FEBS Letters

FEBS PRESS science publishing by scientists

RESEARCH LETTER

Diphthamide synthesis is linked to the eEF2-client chaperone machinery

Lars Kaduhr[1], Klaus Mayer[2], Raffael Schaffrath[1], Johannes Buchner[3] and Ulrich Brinkmann[2]

1 Department of Microbiology, Kassel University, Germany
2 Roche Pharma Research and Early Development (pRED), Large Molecule Research, Roche Innovation Center Munich, Penzberg, Germany
3 Center for Protein Assemblies (CPA), Department of Bioscience, School of Natural Sciences, Technical University of Munich, Garching, Germany

Correspondence
U. Brinkmann, Roche Pharma Research and Early Development (pRED), Large Molecule Research, Roche Innovation Center Munich, Penzberg, Germany
Tel: +89-8856-604753
E-mail: ulrich.brinkmann@roche.com

Lars Kaduhr and Klaus Mayer contributed equally to this article

(Received 28 October 2024, revised 26 November 2024, accepted 15 December 2024, available online 18 January 2025)

doi:10.1002/1873-3468.15095

Edited by Michael Ibba

The diphthamide modification of eukaryotic translation elongation factor (eEF2) is important for accurate protein synthesis. While the enzymes for diphthamide synthesis are known, coordination of eEF2 synthesis with the diphthamide modification to maintain only modified eEF2 is unknown. Physical and genetic interactions extracted from BioGRID show a connection between diphthamide synthesis enzymes and chaperones in yeast. This includes the Hsp90 co-chaperones Hgh1 and Cpr7. The respective co-chaperone deletion strains contained eEF2 without diphthamide. Notably, strains deficient in other co-chaperones showed no defect in the eEF2-diphthamide modification. Our results demonstrate that diphthamide synthesis involves not only Dph enzymes but also the eEF2-interacting co-chaperones Hgh1 and Cpr7 and may thus require a conformational state of eEF2 which is maintained by specific (co-)chaperones.

Keywords: chaperone; Cpr7; diphthamide; elongation factor 2 (eEF2); Hgh1; J-protein

Diphthamide on eukaryotic translation elongation factor 2 (eEF2) is important for accurate protein synthesis [1–3]. Ribosomes that harbor eEF2 without diphthamide can generate mistranslated proteins due to higher incidences of −1 translational frameshifts [4–6]. Biological consequences of diphthamide deficiency include cellular stress responses, developmental defects in humans, and complete absence of diphthamide is embryonal/perinatal lethal in mice [7–14].

The diphthamide modification and its synthesis pathway are conserved in eukaryotes. At least 7 proteins (Dph1-Dph7 + Dph8 in yeast) are necessary to synthesize diphthamide on His715 of human eEF2 or His699 of yeast eEF2 [2,15–17]. This synthesis pathway covers four successive enzymatic steps with eEF2-ACP, -CH3 diphthine and -diphthine as reaction intermediates, and eEF2-diphthamide as the final product.

The expression of eEF2 depends on growth conditions and the metabolic state of the cell, and eEF2 expression levels can therefore be variable. But despite variable eEF2 expression levels and the complex diphthamide synthesis pathway, relevant amounts of unmodified or partially modified eEF2 intermediates are not observed in cells [1,9]. This suggests that the diphthamide modification on eEF2 occurs in a controlled manner, and/or is synchronized with *de novo*

Abbreviations
ACP, 3-amino-3-carboxypropyl group; CCT/TriC, T-complex protein Ring Complex; DPH1-8, protein/enzyme necessary for diphthamide synthesis 1–8; eEF2, eukaryotic translation elongation factor 2; HSP, heat shock protein.

FEBS Letters **599** (2025) 1260–1268 © 2025 The Author(s). *FEBS Letters* published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.

synthesis of eEF2, preventing the release of unmodified or partially modified eEF2.

Nascent and/or partially unfolded eEF2 has been shown to interact with the co-chaperones Cns1, Hgh1, and Cpr7 with a link to the chaperones Hsp90 and CCT/TriC, which enable the proper folding and/or stabilization of eEF2 [18,19]. This raised the question, whether and how the synthesis of diphthamide on eEF2 is integrated in this scenario. Specifically, we explored the possibility that eEF2-client co-chaperones may contribute to the diphthamide modification of eEF2.

To address this possibility, we extracted interaction networks between yeast chaperones and diphthamide synthesis factors from the BioGRID database [20] and analyzed to what degree eEF2-related chaperones affect diphthamide synthesis. We show that yeast strains which lack the eEF2-related chaperones Hgh1 and Cpr7 exhibit deficiencies in the diphthamide modification as they contain unmodified eEF2. Thus, chaperones that ensure the folding and structural integrity of eEF2 also support its decoration by diphthamide. This suggests a link between the chaperone and diphthamide synthesis machineries, which together produce correctly folded diphthamide-modified eEF2.

Materials and methods

Database analyses

The BioGRID database (https://thebiogrid.org/) compiles published physical interactions (affinity capture-MS or—Western, complexes, 2-hybrid) as well as genetic interactions (negative genetics, synthetic growth defects) of individual proteins/genes to each other [20]. To extract interactions, Protein/gene identifiers for diphthamide synthesis factors, eEF2 and chaperones were submitted to the *Saccharomyces cerevisiae* or *Homo sapiens* data collections in October 2024, noting numbers of individual interactors and links between the diphthamide synthesis pathway and chaperones.

Yeast strains and assessment of their sensitivity to hygromycin, sordarin, and diphtheria toxin

BY4741 WT and—derivatives that lack co/chaperones or diphthamide synthesis genes were obtained from the Euroscarf collection distributed by Scientific Research and Development GmbH (Oberursel, Germany, Table S4) and cultivated using standard techniques. Hygromycin and sordarin sensitivity assays were performed by spotting and incubating 10-fold serial dilutions of the strains for 2–4 days at 30 °C on YPD media plates lacking or containing hygromycin or sordarin. Diphtheria toxin sensitivity was similarly tested by inducing expression of the cytotoxic ADP-ribosylase domain of DT from the galactose-inducible vector pSU9 on synthetic media lacking uracil, as previously described [1,11,12].

Detection of eEF2 with and without diphthamide modification

To detect the presence of unmodified eEF2, total cell protein samples were subjected to reducing SDS/PAGE and analyzed by western blotting using an anti-EF2 (no diphthamide) antibody that specifically detects unmodified eEF2, anti-EF2(pan) antibody that binds eEF2 irrespective of its modification, and an anti-Cdc19 antibody (loading control) as described before [1,9,21]. For comparison of eEF2 levels in different strains, the western blot signals were quantified using IMAGEJ [22]. The signal ratio of anti-eEF2(pan) to anti-Cdc19 of the parent strain was set to 100% and the signal ratio %-values observed for the other strains were calculated relative to that. The presence of diphthamide-modified eEF2 was analyzed by ADP-ribosylation (ADPR) assays. Diphtheria toxin (DT) targets exclusively diphthamide-modified eEF2 and accepts biotinylated NAD (bio-NAD) as ADP-donor for ADPR. Reactions with DT and bio-NAD thus generate biotinylated eEF2 which is detected in western blots with labeled streptavidin as previously described [1,23].

Results

Physical and genetic links of diphthamide synthesis factors and chaperone networks

Conversion of nascent eEF2 to full functionality requires proper folding and diphthamide modification. It has been demonstrated recently that eEF2 folding involves a network of chaperones [18,19], and diphthamide modification requires the coordinated activity of a set of diphthamide synthesis enzymes [2,15,16]. Because barely any unmodified or intermediate-carrying eEF2 is observed in cells, we hypothesized that both machineries may be linked to each other.

To address that question, we extracted from the BioGRID database ([20], as of October 2024) physical interactions (affinity capture-MS or—Western, complexes, 2-hybrid) as well as genetic interactions (negative genetics, synthetic growth defects) between chaperones, eEF2 and diphthamide synthesis enzymes (Dph) in yeast (Fig. 1 and Table S1).

As expected, the results reveal physical contacts of several Dph enzymes to eEF2 and to each other: eEF2 is physically linked with all Dph enzymes except for Jjj3/Dph4. Dph1, Dph2, and Dph3 are linked to each other, and Dph3 additionally interacts with Dph8. These links agree well with structural data and our

current knowledge about the diphthamide synthesis pathway [15,16].

Physical interactions also exist between the eEF2/Dph network and chaperones. eEF2 and Dph2 are each reported to be physically linked to the Hsp70 isoforms Ssa1 and Ssa4. Dph1 and Dph5 and eEF2 are each linked to yeast Hsp90 and the CCT/TRiC subunit Cct3. The general (not client specific) chaperone Ssb2, which is connected *via* the ribosome-bound RAC complex to nascent proteins [24,25], appears to be a pronounced physical interactor between chaperones and the diphthamide synthesis machinery. It is physically connected to eEF2 (Dph-substrate), Dph1 (1st step of diphthamide synthesis), Dph5 (2nd step), Dph7 (3rd step), and Dph6 (4th final step). Interestingly, its closely related paralog Ssb1 does not reveal any links to Dph proteins or the diphthamide synthesis network in BioGRID.

Genetic interactions show additional relations between eEF2, Dph enzymes, and co-chaperones (Fig. 1B). Dph proteins connect not only to eEF2 and other Dph's (and 'general' chaperones that are predominant in the physical interactions above, Fig. 1A), but also to a set of special co-chaperones. These include Hgh1 and Cpr7, which together with yeast Hsc90/Hsp90, support the folding of eEF2 [18,19]. Hgh1 is linked to Dph1 and Dph2, Cpr7 is linked to Eft2, Dph2, Dph5, Dph6, and yeast Hsc90 to Eft2, Dph6, Dph7, and Dph8. Most of those chaperones are also part of a network of physical links between each other (Fig. 1C).

The combined data of many physical and genetic interaction experiments compiled in BioGRID thus provide evidence for a link between the diphthamide synthesis pathway and chaperones.

HGH1 and *CPR7* gene deletion strains contain eEF2 pools without diphthamide

Hgh1 and Cpr7 are co-chaperones that form a complex with nascent eEF2, which during its synthesis exits the ribosome without carrying the diphthamide modification [18,19]. To analyze if specific co-chaperones influence the synthesis of diphthamide, we assessed the presence or absence of unmodified eEF2 in wild-type and yeast knock out strains with antibodies that detect either total eEF2, or eEF2 without the diphthamide modification [1,9]. As previously reported, western blots (WB, Fig. 2) show that in the wild-type strain, diphthamide-modified eEF2 was readily detected while only background signals were found for unmodified eEF2. Next, we tested how the deletion of seven different co-chaperones affects the diphthamide modification state of eEF2. We chose Sti1 for which the BioGRID database reveals synthetic negative phenotypes when combined with *CPR7* or *HGH1* gene deletions, Cpr1 and Cpr6 which are potentially redundant homologs to Cpr7, as well as Apj1, Aha1, Snl1, and Sba1 (Table S2). While we observed some reductions in panEF2 signals which may indicate an effect of those enzymes on eEF2 levels (Fig. 2), changes in the levels of diphthamide-modified eEF2 were not observed in those strains. Thus, these co-chaperones are not essential for diphthamide synthesis. However, the situation was different for the co-chaperones Hgh1 and Cpr7. *HGH1* and *CPR7* deletion strains showed reduced levels of eEF2 (quantification in Fig. 2), confirming previous observations [18,19]. And importantly, in these mutant backgrounds additional signals for unmodified eEF2 were detected with intensities similar to those observed in bona fide diphthamide null mutants *dph1*Δ and *jjj3*/*dph4*Δ [1]. Because the antibody specifically recognizes unmodified eEF2 but not intermediates [9], yeast strains deficient in Hgh1 and Cpr7, appear to harbor 'naked' eEF2, which indicates a deficiency in the 1st step of diphthamide synthesis.

Together with the lack of an effect on diphthamide synthesis observed for all the other co-chaperones tested, we conclude that the defect observed for *hgh1*Δ and *cpr7*Δ strains is not a consequence of general interference with chaperone machineries but reflects a specific function in the modification of eEF2 with diphthamide.

The results show that strains lacking either Hgh1 or Cpr7 harbor unmodified eEF2. But our antibody detects just unmodified eEF2 and the WB analysis hence lacks a diphthamide-specific probe. We therefore additionally subjected *hgh1*Δ and *cpr7*Δ strains to ADP-ribosylation (ADPR) assays that base on the specificity of diphtheria toxin to modify (and thereby label) exclusively diphthamide-modified eEF2 [23]. The results of these assays (Fig. 3) show a positive ADPR reaction with eEF2 for the wild-type strain and a lack of signal for the diphthamide null mutants *dph1*Δ and *jjj3*/*dph4*Δ. As for the null mutants *hgh1*Δ and *cpr7*Δ, reduced ADPR signals on eEF2 were observed. Together, the western blot and ADPR assays thus indicate that *hgh1*Δ and *cpr7*Δ strains harbor unmodified eEF2 as well as diphthamide-modified eEF2. Therefore, interference with eEF2 chaperoning reduces but does not completely abrogate diphthamide synthesis.

HGH1 and *CPR7* gene deletion strains are hygromycin and sordarin hypersensitive

Known phenotypes of yeast strains that are completely deficient in diphthamide synthesis *via* inactivation of

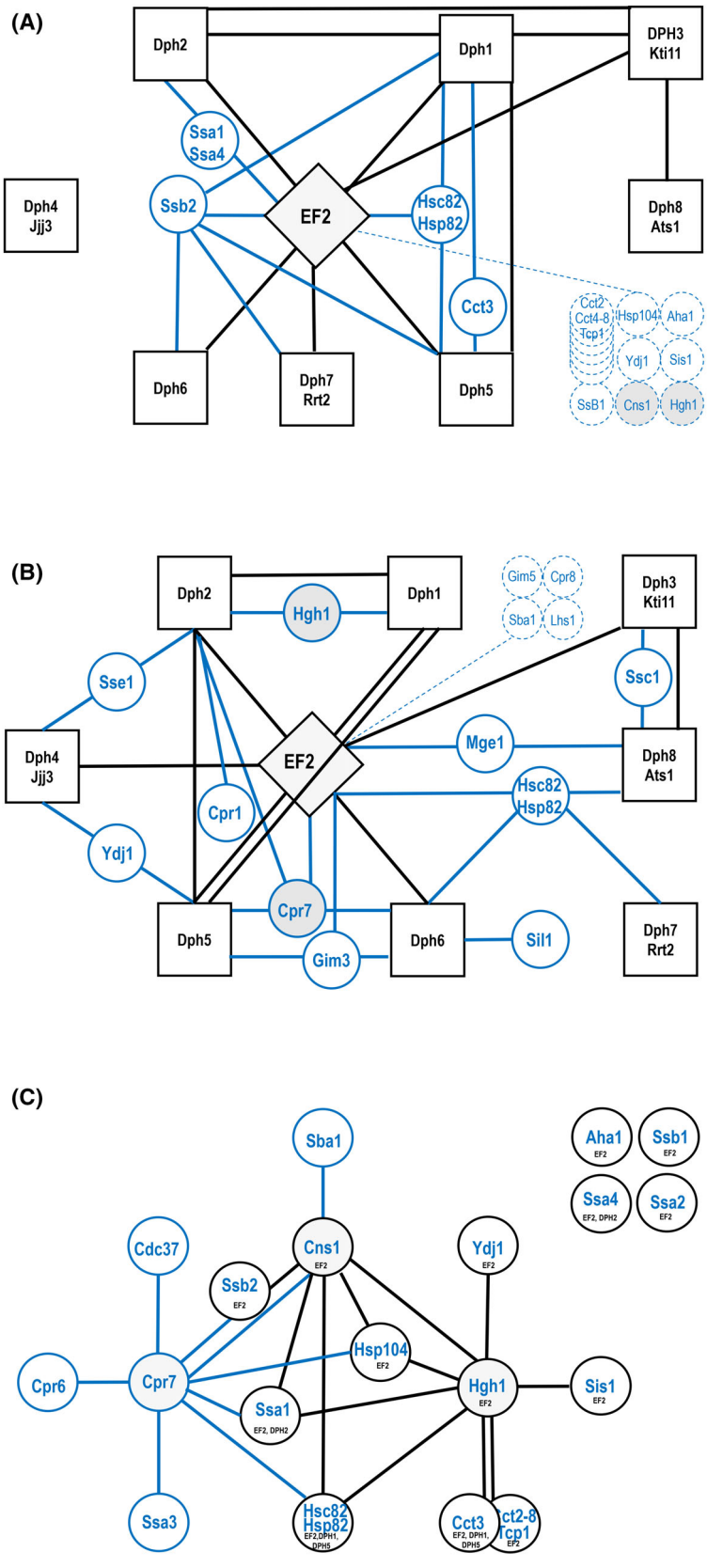
(A)
Dph2
Dph1
DPH3
Kti11
Ssa1
Ssa4
Dph4
Jjj3
Ssb2
EF2
Hsc82
Hsp82
Dph8
Ats1
Cct3
Cct2
Cct4-8
Tcp1
Hsp104
Aha1
Ydj1
Sis1
SsB1
Cns1
Hgh1
Dph6
Dph7
Rrt2
Dph5
(B)
Dph2
Hgh1
Dph1
Gim5
Cpr8
Sba1
Lhs1
Dph3
Kti11
Sse1
Ssc1
Dph4
Jjj3
EF2
Mge1
Dph8
Ats1
Hsc82
Hsp82
Cpr1
Ydj1
Cpr7
Dph5
Dph6
Sil1
Dph7
Rrt2
Gim3
(C)
Sba1
Aha1
EF2
Ssb1
EF2
Ssa4
EF2, DPH2
Ssa2
EF2
Cdc37
Cns1
EF2
Ydj1
EF2
Ssb2
EF2
Hsp104
EF2
Cpr6
Cpr7
Hgh1
EF2
Sis1
EF2
Ssa1
EF2, DPH2
Ssa3
Hsc82
Hsp82
EF2, DPH1, DPH5
Cct3
EF2, DPH1, DPH5
ct2-8
Tcp1
EF2

Fig. 1. Physical and genetic links of diphthamide synthesis factors and chaperones extracted from the BioGRID database ([20]) (A) Physical and (B) genetic links between the eEF2/Dph network and chaperones. Black lines in (A) and (B) reflect links between proteins of the core diphthamide synthesis machinery, blue links of those to the chaperone network, broken lines indicate physical or genetic links between just eEF2 and chaperones, which however are not connected to diphthamide synthesis proteins. (C) Physical links between eEF2-associated chaperones. In (C), black circles and lines indicate co-chaperones with direct physical links to Dph proteins (see panel A), blue indicates physically linked co-chaperones that are not direct Dph-binding partners. Individual links are summarized in Table S1.

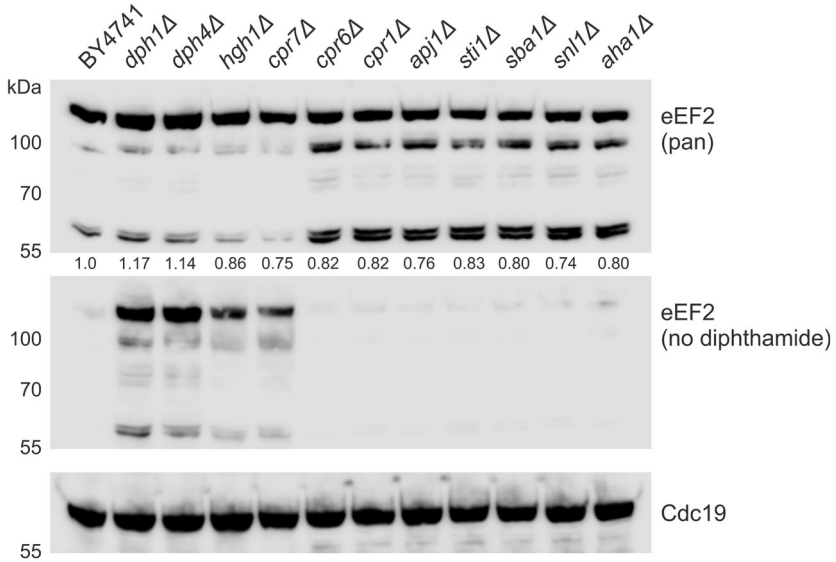

Fig. 2. Detection of eEF2 diphthamide modification in yeast Hgh1 and Cpr7 deficient strains and control strains: Cell extracts of parent wild-type BY4741 and indicated yeast deletion strains were subjected to reducing SDS/PAGE/western blot analyses (see Materials and methods section) with antibodies that specifically detect eEF2 irrespective of modification status (anti-eEF2 pan, top panel) or specifically unmodified eEF2 (anti-eEF2 no diphthamide, middle panel). Detection of pyruvate kinase Cdc19 served as loading control (anti-Cdc19, bottom panel). Data shown are representative of more than three independent western blot analyses with similar results Quantification of total eEF2 levels is indicated below the anti-eEF2 pan blot with values normalized to parent wild-type BY4741.

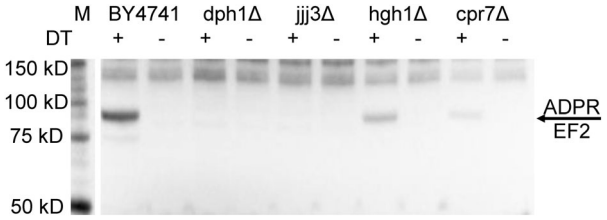

Fig. 3. ADPR of diphthamide-modified eEF2 in *HGH1* and *CPR7* deletion strains and controls. DT-mediated ADPR occurs only at diphthamide-modified eEF2. Because DT also accepts biotinylated NADP as substrate, ADPR transfers bio-ADP-ribose to diphthamide-containing eEF2; bio-ADP-eEF2 is detected *via* labeled streptavidin on reducing SDS/PAGE/western blot membranes (see Materials and methods section and [23]). The data are representative for three independent analyses with consistent results.

Dph enzymes include hypersensitivity to hygromycin and reduced sensitivity to sordarin and DT [1]. We therefore assessed these phenotypes in the *hgh1*Δ and *cpr7*Δ null mutants and compared them to wild-type and chaperone-control strains.

Figure 4 shows that the *hgh1*Δ strain is hypersensitive to hygromycin, to the same degree as the diphthamide-deficient *dph1*Δ and *jjj3*/*dph4*Δ controls. Hypersensitivity toward hygromycin was also demonstrated for a *cpr7*Δ mutant raised in the BY4741 background confirming previous observations in S288C [26–28]. All control strains, parent BY4741 as well as derivatives with deletions of other co-chaperone genes including the *CPR7* homologs *CPR1* and *CPR6* showed no evidence for increased hygromycin sensitivity. Thus, hygromycin hypersensitivity is not associated with general inactivation of co-chaperones but may be (as previously observed in Dph deletion strains [1,2,21]) a consequence of diphthamide deficiency in the *hgh1*Δ and *cpr7*Δ null mutants.

Decreased sensitivity to sordarin and DT are additional phenotypes that were previously observed for cells with diphthamide deficiency [1]. Figure 4 shows

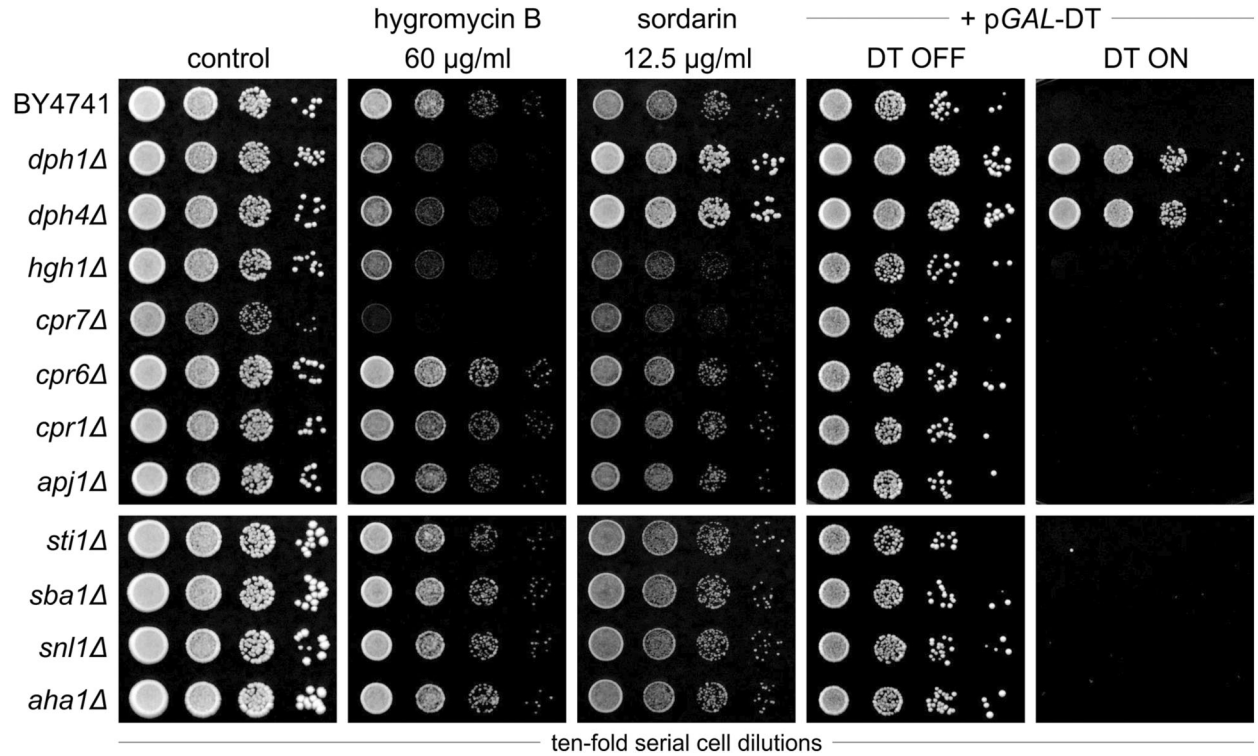

Fig. 4. Sensitivity of *HGH1* and *CPR7* deletion strains and controls to growth inhibition by hygromycin B, sordarin, and DT. Growth of parent wild-type BY4741 and indicated yeast deletion strains was monitored without (control) or with the indicated hygromycin B or sordarin doses. Yeast strains carrying a galactose-inducible diphtheria toxin expression vector (pGAL-DT) were grown in the absence (DT OFF) or presence (DT ON) of galactose in the cultivation medium. Sensitivity assays were replicated independently more than three times with consistent results.

that the *hgh1*Δ and *cpr7*Δ strains are hypersensitive to sordarin and sensitive to DT. Sordarin sensitivity was not altered in strains that had other co-chaperones deleted. Thus, increased sordarin sensitivity of yeast strains deficient for either Hgh1 or Cpr7 may be related to eEF2 folding, but (because *dph1*Δ and *jjj3*/*dph4*Δ are in contrast less sensitive to sordarin) it is not a stringent indicator for eEF2 without diphthamide.

The analysis of the response to DT, a corynebacterial toxin that targets only diphthamide-modified eEF2 by ADPR (see above), revealed resistance of the *dph1*Δ and *jjj3*/*dph4*Δ null mutants, which do not harbor any diphthamide, but sensitivity of all other analyzed strains including the *HGH1* and *CPR7* gene deletion strains (Fig. 4). The *hgh1*Δ and *cpr7*Δ null mutants thus harbor unmodified eEF2 but still are DT sensitive, likely because (a) of their overall reduction in eEF2 levels and/or (b) just a fraction of their eEF2 lacks diphthamide while another is modified and targeted for ADPR by DT. That remaining, protected eEF2 fraction seems insufficient to enable survival of the *hgh1*Δ and *cpr7*Δ null mutants in the presence of DT.

The results of these analyses reveal that *hgh1*Δ and *cpr7*Δ null mutants display hygromycin hypersensitivity, a phenotype associated with defects in diphthamide synthesis, but maintain insufficient levels of unmodified eEF2 to provide DT resistance. Sordarin hypersensitivity likely indicates modulation of eEF2 functionality in strains with deletions for genes that encode the eEF2-client co-chaperones Hgh1 or Cpr7.

Discussion

The yeast co-chaperones Hgh1 and Cpr7 are known to be involved in the folding and prevention of aggregation of eEF2. Yeast strains deficient in those chaperones harbor reduced levels of functional stable eEF2 and increased levels of eEF2-aggregates [18,19,26]. Our current work shows that Hgh1- and Cpr7-deficient strains harbor eEF2 without diphthamide, indicating that these co-chaperones are also relevant for the diphthamide modification of eEF2.

One explanation for the observation that eEF2 from *HGH1* and *CPR7* gene deletion strains lacks a

significant amount of the diphthamide modification could be that the enzymes dedicated in the modification pathway require properly folded eEF2 as a substrate. Interference with eEF2-folding might thus limit the provision of the substrate for the Dph enzymes in the *hgh1*Δ and *cpr7*Δ null mutants.

The contributions of Hgh1 and Cpr7 to eEF2 folding and stabilization/prevention of aggregation are in line with the observation of reduced eEF2 levels upon deletion of the two co-chaperones ([18,19], Fig. 2). In contrast, reduced eEF2 levels are not observed in strains with defects in Dph1 and Jjj3/Dph4 or other previously characterized members of the diphthamide synthesis network [1]. But if only properly folded eEF2 serves as substrate, Hgh1 and Cpr7-deficient strains would in consequence harbor a reduced overall pool of eEF2, part of which being additionally diphthamide deficient and hence prone to cause mistranslation. Thus, translating ribosomes in *hgh1*Δ and *cpr7*Δ mutants might face limited availability of functional eEF2. However, these strains are viable and hence should contain sufficient levels of functional eEF2. Lack of folded eEF2 as a substrate for proper diphthamide synthesis on eEF2 may thus not be the sole explanation for the diphthamide defects observed in the *hgh1*Δ and *cpr7*Δ strains.

Another possible explanation suggests that Hgh1 and/or Cpr7 binding to eEF2 during *de novo* folding is a prerequisite for the diphthamide modification. The binding site for Hgh1 in eEF2 domain III in proximity to the sordarin site [18,19] precedes but does not overlap with the diphthamide acceptor site at position His699.

The chaperoning role of Hgh1 may preferentially target *de novo* folding of eEF2 as properly folded eEF2 has a similar stability in parent and *hgh1*Δ strains [19]. It can be assumed that Cpr7 is also involved in prevention of aggregation (i.e., in stabilization) during *de novo* folding of nascent eEF2 [18]. Considering that diphthamide synthesis may accompany *de novo* folding, Hgh1 and Cpr7 may be co-factors that serve eEF2-folding as well as diphthamide modification, or may recruit Dph enzyme complexes to nascent or partially folded eEF2. The diphthamide synthesis supporting activity of Cpr7 is not solely as a 'nonspecific' proline isomerase but rather specific because the lack of the close proline isomerase homologs Cpr1 and Cpr6 does not interfere with diphthamide synthesis in the respective deletion strains (Figs 2–4).

Cns1 is another co-chaperone relevant for eEF2 folding [18,19]. Inactivation of Cns1 is also associated with hygromycin hypersensitivity and it may therefore contribute to diphthamide synthesis. Cns1-deficient yeast are not viable [18,26,29], and thus, the eEF2-modification data derived from stalled or dying cells cannot be meaningful interpreted. A similar synthetic negative phenotype results from combining Hgh1 and Cpr7 mutants [18,30–32]. We therefore refrained from analyzing the eEF2 diphthamide modification following conditional Cns1 or combined Cpr7 and Hgh1 inactivation.

Another chaperone long known to contribute to diphthamide synthesis is the class3 J-domain protein (JDP) Jjj3 alias Dph4 [29]. Its C-terminal metal-binding domain is homologous to Dph3 which transfers electrons to the Dph1/Dph2 heterodimer [33–35]. Dph-functionality requires both Jjj3/Dph4 domains because mutation of the J-domain HPD motif, and also of the Fe-binding motif, are known to abrogate diphthamide synthesis [36,37]. In contrast to the partial deficiencies that we observed with the *HGH1* and *CPR7* deletion strains, in a Jjj3/Dph4-deficient yeast mutant the diphthamide modification is completely absent [13,38] (note that we used the *jjj3*/*dph4*Δ strain as a diphthamide deficient control). Another difference to Hgh1 and Cpr7 is the absence of described physical interactions of Jjj3/Dph4 with eEF2 or Dph proteins or other chaperones (BioGRID, Table S3). The contribution of Jjj3/Dph4 to diphthamide synthesis may therefore more likely be related to electron transport or FeS cofactor chaperoning for Dph1/Dph2 [36,37] rather than direct involvement in eEF2 chaperoning through Hgh1 and/or Cpr7.

In summary, our study reveals an intricate connection of specific co-chaperones of the Hsp90 chaperone machinery not only to the folding of eEF2 but also to the unique diphthamide modification of eEF2. This opens the possibility that molecular chaperones may be more widely involved in the posttranslational modification of proteins.

Acknowledgements

We thank Jeremy Thorner (University of California, USA) for kindly providing the anti-Cdc19 antibody and Gabriel Karrillon (Universite Paris Cite, France) for his assistance in data replication experiments. This work was in part supported by Pilot grant *Diphthamide* to RS (#2887) from the ZFF (Zentraler Forschungsfonds, Kassel University, Germany), by the DFG (Deutsche Forschungsgemeinschaft, Bonn, Germany) Priority Program 1927 *Iron-Sulfur for Life* to RS (SCHA750/21-1), and the SFB1035 A03 to JB.

Conflict of interest

UB and KM are employed by Roche Pharma Research & Early Development, UB is co-inventor on

patent applications that cover assays to detect presence or absence of diphthamide. Roche is interested in targeted therapies and diagnostics. All other authors declare no conflict of interest.

Author contributions

UB, RS, and JB: concept, experimental strategy and design and data interpretation; KM and LK: experimental strategy and design, data generation and interpretation.

Data accessibility

All supporting data are contained in this manuscript, provided in the Supporting Information section.

References

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

2 Schaffrath R and Brinkmann U (2024) Diphthamide – a conserved modification of eEF2 with clinical relevance. *Trends Mol Med* **30**, 164–177.

3 Zhang H, Quintana J, Ütkür K, Adrian L, Hawer H, Mayer K, Gong X, Castanedo L, Schulten A, Janina N *et al.* (2022) Translational fidelity and growth of Arabidopsis require stress-sensitive diphthamide biosynthesis. *Nat Commun* **13**, 4009.

4 Liu S, Bachran C, Gupta P, Miller-Randolph S, Wang H, Crown D, Zhang Y, Wein AN, Singh R, Fattah R *et al.* (2012) Diphthamide modification on eukaryotic elongation factor 2 is needed to assure fidelity of mRNA translation and mouse development. *Proc Natl Acad Sci U S A* **109**, 13817–13822.

5 Shin BS, Ivanov IP, Kim JR, Cao C, Kinzy TG and Dever TE (2023) eEF2 diphthamide modification restrains spurious frameshifting to maintain translational fidelity. *Nucleic Acids Res* **51**, 6899–6913.

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

7 Mayer K, Mundigl O, Kettenberger H, Birzele F, Stahl S, Pastan I and Brinkmann U (2019) Diphthamide affects selenoprotein expression: diphthamide deficiency reduces selenocysteine incorporation, decreases selenite sensitivity and pre-disposes to oxidative stress. *Redox Biol* **20**, 146–156.

8 Mateus-Seidl R, Stahl S, Dengl S, Birzele F, Herrmuth H, Mayer K, Niederfellner G, Liu XF, Pastan I and Brinkmann U (2019) Interplay between reversible phosphorylation and irreversible ADP-ribosylation of eukaryotic translation elongation factor 2. *Biol Chem* **400**, 501–512.

9 Stahl S, da Silva Mateus Seidl AR, Ducret A, Kux van Geijtenbeek S, Michel S, Racek T, Birzele F, Haas AK, Rueger R, Gerg M *et al.* (2015) Loss of diphthamide pre-activates NF-kappaB and death receptor pathways and renders MCF7 cells hypersensitive to tumor necrosis factor. *Proc Natl Acad Sci U S A* **112**, 10732–10737.

10 Shankar SP, Grimsrud K, Lanoue L, Egense A, Willis B, Hörberg J, AlAbdi L, Mayer K, Ütkür K, Monaghan KG *et al.* (2022) A novel DPH5-related diphthamide-deficiency syndrome causing embryonic lethality or profound neurodevelopmental disorder. *Genet Med* **24**, 2207.

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

12 Shankar SP, Grimsrud K, Lanoue L, Egense A, Willis B, Hörberg J, AlAbdi L, Mayer K, Ütkür K, Monaghan KG *et al.* (2022) A novel DPH5-related diphthamide-deficiency syndrome causing embryonic lethality or profound neurodevelopmental disorder. *Genet Med* **24**, 1567–1582.

13 Webb TR, Cross SH, McKie L, Edgar R, Vizor L, Harrison J, Peters J and Jackson IJ (2008) Diphthamide modification of eEF2 requires a J-domain protein and is essential for normal development. *J Cell Sci* **121**, 3140–3145.

14 Yu YR, You LR, Yan YT and Chen CM (2014) Role of OVCA1/DPH1 in craniofacial abnormalities of Miller-Dieker syndrome. *Hum Mol Genet* **23**, 5579–5596.

15 Schaffrath R, Abdel-Fattah W, Klassen R and Stark MJR (2014) The diphthamide modification pathway from *Saccharomyces cerevisiae* – revisited. *Mol Microbiol* **94**, 1213–1226.

16 Arend M, Ütkür K, Hawer H, Mayer K, Ranjan N, Adrian L, Brinkmann U and Schaffrath R (2023) Yeast gene KTI13 (alias DPH8) operates in the initiation step of diphthamide synthesis on elongation factor 2. *Microb Cell* **10**, 195–203.

17 Dong M, Kathiresan V, Fenwick MK, Torelli AT, Zhang Y, Caranto JD, Dzikovski B, Sharma A, Lancaster KM, Freed JH *et al.* (2018) Organometallic and radical intermediates reveal mechanism of diphthamide biosynthesis. *Science* **359**, 1247–1250.

18 Schopf FH, Huber EM, Dodt C, Lopez A, Biebl MM, Rutz DA, Mühlhofer M, Richter G, Madl T, Sattler M *et al.* (2019) The Co-chaperone Cns1 and the recruiter protein Hgh1 link Hsp90 to translation elongation via chaperoning elongation factor 2. *Mol Cell* **74**, 73–87 e8.

19 Monkemeyer L, Klaips CL, Balchin D, Körner R, Hartl FU and Bracher A (2019) Chaperone function of Hgh1 in the biogenesis of eukaryotic elongation factor 2. *Mol Cell* **74**, 88–100 e9.
20 Oughtred R, Rust J, Chang C, Breitkreutz BJ, Stark C, Willems A, Boucher L, Leung G, Kolas N, Zhang F *et al.* (2021) The BioGRID database: a comprehensive biomedical resource of curated protein, genetic, and chemical interactions. *Protein Sci* **30**, 187–200.
21 Utkur K, Mayer K, Khan M, Manivannan T, Schaffrath R and Brinkmann U (2023) DPH1 and DPH2 variants that confer susceptibility to diphthamide deficiency syndrome in human cells and yeast models. *Dis Model Mech* **16**, dmm050207.
22 Schneider CA, Rasband WS and Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671–675.
23 Mayer K, Schröder A, Schnitger J, Stahl S and Brinkmann U (2017) Influence of DPH1 and DPH5 protein variants on the synthesis of diphthamide, the target of ADPRibosylating toxins. *Toxins (Basel)* **9**, 78.
24 Jaiswal H, Conz C, Otto H, Wölfle T, Fitzke E, Mayer MP and Rospert S (2011) The chaperone network connected to human ribosome-associated complex. *Mol Cell Biol* **31**, 1160–1173.
25 Rakwalska M and Rospert S (2004) The ribosome-bound chaperones RAC and Ssb1/2p are required for accurate translation in *Saccharomyces cerevisiae*. *Mol Cell Biol* **24**, 9186–9197.
26 Tenge VR, Zuehlke AD, Shrestha N and Johnson JL (2015) The Hsp90 cochaperones Cpr6, Cpr7, and Cns1 interact with the intact ribosome. *Eukaryot Cell* **14**, 55–63.
27 Dudley AM, Janse DM, Tanay A, Shamir R and Church GM (2005) A global view of pleiotropy and phenotypically derived gene function in yeast. *Mol Syst Biol* **1**, 2005 0001.
28 Albanese V, Yam AY-W, Baughman J, Parnot C and Frydman J (2006) Systems analyses reveal two chaperone networks with distinct functions in eukaryotic cells. *Cell* **124**, 75–88.
29 Thakur A, Chitoor B, Goswami AV, Pareek G, Atreya HS and D'Silva P (2012) Structure and mechanistic insights into novel iron-mediated moonlighting functions of human J-protein cochaperone, Dph4. *J Biol Chem* **287**, 13194–13205.
30 Kuzmin E, VanderSluis B, Wang W, Tan G, Deshpande R, Chen Y, Usaj M, Balint A, Mattiazzi Usaj M, van Leeuwen J *et al.* (2018) Systematic analysis of complex genetic interactions. *Science* **360**, eaao1729.
31 Costanzo M, VanderSluis B, Koch EN, Baryshnikova A, Pons C, Tan G, Wang W, Usaj M, Hanchard J, Lee SD *et al.* (2016) A global genetic interaction network maps a wiring diagram of cellular function. *Science* **353**, aaf1420.
32 Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS, Ding H, Koh JLY, Toufighi K, Mostafavi S *et al.* (2010) The genetic landscape of a cell. *Science* **327**, 425–431.
33 Dong M, Dando EE, Kotliar I, Su X, Dzikovski B, Freed JH and Lin H (2019) The asymmetric function of Dph1-Dph2 heterodimer in diphthamide biosynthesis. *J Biol Inorg Chem* **24**, 777–782.
34 Dong M, Su X, Dzikovski B, Dando EE, Zhu X, du J, Freed JH and Lin H (2014) Dph3 is an electron donor for Dph1-Dph2 in the first step of eukaryotic diphthamide biosynthesis. *J Am Chem Soc* **136**, 1754–1757.
35 Liu S, Wiggins JF, Sreenath T, Kulkarni AB, Ward JM and Leppla SH (2006) Dph3, a small protein required for diphthamide biosynthesis, is essential in mouse development. *Mol Cell Biol* **26**, 3835–3841.
36 Sahi C and Craig EA (2007) Network of general and specialty J protein chaperones of the yeast cytosol. *Proc Natl Acad Sci U S A* **104**, 7163–7168.
37 Verma AK, Sharma P, Islam Z, Biswal AK, Tak Y and Sahi C (2024) Arabidopsis Dph4 is an Hsp70 cochaperone with iron-binding properties. *ACS Omega* **9**, 37650–37661.
38 Liu S, Milne GT, Kuremsky JG, Fink GR and Leppla SH (2004) Identification of the proteins required for biosynthesis of diphthamide, the target of bacterial ADP-ribosylating toxins on translation elongation factor 2. *Mol Cell Biol* **24**, 9487–9497.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Interactions between eEF2, Dph enzymes and chaperones in BioGRID.

Table S2. Co-chaperone and controls addressed in this study.

Table S3. Physical interactions between J-chaperones and chaperones in BioGRID.

Table S4. Yeast strains used in this study.