Loss of diphthamide pre-activates NF-κB and death receptor pathways and renders MCF7 cells hypersensitive to tumor necrosis factor

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The diphthamide on human eukaryotic translation elongation factor 2 (eEF2) is the target of ADP ribosylating diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PE). This modification is synthesized by seven diphthamide biosynthesis proteins (DPH1–DPH7) and is conserved among eukaryotes and archaea. We generated MCF7 breast cancer cell line-derived *DPH* gene knockout (ko) cells to assess the impact of complete or partial inactivation on diphthamide synthesis and toxin sensitivity, and to address the biological consequence of diphthamide deficiency. Cells with heterozygous gene inactivation still contained predominantly diphthamide-modified eEF2 and were as sensitive to PE and DT as parent cells. Thus, *DPH* gene copy number reduction does not affect overall diphthamide synthesis and toxin sensitivity. Complete inactivation of DPH1, DPH2, DPH4, and DPH5 generated viable cells without diphthamide. DPH1ko, DPH2ko, and DPH4ko harbored unmodified eEF2 and DPH5ko ACP- (diphthine-precursor) modified eEF2. Loss of diphthamide prevented ADP ribosylation of eEF2, rendered cells resistant to PE and DT, but does not affect sensitivity toward other protein synthesis inhibitors, such as saporin or cycloheximide. Surprisingly, cells without diphthamide (independent of which the *DPH* gene compromised) were presensitized toward nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) and death-receptor pathways without crossing lethal thresholds. In consequence, loss of diphthamide rendered cells hypersensitive toward TNF-mediated apoptosis. This finding suggests a role of diphthamide in modulating NF-κB, death receptor, or apoptosis pathways.

ADP-ribosylation of eEF2 | *Pseudomonas* exotoxin | diphtheria toxin | translation | *DPH* gene knockout

Eukaryotic translation elongation factor 2 (eEF2) is a highly conserved protein and essential for protein biosynthesis. EEF2 enables peptide-chain elongation by translocating the peptide–tRNA complex from the A- to the P-site of the ribosome (1, 2). The diphthamide modification at His715 of human eEF2 (or at the corresponding position in other species) is conserved in all eukaryotes (3) and in archaeal counterparts. It is generated by proteins that are encoded by seven genes (4). Proteins encoded by dipthamide biosynthesis protein (DPH)1, DPH2, DPH3, and DPH4 (DNAJC24) attach a 3-amino-3-carboxypropyl (ACP) group to eEF2. This intermediate is converted by the methyltransferase DPH5 to diphthine, which is subsequently amidated to diphthamide by DPH6 and DPH7 (5).

Diphthamide synthesis was previously described in yeast and other eukaryotes (4–6). However, the "complete picture" is (with the exception of the yeast pathway) to a large portion is composed of observations made in different cell types on single genes. Many reports related to diphthamide synthesis of mammalian cells describe "partial knockouts" and "partial phenotypes" (i.e., reduced levels but not complete loss of diphthamide modification or toxin sensitivities) (7–9). Because mammalian genomes are more complex than that of yeast, carrying extendend gene families, mammalian cells may compensate—at least to some degree—functional loss of genes that may be unique and essential in yeast. If and to what degree mammalian cells can compensate a partial or complete loss of *DPH* gene functionality (and with what consequences) is unknown to date.

So far, the function of diphthamide on eEF2 also remained rather elusive. Reports indicate that it contributes to translation fidelity (10–13). On the other hand, *DPH* genes or eEF2 can be mutated to prevent diphthamide attachment, yet cells carrying such mutations are viable (5, 11, 14, 15). Animals with heterozygous DPH knockouts (DPHko) can be generated, but homozygous DPH1ko, DPH3ko, and DPH4ko are embryonic lethal (13, 16–18). Because these studies are based on inactivation of individual genes, it is difficult to discriminate between phenotypes caused by gene loss and phenotypes as a consequence of loss of diphthamide.

Significance

Diphthamide is a conserved modification on eukaryotic translation elongation factor 2 (eEF2). Analyses of genetically defined diphthamide-deficient cell lines indicate that this modification determines not only sensitivity of cells to the ADP-ribosylating toxins *Pseudomonas* exotoxin A and diphtheria toxin, but it also modulates nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) and TNF receptor signaling pathways.

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Diphthamide-modified eEF2 is the target of ADP ribosylating toxins, including *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT) (19). These bacterial proteins enter cells and catalyze ADP ribosylation of diphthamide using nictotinamide adenine dinucleotide (NAD) as substrate (20, 21). This inactivates eEF2, arrests protein synthesis, and kills (14). Tumor-targeted PE and DT derivatives are applied in cancer therapies (22–28) and their efficacy depends on toxin sensitivity of target cells. Therefore, information about factors (and their relative contributions) that influences cellular sensitivities toward diphthamide-modifying toxins may predict therapy responses. For example, alterations in OVCA1 (human DPH1) were described for ovarian cancers (16, 29), yet it is not known if and to what degree such alterations would affect sensitivities of tumor cells toward PE-derived drugs.

Here we describe MCF7 breast cancer cell line derivatives with heterozygous or complete *DPH* gene inactivations. These cells are applied to analyze the contributions of individual DPHs not only to diphthamide synthesis and toxin sensitivity, but also to address gene dose effects. Because the set of knockout cell lines is derived from the same parent cell and provides loss of diphthamide as common consequence of inactivation of different genes, these cells can also shed light on the biological relevance of the diphthamide modification.

Results

Generation of MCF7 Cells with Heterozygous or Completely Inactivated *DPH* Genes. Gene-specific zinc finger nucleases (ZFN) (30) were applied to generate MCF7 cells with inactivated *DPH* genes (*SI Text S1–S3*). Plasmids encoding ZFNs were transfected into MCF7. Forty-eight hours later, to enable ZFN binding, double-strand breaks, and mis-repair, mutated cells were identified by either phenotype selection or by genetic analyses, as described in detail in Fig. S1. For phenotype selection, cells were exposed to lethal doses of PE (100 nM) to kill all cells whose eEF2 is a substrate for the toxin. After an additional 48 h, dead cells were removed and the culture propagated in toxin-containing media. This procedure generated colonies of cells transfected with ZFNs for DPH1, -2, -4, and -5. No colonies were obtained under toxin selection in cells that were mock transfected, or with ZFNs that target DPH3, -6, and -7 (Table S1). Genetic analyses of single-cell clones revealed that all resistant isolates contained only defective (out-of-frame) copies of *DPH1*, *DPH2*, *DPH4*, or *DPH5* genes. None of the toxin-resistant cells contained unaltered functional gene copies (*SI Text S1*).

To identify MCF7 mutants without toxin selection, single cells from each transfection were subjected to high-resolution melting (HRM) analyses genes (31). This technique identifies cells that contain two different alleles of the gene to be analyzed, as those generate biphasic or odd-shaped melting curves (*SI Text S1*). Analyses of candidate clones with bisphasic HRM profiles confirmed the presence of different DPH allele sequences. This approach delivered clones that had one gene copy inactivated and another functional wild-type copy for all DPH genes (Table 1 and *SI Text S1*). In addition, clones that had both genes inactivated with a different mutation on each allele were obtained for DPH4 and DPH5. For DPH3, DPH6, and DPH7, clones that had both genes inactivated could not be obtained in repeated attempts even though the ZFNs were effective (generated heterozygotes) and the number of colonies screened delivered several complete knockouts for other *DPH* genes.

Table 1. *DPH* gene inactivation and toxin sensitivity

DPHko	Alleles	IC_{50} nM PE	IC_{50} pM DT	IC_{50} nM CHX	IC_{50} pM TNFα
MCF7	WT	121 ± 12	12 ± 5	1,969 ± 454	1,403 ± 243
DPH1	WT-KO	142 ± 124	14 ± 6	2,026 ± 1,238	1,220 ± 370
	KO-KO	**>3,000**	**>5,000**	1,473 ± 244	**193 ± 65**
DPH2	WT-KO	95 ± 58	10 ± 2	2,158 ± 625	1,760 ± 579
	KO-KO	**>3,000**	**>5,000**	1,945 ± 276	**260 ± 81**
DPH4	WT-KO	38 ± 27	14 ± 1	1,976 ± 1,122	1,765 ± 709
	KO-KO	**>3,000**	**>5,000**	1,626 ± 594	**330 ± 243**
DPH5	WT-KO	133 ± 18	9 ± 3	1,769 ± 132	1,457 ± 599
	KO-KO	**>3,000**	**>5,000**	1,030 ± 129	**134 ± 37**
DPH3	WT-KO	32 ± 19	11 ± 4	1,555 ± 23	1,217 ± 484
DPH6	WT-KO	36 ± 32	18 ± 10	1,579 ± 1,014	985 ± 81
DPH7	WT-KO	107 ± 80	16 ± 6	1,976 ± 1,002	891 ± 49

PE, DT, CHX, or TNF sensitivity was determined by BrdU incorporation assays. IC_{50} values calculated from dose–response curves shown in Fig. 2 and *SI Text S4*. Boldface entries indicate values of homozygous knockout cells.

DPH Gene Inactivation Influences H715 Modification and ADP Ribosylation. Extracts of MCF7 and DPHko cells were subjected to Western blots with a rabbit mAb that we generated and which specifically detects unmodified eEF2 (see *SI Text S2* and Fig. S2 for antibody generation). These analyses (Fig. 1*A*) revealed that unmodified eEF2 is virtually absent (below detection levels) in MCF7. Cells with complete inactivation of *DPH1*, *DPH2*, or *DPH4* genes contained eEF2 without H715 modification as indicated by strong antibody signals. Unmodified eEF2 was also observed in cells with heterozygous DPH2 mutations, albeit to a much lower degree. All other heterozygous cell lysates generated only background signals. Thus, inactivation of DPH1, DPH2, and DPH4 interferes with H715 modification of eEF2.

Mass spectometry (MS) analyses were subsequently applied to extracts of DPH-mutated cell lines to determine the composition and eEF2 His715 modification in detail. This enabled the determination of relative levels of unmodified eEF2, of the ACP intermediate, and of diphthamide (*SI Text S3* and Fig. S3). In wild-type cells, only diphthamide-modified eEF2 is detectable without evidence for unmodified eEF2 or diphthine or ACP modifications (Fig. 1*B*). Cells with complete inactivation of the *DPH1*, *DPH2*, *DPH4*, as well as *DPH5* genes contained no diphthamide-modified eEF2. Thus, these genes are essential for diphthamide synthesis and their inactivation cannot be compensated by other genes. Complete inactivation of DPH1, DPH2, or DPH4 generated cells in which only unmodified eEF2 and no other modified form was detectable. Complete inactivation of DPH5 generated the ACP intermediate (eEF2 with this intermediate is not recognized by the antibody applied in the preceding Western blots) (Fig. 1*A*). The major eEF2 species in cells with one inactivated and one functional copy of DPH1 to -7 is diphthamide-modified eEF2. In contrast to parent MCF7, however, unmodified eEF2 was detectable in different amounts (up to 25% of the total eEF2) (Fig. 1*B*) upon heterozygous inactivation of DPH1 and DPH2. This finding demonstrates that gene-dose reduction by inactivation of one allele of DPH1 or DPH2 is insufficient to prevent diphthamide synthesis, but sufficient to modulate the amount of unmodified eEF2.

In vitro ADP ribosylation adressed the impact of partial or complete DPH inactivation: cell extracts were incubated with PE as enzyme and biotinylated NAD as substrate, followed by detection of Bio-ADPR-eEF2. This method reliably detects ADP ribosylation; however, it cannot be used for quantification of slight differences (32). Fig. 1*C* shows that eEF2 of parental MCF7, and of all seven heterozygote-inactivated MCF7 derivatives (DPH1–7) becomes ADP ribosylated by PE. In contrast, eEF2 from cells that have completely inactivated *DPH1* or *DPH2* or *DPH4* or *DPH5* genes is not amenable to ADP ribosylation. Only eEF2 with diphthamide, but not without modification (DPH1, -2, -4) or with partial modification (ACP in DPH5) serves as substrate for ADP ribosylating toxins. There is no other remaining toxic activity toward cells carrying these eEF2 forms.

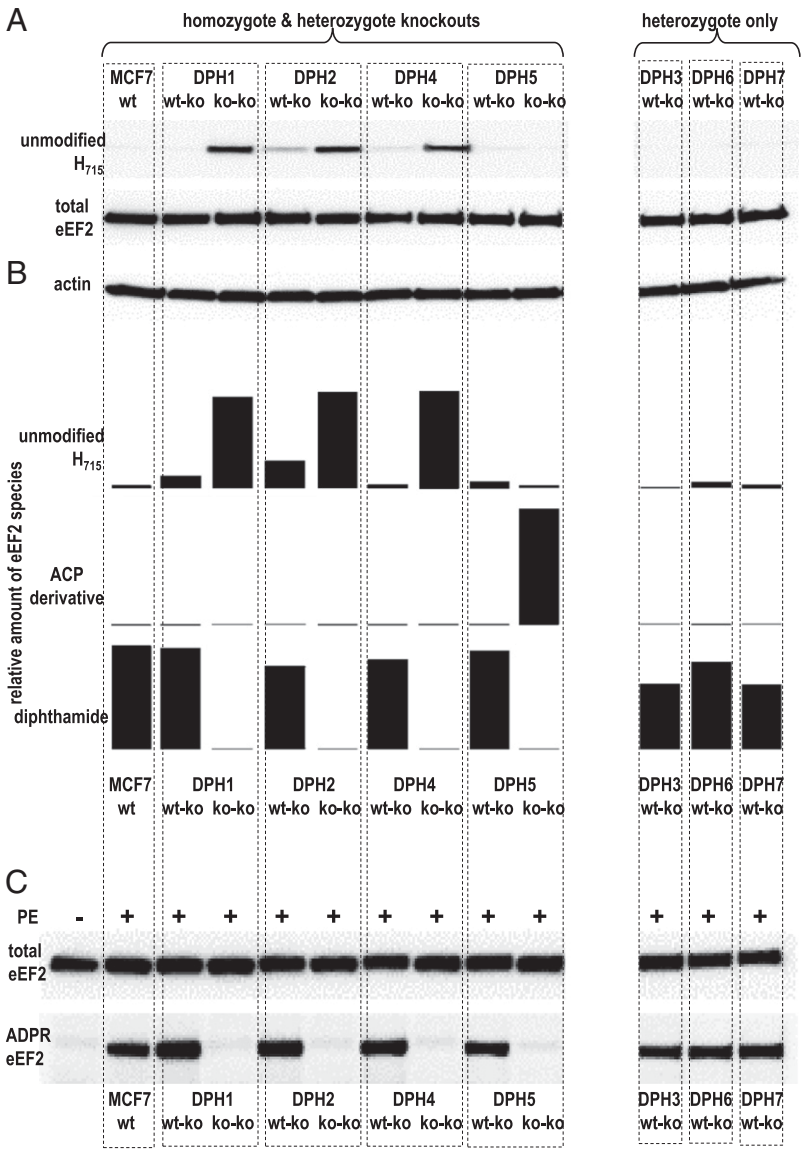

Fig. 1. Diphthamide modification and ADP ribosylation of eEF2. (*A*) Detection of unmodified eEF2 in parent and mutated MCF7 by a rabbit mAb that specifically detects eEF2 without diphthamide (*SI Text S2*). (*B*) Semiquantitative assessment of eEF2 modifications by MS (46) (*SI Text S3*). (*C*) ADP ribosylation of eEF2 was assessed in extracts exposed to PE Bio-NAD, followed by Western blot detection of Bio-ADPR-eEF2 (32).

Influence of Heterozygous and Complete DPH Inactivation on Toxin Sensitivity. Susceptibilities toward PE and DT were determined for MCF7 derivatives with heterozygous or complete *DPH* gene inactivations. Fig. 2 and Table 1 show that complete inactivation of *DPH1*, *DPH2*, *DPH4,* or *DPH5* genes confer absolute resistance of MCF7. Even toxin concentrations exceeding the doses that kill parent MCF7 by more than 10,000-fold did not influence cell growth or proliferation of mutant cells. Absolute resistance correlates with complete absence of diphthamide-modified eEF2 and lack of ADP ribosylation (Fig. 1). Thus, cytotoxicity inferred by PE and DT is solely a result of diphthamide-dependent ADP ribosylation of eEF2 without any other cytotoxic modality. In contrast to cells without functional *DPH* gene copy, MCF7 derivatives that contained one inactivated and one functional *DPH* gene (*DPH1-DPH7*) remained fully sensitive to PE and DT (Fig. 2). As all heterozygous DPH-inactivated MCF7 derivatives contain as the major species diphthamide-modified eEF2, toxin sensitivity correlates with the presence of diphthamide. Heterozygous DPH3, -4, -6, and -7 knockouts contain almost exclusively diphthamide-modified eEF2, indicating that loss of one functional allele of these genes does not affect efficacy of diphthamide synthesis. Heterozygous DPH1 or DPH2 cells contain detectable levels of unmodified toxin-resistant eEF2 (up to 25% in DPH2ko). Thus, *DPH1* and *DPH2* gene doses can influence diphthamide synthesis to some degree. Interestingly, despite harboring some toxin-resistant eEF2, such cells remained as toxin-sensitive as wild-type cells. Thus, *DPH1* and *DPH2* gene-dose modulates the relative amount of unmodified eEF2 in MCF7, but does not affect toxin sensitivity.

Loss of Diphthamide Has only a Minor Impact on Cell Growth and Does Not Alter Sensitivity Toward Protein Synthesis Inhibitors, Which Do Not Target eEF2. The common denominator of all MCF7 derivatives with different *DPH* gene defects is loss of diphthamide. Thus, this cell line set cannot only elucidate the relative impact of each gene on toxin sensitivity, but also address the biological function of the diphthamide modification itself. To eliminate variations between individual clones, we have analyzed at least two independent

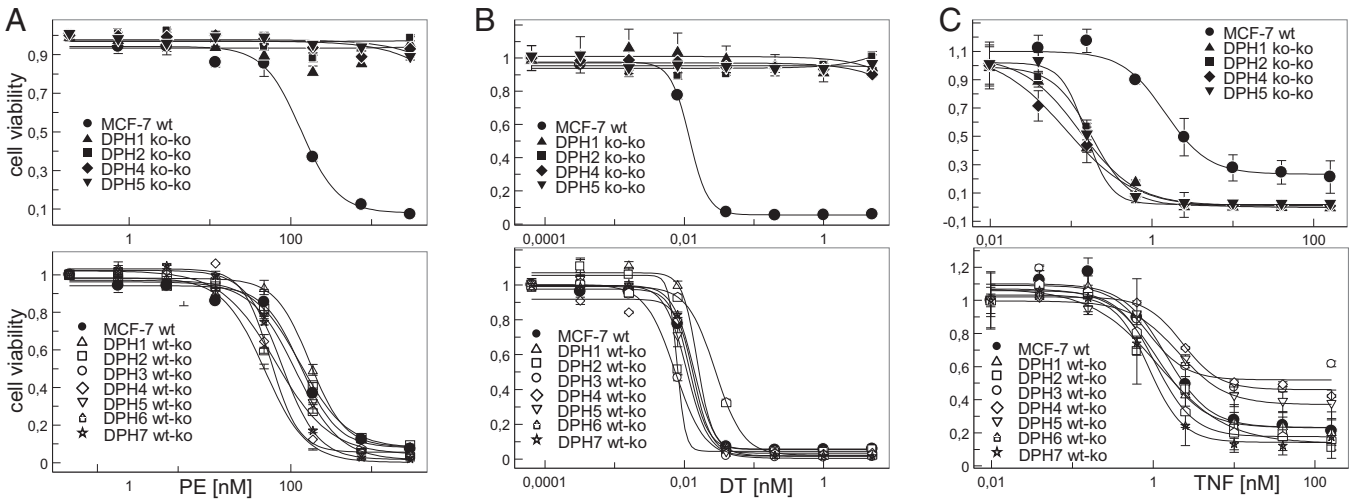

Fig. 2. Influence of DPH inactivation on sensitivity to ADP ribosylating toxins and TNF. Dose–responses of cells with inactivation of DPH1, DPH2, DPH4, and DPH5 in comparison with parent MCF7 (bold line), exposed to PE (*A*), DT (*B*) or TNF (*C*). Complete knockouts (*Upper*) and partial knockous (*Lower*) are shown. Parent MCF7 (bold line) and partial knockouts show the same sensitivity to PE, DT, and TNF. Cells with complete inactivation of DPH1, DPH2, DPH4, and DPH5 are resistant to PE and DT and hypersensitive to TNF.

clones for each knockout. Cytology indicated that morphology and chromosome composition of the individual clones did not diverge from that of parent MCF7 cells (*SI Text S4*). Because lack of diphthamide was achieved by inactivating different genes, common biological effects observed in these cell lines are attributable to loss of diphthamide, and not to loss of individual gene function or potential compensatory effects. Under normal growth conditions, we observed no impact of DPH inactivation on growth for all heterozygous clones (Fig. S4*A*). In addition, complete inactivation of DPH1, DPH2, or DPH4 did not cause significant reductions in cell growth or viability (Fig. S4*A*). We observed growth alterations for some clones, but these effects were not attributable to the gene itself because some clones showed differences but others with the same gene affected did not (*SI Text S4*). Cells with complete inactivation of DPH1, DPH2, and DPH4 harbor only unmodified eEF2. Thus, the exclusive presence of unmodified eEF2 by itself does not inhibit the growth of MCF7. Reduced growth rates were observed for all clones with completely inactivated DPH5. These contain ACP-modified eEF2, which occurs only in DPH5-deficient cells and not in other variants. Therefore, we cannot differentiate between growth reduction related to the presence of the eEF2-ACP intermediate or being a consequence of lost DPH5 function in other cellular processes.

Is the action of other protein synthesis inhibitors also affected in cells without diphthamide? Exposure of parent and DPH-inactivated MCF7 cells to saporin and cycloheximide (CHX) revealed no effect of DPH inactivation and loss of diphthamide on sensitivity (*SI Text S4*). Saporin and CHX inhibited the growth and killed DPH-inactivated cells to the same degree as wild-type MCF7 (Table 1, *SI Text S4*, and Fig. S4*B*). This finding indicates that stress posed upon cells by protein synthesis inhibition in general appears not to be aggravated by lack of diphthamide.

Loss of Diphthamide Activates Pathways That Resemble Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells and Death Receptor Signaling, and Renders MCF7 Cells Hypersensitive to TNF. ADP ribosylation of eEF2 stalls protein synthesis and induces apoptosis in MCF7 (33, 34). The CSE1L protein [identified as a toxin modulator (35–37)] influences not only PE and DT cytotoxicity in MCF7, but also sensitivity toward TNF-induced apoptosis (37). Thus, both processes (diphthamide ADP ribosylation and TNF apoptosis) could be linked. We analyzed if loss of diphthamide influences sensitivity toward TNF-mediated apoptosis. Cells that have at least one functional copy of each *DPH* gene, and therefore possess diphthamide, are as sensitive to TNF as parent cells (Fig. 2*C*). In contrast, cells with complete inactivation of DPH1, DPH2, DPH4, or DPH5 show increased TNF sensitivity (Fig. 2*C*). Hypersensitivity was observed for all clones carrying complete inactivation of DPH1 or DPH2 or DPH4 or DPH5. Because the common denominator of all derivatives with DPH defects is loss of diphthamide, hypersensitivity is

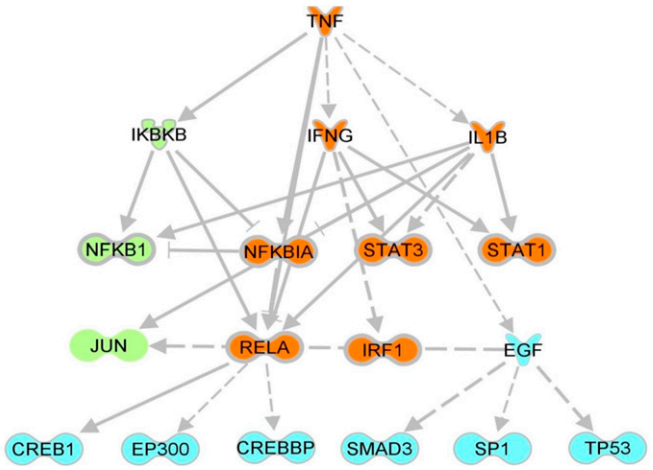

Fig. 3. Inactivation of DPH5 induces NF-κB and death receptor pathways. RNAseq data were obtained for untreated MCF7, TNF treated MCF7, and DPH5 inactivated MCF7 (*SI Text S5*). Genes that are significantly changed in their expression levels (MCF7 vs. TNF-MCF7, MCF7 vs. DPH5ko) were subjected to ingenuity upstream pathway analyses. Interestingly, differentially regulated pathways and regulators in both conditions overlap largely and share a common core of TNF- and IFNG-regulated genes, leading to a pre-activation of death receptor signaling regulators as consequence of DPH5 deficiency and loss of diphthamide (orange: consequence of TNF treatment as well as DPH5ko; green: TNF treatment only; blue: DPH5ko only). Arrows indicate direct activating/inhibiting interactions between two nodes (based on literature findings) and dashed arrows indicate indirect interactions. Different protein types are represented by different symbols.

attributable to loss of diphthamide. This effect is not a result of altered TNF-receptor (TNFR) expression (FACS analyses) (*SI Text S5*). Instead, whole transcriptome analyses by mRNA sequencing of parent and diphthamide-deficient (DPH2ko and DPH5ko) MCF7 cells revealed induction patterns that resemble "preinduction" of nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) death receptor signaling pathways (Fig. 3; see *SI Text S5*, Fig. S5, and Table S2 for experimental details). Thus, cells without diphthamide are presensitized to death receptor signaling, explaining their TNF-hypersensitivity.

Discussion

MCF7 cells exclusively contain diphthamide-modified eEF2; this provides a clean background, above which nonmodified or partially modified eEF2 can easily be detected. Therefore, our set of MCF7 derivatives enables a comprehensive assessment of complete or partial *DPH* gene inactivation, providing insights into gene dose effects, gene essentiality or redundancy, as well as relevance for diphthamide synthesis and toxin sensitivity. Because the MCF7 derivatives have different *DPH* genes affected but generate the common phenotype diphthamide deficiency, they can shed light on the biological function of the diphthamide and of diphthamide deficiency in mammalian cells. Of particular interest is the finding that cells without diphthamide (independent of which the *DPH* gene is being inactivated) are presensitized toward NF-κB and death-receptor associated-pathways, and are hypersensitive toward TNF.

Complete inactivation of DPH1, DPH2, and DPH4 lead to the accumulation of unmodified eEF2 and DPH5 inactivation generates the ACP intermediate. This finding agrees with the known synthesis pathway that assigns DPH1, -2, and -4 to initial modification and DPH5 to diphthine synthesis (4–6). Loss of DPH1, -2, -4, and -5 cannot be compensated by other genes, even though mammalian genomes harbor extended gene families. DPH1-, DPH2-, DPH4-, and DPH5-deficient cells are viable. Thus, these genes are not essential for cell propagation and survival, even though their function is nonredundant. We could not obtain cells with complete inactivation of DPH3, DPH6, or DPH7. These genes appear to be essential but likely in nondiphthamide-related cellular processes because diphthamide-loss per se is not lethal (viable DPH1, -2, -4, -5ko). Loss of *DPH3*, *DPH6*, or *DPH7* is not compensated by other genes. Thus, the whole set of *DPH* genes and functionality appears to be nonredundant. Reduced expression or inactivation of *DPH* genes affects cellular sensitivity to eEF2-ribosylating toxins (7–9). We observed absolute toxin resistance in cells that lack diphthamide. In contrast, gene dose reduction by heterozygous gene knockout has only a minor (*DPH1-2*) or no (*DPH3-7*) impact on diphthamide synthesis and no impact on toxin sensitivity. Compensation of heterozygous gene inactivation suggests mechanisms that sense the presence/absence of diphthamide or DPH activity and regulate the expression of *DPH* genes. Diphthamide-eEF2 is the target for toxin-mediated ADP ribosylation and gene dose effects that influence diphthamidylation could be relevant for tumor therapy with targeted toxins. Alterations of the human *DPH1* (OVCA1) gene are described for various cancers (16, 29, 38–40), but their impact on diphthamide modification and toxin sensitivity has not been quantified so far. Interestingly, partial DPH1ko and DPH2ko generated some gene dose-dependent modulation of the diphthamide content of the cellular eEF2 pool (up to 25% unmodified eEF2), yet without significant impact toward toxin sensitivity. This finding suggests that in addition to direct inactivation of the functionality of eEF2 in translation elongation, ADP ribosylation may trigger (possibly eEF2K-related) signaling events that interrupt protein synthesis, even though non-ADP ribosylated eEF2 is still present.

The set of MCF7 derivatives have the same genetic background, retain cell shape, and (with exception of complete DPH5 deficiencies) good growth properties, yet have different genes inactivated. Cells without DPH1, DPH2, DPH4, and DPH5 cannot produce diphthamide, a common phenotype of inactivating different genes. Common biological effects observed in these cell lines are therefore attributable to lack of diphthamide. This finding enables us to address the function of diphthamide. Inactivation of DPH1, DPH2, or DPH4 has no or only a minor impact on cell growth. Thus, loss of diphthamide or presence of unmodified eEF2 does not severely impact cell growth under normal conditions. Complete inactivation of DPH5 generates viable cells but with reduced growth. Lack of diphthamide in DPH5ko cells by itself does not pose a problem for cells (shown for DPH1ko, DPH2ko, and DPH4ko), indicating that growth reduction upon DPH5 inactivation is a gene-specific phenotype and not related to loss of diphthamide. This gene-specific effect may be explained by intermediate (ACP-eEF2) accumulation, or by a function of DPH5 in other biological processes. Because the diphthamide on eEF2 is highly conserved in all eukaryotes as well as in archea (41), it is surprising that lack of diphthamide synthesis has little overall impact on cell growth. Animals with homozygous DPHko, however, do not survive beyond embryonic stages (13, 16–18). This finding suggests that the diphthamide may be necessary for development.

An explanation for emryonal lethality of KPHko animals may be that NF-κB and death receptor signaling pathways (known to be involved in and necessary for development) are preactivated in diphthamide-deficient cells. These cells are nevertheless viable, as pathway induction does not pass thresholds sufficient to induce apoptosis without additional stimuli. Presensitization becomes phenotypically relevant upon triggering these preinduced pathways: all diphthamide synthesis-deficient cells (independent from target gene knockout) were hypersensitive to TNF-induced apoptosis. This finding indicates that the presence or absence of diphthamide affects NF-κB or death receptor pathways. Pathway preactivation and TNF hypersensitivity upon loss of diphthamide could be explained by modulation of eEF2-targeted stress responses [such as eEF2K-mediated phosphorylation (41–45)] in cells that exclusively contain diphthamide-free eEF2. Diphthamide may also modulate the primary function of eEF2 (i.e., translation elongation). Because eEF2 within MCF7 carries diphthamide, diphthamide-eEF2 enables translation of all essential proteins. eEF2 without diphthamide also supports the translation of essential proteins because cells that lack diphthamide are viable. Diphthamide loss may affect signaling pathways if eEF2 without diphthamide generates some defective or altered proteins (e.g., by allowing translational slippage) (10, 13) (but we did not find *UPR* genes induced in DPHko cells). It is also possible that translation of some nonessential proteins involved in NF-κB and death receptor pathways is different between modified and unmodified eEF2. This finding would explain preinduction of NF-κB and death receptor pathways and TNF-hypersensitivity that we observed in all diphthamide-deficient cells.

Materials and Methods

***DPH* Gene Inactivation.** *DPH* gene inactivation was achieved by ZFN mutagenesis followed by isolation of mutated clones via toxin selection (resistant homozygous DPH1, -2, -4, and -5ko) or by HRM analyses (31) (*SI Text S1*).

Diphthamide Modification and ADP Ribosylation. Diphthamide modification and ADP ribosylation was determined by MS of trypsin-digested cell lysates (46) (*SI Text S3*), by Western blots with antibodies recognizing unmodified eEF2 (*SI Text S2*), or by detection of Bio-ADP ribosylated eEF2 (32).

Cell Proliferation/Cytotoxicity Assays. Cell proliferation/cytotoxicity assays (*SI Text S4*) addressed the consequences of DPH inactivation on toxin and TNF sensitivity.

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