applications including the post-translational modification of tRNAs at the wobble position of the anticodon. Loss of Dph3 disrupts Elongator function and the consequent lack of tRNA modification renders the mutants resistant to the killer toxin zymocin. Therefore dph3 deficient mutants are not only resistant to bacterial toxins but also the yeast killer toxin zymocin. Amongst the five diphthamide synthesis genes, dph3
deletion shows the most severe growth defects suggesting an involvement in multiple pathways (Bar et al. 2008). In line with this, the human homologue of Dph3 is DelGIP1 (DelGEF interacting protein 1), which interacts with deafness locus associated putative guanine nucleotide exchange factor. The Dph3-DelGEF complex is involved in the regulation of the secretion of proteoglycans which increases with the downregulation of human Dph3 (Sjolinder et al. 2002; Sjolinder et al. 2004). In summary, both Dph3 and Dph4 are essential for diphthamide formation but are also involved in multiple biological processes including regulation of the chaperone cycle, tRNA modification and secretion of proteoglycans.

1.9 YBR246w – the new guy in the neighbourhood

As mentioned above, the amidation of the carboxyl group in diphthine is most likely an enzymatic and ATP-dependant reaction with a yet unknown amidase that successfully escaped DT and sordarin screens for more than three decades. Recently, Carette et al. (2009) reported the finding of a novel putative diphthamide biosynthesis gene in human cells: mammalian WDR85 and yeast YBR246w were proposed to act at the first step of the modification by aiding the formation of the ACP intermediate together with Dph1-Dph4. (Carette et al. 2009) identified WDR85 in a genetic screen for mutants resistant to diphtheria and anthrax toxin in a human chronic myeloid leukemia cell. In contrast, more recent work by (Su et al. 2011) suggests the involvement of YBR246w in the final step of diphthamide formation. Mass spectrometric analysis revealed that the diphthamide pathway only progresses as far as diphthine in ybr246wΔ yeast strains. However, the exact role of YBR246w is still unkown. In parallel to the
above-mentioned findings, we identified \textit{YBR246w} as a putative \textit{DPH} gene in an independent search for novel candidate diphthamide genes and investigated its involvement in diphthamide biosynthesis. Our data is in agreement with Su at al. (2011) and will be discussed in chapter 3 and 4.

\textbf{1.10 Sordarin and ricin, two powerful diphthamide indicator drugs}

In addition to being the unique target for ADP-ribosylating bacterial toxins, diphthamide is also associated with the lethal properties of two toxic compounds sordarin and ricin. Not only does this indicate the significance of diphthamide in other biological processes, but also both agents are useful analytical tools for investigating the diphthamidation state of eEF2.

The antifungal compound sordarin is a product of the fungus \textit{Sordaria araneosa}. Similar to bacterial toxins it targets the translation machinery, specifically the fungal elongation step. As mentioned above, eEF2’s translocation activity depends on conformational changes of the protein. Sordarin targets eEF2 directly and binds at the interface between domain III, IV and V (Figure 1.1) (Dominguez et al. 1999; Jorgensen et al. 2003). Upon binding it cross-links eEF2 with the ribosome thereby immobilizing the elongation factor and inhibiting protein synthesis. Interestingly, a chemical genomic screen of the haploid yeast deletion mutant collection revealed that diphthamide formation is necessary for sordarin to target eEF2. Therefore \textit{dph1-dph5} deficient cells lacking diphthamide are not only resistant to DT but also the antifungal translation
inhibitor, sordarin (Dominguez et al. 1999; Baer et al. 2008; Botet et al. 2008). Even though the binding site for sordarin is remote to the tip of domain IV, the lack of diphthamide as a recognition motif allows eEF2 to escape the inhibitory action of sordarin. In contrast to DT, selection for sordarin resistant mutations in *S. cerevisiae* generated a rather large list of genes (Botet et al. 2008). Nevertheless, many of them can be associated with the primary target eEF2 or other ribosomal proteins. Together with DT, sordarin has proven to be a valuable analytical tool for investigating the diphthamide modification. The cross-linking property of sordarin has been used in extensive cryo-EM studies to visualize stalled ribosomes and eEF2 conformations in different environments.

Another cytotoxic protein with relevance to diphthamide is the ribosome inactivating protein (RIP), ricin, from the plant *Ricinus communis*. Similar to the bacterial DT, ETA and cholix toxin it consists of 2 peptide chains, A and B, that harbour the enzymatically active (A) and the receptor-binding (B) properties of the protein toxin. It acts on the ribosome by breaking the 28S ribosomal RNA at a specific adenosine residue (A4324 in rat 28S rRNA). This region, termed sarcin/ricin domain, is also a binding site for eEF2. In fact, higher concentrations of eEF2 were shown to protect the 28S rRNA from the toxic cleavage by ricin (Fernandez-Puentes et al. 1976; Brigotti et al. 1989; Holmberg and Nygard 1994). Recently, Gupta et al. (2008) reported that this protective action of eEF2 is dependant on the diphthamide modification. CHO cells lacking Dph2, Dph3 and Dph5 were shown to be hypersensitive to the cytotoxic action of ricin (Gupta et al. 2008). Complementation and therefore reintroduction of the diphthamide modification regained wild-type levels of ricin tolerance. The difference in
ricin sensitivity of unmodified versus diphthamide eEF2 was threefold. This presents a novel role for diphthamide in cytoprotection of the ribosome towards RIPs.

1.11 Diphthamide mouse models

Despite the fact that Dph1-Dph5 are conserved in all eukaryotic organisms, the physiological role of diphthamide remains elusive. However mouse models of DPH1, DPH3 as well as DPH4 have proven a crucial role for the diphthamidation of eEF2 in early development. All three diphthamide deficient mouse models display distinct phenotypes where the homozygous deletion is embryonically lethal (Chen and Behringer 2004a; Liu et al. 2006; Webb et al. 2008). In agreement with the findings in yeast, dph3/− mice show the most severe phenotype. They show a severe delay in embryonic development (2 - 3 days) and embryonic lethality by day 11.5. Furthermore, the embryos display increased degeneration and necrosis in neural tubes alongside with abnormal placenta development (Liu et al. 2006). dph1/− and dph4/− mice also display developmental delays of about 1 day and prenatal lethality. Interestingly they also share another distinct phenotype that is indicative of errors in the translation of specific proteins: the embryos from both homozygous mutations display preaxial polydactyly (duplication of the digit 1 of the hind foot) (Webb et al. 2008). The mammalian homologue of DPH1, OVCA1 (ovarian cancer gene one), is a tumour suppressor gene and is closely linked to the formation of ovarian and other cancers. The heterozygous Ovca1+/− shows an increase in tumour development, predominantly lymphomas in contrast to ovarian tumours in humans. Consistent with this, mouse embryonic
fibroblasts (MEFs) from a $Ovca1^{+/−}$ background show cell proliferation defects (Chen and Behringer 2004a). In general, the role of diphthamide seems to become more apparent during early development, where cell proliferation is maintained at a maximum rate and is dependent on high translation levels.

1.12 $OVCA1$, tumour suppressor gene

The down-regulation of tumour suppressor genes (TSG) can be caused by gene deletion events, in particular LOH (loss of heterozygosity). The identification of the TSG, $OVCA1$ (Ovarian Cancer Gene 1), has given rise to hope for new and specific treatment for ovarian cancer (Phillips et al. 1993; Phillips et al. 1996). Studies of frequent LOH events in human cancer established chromosome 17 as a hotspot for chromosomal aberrations, particularly in ovarian and breast cancer (Phillips et al. 1996; Schultz et al. 1996; Jorgensen et al. 2003). $OVCA1$ on chromosome 17p13.3 is located in close proximity of $P53$ and $HIC-1$ (Hypermethylated in Cancer 1), which are also associated with various cancers (Schuijer and Berns 2003). Even though $P53$ is the most frequently mutated gene (~50-70%) in epithelial ovarian tumours, it is only seen in advanced stages of ovarian cancer (Berchuck et al. 1994a; Berchuck et al. 1994b). Therefore the discovery of other genes involved in the onset of the disease is the main objective of current research. In this context, Phillips et al. (1993) reported the allelic deletion of human $OVCA1$ to be associated with several types of cancer, in particular primary ovarian tumours. $OVCA1$ expression is deleted or significantly reduced in ~80% (39 of 49) of ovarian tumours, including ~43% low malignant tumours and ~80%
non-metastatic tumours (Phillips et al. 1996). These findings suggest a crucial role for \textit{OVCA1} in the prevention of tumour formation in the early stages of tumourigenesis. The molecular mechanism by which \textit{OVCA1} prevents tumour formation is unclear together with the question of whether this property is dependent on its role as a diphthamide biosynthesis gene. Sequence analysis shows no homology to known functional domains, giving no indication about other diphthamide-independent functions of the protein.

Moreover, (Bruening et al. 1999) reported that the exogenous overexpression of \textit{OVCA1} suppresses colony formation of ovarian cancer cell lines with an increased number of cells arrested in G1 phase, an effect that could be reversed by upregulation of cyclin D1 levels. In agreement with this, a recent finding suggests that \textit{OVCA1} might prevent tumourigenesis by decreasing cyclin D1 and increasing p16 activity at both the mRNA and protein level (Kong et al. 2011). The p16/cyclin D1 cycle is one of the two main pathways that regulate cell proliferation and is implicated in virtually all human tumour types. It would be interesting to clarify if this is linked to the diphthamide modification on eEF2 or if it indicates \textit{OVCA1}’s role in multiple cellular pathways.
1.13 Aims and Objectives

Despite the fact that the diphthamide modification on eEF2 was discovered more than three decades ago its biosynthesis has not been completely understood. Here we focus on the first and the final step of the pathway, where we sought to clarifying Dph1 function as well as identify the amidase that facilitates the conversion of diphthine into diphthamide.

1. Investigating functional domains of Dph1 by a systematic mutagenesis approach:

The systematic truncation of Dph1 from both the N- and C-terminal end in intervals of 30 amino acids was aimed at mapping functional domains of the protein. We sought to identify the minimal function unit of Dph1 and to elucidate its interaction profile with Dph2 and Dph3.

2. Identification of novel DPH genes by data mining of large scale yeast genetic and phenotypic screens:

Using data collected in two independent genetic and phenotypic yeast screens, we sought to identify putative candidate genes that are involved in diphthamide synthesis.

3. Validation of novel candidate Dph proteins using biochemical approaches:

The novel candidate diphthamide genes identified in the previous chapter were biochemically validated in order to elucidate their involvement in the synthesis of the post-translational modification on eEF2. Genetic, phenotypic and biochemical assays to clarify the role of the novel DPH genes in the diphthamide pathway.
CHAPTER 2

Mapping a Dph1 region crucial for the interaction with Dph2 and Dph3
2 Mapping a Dph1 region crucial for the interaction with Dph2 and Dph3

2.1 Introduction

The initial step of diphthamide formation on eEF2 requires the action of four diphthamide biosynthesis proteins, Dph1-Dph4. Together they facilitate the cleavage of an 3-amino-3-carboxypropyl group (ACP) group from S-adenosylmethionine (SAM) and subsequently facilitate its transfer to the C2 position of the imidazole ring of the histidine precursor (Chen and Bodley 1988; Mattheakis et al. 1992). Loss of Dph1 function results in a failure to form diphthamide and protects from diphtheria toxin (DT) as well as the antifungal drug, sordarin. Interestingly, the archael Dph1 homologue, PhDph2 (P. horikoshii Dph2) is an iron-sulfur enzyme, which generates the ACP radical with the help of a bound iron-sulfur cluster. In detail, PhDph2 contains three conserved cysteine residues (Cys59, Cys163 and Cys287, see Figure 2.2) that cluster in the center of the protein to form a triangular mould for binding of the [4Fe-4S] cluster (Zhang et al. 2010; Zhu et al. 2011). PhDph2 is more similar to Dph1 rather than Dph2 in S. cerevisiae, which is highlighted in the fact that ScDph1 carries all three conserved cysteine residues, whereas ScDph2 lacks the second one (Figure 2.2). In fact, yeast Dph1 and Dph2 are homologous to each other and seem to have evolved from the same ancestral protein (Zhang et al. 2010; Zhu et al. 2011).
Data from our own group and others show that Dph1, Dph2 and Dph3 form a complex and co-purify in immunoprecipitation assays (Fichtner et al. 2003b; Liu et al. 2004b; Baer et al. 2008; Zhang et al. 2010). It is very likely that \textit{S. cerevisiae} Dph1 and Dph2 form a heterodimer similar to the PhDph2 homodimer in \textit{P. horikoshii}, however the exact role of Dph3 and Dph4 in the formation of the ACP-intermediate is unclear. Sequence analysis and NMR studies revealed that both Dph3 and Dph4 are redox active and can bind iron and zinc. Therefore it was proposed that in the context of diphthamide synthesis they might act as electron donors for the [4Fe-4S] cluster, thereby keeping it in the reduced state necessary for the cleavage of ACP from SAM (Sun et al. 2005; Wu et al. 2008; Thakur et al. 2012). However, only Dph3 is part of the Dph1-Dph2-Dph3 complex, whereas Dph4 does not co-purify with the other three Dph proteins.

Even though Dph1 and Dph2 are homologous, they are both essential for diphthamidation of eEF2 in yeast and higher organisms. In order to gain further insight into the structure of the Dph1 protein, we decided to truncate \textit{S. cerevisiae} Dph1 in a systematic manner to identify the shortest deletion mutant that confers loss of function, and thus the minimal functional unit of this protein. To do so, progressive N- and C-terminal truncations of Dph1 were generated using a PCR-based approach (see Methods). Figure 2.1 illustrates the genetic dissection of Dph1, with the HA-tagged full-length protein (N=N-terminal and C=C-terminal) and the truncations in intervals of 30 amino acids (N1-N4 and C1-C4). In order to assess the function of the Dph1 variants, the strains were subjected to diphtheria toxin (DT) and sordarin as well as used in co-immunoprecipitation assays. As shown in figure 2.3 and 2.4, the isogenic wildtype strain (W303) is sensitive to DT and sordarin, whereas the \textit{dph1}Δ strain is resistant
which indicates the lack of diphthamide on eEF2. Hence we used both agents as analytic tools to verify the function of the Dph1 truncations. Co-Immunoprecipitation (co-IP) of proteins detects protein-protein interactions such as between Dph1, Dph2 and Dph3, which strongly interact with each other in a complex and co-purify. Here, we aimed to investigate if the Dph1 truncations are still able to interact with Dph2 and Dph3. In order to verify Dph1 binding activity, the mutagenesis was performed in two different strains carrying either Dph2-c-myc or Dph3-c-myc, which allowed us to study the interaction of the HA-tagged Dph1 with Dph2 and Dph3.

Immunoblot detection of the constructs is presented in Figure 2.1, where both Dph2-c-myc and Dph3-c-myc strains co-express wt-Dph1-HA and the N-terminal (N1-N4) as well as the C-terminal truncations (C1-C4). This allowed us to investigate which mutation causes loss of function of Dph1 and at the same time whether the mutation also affected protein-protein interaction between Dph1 and its partner proteins Dph2 and Dph3. Figure 2.2 highlights the position of the truncations in relation to the conserved cysteine sites of PhDph2 and homologues from other species (C. griseus CgDph2, H. sapiens HsDph2). We predicted that the N-terminal truncation upstream of the first cysteine residue (N1-N3) as well as the C-terminal truncation downstream of the region containing the third cysteine residue (C1) might not affect Dph1 function significantly, since all three conserved Cys residues are available for binding of the iron-sulfur cluster. The N-terminal truncations were expressed under the control of the conditional GAL1 promoter and were expressed at a similar level as the full-length protein (Figure 2.2). The C-terminal truncations were expressed from the native promoter with the C2 and C3 truncation showing a significant decrease in expression.
compared to the full-length Dph1, indicating an unstable protein. Alternatively, C2 and C3 levels might be lowered as a result of increased in mRNA decay. Interestingly, the C4 truncation lacking 120 amino acids at the C-terminus was detected as a clear band, but expressed at lower levels compared with C1 and C. Both HA-tagged full-length Dph1 (N and C) and the smallest truncations N1 and C1, were accompanied by smaller degradation products. Taken together, these experiments were designed to find the shortest non-functional deletion mutant of Dph1 as well as mapping a Dph1 region crucial for the interaction with Dph2 and Dph3.
Figure 2.1. Systematic truncation of Dph1. N-terminal and C-terminal truncations of Dph1 in 30aa intervals (N1-N4 and C1-C4) as well as the HA tagged full-length Dph1 (N: N-terminal and C: C-terminal) are presented in a schematic diagram. Dph1 mutagenesis was performed in 2 strains: Dph2-c-Myc and Dph3-c-Myc. Expression of Dph1 variants are detected in western blot analysis using anti-HA antibody.
**Figure 2.2. Sequence alignment of ScDph1 and homologues.** Archaeal PhDph2 (*P. horikoshii*), mammalian chinese hamster (*C. griseus*) CgDph2, human HsDph1 and HsDph2, and *S. cerevisiae* ScDph2 are included for comparison of conserved regions. The highest degree of conservation between the six sequences is marked in dark blue. The three conserved cysteine residues in PhDph2, which bind the iron-sulfur cluster (Cys59, Cys163 and Cys287) are marked with a red triangle (Zhang et al. 2010; Zhu et al. 2011). The position of N-terminal truncations (N1-N4) as well as C-terminal truncations (C1-C4) of ScDph1 are highlighted. Sequence alignment was performed using Jalview.
2.2 Results

2.2.1 DT phenotype of Dph1 constructs

In order to investigate the DT phenotype of the Dph1 variants, the strains were exposed to endogenous expression of the DT F2 fragment from the plasmid pLMY101 under the control of the conditional GAL1 promoter. 10-fold serial dilutions were spotted on plates containing glucose (control), which represses the GAL1 promoter as well as galactose-containing media to turn on DT expression. The isogenic wt and dph1Δ strains were included as controls highlighting that the diphthamide containing wt strain is sensitive to DT, whereas the diphthamide-minus dph1Δ strain is resistant to the bacterial toxin. N- and C-terminal HA-tagging of full length Dph1 retained the DT sensitive phenotype and confirmed the presence of a functional protein, which is not affected by the HA tag. However, all truncations from the N-terminus as well as the C-terminus resulted in a DT resistant phenotype similar to a dph1Δ strain, indicating that diphthamide could not be formed. Seeing that all constructs apart from C2 and C3 are stably expressed, the lack of diphthamide is considered a direct result of the Dph1 truncations. These findings were confirmed in both background strains (DPh2-c-myc as well as Dph3-c-myc).
**Figure 2.3. Dph1 truncations fail to form diphthamide.** 10-fold serial cell dilutions of isogenic wt, *dph1Δ* as well as Dph1 truncations were spotted on YPD plates supplemented with glucose (toxin off) as well as galactose (toxin on). N-terminal truncations (N1-N4) as well as C-terminal truncations (C1-C4) are resistant to diphtheria toxin (DT). Full-length HA-tagged Dph1 remains sensitive to the toxin, similar to the wt strain. DT phenotype of Dph1 variants are confirmed in both background strains (Dph2-c-Myc and Dph3-c-Myc).
2.2.2 Sordarin phenotype of Dph1 constructs

To address the functionality of the Dph1 truncations with a second diphthamide indicator drug, the constructs were exposed to the antifungal, sordarin. To do so, 10-fold serial cell dilutions were spotted on YPD plates supplemented with 20 µg/ml sordarin. As mentioned above, N-terminal truncations are under the control of the GAL1 promoter and therefore required galactose to be expressed, which is supplemented in the YPD plates. However, when the strains were spotted on GalYPD containing 20 mg/ml sordarin, none of the strains grew including the dph1Δ mutant, a sordarin resistant strain. Glucose is the preferred carbon source for S. cerevisiae, which therefore show a slower growth on galactose. The combination of sordarin and galactose seems to potentiate toxicity dramatically and therefore the optimum concentration of sordarin in combination with galactose was identified as 5mg/ml where dph1Δ could grow and the wt strain was killed. All Dph1 variants survived in the presence of sordarin, which confirms the findings from the DT assay (Figure 2.4). Only the full length HA-tagged Dph1 constructs and the isogenic wt strain were killed by the translational inhibitor, which further indicates that none of the N- or C-terminal truncations could form diphthamide on eEF2.
Figure 2.4. Sordarin assay confirms the lack of diphthamide formation due to the truncation of Dph1. 10-fold serial cell dilutions of isogenic wt, dph1Δ as well as Dph1 variants (N1-N4 and C1-C4) were spotted on YPD plates (control) as well as YPD media supplemented with 20mg/ml or 5mg/ml sordarin. N-terminal truncations (N1-N4) were spotted on GalYPD to switch on the expression of the constructs from the conditional GAL1 promoter. Full-length HA-tagged Dph1 remains sensitive to the antifungal sordarin, similar to the wt strain. The sordarin phenotype of Dph1 variants were confirmed in both background strains (Dph2-c-Myc and Dph3-c-Myc).
2.2.3 Co-Immunoprecipitation assay reveals interaction profile of Dph1

2.2.3.1 C-terminal truncations

Interaction of full-length Dph1 and the truncated variants with Dph2 and Dph3 was investigated using Co-IP assays. Due to the DT and sordarin results, we hypothesized that only the full-length Dph1 construct would be able to interact with Dph2 and Dph3. Interestingly, C1-Dph1 was able to bind both Dph2 and Dph3, in fact when compared to binding of the full-length Dph1, this deletion does not alter binding to either proteins. As mentioned above, both C2 and C3 variants are expressed at low levels and are most likely unstable variants of Dph1, therefore it is not surprising that they do not co-purify with Dph2 or Dph3. C4 on the other hand is a stable construct but completely abolished the binding properties of Dph1. These data show that the removal of 30 amino acids from the C-terminus of Dph1 does not disrupt interactions between Dph1 and its partner proteins Dph2 and Dph3.
**DPH2-c-myc**

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**DPH3-c-myc**

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<td>DPH+HA (C3 truncation)</td>
<td>[Image]</td>
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<tr>
<td>DPH+HA (C4 truncation)</td>
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*IP* indicates immunoprecipitation and *pre IP* indicates pre-immunoprecipitation.
**Figure 2.5. C1 truncation of Dph1 does not affect the interaction with Dph2 or Dph3.** Co-Immunoprecipitation (co-IP) of C-terminal HA-tagged full-length Dph1 as well as truncations (C1-C4) with either Dph2-c-myc or Dph3-c-myc are shown. Anti-HA antibody is used for detection of the Dph1-HA constructs and anti-c-MYC antibody for Dph2-c-myc as well as Dph3-c-Myc. Co-IP analysis was performed with anti-c-Myc antibody. Co-purification of full-length Dph1-HA and C1-Dph1-HA with Dph2-c-Myc is presented in the top two panels on the left and with Dph3-c-Myc in the top two panels on the right. The bottom two panels represent immunoblot detection of the constructs in crude protein extracts prior to co-IP.
2.2.3.2 **N-terminal truncations**

Seeing that all N-terminal truncations of Dph1 proved to be DT and sordarin resistant we again hypothesized that they would fail to interact with Dph2 and Dph3. However, co-purification of N1-N4 with Dph2 and Dph3 revealed an even more surprising interaction profile. N1 and N3 variants co-purify with Dph2 and N1, N2 and N3-Dph1 interact with Dph3. The smallest truncation of 30 amino acids from the N terminus does not affect binding of Dph1 to either Dph2 or Dph3, as can be seen by the similar levels of interaction compared to the full length Dph1 construct. N2-Dph1 and N3-Dph1 lacking 60 and 90 amino acids, respectively, co-purified with Dph3, though weaker than N1-Dph1. However it is puzzling, that N3-Dph1 interacts with Dph2 but not N2-Dph1, which is 30 amino acids longer. Taken together the co-IP results indicate that the N-terminal region of Dph1 is less important for binding to Dph2 and Dph3 as compared with the C-terminus.
Figure 2.6. Dph1 N-terminal truncations interact with Dph2 and Dph3. Co-Immunoprecipitation (co-IP) of N-terminal HA-tagged full-length Dph1 as well as truncations (N1-N4) with either Dph2-c-myc or Dph3-c-myc are shown. Anti-HA antibody is used for detection of the Dph1-HA constructs and anti-c-MYC antibody for Dph2-c-myc as well as Dph3-c-Myc. Co-IP analysis was performed with anti-c-Myc antibody. Co-purification of full-length Dph1-HA as well as N1 and N3 variants with Dph2-c-Myc is presented in the top two panels on the left. Interaction of Dph3-c-Myc with full-length Dph1 and truncations N1-N3 are highlighted in the top two panels on the right. The bottom two panels represent immunoblot detection of the constructs in crude protein extracts prior to co-IP.
2.3 Conclusion

The aim of the experiments described in this chapter was to map functional regions of Dph1 by systematic truncation of the protein from both N- and C-terminal ends and to identify the smallest deletion mutant that confers resistance to DT and sordarin. Phenotypic assays with diphthamide indicator drugs DT and sordarin revealed that a removal of only 30 amino acids from either end of Dph1 renders the protein inactive in terms of diphthamide biosynthesis. With the exception of C-terminal truncations C2 and C3, lacking 60 and 90 amino acids respectively, all truncations were expressed in similar levels to the full-length Dph1 and were readily detected in protein immunoblots. Full-length Dph1-HA construct was shown to be functional and therefore it seems unlikely that the HA tag is interfering with Dph1 function. Hence we conclude that the genomic truncation of Dph1 resulted in the diphthamide-minus phenotype and that the full-length construct is necessary for proper Dph1 function.

Mapping the interaction profile of Dph1 with Dph2 and Dph3 revealed a different outcome to the above mentioned drug phenotypes. The truncated Dph1 variant C1 was still able to bind Dph2 as well as Dph3. Similarly N1, N2 and N3 can interact with Dph3 and truncations N1 and N3 with Dph2, though they all fail to promote diphthamidation of eEF2. Figure 2.2 highlights the deleted region in C1-Dph1, which is downstream of the third conserved cysteine residue of PhDph2, whereas the C2 constructs is upstream of S. cerevisiae Cys368. N-terminal truncations however are located upstream of the first conserved cysteine residue, except for N4 lacking 120 N-
terminal amino acids. Therefore mapping interaction regions of Dph1 indicates that the conserved regions, which contain the first and third [4Fe-4S] cluster binding cysteine residues are also crucial for the interaction between Dph1 and Dph2 or Dph3. In other words, C1 as well as N1-N3 variants are able to interact in the Dph1-Dph2-Dph3 complex, but fail to form diphthamide. Zhu et al. (2011) reported that the activity of a single iron-sulfur cluster bound to one of the PhDph2 subunits in the dimer is sufficient to facilitate the formation of the ACP-intermediate. Furthermore, mutating one out of the three cysteine residues did not affect PhDph2 activity, which still could bind the iron-sulfur cluster (Zhu et al. 2011). We hypothesize that the smallest deletion of 30 amino acids changes the structure of Dph1, which might not be able to form the triangular mold necessary for binding of a [4Fe-4S] cluster, even though it can still interact with Dph2 and Dph3.

Furthermore, Roy et al. (2010) recently reported a Dph2 mutant present in Chinese hamster ovary cells, Dph2(C-), which lacks 91aa from the C-terminus and fails to form diphthamide and therefore cannot be ADP-ribosylated in vivo when exposed to DT or ETA, or in vitro when incubated with DT in the presence of radiolabeled NAD. Interestingly, two other mutants, Dph2(N-) with a deletion of 158aa from the N-terminus as well as Dph2(Z-) lacking a putative leucine zipper motif in position 160-181, retained their enzymatic activity and were phenotypically normal (Roy et al. 2010). These findings do not support the hypothesis that the 2 conserved cysteine residues are the main regions of interest for Dph2 function in rodents. In fact the diphthamide-minus Dph2(C-) deletion contains both cysteine residues, whereas the N-terminal truncation of 158 amino acids in Dph2(N-) removes the first and only leaves one cysteine residue,
however the former is non-functional and the latter does not alter diphthamide formation. These findings highlight the importance of the C-terminal end for CgDph2 function in diphthamide formation, however independent of the regions containing the conserved cysteine residues identified in PhDph2. Hence CgDph2 activity does not agree with the iron-sulfur enzyme model proposed by Zhu et al., which clearly shows the minimum requirement of 2 cysteine residues for binding of an iron-sulfur cluster (Zhu et al. 2011). In contrast, our data suggest that the smallest truncation of Dph1 fails to form diphthamide, hence we believe that Dph1 is the main iron-sulfur enzyme in yeast diphthamide synthesis. Even though the wt Dph2 could still bind a [4Fe-4S] cluster, by mutating Dph1, we abolish diphthamidation. In summary, even though Dph1 and Dph2 are fundamental to diphthamide synthesis, our data suggest that Dph1 is the main [4Fe-4S] enzyme and that conformational changes impair binding of a [4Fe-4S] cluster, hence render the truncated Dph1 variants inactive.

Some non-functional variants (N1-N3 as well as C1) resulting in a diphthamide-minus strain in phenotypic assays can still interact with Dph2 and Dph3 as shown by immunoblot. A possible explanation is that even though these mutant proteins can interact with other components of the Dph1-Dph2-Dph3 complex, they are unable to bind a [4Fe-4S] cluster and thus cannot generate the ACP radical necessary for the initiation of the diphthamide pathway. Interestingly, a PhDph2 heterodimer, containing one wt and one mutated PhDph2 copy, is more stable than the homodimer (Zhu et al. 2011). Here only one PhDph2 subunit can bind an iron-sulfur molecule. If the binding of only one iron-sulfur cluster indeed displays an advantage for the stability of the dimer, this might explain why in eukaryotes the homologues Dph1 and Dph2 evolved
differently and prompts the hypothesis that Dph2 might not act as an [4Fe-4S] cluster-binding enzyme.
CHAPTER 3

Identification of novel putative diphthamide biosynthesis genes
3 Identification of novel putative Diphthamide Biosynthesis genes

3.1 Introduction

In yeast approximately 80% of haploid mutations does not affect viability and it is possible to look into the phenotype of the deletion mutants, hence *S.cerevisiae* is an attractive model organism for large-scale studies assessing the biological role of genes (Hillenmeyer et al. 2008; Dixon et al. 2009). Exposure of the ~5000 non-essential gene deletions to genetic, chemical and environmental stresses and the resulting phenotype gives insight into the function of the deleted gene. In detail, screens for synthetic lethality have proven to be a powerful tool for identifying genetic interactions. Synthetic lethality arises when the combination of two mutations leads to an inviable organism, whereas the single mutation does not (Guarente 1993). By scoring the colony size of the double mutant it is possible to deduce genes whose products buffer one another and act in the same essential pathway.

Furthermore, the exposure of the same set of non-essential gene deletions to chemical and environmental stresses allows the identification of related genes that have similar phenotypic profiles (Hillenmeyer et al. 2008).

Here we use data from two large-scale screens to identify novel putative diphthamide biosynthesis genes. In collaboration with Prof. Charles Boone and Dr. Michael Costanzo, University of Toronto, who use the collection of viable *S.cerevisiae*
gene deletions for synthetic genetic array (SGA) analysis we aimed to identify genes that interact with known diphthamide biosynthesis genes, *DPH1-DPH5*. The Boone lab is in the process of generating 12.5 million different double mutations and scoring synthetic lethality or sickness via the colony size of each combination with the help of computational array analysis (Costanzo et al. 2010). To do so, a query strain containing a single mutation, e.g. *dph1Δ*, is crossed with the array of up to 4000 gene deletions. The diploid progeny are allowed to sporulate and the haploid cell containing both mutations is identified via selection for the appropriate markers (Tong et al. 2001; Tong et al. 2004). The resulting ~4000 double mutants are then scored for fitness defects (Figure 3.1). This process is repeated three times to ensure the significance of the outcome. The so-called interaction profile for each query strain, containing all negative and positive regulator genes, can be reviewed on the publically available database, DRYGIN (http://drygin.ccbr.utoronto.ca/; (Koh et al. 2010). Not surprisingly, genes that are biologically related have overlapping interaction profiles (Costanzo et al. 2010). So far, just over 5.4 million gene interactions have been studied highlighting that genes with related functions have highly related interaction profiles. We collaborated with Prof. Boone’s lab to mine their SGA data for novel factors involved in the diphthamide pathway.

As mentioned above, the yeast single gene deletion collection not only serves as a starting point for genetic interaction studies, but also provides the basis for extensive chemical and environmental screens performed by Hillenmeyer et al. (Hillenmeyer et al. 2008; Hillenmeyer et al. 2010). Here the query strains were exposed to 1144 different chemical and environmental stresses. It was reported that even though only
20% of *S. cerevisiae* genes are essential for viability, approximately 97% of the yeast deletions conferred a phenotype under certain stress conditions and are therefore necessary for optimal growth (Hillenmeyer et al. 2008). Similar to the SGA analysis, related genes were shown to have related phenotype profiles, i.e. fitness defects towards specific agents were overlapping in gene deletions that are functionally related. The data is publically available in the Yeast Fitness Data Base (FitDB, http://fitdb.stanford.edu/) and was used to identify novel diphthamide related genes.
Figure 3.1. Simplified schematic representation of synthetic genetic array (SGA) methodology. *MATa* query strain *dph1Δ* (*natMX* selectable marker) is crossed with *MATa* deletion mutant array containing viable mutations linked to *kanMX* marker. The diploid zygote is sporulated and the haploid double mutant is selected for the dominant markers, *dph1Δ::natR* and *xxxΔ::kanR*, against nourseothricin and geneticin. Growth defects of the double mutant compared to the wild-type are measured. Schematic representation adapted from (Tong and Boone 2005).
3.2 Results

3.2.1 SGA analysis reveals novel candidate diphthamide biosynthesis genes

In order to identify novel genes involved in diphthamide biosynthesis we exploited the DRYGIN database to generate correlation profiles for *bona fide* diphthamide synthesis genes, *DPH1*, *DPH2*, *DPH4* and *DPH5*. Figure 3.2 contains the top ten hits of genetically correlated profiles of our query strains. The correlated profiles represent genes that have the closest genetic interaction profile for our query strains. In detail, *DPH1*, *DPH2*, *DPH4* and *DPH5* query strains all contain correlated profiles of each other, i.e. query strain *DPH1* top ten correlated profiles include *DPH2*, *DPH4* and *DPH5*. The same can be observed for query strains *DPH2*, *DPH4* and *DPH5*. This indicates a tightly clustered SGA-based network for diphthamide biosynthesis genes. The dipthamide gene *DPH3* was not included in this screen due to the mutant strain displaying a severe growth phenotype related to cumulative defects in pathways other than diphthamide, including tRNA wobble uridine modification (Fichtner and Schaffrath 2002; Fichtner et al. 2003b; Liu et al. 2004b; Baer et al. 2008; Zabel et al. 2008; Zhang et al. 2010). Furthermore, at least one parologue copy of eEF2 encoding genes, *EFT1* or *EFT2*, scored amongst the top ten correlated profiles of *DPH1*, *DPH2* and *DPH4*. Strikingly, two uncharacterized ORFs, *YLR143w* and *YBR246w*, repeatedly scored highly in the correlated profiles of all diphthamide synthesis genes. This prompted us to look into query analyses using strains deleted for either *YLR143w* and *YBR246w*. Not surprisingly, the top ten correlated profiles for both ORFs contained all
diphthamide synthesis genes included in the SGA analysis (DPH1, DPH2, DPH4 and DPH5). Interaction profiles are correlated and ranked according to the highest Pearson Correlation Coefficient (PCC). Depending on the stringency of the PCC limit, a genetic landscape for each query strain can be designed. Figure 3.3 displays the genetic interaction landscape for YLR143w with a PCC cut-off ranging from <1.0 to <0.14. The former includes a wider landscape of correlated interactors together with diphthamide genes DPH1-DPH5, the novel candidate ORF YBR246w as well as eEF2 encoding genes EFT1 and EFT2. By increasing the PCC cut-off to <0.14, it becomes apparent how closely related YLR143w and YBR246w are to each other and to the DPH1-DPH5 network, which form a tightly related gene family cluster. These highly correlated profiles suggest that YLR143w and YBR246w are candidate genes related to the diphthamide pathway.
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56
Figure 3.2. SGA-based correlated profiles for $DPH1$-$DPH5$ and related ORFs $YLR143w$ and $YBR246w$. Top 10 correlated profiles of the indicated query strains extracted from SGA-based DRYGIN database are ranked according to Pearson Correlation Coefficient determination (PCC) (see methods). Note that $DPH1$-$DPH5$ genes repeatedly score highly and indicate a tightly clustered SGA-based diphthamide synthesis network. For clarity, $DPH1$-$DPH5$, $EFT1$ and $EFT2$ as well as related ORFs $YLR143w$ and $YBR246w$ are shown in bold.
Figure 3.3. *YLR143w* genetic interaction landscape. Schematic representation of interaction landscape of *YLR143w* according to SGA-based correlated profiles. Pearson Correlation Coefficient determination (PCC) cut offs (0.1 - 0.32) indicate how closely related the ORFs are to query strain *YLR143w*. PCC cut off <0.1 (red circle) displays the wider landscape of *YLR143w* containing 29 related ORFs, whereas at a slightly increased PCC <0.11 (black circle) eEF2 gene copies, *EFT1* and *EFT2*, are more closely related to *YLR143w*. The superimposed green square on the right includes all ORFs at a PCC <0.14, which only includes diphthamide synthesis genes *DPH1-DPH5* and closely related ORFs *YBR246w* and *YLR143w*. Figure kindly provided by Dr. Michael Costanzo.
3.2.2  *YLR143w* and *YBR246w* phenocluster with *bona fide* diphthamide genes

In order to find more evidence linking *YLR143w* and *YBR246w* to diphthamide synthetis, we examined data deposited at the FitDB for novel ORFs displaying correlated fitness defects to *DPH1-DPH5*. To do so, we collected the top ten hits of genes that phenocluster with query strains *DPH1, DPH2, DPH4, DPH5, YLR143w* and *YBR246w*. Hillenmeyer *et al.* (2008, 2010) generated these correlated fitness (co-fitness) profiles by comparing scored phenotypes across all experiments and ranked them according to their co-fitness values. Again, *DPH3* is not included in the database for the same reasons mentioned in the previous paragraph. Figure 3.4 contains the top ten sensitivity inducing conditions for *DPH1, DPH2, DPH4, DPH5, YLR143w* and *YBR246w* deletion strains and figure 3.5 lists the correlated fitness profiles for the above mentioned genes. The top ten hits are colour coded for *bona fide* and novel candidate diphthamide genes (yellow), genes that are shared by either two (blue) or four queries (green) and genes typical for only one query (transparent). With the exception of *DPH1*, all other query ORFs phenocluster with at least one diphthamide gene. Interestingly, *YBR246w* phenoclusters with *DPH2, DPH4* and *DPH5* while *YLR143w* phenoclusters with *DPH2, DPH4, DPH5* and *YBR246w*. The enriched GO terms for both ORFs listet in Figure 3.4 assign the process of peptidyl-diphthamide biosynthesis from peptidyl-histidine with P-values ranging from 2×10⁻³ (*YLR143w*) to 9×10⁻⁴ (*YBR246w*).
### DPH1

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### YLR143w

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**Figure 3.4. Top ten sensitivity inducing conditions.** Data obtained from genome-wide yeast fitness screen ([http://fitdb.stanford.edu](http://fitdb.stanford.edu), Hillenmeyer et al., Science 2008) in which homozygous and heterozygous gene deletions were exposed to 1144 different chemical assays and scored for fitness. Top 10 conditions that induce sensitivity in homozygous *DPH1, DPH2, DPH4, DPH5, YLR143w* and *YBR246w* deletion strains are listed.
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Likelihood (P-values) for GO terms related to YLR143w (2e-03) and YBR246w (9e-04):
- peptidyl-histidine modification
- peptidyl-diphthamide metabolism
- peptidyl-diphthamide biosynthesis from peptidyl-histidine

Gene interaction types:
- **Typical of 1 query only**
- **Shared by 2 & 4 queries**
- **DPH-specific (5/6 queries)**
Figure 3.5. FitDB based co-fitness values of bona fide DPH genes and correlated ORFs YLR143w and YBR246w. Collection of top 10 hits of genes that phenocluster with the indicated query ORFs (DPH1, DPH2, DPH4, DPH5, YLR143w and YBR246w). Data is derived from FitDB and is based on genome-scale co-fitness defect analysis of homozygous yeast deletion mutants in response to 1144 different conditions. Enriched GO terms for diphthamide related ORFs YLR143w and YBR246w are indicated with p-values ranging from $2 \times 10^{-3}$ (YLR143w) to $9 \times 10^{-4}$ (YBR246w). Genes, whose deletions most significantly correlate with the indicated query strains (yellow, central nodes) are grouped according to the type of interactions they undergo, e.g. unique (white), shared (blue/green) and DPH-specific profiles (yellow).
3.3 Conclusion

Genetic interaction studies are useful tools for investigating the function of uncharacterized genes and allocating them to specific cellular pathways. Complementary to this are the fields of chemical-genetic interactions as well as environmental-genetic interactions. Gathering data for gene-gene interactions together with gene deletion responses to chemical and environmental stresses has resulted in an immense collection of genetic data available to date that culminates in a genetic interaction network of a cell (Costanzo et al. 2010; Hillenmeyer et al. 2010).

Here we report that data mining of two different screens using yeast ORF deletion collections resulted in the finding of two novel candidate genes for diphthamide synthesis. Initially, genetic interaction studies with the help of SGA analysis provided first clues for a subset of genes that are related to diphthamide synthesis including \textit{DPH1-DPH5} and two novel ORFs, \textit{YLR143w} and \textit{YBR246w} (Koh et al. 2010). The findings were reinforced with the help of the FitDB containing data from approximately 6 million single events of chemical and environmental stress responses (Hillenmeyer et al. 2008).

Both protein products of \textit{YLR143w} and \textit{YBR246w} are located in the cytoplasm like Dph1-Dph5 (Huh et al. 2003). While no specific function has been proposed for \textit{YLR143w} yet, (Botet et al. 2008) reported that the deletion mutant is a strong sordarin suppressor. \textit{YBR246w} on the other hand, has been implicated in several distinct pathways. Sequence analysis revealed that \textit{YBR246w} contains four WD40 domains and is involved in endosomal recycling as the product of \textit{ERE1} that partners with the protein
encoded by the *ERE2* gene (Shi et al. 2011) as well as the regulation of levels of ribosomal DNA transcription aka *RRT2* (Hontz et al. 2009; Shi et al. 2011), (Shi et al. 2011). Neither pathway seems to be directly connected to diphthamide synthesis, however it could indicate a multiple role for *YBR246w* in cellular processes. Furthermore, while our research was ongoing, the putative human homologue of *YBR246w*, WDR85 (WD repeat domain 85), was suggested to be involved in the diphthamide synthesis pathway (Carette et al. 2009) and very recently was reported to be necessary for the final amidation step converting diphthine into the end product of the modification pathway, diphthamide ((Su et al. 2011). 

Analysis of the protein sequence of YLR143w on the other hand revealed an N-terminal Alpha_ANH_like_IV domain, which is predicted to bind ATP. Seeing that the amidation of diphthine is likely ATP-dependant, this might highlight a central role for *YLR143w* in the final step of diphthamide formation.

Taken together, the FitDB and DRYGIN profiles emphasize a shared phenotypic pattern for the *DPH* genes together with *YBR246w* and *YLR143w*, which are tightly clustered within the diphthamide gene network. Thus in the subsequent experiments we chose to further investigate the role of *YLR143W* and *YBR246w* in diphthamide synthesis.
CHAPTER 4

Investigating the role of \textit{YLR143w} and \textit{YBR246w} in diphthamide biosynthesis
4 Investigating the role of YLR143w and YBR246w in diphthamide biosynthesis

4.1 Introduction

As described in the previous chapter, data mining of two independent genetic interaction screens indicated that two novel ORFs, YLR143w and YBR246w, are closely related to bona fide diphthamide synthesis genes, DPH1-DPH5. The aim of the experiments described in this chapter was to investigate a putative role for both candidate genes in the post-translational modification of His699 on eEF2 in S.cerevisiae.

In order to address this question we exposed YLR143w and YBR246w mutant strains to in vivo and in vitro assays involving diphthamide indicator drugs, diphtheria toxin (DT) and the antifungal, sordarin, to investigate whether they phenocopy bona fide DPH genes. Furthermore, epitope tagged eEF2 from YLR143w and YBR246w mutant strains was analyzed via mass spectrometry to identify whether the lack of either gene affects diphthamide formation. A putative interaction between Ylr143w and Ybr246w and known diphthamide synthesis proteins, Dph1, Dph2 and Dph5, as well as eEF2, the protein carrying the posttranslational modification, was analyzed by co-immunoprecipitation. As mentioned in chapter 1 (section 1.5), the lack of diphthamide on eEF2 was shown to play a role in translational fidelity (Ortiz et al. 2006a; Bar et al.
2008). Hence, the level of frameshifting in cells lacking either of our candidate ORFs was compared to *DPH* deletion strains and the isogenic wild-type.

At the point of this research we were not aware of any findings regarding the direct involvement of *YBR246w* in diphthamide formation, hence for the purpose of readability our data will be presented without considering recent findings by Su et al. (2011) mentioned in the introduction (section 1.9). However, in the discussion we will present recent developments in identifying the function of *YBR246w* within the diphthamide pathway and compare our findings to the published work of Su et al. (2011).
4.2 Results

4.2.1 YLR143w and YBR246w phenocopy diphthamide synthesis genes

The unmodified His$_{699}$ on yeast eEF2 protects from diphtheria toxin (DT) as well as sordarin, therefore we assayed $YLR143w$ and $YBR246w$ for their response towards both cytotoxic agents in order to gain insight into their diphthamidation properties. In order to assay for DT, the strains were transformed with vector pLMY101 carrying the F2 fragment (the cytotoxic ADP-ribosylase fragment of the toxin, DTA) under the expression of the conditional GAL1 promoter. Ten-fold serial cell dilutions were spotted on selective media containing either galactose, to induce DTA expression from the $GAL1$ promoter, or glucose, which serves as the control and inhibits the release of the toxin. Figure 4.1A shows that $YLR143w$ and $YBR246w$ are sensitive to the endogenous expression of DTA from the strong $GAL1$ promoter, in contrast to $dph1\Delta$, which cannot be killed by the toxin. In vivo DTA expression from the multi copy vector, pLMY101, under the control of the strong $GAL1$ promoter, results in the intracellular release of high levels of the toxin. We therefore decided to downregulate endogenous DT expression by cloning the DTA fragment from pLMY101 into the single copy pGALS vector, p415-GALS (Mumberg et al. 1994). This $GALS$ promoter is truncated and lowers expression levels up to 50 fold compared to the $GAL1$ promoter. By exchanging the strong promoter for a weak one together with switching from a multi copy to a single copy vector, we sought to reduce the expression of endogenous DTA to a level which might result in a reduced toxin sensitivity of $YLR143w$ and $YBR246w$
compared to the wild-type strain. In collaboration with Dr. Christian Baer, University of Kassel, we altered the level of galactose from 2% to 0.1% while simultaneously replacing it with raffinose as a carbon source, which does not induce the GALS promoter, thereby downregulating the GALS promoter and further decreasing DTA expression. Figure 4.1B clearly demonstrates an increased resistance towards very low levels of DT in the YLR143w and YBR246w strain compared to the isogenic wild-type. These results further support our hypothesis that deletion of either candidate ORF leads to the accumulation of diphthine, which even though a target for DT is less efficiently ADP-ribosylated compared to diphthamide.

When exposed to another diphthamide indicator drug the lack of Ylr143w and Ybr246w showed a significant resistance to levels of 10 µg/ml sordarin, which is cytotoxic to the wild-type strain (Figure 4.1C). In fact, ylr143w and ybr246w mutant strains are as insensitive to the action of sordarin as bona fide DPH genes, DPH1- DPH5. Though we are aware that the list of sordarin resistant yeast deletion mutants is rather large and that the resistance of both candidate ORFs towards sordarin might be independent of the diphthamide pathway, we decided to further investigate their potential as diphthamide biosynthesis genes.

The action of both compounds, DT and sordarin, on yeast eEF2 is dependent on the formation of diphthamide. Unmodified His$^{699}$ cannot be targeted by either agent, whereas the mature modification, diphthamide, serves as a potent recognition motif on eEF2 and results in its inactivation by DT and sordarin. Interestingly, the acid hydrolysis product of diphthamide, diphthine, which is the second intermediate in the pathway, can still be recognized and targeted by DT even though less efficiently than
the complete diphthamide modification. Therefore we hypothesized that the DT sensitive phenotype of *YLR143w* and *YBR246w* towards endogenous expression of high levels of the toxin might be due to the accumulation of diphthine rather than diphthamide, which would explain the dose-dependant resistance towards DT.
Figure 4.1. *DPH6* and *DPH7* deletion strains copy traits typically related to the bona fide diphthamide mutants *dph1-dph5*.

(A) DT phenotype. Indicated strains were tested for sensitivity to intracellular expression of DTA, the cytotoxic ADP ribosylase fragment of DT. This assay involved galactose-inducible expression of DTA from the multicopy vector pLMY101 (see text for details). Serial cell dilutions were replica plated onto glucose (glc) as a control and galactose (gal), which induces expression of DTA. Growth was for 3 days at 30°C. DTA sensitive (S) and resistant (R) phenotypes are indicated. (B) Downregulation of DTA expression. As indicated, yeast *dph* mutants and wild-type control (wt) were tested for sensitivity to intracellular expression of DTA. 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. (C) Sordarin resistance. Ten-fold serial cell dilutions of the indicated yeast strains, *BY4741* wild-type (wt) background and its *dph1-dph7* gene deletion derivatives 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.
4.2.2 YLR243w and YBR246w cannot be ADP-Ribosylated in vitro

Seeing that the cytotoxicity of DT is specifically dependant on the diphthamide modification (in contrast to sordarin, which can be blocked by other pathways as indicated by the collection of sordarin resistant yeast gene deletions), we decided to confirm the in vivo DT sensitivity of YLR143w and YBR246w in an in vitro assay. In collaboration with Dr. Shihui Liu (National Institute of Health, Bethesda, USA) we performed an in vitro ADP-ribosylation assay in presence of DT (20 nM) with biotin labeled NAD as the cofactor. Isogenic wt strain together with gene deletions of DPH1, DPH5, YLR143w and YBR246w were assayed for their ADP-ribosylation property (Figure 4.2). Interestingly, similar to the DPH genes, neither YLR143w nor YBR246w could be ADP-ribosylated in vitro. Uptake of biotin labeled NAD was only observed in the wt strain, showing that eEF2 from YLR143w, YBR246w as well as DPH1 and DPH5 could not be ADP-ribosylated by DT in vitro. In concert with our in vivo results, these findings imply that YLR143w and YBR246w display a defect in diphthamide synthesis.
Figure 4.2. *ylr143w* and *ybr246w* demonstrate a lack of *in vitro* ADP-ribose acceptor activity.

Cell extracts obtained from *dph1*, *dph5*, *ylr143w* and *ybr246w* 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. Data generated by Dr. Shihui Liu.
4.2.3 eEF2 tandem affinity purification

In order to clarify the involvement of our candidate ORFs in diphthamide synthesis we isolated eEF2 from yeast deletion strains and analyzed the modification on His$^{699}$ via mass spectrometry. To address the question, deletions of *DPH1* and *YLR143w* were tested in an *EFT2*-TAP (*Euroscarf*) strain, which allows the tandem affinity purification (TAP) of the protein. The TAP tag contains a protein A binding site followed by a CBP (calmodulin binding protein) domain. These two distinct motifs are separated by a TEV linker domain and allow two consecutive purification steps, which enable the isolation of proteins from crude extracts (for details see Methods). Seeing that TAP purification is a very elaborate process we decided to first investigate *YLR143w* in order to verify that the method is applicable for our strains. The TAP results are demonstrated in Figure 4.3, which highlights how the impurities of the first elution (after TEV protease cleavage) disappear after the second purification step and result in a single band of isolated TAP-tagged eEF2 protein. Though with a lower yield compared to the first elution, the purified protein can be directly trypsin digested in liquid. We isolated eEF2-TAP from wild-type strain and *DPH1* and *YLR143w* mutant strains and performed mass spectrometry, which detected His$^{699}$ in the tryptic peptide 686-VNILDVTLHADAIHR-700. Unfortunately, we failed to detect any modification on His$^{699}$ from any of the samples (MS spectra of unmodified wt, *dph1* and *ylr143w* peptide are included in the appendix). This was expected for the *DPH1* mutant, which impairs diphthamide formation completely, but not for the wild-type sample. After repeating the TAP isolation of eEF2 on new samples and switching from in liquid trypsinisation to in gel digest we still were unable to detect diphthamide in the wild-type
or any other modification of the histidine precursor. It was possible that the large size of the TAP tag (~21kDa) might interfere with the diphthamide formation on eEF2 given that the C-terminal end of the protein is in close proximity to His<sup>699</sup> in the folded protein (see Figure 1.1 in the introduction). Therefore we decided to repeat the experiment with a smaller tag, which would avoid interference with diphthamide formation.

**Figure 4.3. Tandem affinity purification (TAP) of eEF2.**

Total protein extracts of wild-type (wt), <i>dph1</i> and <i>ylr143w</i> variants of an <i>EFT2-TAP</i> strain were used for TAP isolation of tagged eEF2 constructs. The first purification step with IgG coated sepharose beads binds the protein A domain of the TAP tag and is followed by the first elution in presence of TEV protease. The second purification step involved calmodulin affinity beads, which bind to the CBP (calmodulin binding protein) domain of the TAP tag and results in the isolation of a single band of eEF2. TAP eluates were run on SDS-PAGE and visualized with coomassie blue staining.
4.2.4 Mass spectrometry identifies *YLR143w* and *YBR246w* as diphthamide synthesis genes

In order to clarify the involvement of the candidate ORFs, *YLR143w* and *YBR246w*, in diphthamide synthesis we decided to isolate his-tagged eEF2 from strains lacking both genes and analyzing the modification of His$^{699}$ via mass spectrometry. As a control, eEF2 from wild-type strain as well as *dph1Δ* (blocking the modification at the initial step) and *dph5Δ* (arresting diphthamidation after the formation of the ACP-intermediate) were also investigated. The eEF2-His6 construct expressed from plasmid pTKB612 was previously shown to complement an *eft1eft2* double mutant and therefore considered biologically functional. Isolation of His-tagged eEF2 from crude protein preps was performed using magnetic anti-His tag Dynabeads® (Invitrogen). Compared to the TAP-purification method above, His6-tag isolation in *S.cerevisiae* is not very clean, however when samples were run on an SDS-PAGE 4-12% Bis-Tris precast gel (Invitrogen) an enriched band with the right size of ~100kDa for eEF2 was detected (Figure 4.4). A section of the eEF2 band was excised and in gel digestion with trypsin was performed (see Methods). Mass spectrometry and data analysis was performed in collaboration with Prof. Mike Stark and Dr. Sara ten Have, University of Dundee.

Tryptic peptides were fractionated by LC-MS (Liquid Chromatography-MS) linked up to the mass spectrometer. They enter the first stage, which performs a survey scan for parent ions (m/z 335-1800). The top 15 most intense ions from each survey are then sent to the second stage, where they are fragmented to give the b and y ion series and a spectrum showing the ions that are obtained is produced. The actual spectra that
we are presenting are composites made by combining the signal from all spectra obtained for each parent ion, i.e. a particular ion may appear many times in a series of survey scans as its peak elutes from the LC step and be sequences many times as a result. Normally the spectra are identified and annotated by the software. In our case this had to be done manually by Dr. Sara ten Have by sifting through the data to identify parent ion masses that matched what we expected (see below) and then looking in the MS/MS spectrum for ions matching what was predicted. This was probably due to complications associated with the type of modification and the effect on the m/z. Parent ions can be singly or multiply charged, which gives a series of parent ion masses such as [M+H]+, [M+2H]2+, [M+3H]3+ etc. Dr. ten Have then used molecular mass calculator (MolE, http://library.med.utah.edu/masspec/mole.htm) to calculate the exact mass from a formula (see Table 4.1.). The assignment of each modification is by matching multiple lines in the MS/MS spectrum to predicted values for one modification and verifying whether they are different from any other modification or not.
<table>
<thead>
<tr>
<th></th>
<th>[M+H]+ (mono)</th>
<th>[M+2H]2+ (mono)</th>
<th>[M+3H]3+ (mono)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>1686.9285</td>
<td>843.9682</td>
<td>562.9814</td>
</tr>
<tr>
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<td>1829.0397</td>
<td>915.0238</td>
<td>610.3518</td>
</tr>
<tr>
<td>Diphthamide-(CH3)3HN+</td>
<td>1769.9656</td>
<td>885.4867</td>
<td>590.6604</td>
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<tr>
<td>Diphthine</td>
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<td>915.5158</td>
<td>610.6798</td>
</tr>
<tr>
<td>Diphthine - (CH3)3HN+</td>
<td>1770.9496</td>
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<tr>
<td>ACP intermediate</td>
<td>1787.9762</td>
<td>894.4920</td>
<td>596.6639</td>
</tr>
</tbody>
</table>

**Table 4.1. Expected m/z values for the different stages of His699 modification.**

Molecular mass calculator (MolE, http://library.med.utah.edu/masspec/mole.htm) was used to calculate the m/z values of singly or multiply charged parent ion masses ([M+H]+, [M+2H]2+, [M+3H]3+). The different stages of yeast eEF2 His699 modifications are listed with the diphthine and diphthamide values before and after the neutral loss of the trimethylamino group. Data provided by Prof. Mike Stark and Dr. Sara ten Have.
Figure 4.4. eEF2-His6 isolation and immunodetection.

Total protein extracts of wt, dph1, dph5, ylr143w and ybr246w variants of the background strain eft1+pEFT2-(His)$_6$ were used for anti-His isolation with magnetic beads (Dynabeads®, Invitrogen). The enriched eEF2-His6 s were run on SDS-PAGE (top panel) and visualizes with coomassie blue staining. The bottom panel demonstrates immunoblot detection of the eft1+pEFT2-(His)$_6$ construct with anti-His6 antibody.
The modified histidine in eEF2 is located in the tryptic peptide 686-VNILDVTLHADAIHR-700 (Figure 4.5A) and, as expected, unmodified versions of this peptide were readily detected in eEF2 prepared from the $dph1\Delta$ strain (Figure 4.5C). Unmodified peptide was also found in eEF2 prepared from the $dph5\Delta$, $ybr246w\Delta$ and $ylr143\Delta$ strains as well as in eEF2 from the wild-type strain (Figure 4.5). Although we cannot accurately quantitate the relative levels, examination of extracted ion chromatograms for the doubly and triply charged species of the unmodified peptide suggests that by far the highest level was seen in the $dph1\Delta$ strain, with lower, though highly significant levels, in the other three mutants and less in the wild-type strain (Figure 4.6).
Figure 4.5. MS/MS spectra of unmodified eEF2 peptide 686-VNILDVTLHADAIHR-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. Data generated by Dr. Sara ten Have.
Figure 4.6. Extracted ion chromatograms of unmodified eEF2 peptide 686-VNILDVTLHADAIHR-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. Data generated by Dr. Sara ten Have. Data generated by Dr. Sara ten Have.
In addition to the unmodified peptide, we readily detected diphthamide-modified peptide in eEF2 prepared from the wild-type yeast strain (Figure 4.7A), but failed to detect this in any of the mutants. Instead, ACP-modified peptide was found in eEF2 prepared from the dph5Δ strain (Figure 4.7B), as expected, given its known role in generating diphthine from the ACP intermediate (Zhu et al. 2010). In contrast, eEF2 from the ybr246wΔ 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 diphthine rather than diphthamide modification (Figure 4.7C). Furthermore, the quite different elution times of the diphthine-modified and diphthamide-modified peptide evident from the extracted ion chromatograms (Figure 4.8) is consistent with differently modified forms.
Figure 4.7. MS/MS spectra of diphthamide, ACP and diphthine-modified eEF2 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 ybr246wΔ strain. In (A) and (C) * indicates neutral loss of trimethylamino during MS/MS. The insets in (C) show greater detail for the more crowded part of the MS/MS spectrum. The cartoon in the bottom right corner indicates how the B and Y ions are derived from the peptide sequence. Data generated by Dr. Sara ten Have.
Figure 4.8. Extracted ion chromatograms of modified eEF2 peptide 686-VNILDVTLHADAIHR-700. (A) Peaks corresponding to triply-charged ions (m/z diphthine-modified peptide 610.68, m/z diphthamide-modified peptide 610.35, extracted masses 610.2-610.9). (B) Triply-charged ions (m/z ACP-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. Data generated by Dr. Sara ten Have. Data generated by Dr. Sara ten Have.
As noted in previous studies (Zhang et al. 2008), some of the ions in our MS/MS spectra had undergone neutral loss of the trimethylammonium group during MS/MS (Figure 4.7), as indicated by loss of 59.1103 mass units. Two types of spectra corresponding to the peptide with modified His\(^{699}\) were seen when eEF2 from the \(ylr143w\Delta\) strain was analysed. In some spectra (Figure 4.9A), the parent ion m/z and MS/MS data indicated the presence of diphthine as in the \(ybr246w\Delta\) mutant, with some daughter ions again showing neutral loss of the trimethylammonium group during MS/MS as noted above. However, we also detected peptide in which the elimination of the trimethylammonium group had occurred prior to analysis, as indicated by the lower parent ion m/z (Figure 4.9B) and MS/MS spectrum in which all assignable peaks corresponded to ions lacking the trimethylammonium group. Such trimethylammonium group elimination prior to mass spectrometry was observed previously when \textit{Pyrococcus horikoshii} EF2 modified with diphthine was generated in an \textit{in vitro} reaction (Zhu et al. 2010), indicating that the modification at least in \textit{P. horikoshii} might be unstable. In contrast to the \(ylr143w\) deletion strain, we failed to detect any pre-mass spectrometry loss of the trimethylammonium group when eEF2 from the \(ybr246w\Delta\) mutant was analysed, suggesting that the two samples, though both modified with diphthine, are somehow different. Figure 4.8 shows extracted ion chromatograms for ions with m/z values corresponding to the His\(^{699}\)-containing peptide modified with diphthamide or diphthine (Figure 4.8A) or with ACP (Figure 4.8B), indicating that the ACP modified peptide was only present in the \(dph5\Delta\) mutant, the diphthine modified peptide was only present in the \(ybr246w\Delta\) and \(ylr143w\Delta\) mutants and the diphthamide-modified peptide was only seen in the wild-type strain. While we have not accurately
quantitated the relative levels of each modified peptide, the extracted ion chromatograms suggest that the levels of diphthine-modified peptide in the two mutant samples are broadly similar to the level of diphthamide-modified peptide in the wild-type sample (Figure 4.8). Hence we here present clear evidence that deletion of YLR143w as well as YBR246w result in the accumulation of diphthine-modified eEF2 and are therefore involved in the final amidation step of diphthamide synthesis. Based on the above evidence we propose to name the novel ORFs DPH6 and DPH7, respectively.
Figure 4.9. MS/MS spectra of diphthine-modified eEF2 peptide 686-VNILDVTLHADAIHR-700 from ylr143w mutant yeast strains. Spectra are shown for (A) diphthine-modified peptide in the ylr143wΔ strain and (B) diphthine-modified peptide in the ylr143wΔ 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) * indicates neutral loss of trimethylamino during MS/MS. Data generated by Dr. Sara ten Have.
4.2.5 The YLR143w/DPH6 gene product

Analysis of the Ylr143w/Dph6 protein sequence revealed three conserved domains that point towards its function as an enzyme (Figure 4.10). Firstly, the amino-terminal 225 residues contain an Alpha_ANH_like_IV domain (cd1994 in the NCBI Conserved Domain Database (Marchler-Bauer et al. 2011)). This domain, which is a member of the adenine nucleotide alpha hydrolase superfamily, is found in a range of proteins including N-type ATP pyrophosphatases and ATP sulfurylases and is predicted to bind ATP. Two domains (eu_AANH_C1: cd06155 and eu_AANH_C2: cd06166) that are related to the YjgF-YER057c-UK114 family of proteins present in bacteria, archaea, and eukaryotes are located in the C-terminal portion of Ylr143w/Dph6. These domains show sequence and/or structural similarity to several proteins including chorismate mutase and promote homotrimerisation, which forms an inter-subunit cleft that has been proposed to bind small molecule ligands (Sinha et al. 1999; Volz 1999; Burman et al. 2007). Key residues shown to be important for homotrimerisation and ligand binding in human UK114 such as phe-89, asn-93, pro-105 and arg-107 (Mistiniene et al. 2005) appear to be conserved in Ylr143w, and the conserved arg-107 has been shown to form a bidentate salt bridge with the carboxylic acid group of ligands bound to TdcF, an Escherichia coli YjgF-YER057c-UK114 family member with a likely role in 2-ketobutyrate metabolism (Burman et al. 2007). Although many proteins containing these domains are annotated as ribonucleases because of a ribonuclease activity that has been associated with mammalian UK114, these domains are also found in a range of proteins involved in nucleotide and amino acid metabolism (Sinha et al.
1999; Volz 1999; Burman et al. 2007; Lambrecht et al. 2012). It is therefore likely that Dph6 is directly involved in diphthine amidation and that this step is an ATP-dependent process, with perhaps ammonia or glutamine acting as the source of the amide group. The recent finding that *Salmonella enterica* YjgF has an enamine/imine deaminase activity that is conserved even in human UK114 (Lambrecht et al. 2012) suggests that Ylr143w/Dph6 may use one of its YjgF-YER057c-UK114 related domains to generate ammonia for diphthamide formation. It will be interesting to determine why Dph6 contains two such domains. However, definitive proof that Dph6 directly catalyses diphthamide formation will require demonstration of the biochemical activity in an *in vitro* assay system.
Figure 4.10. Conservation of the YLR143w/DPH6 gene product.

(A) Representation of Ylr143w/Dph6 indicating the conserved adenine nucleotide alpha hydrolase (cd1994) and YjgF-YER057c-UK114 related (cd06155, cd06166) domains discussed in the main text. (B) The Ylr143w/Dph6 amino acid sequence was aligned using Clustal with representative examples of putative homologues from other organisms. Sequences are as follows: YLR143w, S. cerevisiae Ylr143w; Sp_mug71, Schizosaccharomyces pombe; At_A_AAH_IV, Arabidopsis thaliana endoribonuclease; Df_A_AAH_IV, Dictyostelium fasciculatum endoribonuclease L-PSP domain-containing protein; Xl_A_AAH_IV, Xenopus laevis ATP binding domain 4; Hs_A_AAH_IV, Human ATP binding domain containing protein 4; Mm_A_AAH_IV, mouse ATP binding domain containing protein; Hs_UK114, human ribonuclease UK114/p14.5/L-PSP; Mm_UK114, mouse UK114/p14.5/L-PSP. Note that the last two sequences appear twice in the alignment so that the sequence relationships to each of the YjgF-YER057c-UK114 related domains in the non-mammalian proteins can be shown. *, conserved residues shown to be important for trimerisation and ligand binding.
4.2.6 The YBR246w/DPH7 gene product

Unlike Dph6, which has not been analysed to this date, two seemingly disparate functions have already been proposed for DPH7/YBR264w, neither of which are obviously related to diphthamide biosynthesis. Firstly, YBR246w has been implicated as a negative regulator of RNA polymerase I and named RRT2 (Hontz et al. 2009). Consistently, the Ybr246w protein interacts with a second protein (Rrt4) identified in the same genetic screen (Hontz et al. 2009). While effects on RNA polymerase I activity might result from changes in eEF2 function associated with its incomplete modification on his-699, no other components of the diphthamide biosynthetic pathway were identified in the same screen (Hontz et al. 2009). Secondly, the YBR246w/DPH7 product has been proposed to function in retromer-mediated recycling of proteins from the yeast endosome back to the plasma membrane and hence named ERE1 (endosomal recycling) to reflect this role (Shi et al. 2011). A variety of evidence supports this role including impaired recycling of the arginine permease (Can1) and the Mup1 methionine transporter in ESCRT-mutant cells when YBR246w was deleted, association of a pool of Ybr246w protein with membranes that increased in ESCRT-mutant cells, and presence of Ybr246w in a complex with internalized Can1 in ESCRT-mutant cells (Shi et al. 2011). The possible connection between retromer-mediated recycling and diphthamide biosynthesis is not immediately clear.

Ybr246w has four clearly-defined WD40 repeats and a secondary structure predicted to consist of exclusively beta-sheet elements. A possible human homologue (WDR85) was previously implicated in diphthamide biosynthesis (Carette et al. 2009).
and has been proposed to function in the first step of the pathway. Figure 4.11 shows an alignment of Ybr246w with human WDR85 and other potential homologues from a range of different eukaryotes. While the predicted protein structure is highly suggestive of a role as an adaptor protein rather than a catalyst of a biochemical transformation, it remains to be demonstrated whether Ybr246w and its homologues function in multiple, disparate processes (as suggested by the yeast studies), or if these functions are somehow linked through effects on eEF2 modification and function.
Figure 4.11. Conservation of the YBR246w/DPH7 gene product.

(A) Representation of Ybr246w/Dph7 showing the location of the conserved WD40 domains. (B) The Ybr246w/Dph7 amino acid sequence was aligned using Clustal with representative examples of putative homologues from other organisms. Sequences are as follows: YBR246w, S. cerevisiae Ybr246w Sp_WD85, Schizosaccharomyces pombe WD repeat protein; At_WD85, Arabidopsis thaliana WD40 domain-containing protein; Dd_WD85, Dictyostelium discoideum WD40 repeat-containing protein; Xt_WD85, Xenopus tropicalis WD repeat-containing protein 85-like; Hs_WD85, Human WD repeat-containing protein 85; Mm_WD85, mouse unnamed protein.
4.2.7 Ylr143w and Ybr246w do not directly interact with each other or other Dph proteins

Given that the mass spectrometry results clearly identified that YLR143w/DPH6 and YBR246w/DPH7 are necessary for the amidation of diphthine, we decided to investigate whether either gene interacts with other diphthamide synthesis genes. To do so, co-immunoprecipitation of tagged constructs were performed with C-terminal TAP-tagged YLR143w/DPH6 and YBR246w/DPH7. Both constructs were readily detected in immunoblot assays where Ylr143w-TAP is accompanied by a smaller degradation product. DPH1, DPH2 and DPH5 were C-terminally HA-tagged, however neither construct co-purified with Ylr143w-TAP (Figure 4.12A). Furthermore, Co-IP of Dph2-HA and Dph5-HA with Ybr246w-TAP showed that they also do not interact directly. As a control for anti-HA immunoblot detection after the co-IP a positive-HA sample was loaded and readily detected at the right size. We therefore conclude that neither YLR143w/DPH6 nor YBR246w/DPH7 directly interact with bona fide diphthamide synthesis genes DPH1, DPH2 and DPH5.

In order to confirm these findings, we repeated the co-IP with c-terminal Myc-tagged YLR143w/DPH6 and YBR246w/DPH7, which is a smaller tag compared to the 21kDa TAP tag. In collaboration with Dr. Christian Baer, University of Kassel, we readily detected Ylr143w-c-Myc and Ybr246w-c-Myc in immuno blots, both of which are accompanied by smaller degradation products (Figure 4.12B). In line with the above findings, Dph5-HA construct did not co-purify with either of the two ORFs. Furthermore, co-IP of Ylr143w-HA with Ybr246w-c-Myc showed that they also do not directly interact with each other. As a positive control, co-IP of Elp2-c-Myc with Kti12-
HA was performed in parallel. Taken together, Co-IP analysis revealed that
*YLR143w/DPH6* and *YBR246w/DPH7* do not directly interact with each other nor with
diphthamide synthesis genes *DPH1, DPH2* and *DPH5*. 
Figure 4.12. Ylr143w and Ybr246w do not interact with each other and bona fide diphthamide synthesis proteins Dph1, Dph2 and Dph5. (A) Failure to detect interaction by co-immune precipitation between Dph6 or Dph7 and either Dph1, Dph2 or diphthine synthase Dph5, factors integral to the first two steps of diphthamide synthesis. Co-immunoprecipitations were performed using magnetic beads (Dynabeads, Invitrogen) coupled 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 DPH1, DPH2 or DPH5. The presence of the respective proteins within the immune precipitates (IP) was assessed using anti-HA and anti-CBP Western blots or anti-HA immune blots on total protein extracts obtained prior to the IP protocol (preIP). (B) Failure to detect Dph6-Dph7 interaction by co-immunoprecipitation. Co-immunoprecipitations 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 DPH5 or DPH6, respectively. A strain co-producing c-Myc- and HA- and tagged versions of the Elp2 subunit (ELP2-c-myc) of the Elongator complex, and Kti12 (KTI12-HA), a protein known to interact with Elongator, was used as internal positive control. The presence of the respective proteins was assessed in individual anti-c-Myc and anti-HA immunoblots both in the IPs (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 B generated by Dr. Christian Baer.
4.2.8 Ylr143w/Dph6 interacts with eEF2 independent of Ybr246w/Dph7

As the sequence analysis (section 1.2.5) implicates a direct role for YLR143w/DPH6 in the conversion of diphthine into diphthamide, we were intrigued to investigate whether it binds to eEF2 during the process. Furthermore, we wished to determine if deletion of YBR246w/DPH7 would affect the putative interaction between eEF2 and Ylr143w/Dph6, given that the protein structure predicts a role in mediating protein-protein interactions (section 1.2.6). Co-immunoprecipitation (co-IP) was performed with His6-tagged eEF2 from plasmid pTKB612 together with C-terminal tagged Dph6-HA (Figure 4.13). Interestingly a faint eEF2-his6 band was detected after co-IP with anti-HA coupled Dynabeads® (Invitrogen). Though the interaction of DPH6 with EFT2 is not very strong, it nevertheless is clearly detectable after co-IP analysis, which indicates that Dph6 binds to a fraction of the isolated eEF2. Furthermore, the direct interaction is not altered by the deletion of DPH7. Taken together, this data suggests that Dph6 directly interacts with eEF2 in a Dph7-independent manner, indicating that it does not mediate the interaction. Furthermore, we also exclude a role for DPH7 in regulating DPH6 gene expression seeing that as Dph6 protein levels are unaltered in the DPH7 deletion strain.
Figure 4.13. eEF2 interacts with Dph6 in a fashion that is independent of Dph7.

Yeast strains co-expressing (His)_6-tagged eEF2 with Dph6-HA in the background of wild-type (DPH7) dph7 mutant strains were subjected to immune precipitations (IP) using the anti-HA antibody. Strains expressing (His)_6-tagged eEF2 on their own served as IP controls (no HA-tag). Subsequently, the precipitates were probed with anti-HA (top right panel) and anti-(His)_6 antibodies (bottom right panel) to check for the content of Dph6-HA. The content of HA-tagged Dph6 as well as (His)6-marked eEF2 in the protein extracts prior to IP (pre-IP) was examined on individual immuno blots using anti-HA (top left panel) and anti-(His)_6 antibodies (bottom left panel), respectively. Absence of Dph7 does not affect the Dph6•eEF2 interaction.
4.2.9 Deletion of DPH7 enhances interaction between Dph5 and eEF2

Interestingly, Carette et al. (2009) reported that lack of WDR85, the putative mammalian homolog of YBR246w/DPH7, increases binding of Dph5 to eEF2. We confirmed this finding by co-immunoprecipitation of HA-tagged Dph5 and His6-tagged eEF2 expressed from plasmid pTKB612. Figure 4.14 highlights that deletion of DPH7 results in a significant increase of interaction between Dph5 and EF2. Therefore we demonstrate that rather than mediating the interaction between Dph6 and eEF2, Dph7 regulates binding of the diphthine synthase, Dph5, to the translation elongation factor. Moreover, the same increase in binding of Dph5 to eEF2 could be observed by deleting DPH1, which lacks the ACP-intermediate that serves as a substrate for Dph5 mediated methylation. Furthermore, the lack of DPH6 also seems to slightly increase Dph5-eEF2 binding. Therefore we conclude that eEF2 carrying an unmodified His^{699} residue (DPH1 mutant) or the diphthine intermediate (DPH7 mutant) displays a significantly increased affinity to Dph5.

Seeing that the interaction between the methyltransferase, Dph5, and the translation elongation factor, eEF2, seems to be altered in diphthamide synthesis mutants, we decided to examine the effect of Dph5 overexpression on dph1-dph5 as well as dph6 and dph7 deletion strains. Figure 4.15 depicts the overexpression of Dph5, which is expressed under the control of the inducible GAL1 promoter (plasmid pGAL-DPH5). While increased Dph5 levels do not show any effect on the wild-type strain and the dph6 mutant, it proved to be detrimental to strains lacking any diphthamide modification (dph1-dph4) as well as the dph7 mutant. This cytotoxic effect of Dph5 overexpression is in line with the above mentioned Dph5-eEF2 interaction profiles.
However, it must be noted that we did not investigate whether the effect of Dph5 overexpression on dph1-dph7 deletion strains was cytocidal or cytostatic.

Figure 4.14. eEF2 interaction with Dph5 is dramatically enhanced by elimination of Dph7 or Dph1. Yeast strains co-expressing (His)$_6$-tagged eEF2 with Dph5-HA in the background of wild-type (DPH) and dph mutant strains (dph7, dph6 and dph1) were subjected to immune precipitations (IP) using the anti-HA antibody. Strains expressing (His)$_6$-tagged eEF2 on their own served as IP controls (no HA-tag). Subsequently, the precipitates were probed with anti-HA and anti-(His)$_6$ antibodies as indicated to check for the content of Dph5-HA and eEF2-(His)$_6$, respectively (all indicated by arrows). The content of HA-tagged Dph5 as well as (His)$_6$-marked eEF2 in the protein extracts prior to IP (pre-IP) was examined on individual immuno blots. Note that Dph5•eEF2 interaction is strongly enhanced by inactivating DPH7 or DPH1. Data generated by Dr. Christian Baer.
Figure 4.15. *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.
4.2.10 Excess Dph6 cannot by-pass the trimethylation step

Even though the diphthamide synthesis pathway has been dissected into three distinct steps, it has not been shown to happen in sequence. Therefore is the trimethylation by Dph5 a necessary step for the final amidation of diphthine? To answer this question we performed a simple test, where we overexpressed DPH6 in a DPH5 deletion strain. Overexpression of DPH6 was achieved by cloning the gene into a multi-copy plasmid carrying the strong GALI promoter. The resulting pSU7 plasmid readily complements the dph6 mutant and increases Dph6 levels in the dph5 mutant in addition to endogenous levels of the protein. Lack of the methyltransferase abolishes the trimethylation of the carboxylgroup in the ACP-intermediate. We next asked whether lack of methylation could be overcome by overexpression of the putative diphthine amidase. To do so, the strains were exposed to sordarin as an indicator drug for diphthamidation of eEF2. Figure 4.16 highlights that increasing levels of Dph6 in a DPH5 mutant strain does not alter its sordarin phenotype and the strain remains resistance to the cytotoxic agent. Therefore we conclude that the putative diphthine amidase, DPH6, cannot by-pass the trimethylation step in order to form diphthamide.
Figure 4.16. *DPH6* overexpression does not rescue the sordarin phenotype of a *
*DPH5* mutant. Ten-fold serial cell dilutions of the indicated yeast strains, wild-type (wt) background and its *dph5* and *dph6* gene deletion derivatives were transformed with either a multi copy *DPH6* vector (mc*DPH6*, pSU7) or an empty control vector (vector) and grown on YPD plates in the absence (control) or presence (+ sor) of 10 µg ml⁻¹ sordarin. Growth was assayed for 2 d at 30°C. Sordarin resistant (R) and sensitive (S) responses are indicated.
4.2.11 Physiological role of diphthamide

Though the exact role for diphthamide on eEF2 has not been defined yet, it has been reported that amino acid substitution of His$^{699}$ confer a thermosensitive growth phenotype (Kimata and Kohno 1994; Ortiz and Kinzy 2005). We observed the growth of $DPH$ mutants, $dph1-dph7$, in liquid as well as on solid media but failed to detect growth defects at normal temperatures (30°C) or at an increased cultivation temperature of 39°C (Figure 4.17). The only exception was the $DPH3$ mutant, which is involved in multiple cellular pathways and displays a severe growth phenotype at 39°C.

Furthermore, diphthamide deficiency has been shown to affect translational fidelity. Data from our own group (Bar et al. 2008) as well as from Ortiz et al. (2006) demonstrates that deletion of $DPH2$, $DPH1$ as well as $DPH5$, which arrests the pathway at the first step and after formation of the ACP intermediate, respectively, results in an increase in -1 frameshifting during translation. Therefore we investigated the lack of $DPH6$ and $DPH7$ on translation fidelity by using the same approach as described by Ortiz et al. (2006). Here we take advantage of programmed ribosomal frameshifting seen during viral translation (Dinman 1995; Farabaugh 1996). In the interest of increasing the efficiency of genome packaging, RNA viruses contain overlapping ORFs, which require the ribosome to switch the reading frame in order to translate the shifted genes. To examine the level of frameshifting we transformed reporter plasmids carrying the $lacZ$ gene in the 0 frame (pJD204.0), +1 frame (pJD204.+1) and -1 frame (pJD204.-1) (Harger et al. 2001; Ortiz et al. 2006a). The level of -1 and +1 frameshifting is measured relative to read-outs from the 0 frame plasmid. Both candidate genes, $DPH6$ and $DPH7$, as well as $DPH1$-$DPH5$ knock out strains were
transformed with the translation reporter plasmids and three different colonies of each strain were assayed for beta-galactosidase activity of the reporter gene (for details see Methods). The readouts from the +1 frameshift reporter plasmid vary within a wide range and therefore are not significantly different from the isogenic wild-type strain (Figure 4.18). -1 frameshifting however was significantly increased in dph6 and all dph mutants, with the exception of dph4 (Figure 4.19). Though together with dph7 dph4 displays an elevated level of -1 frameshifting, it is not significantly different from the wild-type strain. The DPH6 deletion results in the most significant increase of translational fidelity and indicates a central role in the maintenance of eEF2 function during translation regulation.
Figure 4.17. 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. OD600 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 dph3/kti11 mutant, which affects additional biosynthetic pathways apart from diphthamide biosynthesis shows temperature sensitivity (S) while the other dph mutants tolerate high (T) temperatures.
Figure 4.18. Ribosomal frameshift analysis reveals no significant increase in +1 frameshifting. Strains with the indicated genetic backgrounds were transformed with control (pJD240.0) or lacZ +1 frameshift (pJD240.+1) plasmids 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.+1). 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. None of the mutant backgrounds showed a significant increase in ribosomal +1 frameshifting relative to wild-type (wt).
Figure 4.19. Ribosomal frameshift analysis reveals erroneous translation in dph1-dph7 mutants. Strains with the indicated genetic backgrounds were transformed with control (pJD240.0) or lacZ -1 frameshift (pJD240.-1) plasmids 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.-1). 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).
4.3 Discussion

The experiments presented in this chapter were performed to investigate the role of two candidate ORFs, \textit{YLR143w/DPH6} and \textit{YBR246w/DPH7}, in diphthamide synthesis. As mentioned above, while our work was in progress, Su et al. (2011) reported that \textit{YBR246w/DPH7} is required for the final step of diphthamide synthesis. Here we discuss how our data support these recent findings and provide more evidence to further specify its role in the diphthamide pathway. Furthermore, the role of \textit{YLR143w/DPH6} in the amidation of diphthine to complete diphthamide synthesis will be discussed.

Initially, exposing deletion mutants of both ORFs to diphthamide indicator drugs revealed that they phenocopy diphthamide synthesis genes by displaying resistance to the antifungal, sordarin, as well as failing to serve as a target for DT-mediated (20nM) \textit{in vitro} ADP-Ribosylation. However, in contrast to \textit{DPH1-5} mutants, endogenous overexpression of the bacterial toxin inhibits cell growth and renders \textit{YLR143w/DPH6} and \textit{YBR246w/DPH7} sensitive to DT. Lowering the level of endogenous DT expression via a truncated \textit{GALS} promoter showed that both mutants are clearly more resistant to the action of DT compared to the wild-type. Su et al. (2011) recently reported that \textit{YBR246w/DPH7} mutants can be ADP-Ribosylated \textit{in vitro} when exposed to levels of 10µM of toxin, a 500 fold higher dose than used in our assay. As mentioned in the introduction, the second intermediate in the pathway, diphthine, lacking the amide group, can still be ADP-ribosylated, although at a lower rate in comparison to diphthamide-modified eEF2 (Moehring et al. 1984; Chen and Bodley...
The dose-dependant effect of DT on YLR143w/DPH6 and YBR246w/DPH7, which are sensitive to endogenous overexpression of DT but are not affected by low levels of the toxin in vitro, lead to the hypothesis that both genes might be involved in the final step of diphthamide formation.

To address the question whether YLR143w/DPH6 and YBR246w/DPH7 are indeed involved in the diphthamide pathway, we isolated His6 tagged eEF2 from strains lacking either ORF and performed mass spectrometry to identify which post-translational modification was present in the deletion mutants. Our mass spectrometry analysis clearly shows that in yeast strains lacking either YLR143w/DPH6 or YBR246w/DPH7, modification of his-699 progresses only as far as diphthine. Thus Dph6 and Dph7 are both required for the final amidation step required to generate diphthamide. Our work is therefore consistent with that of Su et al. (2011), who while our study was in progress reported that Ybr246w/Dph7 is required for conversion of diphthine to diphthamide. In eEF2 isolated from the ybr246w/dph7 mutant we only detected loss of the trimethylamino group of diphthine during mass spectrometry, in eEF2 isolated from the ylr143w/dph7 mutant we observed loss of the trimethylamino group before analysis of the peptide. This suggests that diphthine-modified eEF2 in the ylr143w/dph6 mutant is somehow more labile and prone to elimination of the trimethylamino group. Previously, it was suggested that similar lability of diphthine on EF2 from the archaon P. horikoshii might be due to the local amino acid context of the modified residue in EF2 and that sequence differences between eukaryal and archaeal EF2 could explain why diphthine-modified eEF2 from yeast appeared stable in the ybr246w/dph7 knockout strain (Su et al. 2011). Furthermore, we detected unmodified
his-699 peptide in eEF2 extracted from all the strains examined and not just in the dph1Δ mutant that is blocked in the first step of the diphthamide biosynthetic pathway. Thus even in wild-type strains, not all eEF2 appears to be modified. The presence of higher levels of unmodified eEF2 in ybr246w/dph7, ylr143w/dph6 and possibly also dph5 in comparison to wild-type yeast could indicate some sort of feedback control mechanism whereby the first step of the diphthamide biosynthetic pathway is downregulated if significant levels of eEF2 with modification intermediates, e.g. ACP or diphthine, accumulate.

Prompted by our mass spectrometry results, we examined whether either novel diphthamide synthesis protein interacts with each other or bona fide DPH genes and eEF2. Co-immunoprecipitation of tagged constructs revealed that YLR143w/DPH6 and YBR246w/DPH7 do not interact with each other or with DPH1, DPH2 and DPH5. However, Ylr143w/Dph6 directly binds to eEF2 suggesting that it is the favored candidate for the amidase, which converts diphthine into diphthamide. In line with this, sequence analysis of YLR143w/DPH6 revealed three conserved domains, which indicate that it functions as an enzyme. The N-terminal region contains a Alpha_ANH_like_IV domain (cd1994 in the NCBI Conserved Domain Database), a member of the adenine nucleotide alpha hydrolase superfamily, which is proposed to bind ATP. The C-terminal region carries two conserved domains related to the YjgF-YER057c-UK114 protein family (eu_AANH_C1: cd06155 and eu_AANH_C2: cd06166). These domains are found in a range of proteins involved in nucleotide and amino acid metabolism (Volz 1999; Burman et al. 2007; Lambrecht et al. 2012). We therefore consider it likely that
Dph6 is directly involved in diphthine amidation and that this step is an ATP-dependent process, with perhaps ammonia or glutamine acting as the source of the amide group.

Sequence analysis revealed that **YBR246w/DPH7** contains four WD40 domains and is involved in two seemingly diphthamide-unrelated processes: firstly it was shown to act in endosomal recycling as the product of the **ERE1** gene (Shi et al. 2011) and secondly as a negative regulator of RNA polymerase I (**RRT2**) (Hontz et al. 2009; Shi et al. 2011). Though the putative mammalian homologue, WDR85, was proposed to act in the first step of diphthamide formation (Carette et al. 2009), our data, in line with recent findings reported by Su et al. (2011), clearly demonstrate that it is necessary for the final step of diphthine amidation. The four conserved WD40 repeats in **YBR246w/DPH7** suggest that it acts in mediating protein-protein interaction. Though it does not bind to Ylr143w/Dph6, our data suggest a regulatory role for Ybr246w/Dph7 in modulating the interaction between Dph5 and eEF2, where the **ybr246w/dph7** mutant shows a significant increase in interaction between the diphthine synthase and the translation elongation factor. We hypothesize that Ybr246w/Dph7 might be necessary to displace Dph5 from eEF2 in order to allow the diphthamide synthesis to progress into the final amidation step. This notion is supported by the finding that the mammalian homolog, WDR85, shows a similar effect on Dph5-eEF2 interaction. Furthermore, we demonstrate that elevated levels of Dph5 are cytotoxic to **dph1-dph4** cells as well as the **ybr246w/dph7** mutant. It appears that the Dph5 is inhibitory to the translation factor when diphthamide synthesis is incomplete. Therefore we conclude that Dph5 does not only act as a methyltransferase but also binds to eEF2 when diphthamidation is incomplete and inhibits its function in translation elongation. In agreement with this, we
demonstrate that the lack of DPH1 resulting in an unmodified His\textsuperscript{699} also promotes binding of Dph5 to eEF2. In addition, the detrimental effect of Dph5 overexpression on the \textit{ybr246w} mutant contributes to our hypothesis that \textit{YBR246w} is necessary to displace Dph5 from eEF2 before diphthine can be converted to diphthamide by Ylr143w/Dph6. Whereas in the \textit{ylr143w/dph6} cell, endogenous levels of Ybr246w are present to antagonize the toxicity of increased Dph5 levels and rescue eEF2 function. This would predict a better accessibility of the His\textsuperscript{699} residue in the \textit{ylr143w/dph6} mutant compared to the \textit{ybr246w} mutant, which is confirmed in the finding that cells lacking \textit{YLR143w/DPH6} are more sensitive to the action of endogenous expression of DT.

Moreover, our mass spectrometry data not only clarifies the involvement of \textit{YLR143w/DPH6} and \textit{YBR246w/DPH7} in diphthamide synthesis but also showed a loss of the trimethylamino group in both mutant strains. While eEF2 analysis from \textit{ybr246w} cells displayed this chemical change only during the mass spectrometry, eEF2 from \textit{ylr143w/dph6} showed partial loss of the trimethylamino group before analysis. Hence the diphthine modification in the \textit{ylr143w/dph6} is more labile than in the \textit{ybr246w/dph7} mutant. It is possible that Ybr246w/Dph7 displaces Dph5 in the \textit{ylr143w/dph6} cell thereby rendering it more prone to elimination, whereas in a \textit{ybr246w/dph7} mutant Dph5 occupies and stabilizes the diphthine modification. The proposed model for Ybr246w/Dph7 function in diphthamide synthesis will need further biochemical examination to confirm our hypothesis, however the previous findings of its involvement in endosomal recycling and regulation of RNA transcription might implicate Ybr246w/Dph7 in multiple cellular processes. Nevertheless, we present extensive data suggesting Ylr143w/Dph6 as the more likely candidate for the amidase
and Ybr246w/Dph7 as a regulator of the interaction between Dph5 and eEF2 and therefore we have named the former \textit{DPH6} and the latter \textit{DPH7}.

Though the pathological relevance of diphthamide as an effector of the cytotoxic drugs, diphtheria toxin, sordarin and ricin, has been extensively studied, its physiological role remains elusive. However, data from our own group and others have demonstrated that \textit{DPH1}, \textit{DPH2} and \textit{DPH5} mutants display a significant increase in -1 frameshifting (Ortiz et al. 2006a; Bar et al. 2008). Furthermore, mouse models of \textit{DPH1}, \textit{DPH3} and \textit{DPH4} indicate a crucial role for the diphthamide modification in embryonic development and tumourignesis (Chen and Behringer 2004a; Liu et al. 2006; Webb et al. 2008). It appears that the function of the posttranslational modification on eEF2 becomes more apparent in higher organisms, where the regulation of translation is a tightly regulated process. In support of this, we examined -1 frameshifting in all \textit{bona fide} diphthamide synthesis genes as well as the novel \textit{DPH6} (\textit{YLR143w}) and \textit{DPH7} (\textit{YBR246w}) ORFs. Our assay resulted in a similar incidence of -1 frameshifting in \textit{DPH2} and \textit{DPH5} cells as reported by Ortiz et al. (2006) and further demonstrates that apart from \textit{DPH4} and \textit{DPH7}, all other diphthamide deficient mutants display a significant increase in -1 frameshifting. Though the ratio of -1 frameshifting in \textit{dph4} and \textit{dph7} is statistically not significant, it is elevated compared to the wild-type. We therefore propose that diphthamide on eEF2 is necessary for maintaining the correct reading frame during translation and that together with other \textit{DPH} genes, \textit{DPH6} is crucial in this capacity.
CHAPTER 5

DISCUSSION
5 Discussion

The research presented here is centered upon diphthamide, a unique post-translational modification on translation elongation factor 2 (EF2) from archaea and eukarya (Moehring et al. 1984; Chen et al. 1985a). As the name implies, eEF2 is a key player in the elongation cycle of de novo protein synthesis and facilitates the translocation of the newly synthesized polypeptide within the ribosome. Since it was first discovered in 1974, diphthamide has been subject to extensive studies revealing its chemical structure and the involvement of at least 5 proteins, Dph1-Dph5, in a three step biosynthesis pathway (Pappenheimer 1977; Liu and Leppla 2003a). However, the physiological role for this complex modification remains elusive. While diphthamide-deficient yeast display mild phenotypes, mouse models of DPH1, DPH3 and DPH4 are embryonically lethal and display developmental delays (Chen and Behringer 2004b; Liu et al. 2006; Webb et al. 2008). Furthermore, DPH1 is allelic with OVCA1, a tumour suppressor gene that is strongly linked to ovarian and breast cancer formation. Mouse embryonic fibroblasts (MEFs) lacking OVCA1 display cell proliferation defects, which could explain the defects in embryogenesis (Chen and Behringer 2004b). Therefore the crucial role of diphthamide on eEF2 seems to be more apparent in multi-cellular organisms, however the conserved diphthamide synthesis pathway allows us to investigate this unique post-translational modification in S. cerevisiae. This research was aimed at dissecting diphthamide biosynthesis further by investigating the first and the final step of the pathway.
5.1 Dph1 mutagenesis reveals a central role for the iron-sulfur enzyme

The initial step of eEF2 diphthamidation is the transfer of an ACP (3-amino-3carboxylgroup) radical from SAM (S-adenosylmethionine) onto the imidazole ring of the histidine precursor and in eukaryotes involves four proteins, Dph1-Dph4 (Chen and Bodley 1988). Our group together from data from other groups demonstrated that Dph1, Dph2 and Dph3 form a complex, where all partner proteins interact with each other and co-purify in protein immunoblots (Fichtner et al. 2003a; Liu et al. 2004a; Bar et al. 2008). Furthermore, the homologous Dph1 and Dph2 form a heterodimer where the archael counterpart of Dph1, PhDph2 (P. horikoshii Dph2), was recently reported to serve as an iron-sulfur containing enzyme that catalyzes the cleavage of the ACP group from SAM. Extensive structural and spectrometric data revealed that PhDph2 has three distinct domains each containing a conserved cysteine residue (Cys59, Cys163 and Cys287), which cluster at the center of the protein and present the binding sites for the iron-sulfur cluster (Zhang et al. 2010; Zhu et al. 2011).

Here we aimed at dissecting the eukaryotic homologue of PhDph2 in S. cerevisiae (ScDph1) in order to map functional regions of Dph1. By introducing progressive truncations in intervals of 30aa from the N- and the C-terminal end we hoped to identify the shortest deletion mutant that confers loss of function and therefore the minimal function unit of this protein. Surprisingly we observed that the smallest
truncation of only 30aa from either end renders Dph1 inactive in terms of diphthamide biosynthesis. Furthermore, the conserved regions containing the first and the third cysteine residue proved to be crucial for the interaction of Dph1 with either Dph2 or Dph3. Despite the fact that all N- and C- terminal truncations (N1-N4 and C1-C4) were not able to form diphthamide, the interaction between Dph1 and Dph2 as well as Dph1 and Dph3 remained intact as long as the conserved regions containing the cysteine residues were present. Since only the full-length Dph1 was able to form diphthamide, we hypothesize that the smallest truncation changes the structure of the protein, which might interfere with the formation of the triangular mold necessary for binding of the iron-sulfur cluster. It appears that the conformation of Dph1 is as important as the three conserved cysteine residues for binding of the [4Fe-4S] in order to facilitate cleavage of the ACP radical and its subsequent translocation to the histidine precursor.

The first step of archael diphthamide synthesis only requires the action of PhDph2 in vitro, which functions in the form of a homodimer. In eukaryotes on the other hand, the homologous Dph1 and Dph2 form a heterodimer with Dph2 only containing the first and the third conserved cysteine residue found in PhDph2 (Zhang et al. 2010). Therefore ScDph1 is the yeast counterpart to bacterial PhDph2, however ScDph2 is likely to have evolved from this same ancestral protein. A similar investigation of the Dph2 protein from Chinese Hamster ovary cells (CgDph2, C. griseus) showed that an N-terminal truncation of 158aa, which removes the first conserved cysteine residue and only leaves the second C-terminal one, has no effect on CgDph2 function and is as active as the wild-type protein (Roy et al. 2010). This finding does not agree with the proposed model by Zhu et al. (2011) who recently
reported that at least two of the three cysteine residues in PhDph2 are necessary for binding of the [4Fe-4S] cluster and that mutation of a single Cys residue does not affect diphthamide formation however the double mutant is inactive. Together with our observation that in contrast to Dph2, Dph1 is sensitive to a truncation as small as 30aa, we believe that Dph2 is not likely to act as the primary iron-sulfur binding enzyme, which generates the ACP radical from SAM. Furthermore, by mutating one of the PhDph2 subunits, Zhu et al. (2011) showed that a heterodimer with one wildtype and one mutated PhDph2 subunit, which could not bind the [4Fe-4S] construct, was more stable than the unaltered homodimer. Though the reason for this is unclear, it was suggested that a single bound iron-sulfur cluster might be thermodynamically more stable than the homodimer with two such clusters. If this proves to be the case, it might provide an explanation as to why archael PhDph2 evolved into the closely related Dph1 and Dph2 in yeast and higher organisms, where the former is likely to act as the main [4Fe-4S] cluster binding enzyme. Though Dph2 activity is essential for diphthamide formation, it might have a structural or regulatory function as opposed to the catalytic activity of Dph1. Since the Dph1-Dph2 heterodimer is fundamental to the diphthamide pathway, one could speculate that Dph2 stabilizes the structure, which is necessary to shape the triangular mold for binding of the iron-sulfur scaffold.

Though X-ray crystallography of PhDph2 has provided valuable insight in the mechanism of action which generates the ACP radical, it will be interesting to elucidate the structure of the Dph1-Dph2 heterodimer as well as the Dph1-Dph2-Dph3 complex in eukaryotes to clarify the function of each protein in diphthamide formation.
5.2 Data mining of genetic interaction database reveals novel

*DPH* genes

The analysis of toxin-resistant mutants in yeast and Chinese hamster ovary cells (CHO) cells identified 5 diphthamide synthesis genes, *DPH1*-*DPH5*, which are involved in the first and second step of the pathway (Pappenheimer 1977; Liu et al. 2004a). However, no specific enzyme was identified for the final conversion of diphthine into diphthamide. While our study was in progress, Su et al. (2011) reported a novel candidate gene, *YBR246w*, which plays a role in the final step. We here present our data, which are in agreement with the above mentioned publication and report the finding of a novel gene, *YLR143w*, which is likely to be the amidase that catalyzes the conversion of diphthine into the final product.

Data mining of two independent yeast screens revealed two candidate ORFs, *YLR143w*/*DPH6* and *YBR246*/*DPH7*, that share genetic and phenotypic traits of *bona fide* diphthamide synthesis genes. As determined by data collected in the DRYGIN database (Koh et al. 2010) *DPH6* and *DPH7* are closely linked to the genetic interaction landscape of *DPH1*, *DPH2*, *DPH4* and *DPH5*. Furthermore, both ORFs phenocluster with Dph1-Dph5 according FitDB, a data collection of fitness defects of yeast deletion mutant (Hillenmeyer et al. 2008). We further looked into the phenotypic similarity by demonstrating that like Dph1-Dph5, Dph6 and Dph7 are resistant to the diphthamide indicator drugs sordarin as well as DT (diphtheria toxin) in low levels. However, the sensitivity to elevated DT levels prompted the question of how Dph6 and Dph7 are
different from Dph1-Dph5 and whether they are indeed involved in the diphthamide pathway. Isolation of eEF2 from dph6 and dph7 mutant strains and subsequent mass spectrometry clearly shows that both gene products are necessary for the progression of diphthine into diphthamide. Therefore our data confirms the findings recently reported by (Su et al. 2011).

To further investigate the role of DPH6 and DPH7 in diphthamide synthesis we investigated whether either protein directly interacts with other Dph proteins or eEF2. Though neither Dph6 nor Dph7 interact with Dph1, Dph2 or Dph5, we observed binding of Dph6 to eEF2 in protein immunoblots. Dph7 on the other hand acts as a modulator of the interaction between Dph5 and eEF2. In other words, Dph5-eEF2 binding is elevated in the absence of Dph7, a finding also observed with its putative mammalian homologue, WDR85 (Carette et al. 2009). In addition, elevated levels of Dph5 are cytotoxic to dph1-dph4 and dph7 mutant strains. We hypothesize that Dph7 is necessary to displace Dph5 from eEF2 and that this is crucial for the progression of the diphthamide pathway from diphthine to the final product. We propose that in the DPH6 deletion strain, endogenous levels of Dph7 rescue the cytotoxic phenotype of Dph5 overexpression by downregulating binding of Dph5 to eEF2. This hypothesis could be confirmed by repeating the same experiment in a dph6/dph7 double mutant, where we would expect to see toxicity conferred by Dph5 overexpression. It is likely that Dph5 not only acts as a methyltransferase, but also serves as a checkpoint enzyme, which is inhibitory to eEF2 and the diphthamide pathway when intermediate forms of the peptide (ACP intermediate or diphthine) are present. In agreement with this, DPH1 mutated strains with unmodified eEF2 promote binding of Dph5 to eEF2. This model (Figure
5.1) is reinforced by our mass spectrometry data, where all samples contained unmodified His$_{699}$, but the highest levels were found in the $dph6$ and $dph7$ and possibly $dph5$ mutant strains. In sum, we demonstrate that the post-translational modification of eEF2 by the seven diphthamide synthesis genes, Dph1-Dph7, is not a linear process but contains some sort of feedback loop which regulates the progression of the pathway.

The findings reported in this study clearly favour Dph6 as the enzyme that catalyzes the final step of the diphthamide pathway. Not only does Dph6 directly bind to eEF2, but it also contains a putative ATP-binding site, which is necessary for the ATP-dependant amidation of diphthine. Structure analysis of Dph7 however, revealed four conserved WD40 repeats, which are indicative of proteins, which mediate protein-protein interaction, a feature that is in agreement with the above mentioned regulatory function. Nevertheless, definite proof that Dph6 is indeed the amidase involved in the final step can be gained by reconstructing the amidation of diphthine in vitro in presence of Dph6 alone.

It should be noted that the protein interaction profiles for Dph6 and Dph7 presented here have been generated by co-immunoprecipitation experiments only. Though this method is widely used it generally detects high affinity protein-protein interactions only and has its limitations. The use of epitope tags might interfere with proper folding of the tagged protein and alter the outcome of the co-immunoprecipitation. In addition, prior to co-purifying interacting proteins the cells are lysed in order to harvest the proteins, which then are processed further in different binding and elution buffers, which might not present an ideal environment for transient protein interactions. Hence, if Dph6 and Dph7 interact with other component of the
diphthamide pathway in a transient manner, they might have escaped our detection via co-immunoprecipitation. In order to address this issue several methods could be explored to verify the findings presented in this study. The traditional yeast-two-hybrid (Y2H) approach is an alternative assay, which allows detection of protein interactions \textit{in vivo} (Berggard et al. 2007). Here, the expression of a downstream reporter gene is dependent on transcription factors (for example \textit{Gal4}), which binds to an upstream activating sequence (UAS). In order to detect protein interactions, the transcription factor is split into a bait and a target sequence, which are used to c-terminally tag the two proteins of interest. If the bait protein and the target protein interact, the transcriptional activator is assembled and drives the expression of the reporter gene, which in turn is a measure for the protein-protein interaction of the bait protein with the target protein. The Y2H method does not only allow detection of protein interactions \textit{in vivo}, but also amplifies the signal by using a reporter gene. Alternatively, chemical crosslinking of interacting proteins with the use of bifunctional reagents, such as carbodiimide, allow to freeze putative protein complexes by coupling the carboxyl group from one protein to the lysine group of another protein (Melcher 2004). This enables the analysis of low affinity interactions before they dissociate. Taken together, co-immunoprecipitation has its limitations for detecting weakly interacting proteins, therefore verifying our results with an alternative method would provide further evidence for our proposed model for the diphthamide pathway.
Figure 5.1. Model for the diphthamide pathway incorporating the proposed novel roles of Dph5, Dph6 and Dph7. Diphthamide pathway showing the interaction of Dph5 with unmodified eEF2 and the proposed role of Dph7 in displacement of Dph5 prior to diphthine amidation.
5.3 The physiological role of diphthamide in normal cellular function

Though the diphthamide pathway has been implemented in cancer formation, embryonic development as well as cell proliferation control, the normal physiological role for this post-translational modification (PTM) is unclear. The three step pathway represents one of the most complicated PTMs and its final product, diphthamide, was first identified as the unique target for bacterial toxins (Robinson et al. 1974; Van Ness et al. 1980b). However, it seems unlikely that seven gene products, Dph1-Dph7, would be recruited to form a complex modification on eEF2, just so it could serve as a target for lethal bacterial toxins. In regard to the physiological role of diphthamide, deletion mutants of \textit{DPH1}, \textit{DPH2} and \textit{DPH5} have previously been shown to increase -1 frameshifting (Ortiz et al. 2006a; Bar et al. 2008). Here we demonstrate that apart from \textit{dph4} and \textit{dph7} all \textit{bona fide} Dph proteins together with the novel diphthamide protein, Dph6, significantly increase the incidence of -1 frameshifting. We therefore conclude that diphthamidation of eEF2 is crucial for maintaining the correct reading frame during translation. These findings are consistent with (Liu et al. 2012), who very recently reported that lack of diphthamide on eEF2 in mammalian cells significantly elevated -1 frameshifting. The diphthamide modification is located at the tip of domain IV of eEF2, a region that protrudes into the ribosomal decoding center and has been suggested to interact with the codon-anticodon interface of tRNA and mRNA (Stark et al. 2000; Spahn et al. 2004). eEF2 function is strictly dependant on conformational changes, which allow its translocation activity. Therefore it is likely that the
diphthamide modification contributes to the maintenance of the eEF2 conformation, which in turn is fundamental to its role in translation. In line with this, Liu et al. (2012) demonstrated that the use of a specific eEF2 antibody, which does not target the site of diphthamidation directly, has an increased reactivity to eEF2 lacking diphthamide in contrast to the wildtype, which indicates conformational changes that influence the accessibility for the antibody.

Despite the elevated -1frameshifting incidence in dph4 and dph7 mutant strains, they were statistically not significant. The uni-reporter plasmids used in our assay with a single reporter gene readout resulted in a wide range of fluctuation for the same strain. The error bars (as shown in figure 4.18 and 4.19) especially for the -1 frameshifting assay of the dph7 mutant could be corrected with a dual-reporter construct, where the reference gene demonstrates the level of total translation and the relative -1 frameshifting incidence is measured from the second reporter gene, which is in -1 frame. This presents a favourable alternative to the uni-reporter assay and we would expect dph4 and dph7 mutant strains to phenocopy the other DPH genes in regard to the incidence of errors in translation.

The specific role of maintaining the fidelity of mRNA translation would explain why the lack of the modification on eEF2 has such a deleterious effect on embryonic development, a stage where protein synthesis is a tightly controlled process. In the unicellular organism S. cerevisiae however, though increased -1 frameshifting is clearly measurable, it is not lethal to yeast as demonstrated by the viability of dph1-dph7 mutants. Therefore the crucial physiological role of diphthamide may only be apparent in multicellular organisms.
Interestingly, frameshifting events are present in organisms from archaea, eubacteria as well as eukarya (Dinman 2006). Though first discovered in RNA-viruses, which routinely use -1 programmed ribosomal frameshifting (PRF) to achieve alternative read-through of overlapping ORFs, it has been demonstrated that higher organisms as well as bacteria apply frameshifting as an intrinsic part of their translation machinery (Brierley 1995; Farabaugh 1996). Computational analyses of the *S. cerevisiae* genome predicted 10340 putative -1 PRF motifs, with 1275 out of the 6353 yeast genes containing at least one statistically significant -1 PRF signal (Jacobs et al. 2007). Out of nine tested motifs eight were shown to result in significant -1 PRF levels *in vivo*. However, unlike viruses yeast frameshifting events do not result in alternative gene products, but redirect ribosomes to premature stop codons, which leads to the degradation of the reporter mRNA via the NMD (nonsense-mediated mRNA decay) pathway. More recently, (Belew et al. 2011) demonstrated that the regulation of mRNA abundance via -1 operational ribosomal frameshifting (-1 RF) in *S. cerevisiae* is not only determined via the NMD pathway but also the no-go decay pathway (NGD). The fact that approximately 11% of yeast genes contain at least one -1 RF indicates that frameshifting is a widely-spread post-transcriptional mechanism for the regulation of mRNA abundance and stability. This is not only the case for yeast genomes, but across 25 analysed eukaryotic genomes so far, 8-12% of genes have been shown to contain at least one -1 RF (data collected in PRF database: PRFdB at [http://prfdb.umd.edu/](http://prfdb.umd.edu/)) (reference) (Belew et al. 2008). In the context of diphthamidation of eEF2, the significant increase of -1 frameshifting in diphthamide-deficient yeast as well as mammalian cells indicates that the post-translational modification of eEF2 is necessary
to regulate the level of -1 frameshifting, thereby affecting the abundance and stability of mRNA. It is likely that in the unicellular yeast deregulation of the affected mRNA levels is less severe compared to higher organisms, which especially during embryonic development are dependant on a tightly controlled translation machinery.

An outstanding issue in the field is how in humans, \textit{OVCA1/DPH1}, and the subsequent lack of eEF2 diphthamidation leads to formation of ovarian and breast cancer. Liu et al. (2012) reported that the loss of function of \textit{OVCA1} is unlikely to have other effects than the lack of diphthamide formation on eEF2. Together with our observation that diphthamide-deficient strains are prone to errors in translation fidelity, it is likely that the lack of diphthamidation of eEF2 deregulates its function and the resulting increase in -1 frameshifting significantly degrades affected mRNA levels, which in turn promote cancer formation. \textit{OVCA1} would therefore acts as a tumour suppressor gene by allowing the post-translational modification of the translation factor and could thereby prevent these frameshift errors during protein biosynthesis. It would therefore be interesting to investigate mRNA levels in \textit{OVCA1} deficient mammalian cells to investigate which specific mRNAs are significantly degraded in absence of the tumour suppressor gene.

5.4 Concluding remarks and future directions

In conclusion, the work presented here contributes to a better understanding of the diphthamide biosynthesis pathway and its role in normal cell physiology. Our data provides a better insight into the role of Dph1 in the first step of eEF2 diphthamidation.
Furthermore, by mining data collected in extensive genetic and phenotypic studies, we identified the novel and until now uncharacterized member of the *DPH* gene family, *DPH6*. We here present genetic, phenotypic and biochemical analyses of Dph6 and Dph7 and present a model for their role in the final step of diphthamide formation.

However, in order to further explore the diphthamide pathway a number of potential experiments have been proposed. These include structural analysis of the Dph1-Dph2-Dph3 complex in eukaryotic cells to clarify how these proteins interact with each other in order to facilitate the initial step of the pathway. *In vitro* reconstruction of the conversion of diphthine to diphthamide with the help of Dph6, would give definite proof that it is indeed the missing amidase as indicated by our study. Furthermore, investigating the putative homologues of *DPH6* and *DPH7* in mammalian cell lines and in a mouse model would clarify if they phenocopy the defects in embryogenesis and cell proliferation seen in *DPH1*, *DPH3* and *DPH4* deficient mice. In addition, a *DPH6* and *DPH7* mouse model would give insight into their role in the context of cancer formation. Given the implication of this unique post-translational modification on eEF2 in cancer formation, embryogenesis and cell proliferation control it is crucial to further our knowledge on the role of diphthamide in translation regulation.
CHAPTER 6

MATERIALS and METHODS
6 Materials and Methods

6.1 Materials

6.1.1 *Escherichia coli* strains

<table>
<thead>
<tr>
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<th>Genotype</th>
<th>Source</th>
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<tbody>
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<td>Gibco BRL</td>
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6.1.2 *Saccharomyces cerevisiae* strains

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CBKY143 kanMX6::pGAL1-::HA-DPH1

This study

YSC1178- BY4741 YLR143w-TAP::His3MX6

Open Biosystems

YBR246w

This study

CBKY1 Y43 ybr246w::URA3

Dr. Christian Baer

CBKY2 Y43 ylr143w::URA3

Dr. Christian Baer

CBKY3 Y43 dph1::URA3

Dr. Christian Baer

CBKY5 BY4741 DPH5-3HA::kanMX6

Dr. Christian Baer

CBKY6 Y43 DPH6(3HA)::kanMX6

Dr. Christian Baer

CBKY7 BY4741 YLR143w-3c-myc::kanMX6

Dr. Christian Baer

CBKY8 BY4741 YBR246w-9c-myc::HIS3MX6

Dr. Christian Baer

CBKY9 BY4741 DPH5-3HA::kanMX6; YBR246w-9c-myc::HIS3MX6

Dr. Christian Baer

CBKY10 BY4741 YLR143w-3HA::kanMX6; YBR246w-9c-myc::HIS3MX6

Dr. Christian Baer

CBKY11 BY4741 YLR143w-3c-myc::kanMX6; DPH5-6HA::HIS3MX6

Dr. Christian Baer

CBKY12 Y43 but ybr246wD::HIS3

Dr. Christian Baer

CBKY13 BY4741 HIS3MX5::pGAL1-3HA-DPH6

Dr. Christian Baer

CBKY14 BY4741 HIS3MX5::pGAL1-3HA-DPH6; dph5D::KanMX4

Dr. Christian Baer

CBKY15 BY4741 HIS3MX5::pGAL1-3HA-DPH6; dph7D::KanMX4

Dr. Christian Baer

SUY1 BY4741 YLR1143w + pTKB612 (eEF^2His)

This study

SUY2 Y4741 YLR1143w + pTKB612 (eEF^2His)

This study

SUY3 DPH5-HA::His3MX6

This study

SUY4 BY4741 dph1D::KIURA3

This study

SUY5 BY4741 dph2D::KIURA3

This study

SUY6 BY4741 dph3D::KIURA3

This study
### 6.1.3 Plasmids

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<td>(Knop et al. 1999)</td>
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pYES2

| AmpR, URA3, 2α, Yeast-E.coli shuttle vector |

+ Invitrogen

pJD204.0

| pRS316, lacZ reporter (control), URA3, CEN-ARS, AmpR |

+ kindly provided by G. Kinzy, Robert Wood Johnson Medical School, Piscataway, USA (Harger et al. 2001)

pJD204-1

| pRS316, lacZ reporter (-1 frameshift), URA3, CEN-ARS, AmpR |

+ kindly provided by G. Kinzy, Robert Wood Johnson Medical School, Piscataway, USA (Harger et al. 2001)

pJD204+1

| pRS316, lacZ reporter (+1 frameshift), URA3, CEN-ARS, AmpR |

+ kindly provided by G. Kinzy, Robert Wood Johnson Medical School, Piscataway, USA (Harger et al. 2001)
### 6.1.4 Oligonucleotides

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RV-koDPH3  CTTATTTCCTATTTGTATTCGATCTAGCCTCTCATCTTTAGGCAGCAGAGCTTTTGCTGC
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FW-koDPH4  CTTTTTTTTGTTGGAATTTTACGCGACGGCCGATGGAATTTCCCGG
          DPH2 gene deletion
RV-koDPH4  GCCATAATCCCTTCCCCCTTTTACGGTGGCTGAGGTGACACGG
          DPH3 gene deletion
FW-koDPH5  ATGCTTTATTTGATCGGACCTTGTTCTCTGTAACAAATGACATTACCGTCAGGCAGGCA
          GTGAATTCCCGG
          DPH3 gene deletion
RV-koDPH5  ATAAAAAGAAACTACACATGAGCGTGTGCATTACCTTTACTCCTGCGAGCTTTGCC
          DPH4 gene deletion
FW-koYLR143w CAATAAGTGTATATGAGCTATAGCAGGTTGGAAGCGACGGCCAGT
          YLR143w gene deletion
RV-koYLR143w CATTGGAGTTAAGAAGCCAAATGCAACCCAAACAGGGCTTTTCAAGCTTTGGCTGC
          YLR143w gene deletion
FW-koYBR246w CTATATCTGCATGTGTTCTCTTGACATATAGGATCTGTCGAGCTTTGCTCAG
          YBR246w gene deletion
RV-koYBR246w CTAAACTATCCCATGTTTCAAGGAATATTACATATAAAAATAGGACATGTGAGCTTGGCTGC
          YBR246w gene deletion
S2-DPH1   GAATATGATACTATTTTTATATACATAGCTAAGGAAAGCAAGTCGACACAAACAAAAAACTAT
          DPH1 epitope tagging
          TTAAATGCGAATTACGCGGCTCG
          (C-terminal)
S3-DPH1   ATCCAAAGGTATATAGGCTAAGCAAAGGAGATCCAAACTCCGAAACACCTCC
          DPH1 epitope tagging
           ACATCGG
          (C-terminal)
S2-DPH2   TAAATAGTTTTAGTAAATATCTTGGATTTATATAGAGTCGAGGGAACACAAATATTAT
          DPH2 epitope tagging
          AAGGAGATCGATAGGCTAGTCG
          (C-terminal)
S3-DPH2   GTATTTAATGCTGTCGACAGTGTTATGAGGTGGGTTATGAGTCGGAAGACTGCTATGAAAGGGAA
          DPH2 epitope tagging
          AACAAACGTCAGTACGGTCGAC
          (C-terminal)
S2-DPH3   TGCGTTGCTAATAGCTACTTCTTCTTTCTTTCTTTCTTTTTGTAATTCTCAGATCTAGGCTTCTCA
          DPH3 epitope tagging
          TCTATCAGATAGATCCAGACCTCG
          (C-terminal)
S3-DPH3   AAGACTTGGCTAGTACTCAGGAAGGAGCCAGGACATCCACCACCCCTAGGGCTATTTGCGCCT
          DPH3 epitope tagging
GCTGCCCGTACGCTGCAGGTCGAC
CTATAAACAGATTATCATGATATGCTCAATTTCCCTCCCTCCCATCGATGAATTGAGCTCG

S2-DPH4  DPH4 epitope tagging (C-terminal)
S3-DPH4  DPH4 epitope tagging (C-terminal)
S2-DPH5  DPH5 epitope tagging (C-terminal)
S3-DPH5  DPH5 epitope tagging (C-terminal)

GAAGGTTAATTTTGACATCGAGGAAGAGCACAAGAAGGACACGTACGTCAGGTCGAC

S2-YPH1  YLR143w epitope tagging (C-terminal)
S3-YPH1  YLR143w epitope tagging (C-terminal)
S3.1-DPH1  DPH1 truncation and epitope tagging (C-terminal)
S3.2-DPH1  DPH1 truncation and epitope tagging (C-terminal)
S3.3-DPH1  DPH1 truncation and epitope tagging (C-terminal)
S3.4-DPH1  DPH1 truncation and epitope tagging (C-terminal)
F4- DPH1  DPH1 truncation and epitope tagging (N-terminal)
R3- DPH1  DPH1 truncation and
| R3.1- DPH1 | TTGTAGTTAGAGGCAATAATTTGGATGGCTTCTCAATTCAACTCTTTGTCAATTGCAGCA CGTAATCTG | epitope tagging (N-terminal) DPH1 truncation and epitope tagging (N-terminal) |
| R3.2- DPH1 | TCACTTATAATCAATGAGTAAATCAGCAACCTCTCCAGGCATCTGTAGGGCTATTCTTTTA GCATTGCAGCA GTGAGCA CGTAATCTG | DPH1 truncation and epitope tagging (N-terminal) |
| R3.3- DPH1 | TCATCAATACAGCATGCACCATGACAACATCCATCCCATTACTAGAGATTTCGAGAGCA CGTAATCTG | DPH1 truncation and epitope tagging (N-terminal) |
| R3.4- DPH1 | AGTACTTTAAATCTTTTGTAACGTAATAGGAACCTAAACGAAATGACGTAATGAGCA CGTAATCTG | DPH1 truncation and epitope tagging (N-terminal) |
6.1.5 Antibodies

All antibodies used in this study were obtained from Santa Cruz. For western blot detection of proteins and for coupling of Dynabeads (Invitrogen) prior to use in co-immunoprecipitation assays the following antibodies were used: monoclonal primary antibodies anti-HA, anti-c-Myc, anti-His6 and anti-CBP. The corresponding secondary antibodies were: anti-mouse, anti-rabbit and anti-goat.

6.1.6 Cultivation of bacterial strains

*E. coli* cultures were grown in Luria-Bertani (LB) medium containing 0.5% (w/v) Bacto yeast extract, 1% (w/v) Bacto tryptone and 0.5% (w/v) sodium chloride. To select plasmids LB was supplemented with ampicillin to a final concentration of 100 µg/ml. *E. coli* strains were grown at 37°C, while the liquid cultures were shaken during incubation.

6.1.7 Cultivation of yeast strains

*S. cerevisiae* strains were routinely grown at 30°C for 2-3 days in YEPD (Yeast Extract, Peptone, Dextrose) medium containing 1% (w/v) Bacto yeast extract, 2% (w/v) dextrose and 2% (w/v) Bacto peptone. Minimal synthetic medium (SD) consisted of 0.81% (w/v) Difco yeast nitrogen base without amino acids and 2% (w/v) dextrose (Sherman 1991). Strains with auxotrophic markers were propagated by addition of the following amino acids to the SD medium: adenine hemi-sulphate (40mg/l), L-leucine (120mg/l), L-histidine (20mg/l), L-tryptophan (20mg/l), L-methionine (20mg/l) and Uracil (20mg/l). 2% (w/v) Agar-Agar were added to the media before autoclaving to prepare plates.
6.2 Methods

6.2.1 Isolation of plasmid DNA from bacterial cultures

Plasmid extraction from over night bacterial culture was performed using Plasmid Mini Kit1 (OMEGA Bio-TEK) according to manufacturer’s instructions.

6.2.2 Isolation of genomic DNA from yeast cultures

Isolation of yeast genomic DNA was performed after the desired strain was grown in 5ml liquid selective culture over night at 30°C. The cells were collected by spinning at 4000 rpm for 2-3 min. The pellet was washed with 1ml distilled water before they were centrifuged at 4000 rpm. The cells were resuspended in 200 µl breaking buffer (2% Triton X100, 1% SDS, 100mM NaCl, 10mM Tris/HCl pH8.0, 0.1mM EDTA), 200 µl Phenol-Chloroform-Isoamyl alcohol and a volume of 250ml of glass beads were added, before breaking the cells open in the bead beater for 1min. This mix was centrifuged for 5 min. at 14000 rpm before 150 µl of the clear phase on top was harvested. The DNA was precipitated by addition of 1ml 96% ethanol and gently inverted before the supernatant was dried and resuspended in 300µl 1x TE (0.1M Tris-HCl pH8 and 1mM EDTA).
6.2.3 Transformation protocols for E.coli

6.2.3.1 *E.coli* transformation (chemical)

1-2 µl of plasmid was added to 120 µl chemically competent *E.coli* cells (DH5α). This mix was incubated on ice for 30 min. before heat shocked at 42°C for 90 sec. Following this, the cells were chilled on ice for 2 min. before the addition of 1 ml Luria Broth. The *E.coli* were incubated at 37°C for one hour while shaking. Finally the cells were collected by centrifugation for 1 min. at 14000 rpm before they were spread on LB/Amp plates containing 100µg/ml Ampicillin. Colonies were allowed to grow over night at 37°C.

6.2.3.2 *E.coli* transformation (electroporation)

1-2 ml plasmid was added to 120 ml electrocompetent *E.coli* and transferred into a cuvette. An electric pulse of 2.5V was applied followed by the addition of 1 ml Luria Broth. The transformed *E.coli* were allowed to incubate at 37°C for one hour before they were collected by centrifugation and spread onto LB/Amp plates containing 100µg/ml Ampicillin. Colonies were allowed to grow over night at 37°C.

6.2.4 *S. cerevisiae* transformation procedures

6.2.4.1 Short transformation protocol for *S. cerevisiae*

This protocol was adapted from (Chen et al. 1992) and was routinely performed for plasmid transformation into yeast. Liquid yeast culture was grown over night and a 250 µl aliquot was pelleted by centrifugation at 4000rpm for 5 min. The pellet was
resuspended in 100 µl of one step transformation mix (800 µl 40% (w/v) PEG (polyethylene glycol, MW 3350), 200 µl 1M lithium acetate, 100 µl 1M DTT), 5 µl of 10mg/ml single stranded salmon sperm DNA (previously denatured at 95°C for 10 min) and 1µg of plasmid DNA. The mixture was vortexed and heat shocked at 42°C for 30min. The transformed yeast cells were collected by centrifugation at 4000rpm for 3 min. and washed with 1 ml sterile water. After centrifugation (4000rpm for 3 min.) the cells were resuspended in 100 µl sterile water and plated on appropriate media. The transformed cells were allowed to incubate at 30°C for 2-3 days.

6.2.4.2 High efficiency transformation protocol for S. cerevisiae

The yeast transformation protocol was based on (Schiestl and Gietz 1989) and was used for endogenous gene disruption or for N- and C-terminal epitope tagging. 40ml of liquid culture (grown from an overnight culture) were incubated at OD\text{600} 0.5 and allowed to grow until OD\text{600} 1.5. The cells were collected at 4000rpm for 5 min. and washed with 30 ml sterile water before they were collected again (centrifugation at 4000rpm for 5 min). The pellet was resuspended in 100 µl Lithium Acetate (100mM) and incubated at 30°C for 30 min while shaking. 100 µl aliquot was used for each single transformation. The cells in the aliquot were collected (centrifugation at 8000rpm for 15sec) and the pellet was resuspended in the transformation mix (240 µl 50% (w/v) PEG (polyethylene glycol), 36 µl Lithium acetate (1M), 50 µl single stranded salmon sperm DNA (previously denatured at 95°C for 10 min) and 34 µl DNA (precipitated and purified PCR product). This transformation mix was incubated at 30°C for 30 min followed by a heat shock step of 50 min at 42°C. The transformed cells were pelleted at 8000 rpm for 15 sec and resuspended in 1 ml fresh YEPD. In order to allow the
antibiotic marker to be expressed the mix was incubuated at 30°C for 2-3 hours while shaking. The cells were harvested and washed with 1 ml sterile water before they were plated on appropriate media and allowed to grow for 2-3 days.

6.2.5 Polymerase chain reaction

6.2.5.1 Taq polymerase PCR protocol

Routine PCR reactions were carried out using KAPA Taq polymerase (Kapa Biosystems) according to manufacturer’s instructions with 1µM of each primer and under normal PCR conditions. Typical cycling conditions were an initial 95°C denaturation step for 1 min; 25-35 cycles of denaturation at 95°C for 30 sec followed by annealing at a primer-dependent temperature for 30 s, an elongation step at 72°C for 1 min/kb and a final extension at 72°C for 10 min. All PCR reactions were carried out using Sesoquest Thermo-Cycler PCR machines.

6.2.5.2 Proofreading PCR

Phusion high-fidelity polymerase and high-fidelity buffer (USB) were used according to manufacturer’s instructions when its proofreading property was desired. In contrast to KAPA Taq polymerase reactions, the elongation step was reduced to 20 sec per kb.
6.2.5.3 Sequencing PCR

Sequencing PCR was performed using the following mix: 2 µl primer, 4 µl plasmid, 1 µl Big Dye (provided by PNACL sequencing service), 3 µl sequencing buffer and 2 µl distilled water. The PCR conditions were: initial denaturation step: 94°C for 5 min, followed by cycles of 96°C for 10 sec and annealing and extension step: 60°C for 4 min, and the final step: 15°C for ∞. The denaturation, annealing and elongation cycle was repeated 29 times. Following sequencing PCR, the product was purified using Performa DTR Gel Filtration Cartridges (Edge BioSystems) according to manufacturer’s instructions before sending for sequencing by PNACL (University of Leicester sequencing service).

6.2.5.4 Yeast colony PCR

In order to test freshly transformed yeast, colony PCR was performed without prior DNA isolation from the colony. A very small amount of yeast cells was picked from a colony and transferred into a 0.2 ml PCR tube containing 10 µl 1x SPZ solution. 1x SPZ solution stock was previously prepared as follows: for a 50 ml SPZ stock 30 ml 2M sorbitol, 4.05 ml (1M) Na2HPO4, 0.95 ml NaH2PO4 and 15ml water was mixed and frozen at -20°C. 2.5 mg/ml Zymolyase 100-T was added to 1x SPZ solution before use. The yeast cells suspended in SPZ solution were incubated for 30 min at 37°C then 5 min 95°C in a PCR Thermo-Cyler. 90 µl sterile water was added and after mixing 2 µl of this template was used for a 20 µl PCR reaction with KAPA Taq polymerase.
6.2.6 Agarose gel electrophoresis

In order to visualize DNA or to separate a pool of different sized DNA fragments from each other, samples were routinely run on a 0.8 – 2 % agarose gel depending on the size of the fragments. To do so the samples were first mixed with 0.25x their volume of 5x loading dye containing 10% (w/v) Ficoll type 400, 0.1 M EDTA, 0.2% (v/v) bromophenol blue and 0.5% (w/v) SDS. Agarose was melted in 1x TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA pH 8.0) and the solution was supplemented with ethidium bromide (0.5 μg/ml) prior to polymerization. 1x TBE buffer was also used as running buffer. The appropriate DNA molecular ladders (GeneRuler, Fermentas) were run in parallel to allow determination of the size of the fragments. After the samples had run for the desired amount of time they were visualized using a UV light transilluminator.

6.2.7 DNA isolation from agarose gels

The elution of DNA bands from agarose gels was performed using Gel Extraction Kit (OMEGA Bio-TEK) according to manufacturer’s instructions.

6.2.8 Restriction Digestion

Restriction digestion of DNA samples was performed using restriction enzymes from Fermentas or New England Biolabs together with the according buffers according to manufacturer’s instructions.
6.2.9 Ethanol precipitation of PCR products

In order to concentrate DNA, ethanol precipitation of PCR products was performed. To do so the PCR product was mixed with 0.1x the starting volume 3M sodium acetate (pH 4.8) and 2x the volume ice cold 100% ethanol. This mixture was incubated at -20°C for 30 min. The precipitated DNA was centrifuged at 4°C and 13000 rpm for 30 min. The supernatant was discarded and the DNA was washed with 1 ml ice cold 70% ethanol and centrifuged again at 4°C and 13000 rpm for 5 min. The ethanol was discarded and the precipitated DNA was dried before it was resuspended in 34 µl 1x TE (0.01M Tris-HCl pH8 and 1mM EDTA).

6.2.10 Ligation

Ligation reactions of blunt-end DNA or sticky-end DNA was performed using T4-DNA ligase (5U/µl) and 10x reaction buffer (Fermentas). The ligation mix (1 µl ligase, 1 µl buffer, 4 µl distilled water, 3 µl insert and 1 µl plasmid DNA) was incubated either at RT for 2 hours or at 16°C over night before transformation into E. coli. Vector and insert DNA were typically used at a 1:3 ratio.

6.2.11 Gene manipulation procedures

6.2.11.1 PCR-based in vivo epitope tagging

N- and C-terminal tagging of a desired gene was performed according to previously published in vivo PCR-based one step epitope tagging protocols by (Knop et al. 1999) using appropriate S3/S2 primers (listed under 6.1.4). S2/S3 primers were
designed with a 50bp 5’ overhang which is homologous to the upstream region of the desired gene and were used to amplify a desired cassette containing a marker gene and a tag. Epitope tagging occurred in vivo homologous recombination after the PCR product was transformed following the high efficiency procedure mentioned in 6.2.4.2. Tagged genes were confirmed via PCR using ORF specific primers (listed under 6.1.4) as well as western blot detection with appropriate antibodies.

6.2.11.2 PCR-based in vivo gene deletion

confirmed via PCR from genomic DNA using ORF specific primers (listed under 6.1.4).

6.2.12 Phenotype assays for *S. cerevisiae*

6.2.12.1 Sordarin phenotype

In order to test *S. cerevisiae* strains for their sordarin phenotype, YEPD agar media was supplemented with 20 µg/ml sordarin sodium salt from *Sordaria araneosa* (Sigma-Aldrich) when glucose was the carbon source and 5 µg/ml sordarin when glucose was the carbon source. The strains were allowed to grow for 2-4 days before they were assessed for their sordarin phenotype.

6.2.12.2 Diphtheria toxin (DT) phenotype

In order to test the DT phenotype *S. cerevisiae* strains they were transformed with the *URA3* plasmid pLMY101, a kind gift from R. John Collier (Harvard Medical School, Boston, Mass.). This plasmid allows DT F2 fragment expression under the transcriptional control of yeast *GAL1* promoter, which allows conditional expression of the toxin on galactose-inducing medium. In order to reduce the level of DT expression, *BamH1* fragment with the DT F2 insert from pLMY101 was cloned into *GAL-S* vectors p415-*GALS* and p416-*GALS* (Mumberg et al. 1994) respectively, which are *CEN-ARS* plasmids carrying a truncated *GALS* promoter. The resulting p415-*GALS*-DT (pSU8) and p416-*GALS*-DT (pSU9), allowed conditional DT expression on galactose-inducing medium.
6.2.12.3 Frameshift reporter assay

Translational frameshift reporter assay involved the lacZ reporter plasmids pJD204.0 (wildtype control), pJD204.-1 (-1 frame) and pJD204.+1 (+1 frame) (Harger et al. 2001) (kindly provided by G. Kinzy, Robert Wood Johnson Medical School, Piscataway, USA). Translational fidelity was assayed as previously described by Ortiz et al. 2006 (Ortiz et al. 2006b). For measurement of β-galactosidase readouts from the lacZ reporter gene, three biological replicates (three different colonies from the plasmid transformations) of each strain were used for two technical replicates. Overnight cultures grown in SD media were used for the assay. 800µl of liquid culture was washed with sterile water and collected by centrifugation at 4000 rpm for 5 min. The pellet was resuspended in 1ml Z-buffer (for a 10ml Z-buffer stock: 60 mM Na₂PO₄, 40 mM Na₂HPO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0 before addition of 50 mM β-mercaptoethanol, 400 µl 0.1% (w/v) SDS 600 µl chloroform). The reaction was started by addition of 200 ml ONPG (4 mg/ml in Z-buffer without β-mercaptoethanol) and incubated at 30°C, while the time was measured until the solution started turning yellow. The reaction was stopped by adding 400 µl 1M Na₂CO₃ and the sample was centrifuged at 4000 rpm for 5 min. The supernatant was collected and the OD₄₀₀ and OD₅₅₀ was measured. β-galactosidase activity was calculated using the following formula:

\[
\text{Activity} = 1000 \times (\text{OD}_{420} - (1.75 \times \text{OD}_{550})) / (\text{time} \times \text{volume} \times \text{OD}_{600})
\]
In order to minimize measurement errors the above protocol was adjusted by using protein extracts instead of the cell solution. Total protein extracts from the yeast strains transformed with the lacZ reporter plasmids were prepared according to the protocol described under 6.2.13.1. The protein extracts were used for measurement of β-galactosidase activity on the same day they were prepared to avoid protein degradation during storage and thawing. 5-10 µl of the protein extract was mixed with 800 µl Z-buffer (without β-mercaptoethanol or chloroform). The reaction was started by addition of 200 µl of ONPG (4 mg/ml in Z-buffer without β-mercaptoethanol or chloroform) and the mix was incubated at 30°C until the solution turned yellow. The reaction was stopped by addition of 400 µl 1 M Na₂CO₃ and the OD₄₂₀ was measured. β-galactosidase activity (unit = nM cleaved ONPG / min / mg protein) was calculated using the following formula:

\[
\text{Activity} = \frac{((\text{OD}_{420} \times 1.4)/0.0045)\text{(amount of protein*extract volume*time)}}
\]

6.2.13 Protein biochemistry

6.2.13.1 Isolation of whole cell protein extract from yeast

To prepare whole cell protein extract yeast strains were grown overnight in the appropriate media. This culture was used to inoculate a fresh 50 ml culture and allowed to grow until OD₆₀₀ 1.5 was reached. The cells were harvested at 4°C and 4000rpm for 5 min and washed twice with 30 ml sterile water. The pellet was resuspended in 400 µl breaking buffer (50mM Heps-KOH pH7.3, 60mM Sodium acetate, 5mM Manganese acetate, 0.1% Triton X100, 10% Glycerol, 1mM Sodium fluoride, 20mM
Glycerophosphate, 1mM DTT) supplemented with Proteinase inhibitor cocktail (Roche). ~300µl volume of glass beads were added and the cells were broken in a bead beater for 1 min. at highest speed. This was followed by centrifugation at 4°C and 13000rpm for 5 min. to separate and harvested the supernatant containing the extracted protein. After another round of centrifugation at 4°C and 13000rpm for 20-30 min. The clear supernatant was collected and used for consequent experiments. The protein concentration of each sample was measured using the NanoDrop spectrometer.

**6.2.13.2 Polyacrylamide gel electrophoresis**

In order to separate a pool of proteins from each other they were run SDS-PAGE gels alongside an appropriate pre-stained protein ladder (PageRuler, Fermentas). If not otherwise stated, SDS-PAGE gels were composed of a 5% stacking gel (5% (v/v) acryl amide, 125mM Tris/HCl pH 6.8, 0.1% (w/v) SDS) and a 12% separation gel (12% (v/v) acryl amide, 375mM Tris/HCl pH 8.8, 0.1% (w/v) SDS). Prior to loading, protein samples were denatured in 1x Laemmli buffer (0.25M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) β-mercaptoethanol, 0.02% (w/v) bromphenol blue, 40% (v/v) glycerol) and heated to 90°C for 10 min. Proteins samples were run in 1x running buffer ((0.025 Tris-HCl, 0.192M glycine, 0.1% SDS) at 200 volts until the desired separation of the pre-stained band in the protein ladder was reached.

**6.2.13.3 Western blotting**

Routinely 60 µg of each protein sample along with an appropriate marker (GeneRuler, Fermentas) was run on SDS-PAGE gels and used in the Western blot
assay. The proteins were transformed from the gel to a PVDF membrane (Millipore) activated by 20% (v/v) methanol in 1x running buffer (0.025 Tris-HCl, 0.192M glycine, 0.1% SDS) and blotted for 1-2 hours at 100 volts using transfer buffer (0.025 Tris-HCl, 0.192M glycine, 0.1% SDS, 20% (v/v) methanol). The transfer was performed in wet conditions using a Bio-Rad apparatus. After the transfer the PVDF membrane was incubated in blocking solution (5%(w/v) milk in 1x TBST (20mM Tris/HCl pH7.6, 137mM Sodium chloride, 0.3% Tween 20) for 30min. Following this the membrane was incubated for 2 hours in 5%(w/v) milk in 1x TBST containing the desired primary antibody in dilution of 1:1000 – 1:10 000 according to manufacturer’s instructions. After three wash steps with 1x TBST for 15min., the membrane was incubated for 2 hours with the appropriate secondary antibody diluted in 5%(w/v) milk in 1x TBST according to manufacturer’s instructions. After three wash steps with 1x TBST for 15min., the protein bands were then visualized using the ECL-Amersham Bioscience according to manufacturer’s instructions.

6.2.13.4 Stripping PVDF membranes

In order to remove the antibodies from proteins blotted onto a PVDF membrane and to repeat immuno detection of the same samples, the membrane was stripped of the bound antibodies by incubation in 1% SDS in 0.1M glycine at pH 2.8. After a 30 min. incubation the membrane was washed four times for 15min. in 1x TBST (20mM Tris/HCl pH7.6; 137mM Sodium chloride, 0.3% Tween 20).
6.2.13.5 Co-Immunoprecipitation

Detection of N- and C-terminal HA and c-myc tagged proteins in co-immune precipitation (co-IP) assays was performed using magnetic Dynabeads (Invitrogen, #101-03D). To do so total protein extracts were prepared as described under 6.2.13.1. A small aliquot of the whole cell protein extract (routinely 50 µl) was set aside for detection of the proteins in the pre-IP sample. First, Dynabeads were coupled with the desired antibody according to manufacturer’s instructions. 3 mg of total protein was applied to Dynabeads and immunoprecipitated according to manufacturer’s instructions. IP and pre-IP samples were loaded on SDS-PAGEs (10-15%) and detected via Western blot using the appropriate antibodies.

6.2.13.6 TAP purification

Tandem affinity purification was performed according to (Rigaut et al. 1999) and (Puig et al. 2001). The TAP tag allows a tandem of two consecutive purification steps: first the Protein A domain at the C-terminal end of the TAP tag is immunoprecipitated with Dynabeads previously coupled with IgG primary antibody. The bound protein is eluted by cleavage of the linker sequence by AcTEV Protease (Invitrogen). The second purification step is performed with CBP-coupled Dynabeads, which binds the CBP domain of the TAP tag.

A total volume of 2 litres of yeast culture was inoculated from overnight cultures and grown to OD600 1.5. The cells were harvested by centrifugation at 4000rpm for 20 min and washed twice with sterile water. Cell pellets were frozen at -80°C overnight before they were disrupted at the following day. The cell pellet was resuspended in 10
ml buffer (10mM K-HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, 0.5mM DTT, 0.5mM PMSF, 2mM benzamidine, Complete Protease Inhibitor Cocktail (Roche) and the cells were disrupted with the bead beater. The crude extract was adjusted 10 200 mM KCl by addition of the appropriate amount of 2 M KCl. Two consecutive centrifugation steps were performed at 4°C: 35000rpm for 30 min followed by 45000rpm for 1hr. The cleared extract was dialyzed against buffer D (20mM K-HEPES pH7.9, 50mM KCl, 0.2mM EDTA pH8.0, 0.5mM DTT, 20% glycerol, 0.5mM PMSF, 2 mM benzamidine) for 4hrs at 4°C. The dialyzed volumes of the samples varied from 7 - 10 ml and was frozen at -80°C overnight before the actual TAP purification was performed.

200 µl IgG Sepharose beads were washed with 5 ml IPP150 buffer (10 mM Tris-Cl at pH 8.0, 150 mM NaCl, 0.1% NP-40) at RT for 5 min. before the IPP150 buffer was removed. Each 10 ml extract buffer was adjusted by addition of 50 µl 2M Tris-HCl at pH 8.0, 200 µl 5M NaCl and 100 µl NP-40. 10ml of this adjusted extract was added to the washed IgG Sepharose beads and incubated at 4°C while gently shaking. The unbound liquid was removed and the beads with the bound protein were washed three times with 10 ml IPP150 buffer. Following this the beads were washed once with 10 ml TEV-cleavage buffer (10mM Tris-HCl at pH8.0, 150mM NaCl, 0.1% NP-40, 0.5mM EDTA and 1mM DTT) at 16°C. The liquid was removed and the bound protein was eluted by incubation with 1 ml TEV cleavage buffer and 100 units of AcTEV Protease (Invitrogen) for 2hrs at 16°C. The eluate was collected and the remaining eluate was washed out with 200 µl TEV cleavage buffer. 200 µl calmodulin affinity beads were prepared by washing with 5 ml IPP150 buffer in a new column. The IPP150 buffer was removed and the eluate was prepared by adding 3 volumes of IPP150 calmodulin-
binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole 2 mM CaCl2 and 0.1% NP-40) and 1 µl of 1M CaCl2 per ml of eluate. The adjusted eluate was transferred to the calmodulin affinity beads and incubated for 2hrs at 4°C while rotating. The unbound material was removed and the beads were washed three times with 10 ml IPP150 calmodulin-binding buffer. The bound protein was eluted in fractions of 200 µl with IPP150 calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole 2 mM CaCl2 and 0.1% NP-40, 2 mM EGTA).

6.2.13.7 In vitro ADP-Ribosylation

In vitro ADP-Ribosylation assays were performed by Dr. Shihui Liu at the National Institute of Health, Bethesda, USA. Yeast cell extracts were prepared as described previously (Liu and Leppla 2003b). 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 50 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 gels (Invitrogen). The proteins were transferred to nitrocellulose membranes and western blotting was performed using streptavidin-IR conjugate (Rockland Immunochemicals, Gilbertsville, PA) and scanned on an Odyssey Infrared Imager (LICOR Biosciences, Lincoln, NE). Two unknown non-specific bands (indicated by *) served as the even loading controls.
6.2.13.8 Expression and purification of His6-tagged eEF2

BY4741 wt strain as well as dph1, dph5, ylr142w and ybr246w mutant strains were transformed with LEU-marked pTKB612 plasmid (Jorgensen et al. 2005), which expresses His-tagged eEF2. Jorgensen et al. showed that the eEF2-His construct can complement a eft1Δ eft2Δ double mutant confirming that the construct is functional. In order to express and purify eEF2 for MS/MS analysis 750ml of yeast culture were grown in YPD to an OD\textsubscript{600} 2.0 and harvested by centrifugation. The pellet was resuspended in 3ml B60 buffer without DTT (50mM Hepes-KOH pH 7.3, 60mM KAc, 5mM MgAc, 0.1% Triton X100, 10% Glycerol, 1mM NaF, 20mM Glycerophosphate, Protease Inhibitor complete tablet (Roche)) and cells were lysed in a bead beater. The lysate was centrifuged twice at 13 500rpm for 30 min. and the protein concentration measured with a NadoDrop spectrophotometer. 5mg total protein was applied to 2mg anti-His-tag-Dynabeads and purified according to manufacturer’s instructions (Invitrogen, #101-03D). The identity of the purified protein was confirmed by Western Blot using anti-6xHis antibody (Abcam, #ab18184).

6.2.13.9 Analysis of Diphthamide pathway modifications of eEF2 by mass spectrometry

Crude yeast eEF2 preparations were separated by SDS-PAGE using 4-12% Bis-Tris precast gels (Invitrogen, Carlsbad, 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 using trypsin, subsequent to reduction and alkylation with dithiothreitol and iodoacetamide, with the resulting peptides cleaned over C18
columns. Peptides were then analysed 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 445.120024. 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 (Cox and Mann 2008) against a proteome database generated from the Saccharomyces Genome database (Cherry et al.) in 2010. Manual annotation of the modified peptide spectra corresponding to the modified eEF2 peptide and generation of extracted ion chromatograms were done using the Thermo Xcalibur software for spectra visualisation. Mass spectrometry analyses was performed and analysed at the University of Dundee in collaboration with Prof. Mike Stark and Dr. Sara ten Have.

6.2.14 Bioinformatic data mining of DPH genes

DRYGIN database (http://drygin.ccbr.utoronto.ca/, (Baryshnikova et al. 2010; Costanzo et al. 2010; Koh et al. 2010), which is based on quantitative genetic interactions of S. cerevisiae derived from the SGA double-mutant arrays conducted in the C. Boone lab, University of Toronto, was used to identify putative Dph synthesis genes.
In addition, Yeast Fitness Data Base, FitDB (http://fitdb.stanford.edu/fitdb.cgi), was used for data mining in order to identify genes that phenocluster with known Dph genes. The gene-gene relationship studies in FitDB are based on homozygous co-fitness profiling according to Hillenmeyer et al. (2008 and 2010) (Hillenmeyer et al. 2008; Hillenmeyer et al. 2010).

6.2.15 Multi-alignment tool

DNA sequences of the desired ORFs were obtained from NCBI website (http://www.ncbi.nlm.nih.gov/gene/) and aligned with the help of the multi-alignment tool, Jalview (http://www.jalview.org/) from the University of Dundee.

6.2.16 Statistical Analysis

The relative values for +1 and -1 frameshifting were statistically analyzed using one-way ANOVA followed by Dunnett’s multiple comparison post test and was performed with Graphpad Prism 5.0 software (Roy et al. 2010).
APPENDICES
7 Appendices

7.1 Appendix 1: Mass spectrometry analyses of TAP-isolated eEF2 from wt, dph1Δ and ylr143wΔ strains

The following spectra from mass spectrometric analysis of TAP-isolated eEF2 tryptic peptide 686-VNILDVTLHADAIHR-700 of wildtype, dph1Δ and ylr143wΔ mutant strains only show unmodified His$_{699}$. 
Figure A2. Mass spectrometry spectra of wildtype unmodified His$_{699}$ in eEF2 tryptic peptide.
Figure A3. Mass spectrometry spectra of \textit{ylr143w}Δ unmodified His\textsubscript{699} in eEF2 tryptic peptide.
Figure A4. Mass spectrometry spectra of dph1Δ unmodified His_{699} in eEF2 tryptic peptide.
7.2 Appendix 2: The amidation step of diphthamide biosynthesis in yeast requires DPH6, a gene identified through mining the DPH1-DPH5 interaction network
The Amidation Step of Diphthamide Biosynthesis in Yeast Requires DPH6, a Gene Identified through Mining the DPH1-DPH5 Interaction Network

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Abstract

Diphthamide is a highly modified histidine residue in eukaryotic translation elongation factor 2 (eIF2) that is the target for irreversible ADP ribosylation by diphthamide 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 (YBL264w/DPH7) in the amidation step. Like dph1-dph5, dph6 and dph7 mutants maintain eIF2α forms that evoke inhibition by DT and sorbinil, a diphthamide-dependent antifungal. Moreover, mass spectrometry shows that dph6 and dph7 mutants specifically accumulate diphthine-modified eIF2, demonstrating failure to complete the final amidation step. Consistent with an expected requirement for ATP in diphthine amidation, Dph6 contains an essential adenine nucleotide hydrolase domain and binds to eIF2. Dph6 is therefore a candidate for the elusive amidase, while Dph7 apparently couples diphthine synthase (Dph5) to diphthine amidation. The latter conclusion is based on our observation that dph7 mutants show drastically upregulated interaction between Dph5 and eIF2, 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.


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Introduction

Regulation of biological processes by posttranslational modification can involve the function, distribution and interaction capabilities of the modified protein [1–5]. Through most modification pathways such as phosphorylation and ubiquitin conjugation target many different proteins, some exceptional ones uniquely target just a single polypeptide [6]. One prominent example is diphthamide formation on eukaryotic translation elongation factor 2 (eIF2) [7]. Strikingly, this modification is pathobiologically important because it is hijacked for eIF2 inhibition by sorbinil fungicides and by diphthamide toxin (DT) produced by pathovarieties of Cryptosporidium 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, viz. masking the diphthamide-modified forms of eIF2 via ribosylation and irreversible ADP ribosylation of eIF2α, respectively [9–12]. Diphthamide itself is a highly modified histidine residue on eIF2α – 2-[3-carboxyamido-l-5-sarcosylbutylamino-propyl]-histidine – which is conserved from yeast (H3H) to man (H3C) (Figure 1) [5,9,13]. Intriguingly, it is absent from the bacterial (E. coli) 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

Diphtamide 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 diphtamide toxin, the disease-inducing agent produced by the pathogen Cosynt bacterium diphtheriae. Why cells require eEF2 to control diphtamide synthase is unclear, although mice unable to make it fail to complete embryogenesis. Cells generate diphtamide 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 diphtamide 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 diphtamine synthase (Dph5), which carries out the second step of diphtamide synthesis, and that Dph6 has a novel role as an eEF2 inhibitor when diphtamide synthesis is incomplete.

mutant strains that confer resistance to growth inhibition by DT and sorcinin. This has led to the identification of the diphtamide synthase genes DPH1- DHPS [7,12,14-16] (Figure 1). The first step in diphtamide synthesis involves transfer of a 3-amino-1-carboxypropyl (ACP) radical from S-adenosylmethionine (SAM) to the histidine amidation ring, generating the ACP-modified intermediate of eEF2 [17-19]. ACP radical transfer requires the protein Dph1-Dph2 [16], where Dph1 and Dph2 are paralogous iron-sulfur cluster containing partner proteins that cooperatively interact with Dph3, potentially as part of a multienzyme complex [6,20-22]. Dph1 and Dph4 are thought to deaminate Dph1-Dph2 by maintaining their iron-sulfur clusters in reduct states required for proper ACP radical generation. In line with this, Dph3 and Dph6 have electron carrier activities [23,24], while Dph5 (also known as Kitl1 [25]) additionally partners with Elongator subunit Elp3 [6,20], an iron-sulfur cluster and radical SAM enzyme with roles in protein and RNA modifications [26-28].

Formation of diphtamine, the second pathway intermediate (Figure 1), requires trimethylation of the amino group in ACP and is catalyzed by diphtamine synthase Dph5, using SAM as methyl donor [29,31]. Intriguingly, reconstitution of archael Dph5’s activity has shown that the trimethylamino group formed in diphtamine is prone to elimination in vitro [32]. Finally, the carbonyl group of diphtamine is amidated by an elusive, ATP-dependent diphtamide synthetase (Figure 1). Once fully modified, diphtamide can be efficiently targeted by NADPH-dependent ADP ribosyltransferase including DT, Polr2a, and the chola toxin [33,34]. However, the intermediate diphtamine is also a very weak substrate for inhibitory ADP ribosylation [23,31]. Together with data showing that growth inhibition by sorcinin also depends on DHPS-DPH1 [8,7], translation factor eEF2 constitutes an ‘Achilles heel’ for yeast, study of which has provided important insight into the pathological relevance of posttranslational protein modification [35].

Physiologically, the function of the diphtamide modification is enigmatic. Yeast mutants unable to synthesize diphtamide confer elevated frequency of ribosomal frameshifting [6,36] but are viable and grow normally [14], although substitution of the modified histidine in eEF2 by other amino acids confers growth defects in some instances [37]. However, loss of diphtamide synthesis leads to delayed development and is embryonic lethal in homozygous DHPS knockout mice [30-40]. Together with the association of mammalian DHPS with tumorigenesis [36,38] as well as neuronal and embryonic development, this indicates that diphtamide modification plays an important biological role. Whether or not this implies structural or regulatory roles for diphtamide-modified eEF2 remains to be seen, but the latter notion is intriguing given the possibility of endogenous cellular ADP ribosylases that target eEF2 [38].

Interestingly, no DT resistant yeast mutants have been identified to date that affect the final amidation step in the pathway, probably because diphtamine is targetable, albeit medi-
Dithiaplate Biosynthesis Requires Dph6 and Dph7

Recently, by ADP ribosylation [29,31]. Thus amitidate-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 dithiaplate biosynthesis has come from recent work on WDR55 and its potential yeast ortholog YEB246a [41,42], while our preliminary investigation of the yeast DPH1 [53] implicated both YEB246a and YEB145a as novel proteins potentially involved in the dithiaplate pathway. Here we further exploit yeast genome-wide genetic interaction and chemical genomics databases [43,44] to demonstrate that YEB145a (DPH6) and YEB246a (DPH7) cluster tightly with all known members of the dithiaplate gene network. We find that DPH6 and DPH7 mutants phototoxicity and DT strain typical of the bona fide DP mutants, which are defective in the first two steps of dithiaplate synthesis. Importantly, we show that DPH6 and DPH7 deletions block the final amidation step of the dithiaplate pathway, cause dithiaplate-modified forms of EF2 to accumulate and consequently abolish ADP ribose acceptor activity upon DT treatment. Thus conversion of dithiaplate to dithiaplate depends on Dph6 and Dph7.

Results

Yeast Gene Interaction Databases Predict Dithiaplate Functions for YEB145a (DPH6) and YEB246a (DPH7)

To identify factors involved in the terminal amidation step of the dithiaplate 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 dithiaplate 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, YEB145a and YEB246a, within the dithiaplate gene network [13], we next examined this network in more detail by mining the SGA DRYGEN database for quantitative s. cerevisiae genetic interactions [46,50]. We compared DPH1, DPH2, DPH4, DPH5, YEB145a and YEB246a gene interactions with every array ORF represented in the SGA network and deposited at DRYGEN, 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 interaction profiles generated for each specific DPH query gene, consistently being ranked among the top ten genetic interactors (Figure 2A). Interestingly, YEB145a and YEB246a were among the top interactors of DPH1, DPH2, DPH4 and DPH5, while the most correlated interactors for YEB145a and YEB246a included each other and several four specific DPH genes (Figure 2A). Such highly correlated interaction patterns suggest that YEB145a and YEB246a are both functionally interrelated and qualify as candidate ORFs of the pathway for EF2 modification by dithiaplate. In line with this notion, the two EF2 encoding genes, EF1 and EF2A, also ranked among the top ten interactors of DPH1, DPH2 and DPH5 (Figure 2A).

For independent validation of these correlations, we searched the FunDB yeast fitness database [51], which contains genome-scale phenotypic profiles for diploid yeast deletion collections in response to more than 1100 different growth conditions [43,52]. Here, scoring gene interaction profiles by homogeneity of sensitivity revealed that among the top few to phenoclicate with YEB145a are DPH1, DPH4 and DPH5, while top interactors of YEB246a include DPH1, DPH4 and YEB145a (Figure 2B). A similar pattern of interaction is shown by DPH5 (Figure 2E), DPH2 and DPH4 (data not shown). Based on correlated interaction profiles, FunDB scores GO terms enriched for processes concerning pyrophosphate, dithiaplate biosynthetic from pyrophosphate to LTR14a and YEB246a with p-values of 2×10^{-3} and 9×10^{-2} respectively (Figure 2C). Collectively, the YEB6 and DRYGEN profiles indicate that the presence of the DPH genes network (Figure 2C). This notion is consistent with a recent report that YEB145a and its mammalian homolog, WDR55, have a dithiaplate related function [41,42]. Since YEB145a is as yet unannotated in the Saccharomyces genome database (SGD), based on the evidence below that YEB145a and YEB246a are indeed dithiaplate synthesis genes we have named them DPH6 (YEB145a) and DPH7 (YEB246a).

DPH6 and DPH7 Deletions Cause Phenotypes Typical of Bona Fide Dithiaplate Mutants

To verify the predicted roles for DPH6 and DPH7 in the dithiaplate pathway, we next examined strains deleted for these ORFs for phenotypes specifically linked to defects in dithiaplate formation on EF2, namely phototoxicity and response to DT [6,7]. Sordarin traps c-E22 on the 80S ribosome [51], blocking mRNA translation elongation and yeast cell growth [54] in a fashion that depends on dithiaplate synthesis [6,7]. As a result, dithiaplate mutants dph1&dph5 efficiently protect against sordarin inhibition [6,7]. Like dph1&dph5, dph4 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 EF2 substitution mutants g5H54L and g5H54L (Figure 3A), which lack the H54L acceptor site for dithiaplate modification [37]. Thus DPH6 and DPH7 are novel sordarin effectors, a feature they share with the dithiaplate synthase gene DPH5 (DPH5).

Dithiaplate modification plays an key effector role for inhibitory ADP ribosylation of c-E2 by DT, hence dph1&dph5 mutants in both yeast and mammalian cell confer resistance towards DT [14,15]. We therefore compared DT-dependent ADP ribosylation of c-E2 in vitro between wild-type cells and dph1, dph4, dph6, dph7 mutants. While the translation factor from wild-type cells was efficiently modified by the toxic ADP ribosylase (Figure 3E), c-E2 extracted from dph1, dph4, dph6 and dph7 mutants could not be ADP ribosylated by agonistically activated DT under the conditions used (Figure 3E). Such lack of ADP ribose acceptor activity in vitro strongly suggests defects in the dithiaplate pathway and that DPH6 and DPH7 encode novel genes required for dithiaplate formation. To further address this experimentally, we assayed the response of dph1 and dph7 mutants to intracellular expression of the ADP ribosylase domain of DT (DTA) using GALL, a transmembrane variant of the GALL promoter [55]. When DTA expression was induced by 0.1% galactose, dph1 and dph7 mutants were indeed found to show some protection against DTA in contrast to wild-type cells (Figure 3G),
Figure 2. Genome-wide gene interaction databases identify additional diphthamide related candidate genes: YLR143w/DPH6 and YBR246w/DPH7. (A) SGD Database (DIP3.0). Genetic interaction profiles among DPH2, DPH4, DPH6, DPH5, YBR246w, and YLR143w query gene deletion strains and 3895 or 4446 array ORF mutants were extracted from data for a total of ~1700 query strain sets deposited at DIP3.0 (for full details, see excel spreadsheet in Table S1). Ranking of top interactors for each query ORF was according to PCC (Pearson correlation coefficient) determination. For simplicity, 15 ORFs (DPH4, DPH6, DPH5, EFT1, YLR143w and YBR246w red circles) are listed that score repeatedly as significantly high interactors of the query ORFs. (B) Yeast Fitness database (YFD). Genes whose deletions phenocast with the six query ORFs above were extracted from YFD, which is based on genome-scale co-fitness defect analysis of homozygous yeast deletion mutants in response to greater than 1144 different conditions. For simplicity, the top ten interactors for the five query ORFs above are depicted. (C) Representation of the tightly clustered and expanded DPH7/DPH6 gene network where nodes (pale blue) correspond to individual DPH gene family members and edges connect gene pairs by PCC>0.14. Enhanced gene interaction strength is proportional to PCC strength. Enriched GO process likelihoods in the diphthamide modification pathway are listed as P-values for the identified candidates DPH6/YLR143w and DPH7/YBR246w.

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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 dph6 and dph7 mutants remained fully resistant (Figure 3C). This suggests that EEF2 from dph6 or dph7 mutants, although not substrates in vitro (Figure 3E), 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 ypl146w/dph7 mutant was shown to be a very weak substrate for ADP ribosylation when treated with 10 mM DTA [42], a 500-fold increase in concentration over that used in our in vitro ADP ribosylation assays (Figure 3E). Thus EEF2 from the dph6 or dph7 mutants is resistant to DTA and shows a vastly reduced ability to be ADP-ribosylated by DTA, strongly suggesting that the diphthamide pathway is defective. Since the intermediate diphtehine can serve as a sub-optimai substrate for ADP ribosylation using excess levels of DTA or upon overexpressing its toxic ADP ribosylase domain from nude 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 diphtehine 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 Diphthamine 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 His92, the EEF2 residue that is modified to

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Figure 3. DPH6 and DPH7 deletion strains copy traits typically related to the bona fide diphthamide mutants dph6Δ-dph8Δ. (A) Serotonin resistance. Ten-fold serial cell dilutions of the indicated yeast strains, BY4741 wild-type (left) background and its dph1Δ-dph8Δ gene deletion derivatives (upper panels) as well as an MXK-derived eEF2 eEF2 double deletion background maintaining plasmid pEF2 wild-type or Hup1p substitution Hup6p N and Hup6p alleles of EFT2 lower panels, were grown on YPD plates in the absence (control) or presence (+ser) of 10 μg/ml serotonin. Growth was assayed for 3 d at 30°C. Serotonin resistant (R) and sensitive (S) responses are indicated. (B) Lack of in vitro ADP-ribose acceptor activity of eEF2. Cell extracts obtained from dph6Δ, dph7Δ, and eEF2 mutant and wild-type (wt) strains were incubated with (+DT) or without (-DT) 20 μM diphthamide (in the presence 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 each sample leading to DT DT phototypes. As indicated yeast dph mutants and wild-type control (wt) were tested for sensitivity to intracellular expression of DTA, the cytosolic ADP-ribose fragment of DT. This in vivo assay involved galactose-inducible expression from vector pSUB (see Materials and Methods). Serial cell dilutions were replica spotted onto rifampicin (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 d at 30°C. DTA sensitive (S) resistant (R), partially resistant (PR) and reduced sensitive (RS) phenotypes are indicated.

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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 ensured the stability of an eEF2 double mutant lacking eEF2 function, and it is thus considered to be biologically active [34]. Strains lacking either DPH1 in which the first step of diphthamide biosynthesis is blocked, or lacking DPH3 (encoding diphthamide synthase), were used respectively. As controls for complete lack of modification and presence of ACP, the first intermediate in the diphthamide pathway [14,15,30,31]. All strains expressed similar levels of His6-tagged eEF2 (data not shown).

The modified histidine in eEF2 (Hup6p) is located in the tryptic peptide E686-VNLDVSLHADAHSF-746, and, as expected, unmodified versions of this peptide were readily detected in eEF2 prepared from the dph6 mutant (Figure S1C). Unmodified peptide was also found in eEF2 prepared from dph1, dph4 and dph7 deletion strains as well as from wild-type cell (Figures S1 and S3). 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 1E), as expected given its known role in generating diphthamines [32] from the ACP intermediate in the pathway.

In contrast, eEF2 from the dph7 mutant generated spectra consistent with the presence of diphthamine on Hup6p, 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 carbonyl group in diphthamine 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 4. MS/MS spectra of diphthamide, ACP, and diphthine-modified EF2 peptide 666-VNLDEYLFYEHADNHK-700 from wild-type and mutant yeast strains. Spectra are shown for (A) diphthamine-modified peptide from the wild-type yeast strain; (B) ACP-modified peptide from the dpht6 mutant; (C) diphthamine-modified peptide in the dpht6/strain; (D) diphthamine-modified peptide in the dpht7/strain with loss of the trimethyllysine 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 trimethylamine during MS/MS. The inset in (C) shows greater detail for the more crowded part of the MS/MS spectrum. Figure 5A shows how B and Y ions are derived from the peptide sequence.

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Different effusion times of the diphthine-modified and diphthamine-modified peptide that are evident from the extracted ion chromatograms (Figure S5) are consistent with differently modified forms of EF2. As noted in previous studies [25,33,34], some of the ions in our MS/MS spectra had undergone neutral loss of the trimethyllysine group during MS/MS, as indicated by loss of 59,110 mass units.

Two types of spectra corresponding to the peptide with modified H3K9t were seen when EF2 from the dpht6 mutant was analyzed. In some spectra (Figure 6A), the parent ion m/z and MS/MS data indicated the presence of diphthine as in the dpht7 mutant, with some daughter ions again showing neutral loss of the trimethyllysine group during MS/MS as noted above. However, we also detected peptide forms in which elimination of the trimethyllysine group had occurred prior to analysis, as indicated by the lower parent ion m/z (Figure 6C) and an MS/MS spectrum in which all assignable peaks corresponded to ions lacking the trimethyllysine group. Such trimethyllysine elimination prior to mass spectrometry was observed previously when diphthine-modified Pyrococcus horikoshii EF2 was generated in an in vitro reaction [35], indicating that this modification might be unstable. However, we failed to detect any pre-mass spectrometry loss of the trimethyllysine group when EF2 from the dpht6 mutant was analyzed. Thus while EF2 from both mutants carries diphthine, the modification appears to be more labile in the dpht6 mutant and may be protected from trimethyllysine elimination by the absence of Dph7.

Figure 5A shows extracted ion chromatograms for ions with m/z values corresponding to the H3K9t-containing peptide modified with diphthamine, diphthine or with ACP, indicating that the ACP-modified peptide was only present in the dpht5 mutant, the diphthine-modified peptide was only present in dpht5 and dpht7 mutants, and diphthamine-modified peptide was only seen in wild-type cells. Our mass spectrometry analyses therefore show that in yeast strains lacking either DPH6 or DPH7, modification of H3K9t progresses only as far as diphthine. Thus both tests indeed qualify as novel diphthamine synthesis genes with likely roles in conversion of diphthine to diphthamine.

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

Although Dph6 and Dph7 appear to function within the same step of the diphthamine synthesis pathway, using co-immune precipitation they were not found to interact either with one another or with Dph5 and Dph6, players involved in the two earlier pathway steps (Figure 6C, Figure 5A) and data not shown. However, in support of our evidence that Dph5 is a diphthamine biosynthetic factor, we observed by co-immune precipitation that Dph6-HA bound to a fraction of H3K9t-tagged EF2 (Figure 5A). Interestingly, this interaction was independent of Dph7 (Figure 5A), suggesting Dph7 may not mediate interaction between Dph5 and the translation factor. Dph7 is also unlikely to play an indirect role through regulation of DPH6 gene expression because Dph6 protein levels were unaltered in the DPH7 deletion strain (Figure 5A).

Figure 5. Co-immune precipitations reveals eEF2 interactions with Dph6 and Dph5. (A) eEF2 interacts with Dph6 in a fashion that is independent of Dph7. (B) eEF2 interaction with Dph5 is dramatically enhanced by elimination of Dph7 or Dph5. Yeast strains co-expressing (His6)-tagged eEF2 with Dph6-HA (A) or Dph5-HA (B) in the background of wild-type (A, DPH7) and B. w30 and diphthine mutants (A, dpht5; B, dpht5, dpht6 and dpht7) were subjected to immune precipitations (IP) using the anti-HA antibody. Strains expressing (His6)-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 right panel; B, top panel) and anti-His6 antibodies (A, bottom left panel; B, bottom panel) to check for the presence 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 His6-tagged 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-His6 antibodies (A, bottom right panel; B, third panel), respectively. While absence of Dph7 hardly affected the eEF2 interaction (A), Dph6/eEF2 interaction was strongly enhanced by inactivating DPH5 or DPH7 (B).

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mutant (Figure 3E). This strongly suggests a conserved role for Dph4/17/20/85 as a regulator of the Dph5-eEF2 interaction. Remarkably, we also found similarly enhanced binding of Dph5 to eEF2 in the dph4 mutant, which has a defect in the first step of the diphthamide pathway and therefore lacks the acyl modification that is the immediate substrate of diphthamide synthesis (Figure 3E). Strikingly, DPH5 overexpression from a galactose-inducible promoter was found to be highly detrimental to cells deleted for DPH7 and to all mutants blocked at the first step of the pathway, but had little effect on the dph5 mutant and no effect on wild-type or dph5 cells (Figure 6A). Intriguingly, this cytotoxicity was seen in hand with the enhanced Dph5-eEF2 interaction profiles we observed in dph5, dph5 and dph5 cells under conditions of NAC deletion strain displayed altered responses to translation elongation indicator drugs such as hygromycin, anisomycin or paromomycin (Figure 5). In conclusion, our data indicate that diphthamide mutants such as dph5 increase ribosomal errors typical of −1 translational frameshifts and that the diphthamide modification function of Dph6, which is required for completion of diphthamide synthesis, is likely to assist eEF2 in reading frame maintenance during translation.

Discussion

We have presented genetic, phenotypic, mass spectrometric and biochemical analyses that clearly identify Dph4 as a novel protein required for the final step of diphthamide biosynthesis and that confirm a similar role for Dph7 as reported recently [11,12]. Thus, in yeast strains lacking either DPH5 or DPH7, modification of His804 on eEF2 progresses only as far as diphthamine and these gene products are required for utilization of diphthamine to generate diphthamide. Our findings are consistent with a recent bioinformatics analysis that predicted a role for Dph6 in the diphthamide to diphthamide conversion [60] and with the identification of Dph6 as yeast diphthamide synthase reported by Xu et al. [61] while we were revising our manuscript.

Dph6

Dph6 contains three conserved domains consistent with it functioning as an enzyme (Figure S8). The amino terminal 225 residues constitute an Alpha, ANL-like IV domain (α1994 in the NCBI Conserved Domain Database [62], also known as DPH7), a member of the adenine nucleotide alpha hydrolase superfamily that is predicted to bind ATP. Many DUF71 proteins from archaebacteria to mammals contain the highly conserved motif HxxGxxG (Dph6 numbering), which has been proposed to be involved in substrate binding and catalysis and which is replaced by ENGER/VDH in a group of related DUF71 proteins implicated in biosynthetic [60]. Based on this, we generated a dph6 allele encoding two substitutions in this region (G216E, E220A) and tested its functionality by monitoring complementation of reconstituted strain in yeast dph6 knockout strain. Figure 7 clearly shows that this small change completely inactivates the function of Dph6, demonstrating that the Alpha, ANL-like IV domain is critical for the conversion of diphthamine to diphthamide. The N-terminal portion of Dph6 contains two domains related to the Yrog-YER057c-UK114 protein family (α2, ANL-Like IV; α3, ANL-Like IV) that may promote homostabilization and formation of an inter-subunit cleft that has been proposed to bind small molecule ligands (α3−α4). Several key residues in human UK114 required for homostabilization and ligand binding [62] are present in Dph6 (Figure S8) including Arg 107, which in E. coli TdcF forms a hydrogen bond with the carbonyl side group of bound ligand [59]. Deletion of residues 353−425 encompassing much of the Yrog-YER057c-UK114 region abolished the function of Dph6 as measured by reconstituted strain (Figure 7), while truncation of Dph6 at the first of the two conserved domains by insertion of a stop tag also eliminated Dph6 function (Figure 7) despite detectable expression of the truncated polypeptide (data not shown), indicating that the Yrog-YER057c-UK114 domains are also important for Dph6 function and that the Alpha, ANL-like IV domain is nonfunctional on its own. Since Saccharomyces cerevisiae Yrog has an enzyme/substrate domain activity that is conserved in human UK114 [67] it is possible that the Yrog-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 pGAL5-DPH5 for galactose inducible overexpression of diphthine synthase Dph5 were serially diluted and replica spotted onto glucose (5% 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 (−) and sensitive (S) responses are indicated. Note that dph1-dph4 and dph7 mutants are extremely sensitive to DPH5 overexpression. (B) Ribosomal frameshift analysis reveals error-prone translation in dph1-dph4 mutants. Strains with the indicated genetic backgrounds were transformed with control (pID488.D) or deF−1 frameshift (pID488.D−1) plasmids (5×) to monitor deF expression through β-galactosidase (β-Gal) production using D-nitropheno-D-galactopyranoside assays and to score translation efficiency (pID440.D) and fidelity (pID440−1). 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; n.s. = not significant).

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Figure 7. Both the Alpha_ANH like IV and Yfp1-YER057c-UK114 domains in Dphpa are essential for its functionality. A) Diagram showing the Dphpa wild-type and mutant constructs tested in B, indicating the Alpha_ANH like IV (ANH) and Yfp1-YER057c-UK114 (UK114) domains and the position of point mutations, an in-frame deletion (--- - - - -) and triple myc epitope tag (myc3) as appropriate. B) Tenfold serial dilution of a dphpa deletion strain carrying the constructs shown in A or the corresponding empty vector (top panel), pU56 (wt Dphpa), lower panel, pU57 (wt Dphp3); Table S3) were spotted onto SC-ura plates with or without 10 µg/ml sorafide and grown at 30°C for 3 days.

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Taken together, these properties suggest a direct, ATP-dependent role for Dphpa in diphthamide maturation proceeding via an idenitified intermediate and with an unusual active as the source of the catalytic group. Such a direct role has now been demonstrated by Su et al., who have used an in vivo assay system to show that Dphpa has diphthamide synthetase activity [61]. Although proteins showing Dphpa-like domain organization are readily identified in fungi, plants, amphibians and insects (Figure S8), they are largely absent from archaeb and mammalian proteomes. However, mammalians and archaea have separate proteins showing strong similarity to either the condense molecule alpha hydrolase domain or to the Ygp1-YER057c-UK114 related regions (Figure S8 and data not shown), suggesting Dphp3 functionality may be split between different polypeptides in these cases. It is therefore surprising that expression of the human Dphpa ortholog in a yeast dhps mutant can dephthamidase biogenesis [61] despite lacking the Ygp1-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 Ygp1-YER057c-UK114 domains in Dphp3 from lower eukaryotes.

Dphp3 has four well-defined WD40 repeats (Figure S9) and its predicted structure consists of essentially β-sheet elements [41,61]. Although its human homolog WDR55 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 diphthamide in the absence of Dphp3 and that the block is therefore in conversion of diphthamide to diphthamide. Furthermore, this block cannot be bypassed simply by introducing Dphp3 on a multicopy plasmid to increase the level of diphthamide synthetase (data not shown). How then might Dphp3 contribute to diphthamide maturation? Its domain structure suggests it could act as an adaptosome molecule for diphthamide maturation [42], but this notion is at odds with our failure to detect interaction between Dphp3 and Dphp3 (see above). Our intriguing finding that E2F binds much more Dphp3 in the absence of Dphp3 suggests an alternative role, namely that Dphp3 is needed to displace Dphp3 from diphthamide-modified E2F2 to allow the maturation reaction to occur. Similar findings in mammalian cells upon inactivation of WDR55 support this notion [41]. Together with our data showing that viability of dhps-dphp3 and dhps cells is extremely sensitive to excess Dphp3 in comparison to wildtype or dphp3 cells, it appears that binding of Dphp3 to E2F2 is inhibitory to the function of the translation factor and negatively interferes with cell growth unless E2F2 carries the completed diphthamide modification. Perhaps in addition to catalyzing methylation of ACP-modified E2F2, Dphp3 binds to newly-synthesized E2F2 to exclude it from functioning in translation until the diphthamide maturation step takes place (Figure 5).

Consistent with this proposal is our observation that the level of Dphp3-associated with E2F2 in the dhps mutant, in which modification of H1073 cannot be initiated, is drastically increased and virtually indistinguishable from the enhanced Dphp3-E2F2 interaction seen when Dphp3 is absent. Dphp3 may be needed to displace Dphp3 once diphthamide has been generated so that Dphp3 can carry out the diphthamide to diphthamide conversion (Figure 6), a notion consistent with the sensitivity of the dhps mutant to Dphp3 overexpression. In contrast, the dhps mutant may tolerate Dphp3 overexpression because Dphp3 is present to displace it.

Two other seemingly unrelated functions have been previously proposed for Dphp3. Firstly, it emerges from a genetic screen as a potential negative regulator of RNA polymerase I (Rpi2), although no other Dphp3 genes were similarly identified [63]. Secondly, Dphp3 has been implicated in repressor mediated endosomal recycling and named ERR2 [65]. The connection between endosomal recycling and diphthamide biogenesis is currently unclear and it remains to be determined whether Dphp3 is multifunctional or if these other roles are linked to its E2F2 modification function.

Diphthamide on E2F2 is the target for bacterial ADP-ribosyltransferase toxins and also affects toxicity of neutrophil and macrophage inhibiting protein toxins from plants [70]. Although this emphasis its pathologival 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 [35] 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 decreased translational frame-shuffling, 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 synthetase gene affects cell proliferation and development: inactivation of Dphp3/XIU1 is associated with RNA modification defects and neuro-degeneration and mutations in Dphp3/VEGFA1 revealed a tumor suppressor role for this diphthamide synthetase 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 amimation step (Figure 8). While it is now clear that Dph6 is diphthamide synthase [61], the true role of Dph7 is now being explored in the context of the whole group.

Materials and Methods

Strains, Media, Growth Conditions, and Growth Assays

Three strains used in this study are listed in Table S2 and plasmids in Table S3. Culture was 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 spectinomycin sodium salt (Sigma-Aldrich). Yeast transformations with plasmid DNA were performed following the lithium acetate protocol [74]. Diphthamide toxin (DT) growth assays in vivo involved expression of the toxin via a cytosolic ADP-ribosyltransferase (ADPRT) from vector pGUS [p415-GAL1-ADPRT], essentially as previously described for dph1-dph5 mutants [60]. pGUS was made by cloning the GroESL fragment encoding DTA from pMY101 [50] into plasmid p415-GAL1, a single-copy K. lactis yeast shuttle vector with a truncated GUS promoter [55]. The translational frame-shift reporter assay essentially involved previously published protocols together with the described fUS reporter plasmid pJ2040.0 [wild-type control], pJ2040.1 (-1 frame) and pJ2040.1(+1 frame) [56,58]; the pJ2040 plasmid series was kindly provided by T. Kinney (UMDNJ, USA). The relative values for -1 and +1 frame-shifts were statistically analyzed 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 Deletion and Epitope Tagging

Details of all primers used in numerous PCR-dependent genomic manipulations experiments can be found in Table S8. Gene deletions were performed using in vivo PCR-based one step gene disruption protocols in combination with marker plasmids YEpKl4, YEpGalU or YEpSpH [76] and knockout primers (Table S4) including those previously described [6,25,77]. Gene deletions were confirmed via diagnostic PCR on genomic DNA preparations using target ORF-specific primer pairs (Table S4) as well as Southern blot assays. C-terminal tagging of DPH1, DPH2, DPH5, DPH7 and DPH7/HER1/161 and DPH7/HER1/161 was performed according to previously published in vivo PCR-based epitope tagging protocols [19] using appropriate BglII/SphI 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, Dph7 and Dph7 as well as Dph5 and Epl2 in co-immune precipitation [Co-IP] assays performed as previously described [6,73,77].

DPh6 Constructs

p5S6 was generated by insertion into YCplac111 [80] of a genomic PCR fragment including DPH6 plus with 299 bp of upstream and 59 bp of downstream sequence flanked by KpnI and BamHI sites incorporated using PCR primers (Table S4). The insert was verified by sequencing and shown to complement a dph6 knockout strain, p5S7 was made by closing the DPH6 insert in p5S6 into YEpplac181 [80]. To generate a 2G1EN N220A dph6 mutant, p5S6 was digested with ApaI and BamHI and the small DPH6 fragment replaced by an identical synthetic fragment (Integrated DNA Technologies) carrying the 2G1EN N220A mutations, generating independent clones p5M61 and p5M62. The replaced region was verified by DNA sequencing. p5M61 and p5M62 were generated from p5S6 by replacing the BamHI fragment carrying the C-terminal region of DPH6 and downstream sequence with a synthetic BamHI-SalI fragment in which codons 335-465 were replaced by sequence encoding the linker and triple myc tag from pMY23 [81]. To generate p5M72, the smaller NdeI-EcoRI fragment of p5S7 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 50 μg of yeast extract, 50 μg of 125 I-labeled [125I]ADP and 10 μM G63-64-NAD (Trevigen). Samples were then mixed with SDS sample buffer, boiled for 5 min and run on 4-25% SDS-PAGE gradient gels. The proteins were transferred to nitrocellulose.
membrane and Western blotting was performed using streptavi-
din-IR conjugate (Rockland Immunoclticals, Gilbertsville, PA) and scanned on an Odyssey Infrared Imaging (LI-COR Biosciences, Lincoln, NE).

Expression and Purification of Affinity-Tagged eEF2-(His)₆  
BY4741 wild-type yeast cells as well as ΔphlΔphcΔphbΔphb6 and Δphb2Δphb3Δphb46 mutants thereof carrying an gfp mini-allele were transformed with plasmid pTK121 (a kind gift from A. R. Merrill, University of Guelph, Ontario, Canada), which expresses a His₆-tagged form of translation elongation factor eEF2 (Table S5) that is fully functional and able to complement an gfpΔgfp double mutant [56]. In order to express and purify His₆-tagged eEF2 for MS/MS analysis, 750 ml of yeast cultures were grown in YPD to an OD₆₀₀ of 2.0 and harvested by centrifugation. The pellet was resuspended in 3 ml TBS buffer (50 mM HEPES-KOH pH 7.3, 60 mM KOAc, 5 mM MgOAc₂, 0.1% Triton X100, 10% (v/v) glycerol, 1 mM NaF, 20 mM glycerophosphate, 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-His₆ agarose Dynabeads (Invitrogen, #065-05D) and purified according to manufacturer's instructions. The identity of purified eEF2 fraction was confirmed by SDS-PAGE and Western blot analyses using an anti-His₆ antibody (Abcam, ab11184).

Analysis of Diphtamide Pathway Modifications on eEF2 by Mass Spectrometry  
Crude yeast eEF2 preparations from wild-type and Δphb strains were separated by SDS-PAGE using 4–12% Bis-Tris precast gels (Invitrogen, Carlsbad, USA) and the area of the gel containing eEF2 was excised after staining with Instant Blue Coomassie (Expedeon, Cambridge, UK). In-gel digestions were performed using trypsin, subsequent to reduction and alkylation with diethylthiodiisocyanate, with the resulting peptides cleaned over C₁₈ columns. Peptides were then analyzed via HPLC/MS/MS using a Dionex U3000 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 500 nl/min over 120 min using a linear 0–90% (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 465.12082. The initial survey scan was performed at 60000 resolution, FTMS scanning from 355–1800 Da. The top 15 most intense ions were selected for MS/MS sequencing, using collision-induced dissociation (CID). MS/MS charge state 1 ± 1, ± 2 accepted. Protein identification was performed using MaxQuant 1.2.12.2 [52] against a proteome database generated from the Saccharomyces Genome database [53]. Manual annotation of the modified peptide spectra corresponding to the modified eEF2 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 606- 610 (H697L701HDAHR-701) 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-697. V1 to V13 and B14 ions contain His-697 and their m/z values are therefore informative regarding the modification state of His-697. (B) MS/MS spectra of unmodified peptide in eEF2 obtained from the indicated yeast strain: the parent ion m/z and charge state is indicated in each case.

Figure S2 Extracted ion chromatograms of unmodified eEF2 peptide 606-VNLDDVLHADAHIR-701. In (A), peaks corresponding to doubly-charged ions (m/z unmodified peptide 562.98, extracted mass range 563.5–563.2) are shown while triply-charged ions (m/z unmodified peptide 562.98, extracted mass range 562.3–562.0) 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 wet sample. Peak annotations are as follows: RT, retention time; AA, peak area; RP, parent ion m/z.

Figure S3 Extracted ion chromatograms of modified eEF2 peptide 606-VNLDDVLHADAHIR-701. (A) Peaks corresponding to triply-charged ions (m/z diphtamide-modified peptide 610.68, m/z diphtamide-modified peptide 599.55, extracted masses 610.2–610.9). (B) Triply-charged ions (m/z acp-modified peptide 596.66, extracted masses 596.2–596.8). Peak annotations are as follows: RT, retention time; AA, peak area; RP, parent ion m/z.

Figure S4 Failure to detect interaction by TAP-based co-immunoprecipitation between Diphtb or Dphb7 and either Dphb or diphtamine synthase Dphb5, factors integral to the first two steps of diphtamine synthesis. Co-immunoprecipitations were performed using magnetic beads (Dynabeads, Invitrogen), coupled to anti-CBP antibody (Santa Cruz Biotechnology) specific for the calmodulin binding peptide (CBP) of the TAP tag. The indicated strains expressed DPHF-TAP or DPH7-TAP in conjunction with HA-tagged versions of either DPHF or DPH7. A strain co-expressing respectively, HA- and TAP-tagged variants of Dphb1 and Dphb3, 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 precipitate (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 (Suppl. B). Asterisks indicate breakdown products of Dphb3-HA, TAP-Dphb7 and Diphtb-TAP.

Figure S5 Failure to detect Dphb-Dphb7 interaction by co-immunoprecipitation. Co-immunoprecipitations using the anti-AHA-antibody were performed with the indicated strains expressing DPH7-myc or DPHF-myc on their own or in parallel with HA-tagged versions of DPHF or DPHF, respectively. A strain co-expressing eMyc and HA- and tagged versions of the Eip2 subunits (EIP2-myc) of the Elongator complex, and Knt2 (KNT2- HA), a protein known to interact with Eip2 [84], was used as internal positive control. The presence of the respective proteins was assessed in individual anti-eMyc and anti-HA Western blots both in the IPs (top two panels) and crude extracts (pre IP, bottom two panels). The asterisk denotes an ump-clip band that originates from the anti-HA antibody present in the IPs.

Figure S6 Lack of effect of Δphb1-Δphb46 gene knockouts on growth performance and viability. (A) The wild-type parental strain and diphtamine deficient mutants Δphb1, Δphb46 and Δphb46 were grown in YNB minimal media supplemented with His, Met, Ura, Lou to
cover the astrophysical markers (Table S2) under standard laboratory conditions over a period of 50 h. OD500 was monitored at 2 h intervals. 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 35°C. Note that only the dfh6Δ/dfh6Δ mutant, which affects additional biosynthetic pathways [6,57] apart from diaphanous biosynthesis [11] shows temperature sensitivity (S) (S) while the other dfh7Δ mutants tolerate higher temperatures (T) (T).

Figure S7: Altered growth performance of dfh6Δ/dfh6Δ mutants in response to translation elongation inhibitor drugs under standard or elevated cultivation temperatures. Ten-fold serial cell dilutions of wild-type parental strain as well as diaphanous mutants dfh6Δ/dfh6Δ were spotted on YPD plates without (control) and supplemented with hygromycin (20 µg/ml), amycin (20 µg/ml) or paromomycin (5 µg/ml) and grown at 30°C or 15°C [B]. Reduced or improved performance of the dfh6Δ mutants relative to wild-type behavior reflects respectively, enhanced sensitivity or improved tolerance towards the drug in question respectively. (TDF)

Figure S8: Conservation of the DFPH6 gene product, Dph6. (A) Representation of Dph6 indicating the conserved adenine nucleotide alpha hydrolase (cdh994) and Yfg1/YER057c-UK114 related (J006155, J006166) 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): DFPH6: S. cerevisiae Dph6p (Sp_3p_00047), Schizosaccharomyces pombe (NP_055310); Aa_AAH_IV, Adipose tissue aminoacylase (NP_197098); Dc_AAH_IV, Dicyostelium discoideum aminoacylase L-ATP binding domain 4 (NP_05003655); Hs_AAH_IV, Human ATP binding domain containing protein 4 (NP_05003655); Mm_AAH_IV, Mouse ATP binding domain containing protein 4 (NP_05003655); Hs_UK114, Human ribonucleases/tRNA-related ATP binding domain 4 (NP_05003655); Mm_UK114, Mouse ribonucleases/tRNA-related ATP binding domain 4 (NP_05003655). Note that the last two sequences appear before the alignment so that the sequence relationships to each of the Yfg1/YER057c-UK114 related domains in the non-mammalian proteins can be shown. (TDF)

Figure S9: Conservation of the DFPH7 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): DFPH7: S. cerevisiae Dph7p/YER364w Sp_3p_00047, Schizosaccharomyces pombe WD repeat protein (CAA12349); At_WD55, Arabidopsis thaliana WD40 domain-containing protein (NP_201166); Dd_WD55, Dicyostelium discoideum WD40 repeat-containing protein (NP_646503); Xl_WD55, Xenopus tropicalis WD repeat-containing protein 5-28 like (XP_002942023); Hs_WD55, Human WD repeat-containing protein 5-28 (NP_62103); Mm_WD55, Mouse unannotated protein (FAE_82074).

Table S1: SSA database for comprehensive presentation of genetic interactions between query genes DFPH6, DFPH7, DFPH5, DFPH9/YER146c and DFPH11/YER146a and array ORFs totaling 3835 (DFPH6, DFPH7, DFPH9/YER146c and DFPH11/YER146a) and 4410 (DFPH6 and DFPH7). Genetic interaction profiles among the six queries were ranked according to Pearson Correlation Coefficient (PCC) Scores of the top ten interactions identified with each query gene identified a tightly clustered and highly robust, SSA-based DFPH gene network (Figure 2C).

Table S2: Strains used or generated for this study.

Table S3: Plasmids used or constructed for this study.

Table S4: Primers and oligonucleotides used for this study.

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Author Contributions
Conceived and designed the experiments: ST CR SL FG MJRS RS. Performed the experiments: ST CR VS SL BS. Analyzed the data: ST CR VS SL BS FG MJRS RS. Contributed reagents/materials/analysis tools: ST CR VS SL BS FG MJRS RS. Wrote the paper: FG MJRS RS.

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Diaphanous Biosynthesis Requires Dpbb and Dpbl7

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Diphthamide Biosynthesis Requires Dph4 and Dph7


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