

Research paper

Diphthamide affects selenoprotein expression: Diphthamide deficiency reduces selenocysteine incorporation, decreases selenite sensitivity and predisposes to oxidative stress

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ABSTRACT

The diphthamide modification of translation elongation factor 2 is highly conserved in eukaryotes and archaeobacteria. Nevertheless, cells lacking diphthamide can carry out protein synthesis and are viable. We have analyzed the phenotypes of diphthamide deficient cells and found that diphthamide deficiency reduces selenocysteine incorporation into selenoproteins. Additional phenotypes resulting from diphthamide deficiency include altered tRNA-synthetase and selenoprotein transcript levels, hypersensitivity to oxidative stress and increased selenite tolerance. Diphthamide-eEF2 occupies the aminoacyl-tRNA translocation site at which UGA either stalls translation or decodes selenocysteine. Its position is in close proximity and mutually exclusive to the ribosomal binding site of release/recycling factor ABCE1, which harbors a redox-sensitive Fe-S cluster and, like diphthamide, is present in eukaryotes and archaea but not in eubacteria. Involvement of diphthamide in UGA-SECIS decoding may explain deregulated selenoprotein expression and as a consequence oxidative stress, NFκB activation and selenite tolerance in diphthamide deficient cells.

1. Introduction

The post-translational diphthamide modification of eukaryotic translation elongation factor eEF2 is highly conserved in eukaryotes as well as in the archaeal eEF2 counterpart [1–5]. It consists of a histidine in elongation factor 2 (His 715 in human eEF2), modified by the concert action of diphthamide synthesis enzymes encoded by DPH genes DPH1–7 in humans, [6–12]. High conservation of diphthamide and -synthesis genes would suggest that this modification may be rather important for eEF2 functionality and hence for protein synthesis. Reports indicate that it contributes to translation fidelity and avoidance of frameshifting during elongation [13–16], as well as IRES-dependent translation events [41]. Because diphthamide deficient cells are viable [17], lack of diphthamide does not generally affect the synthesis and function of proteins essential for metabolism, propagation and replication of cells.

We have recently generated a set of MCF7 derivatives which lack

diphthamide as consequence of gene editing inflicted destruction of individual DPH genes [12,17]. All copies of the DPH1 gene were inactivated in MCF7-DPH1ko, all copies of DPH2 in MCF7-DPH2ko, of DPH4 in MCF7-DPH4ko, and of DPH5 in MCF7-DPH5ko. Because diphthamide deficiency is inflicted by knockout of different genes, this set of cell lines can be applied to address the function of diphthamide by itself: phenotypes that are common among all these cell lines are attributable to diphthamide deficiency itself and not to potential other functionalities of the different inactivated individual genes.

One common observation in all these cell lines was resistance to Diphtheria and Pseudomonas toxin, an expected phenotype as diphthamide is the molecular target of toxin-mediated eEF2 inactivation. In addition, all diphthamide deficient cell lines had NFκB pathway genes activated (without active stress-trigger) and all were hypersensitive to TNF. TNF hypersensitivity is probably due to NFκB pathway activation, but the underlying reason for diphthamide mediated modulation of NFκB activity is still unexplained [17].

Abbreviations: eEF2, eukaryotic translation elongation factor 2; DPH gene, diphthamide synthesis gene; SeCys, selenocysteine; SECIS, selenocysteine incorporation stemloop; DT, diphtheria toxin; NFκB, nuclear factor kappa B; Dio1, deiodinase 1

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Diphthamide is part of the translation elongation factor eEF2. ‘Occam’s razor’ (simplest explanation most likely being the right one) would therefore imply effects on protein translation as likely explanation for diphthamide deficiency associated phenotypes. Because overall protein synthesis and generation of proteins essential for cell growth was not affected by diphthamide deficiency, we considered the possibility that expression of only certain proteins or of a subset of ‘special’ proteins is affected by diphthamide deficiency. Such proteins would not be essential for general growth, survival and propagation, yet (as that is a phenotype of lack of diphthamide) nevertheless be involved in NFκB signaling.

Selenocysteine containing proteins are a small group of proteins whose translation involves a special incorporation process at the elongation-vs-termination decision point [18–23]. Their mRNAs harbor UGA codons which are not recognized as termination signals but instead decoded as selenocysteine, dependent on presence of 3’UTR-SECIS elements. Most selenoproteins are involved in redox-processes to manage or detoxify oxidative stress [21–23] and oxidative stress is a known trigger of NFκB activation [24–26]. Therefore, we have analyzed if diphthamide deficiency influences the expression of selenoproteins and if it affects (as downstream consequence) cellular sensitivity towards oxidative stress.

2. Results

2.1. Diphthamide deficiency affects transcript levels of cytoplasmic amino acid tRNA-synthetase genes and of genes that encode selenoproteins

A common phenotype of cells carrying DPH gene knockouts resulting in lack of diphthamide is induction of NFκB pathway-associated genes under normal growth conditions. This phenotype correlates with TNF hypersensitivity due to NFκB triggered induction of TNF-sensitivity genes [17]. The underlying reason for the association of diphthamide deficiency with NFκB activity, however, is still unexplained. Diphthamide is part of the essential translation elongation factor eEF2, yet its deficiency does not interfere with overall protein synthesis. Diphthamide may, however, play a role in regulating translation. NFκB induction would be a consequence of direct or indirect modulation of protein synthesis in diphthamide deficient cells. Evidence that diphthamide deficiency affects some aspects of protein synthesis is provided by transcriptional profiling of different DPH-knockout derivatives in comparison to parent MCF7 cells. Table 1A shows that the transcript levels of nuclear encoded cytoplasmic tRNA-synthetases are changed significantly. Alteration of tRNA-synthetase transcript levels was observed in all analyzed diphthamide deficient cell lines (DPH1, DPH2, DPH4 or DPH5). We found changes in > 50% of the synthetases that charge cytoplasmic tRNAs (i.e. those that utilize eEF2 for incorporation by 80 S ribosomes). The general pool of tRNA-synthetases for eEF2-independent mitochondrial protein synthesis was not affected when compared to overall transcriptional changes (the only mito-synthetase affected was prolyl-tRNA synthetase). Also, the levels of tRNA-synthetase pseudogene transcripts were not changed. In summary, diphthamide deficiency triggers changes in transcript levels of those tRNA-synthetases that utilize diphthamide-eEF2 for chain elongation.

Another set of genes whose mRNA levels change in diphthamide deficient cells encode selenoproteins (Table 1B): mRNA levels for 7 out of 23 selenoproteins were altered in DPH1ko cells, 8 were altered in DPH2ko cells, 5 were altered in DPH4ko cells and 9 were altered in DPH5ko cells. Thus, diphthamide deficiency changes the transcript levels of a large portion of selenoproteins.

2.2. Diphthamide deficiency affects the translation of selenocysteine containing proteins

Because diphthamide is not part of the transcriptional machinery, altered selenoprotein transcript levels in diphthamide deficient cells

may be a feedback/compensation in response to altered selenoprotein translation. Selenocysteine is incorporated at UGA codons of mRNAs that carry SECIS elements in their 3’ untranslated region. We expressed UGA-selenocysteine containing cDNAs accompanied by 3’ SECIS sequences in parent MCF7 and DPH1ko cells and compared their translation products. Fig. 1 shows Western blot analyses of deiodinase 1 (Dio1) protein [27] in extracts of MCF7 cells, and in DPH1ko cells that lack diphthamide. Dio1 is composed of 249 amino acids with a selenocysteine at position 126. Termination at that position generates a ~15 kDa truncated protein; successful selenocysteine incorporation results in a ~30 kDa full length protein. To facilitate detection of full length Dio1, we have added a His6 tag to its C-terminus, only full length Dio1 carries this tag (Fig. 1A). Detection of full length Dio1 via His6-binding antibody (Fig. 1B) indicates that full length Dio1 translation is strictly dependent on presence of the SECIS element. Cells carrying constructs without SECIS do not produce His-tagged Dio1. A comparison of the levels of expressed Dio1-His6 revealed higher levels of full length Dio1 in parent MCF7 compared to DPH1ko cells. Fig. 1C shows additional Western blots that were probed with antibodies that detect full length as well as truncated Dio1 protein. Polyclonal antibodies from two different sources revealed differences in Dio1 protein content and composition between parent MCF7 and DPH1ko cells. The 30 kDa Dio1 protein band corresponding in size to full length protein was more pronounced in extracts of MCF7 cells than in DPH1ko extracts and the 15 kDa Dio1 fragment (indicative of premature termination at SeCys-UGA) was more pronounced in DPH1ko than in parent cells.

To confirm the influence of diphthamide on selenoprotein expression, we compared the expression of a different selenoprotein in MCF7 and DPH1ko cells. SelenoMabs are antibody derivatives that contain in their mRNA an UGA codon and 3’SECIS for selenocysteine incorporation, for site-directed payload attachment, [28,29]. SelenoMabs are secreted into culture supernatants and can be purified by protein A-chromatography. This allowed us to assess and quantify selenocysteine incorporation on purified protein samples. Fig. 2A shows the composition of the SelenoMab and of the expression cassettes used for its production. Selenocysteine-UGA is positioned at the C-terminus of the L-chain, followed by a His6 stretch and the 3’-SECIS element of human Dio1 mRNA. Reading UGA at this position as ‘stop’ generates a normal IgG, selenocysteine incorporation generates a SeCys-His6 extended antibody. Both antibody forms (irrespective of presence or absence of His6) are secreted into medium from which they were purified by proteinA and subsequently quantified (Fig. 2). Total IgG protein can subsequently be separated via NiNTA-chromatography into a ‘normal IgG’ fraction (UGA is read as ‘stop’, protein does not bind to NiNTA) and a fraction with His6 extension (captured on NiNTA).

Quantification of total IgG and individual fractions revealed that MCF7 as well as DPH1ko cells produced similar amounts of total IgG. This confirms that overall protein synthesis is not significantly affected in diphthamide deficient cells. A comparison of the relative content of normal and His6 extended IgG showed that in parent MCF7 cells approximately 4% of total IgG had the UGA codon decoded followed by His6. This ratio of read-through vs stop is similar in order of magnitude as previously reported for SelenoMabs (approx.10% in systems optimized for expression, [29]). In contrast to that, DPH1ko cells (despite expressing the same overall amount of IgG) contained almost exclusively IgG terminated at UGA. Less than 1% of the total IgG contained His6. Thus, diphthamide deficient cells decode selenocysteine codons (UGA-SECIS) less efficient than parent MCF7 cells. Reduced production of full length Dio1 and reduced selenocysteine-UGA read-through of SelenoMabs in diphthamide deficient cells indicates that diphthamide plays a role in the ‘special’ translation of selenoproteins.

To determine if diphthamide deficiency affects chromosome encoded selenoprotein transcripts, we analyzed chromosome encoded selenoprotein P (SELENOP, SEPP1) secreted into supernatants of MCF7 cells and of DPHko derivatives. SELENOP harbors multiple selenocysteins as well as an oligoHis stretch. The latter enables enrichment of full

Table 1

Diphthamide deficiency affects mRNA levels of amino-acid tRNA-synthetases and selenoproteins. (A) mRNA levels of amino-acid tRNA-synthetases in diphthamide deficient cells compared to wildtype cells. (B) Levels of selenocysteine encoding mRNAs in diphthamide deficient cells.

A cytoplasmic tRNA synthetase genes		DPH1 ko		DPH2 ko		DPH4 ko		DPH5 ko	
		log ratio	p-value	log ratio	p-value	log ratio	p-value	log ratio	p-value
AARS	alanyl-tRNA synthetase	1,33	1,4E-30	1	2,57E-24	1,664	2,23E-56	1,014	2,18E-12
CARS	cysteinyl-tRNA synthetase	1,221	9,42E-25	1,284	2,29E-34	1,612	3,82E-47	1,098	9,99E-15
GARS	glycyl-tRNA synthetase	1,019	2,76E-19	0,932	3,98E-22	1,351	5,27E-39	0,965	1,29E-12
IARS	isoleucyl-tRNA synthetase	0,628	3,13E-07	0,454	0,000012	0,91	1,09E-17	0,578	0,000252
MARS	methionyl-tRNA synthetase	1,322	1,69E-31	1,242	1,26E-38	1,743	4,82E-64	1,181	5,11E-18
SARS	seryl-tRNA synthetase	1,194	8,29E-26	1,043	3,26E-27	1,307	1,6E-36	0,975	7,38E-12
TARS	threonyl-tRNA synthetase	0,759	5,21E-11	1,262	7,26E-40	1,022	3,1E-23	0,808	2,49E-09
WARS	tryptophanyl-tRNA synthetase	0,757	2,83E-10	0,589	9,01E-09	0,964	4,6E-19	0,657	0,0000255
YARS	tyrosyl-tRNA synthetase	1,036	1,75E-19	0,89	9,76E-20	1,569	1,16E-50	1,051	1,06E-14
SEPSECS	selenocysteine tRNA synthetase	1,334	5,92E-23	1,238	1,01E-23	0,546	0,0000364	1,054	1,42E-11
NARS	asparagyl-tRNA synthetase	0,263	0,0761	0,355	0,000837	0,55	8,71E-07	0,19	0,403
EPRS	glutamyl-prolyl-tRNA synthetase	0,379	0,0052	0,101	0,527	0,535	0,00000206	0,171	0,531
GATB	glutamyl-tRNA(Gln) amidotransf. B	-0,021	1	0,132	0,523	-0,239	0,199	0,813	0,00000099
DARS	aspartyl-tRNA synthetase	0,037	0,973	-0,029	1	-0,035	1	0,007	1
LARS	leucyl-tRNA synthetase	0,171	0,386	0,114	0,446	0,153	0,379	-0,076	0,864
VARS	valyl-tRNA synthetase	-0,211	0,185	-0,23	0,0584	-0,061	0,893	-0,347	0,00943
HARS	histidyl-tRNA synthetase	-0,384	0,00337	-0,133	0,45	-0,029	1	-0,163	0,398
QARS	glutamyl-tRNA synthetase	-0,294	0,0273	-0,066	0,793	-0,25	0,0628	-0,265	0,0814
FARSB	phenylalanyl-tRNA synthetase, beta	-0,32	0,0271	-0,139	0,482	0,037	0,958	-0,12	0,737
KARS	lysyl-tRNA synthetase	-0,411	0,00245	-0,463	0,0000354	-0,331	0,0122	-0,697	0,00000162
RARS	arginyl-tRNA synthetase	-0,644	5,07E-08	-0,743	2,95E-13	-0,497	0,0000138	-0,571	0,0000273
GATC	glutamyl-tRNA(Gln) amidotransf. C	-0,441	0,00307	-0,404	0,00311	-0,467	0,000917	-0,371	0,0255
FARSA	phenylalanyl-tRNA synth. alpha	-0,659	2,69E-08	-0,732	2,55E-13	-0,436	0,00018	-0,744	5,87E-09
cut-off: p-value = 0.0001									
deregulated tRNA-synthetases (% of 23 transcripts)		12 (52%)		13 (57%)		12 (52%)		14 (61%)	
deregulated overall (% of all 21893 transcripts)		2010 (9%)		2568 (12%)		1675 (8%)		2025 (9%)	
significance tRNA synthetases vs overall*		p=1.79E-7		p=2.82E-7		p=2.36E-7		p=1.18E-9	
significance DPH1+2+4+5 (diphthamide) effect**		p=2.46E-28							
mitochondrial tRNA synthetase genes & cytoplasmic tRNA synthetase pseudogenes									
PARS2	prolyl-tRNA synthetase 2, mito	0,853	0,000023	0,819	0,0000285	0,722	0,000317	0,741	0,00211
DARS2	aspartyl-tRNA synthetase 2, mito	0,267	0,0716	0,767	3,32E-15	0,364	0,00268	0,506	0,000881
SARS2	seryl-tRNA synthetase 2, mito	-0,136	0,635	0,452	0,00258	-0,067	0,882	0,425	0,047
CARS2	cysteinyl-tRNA synthetase 2, mito	-0,299	0,0804	0,601	0,0000087	0,001	1	-0,147	0,499
TARS2	threonyl-tRNA synthetase 2, mito	0,164	0,427	0,349	0,00174	-0,013	1	0,345	0,0742
AARS2	alanyl-tRNA synthetase 2, mito	0,24	0,181	0,028	0,985	0,087	0,851	0,139	0,829
EARS2	glutamyl-tRNA synthetase 2, mito	-0,026	1	0,17	0,248	0,107	0,74	0,261	0,263
FARS2	phe-tRNA synthetase 2, mito	-0,213	0,348	-0,223	0,247	-0,29	0,106	-0,15	0,594
HARS2	histidyl-tRNA synthetase 2, mito	-0,16	0,407	-0,083	0,732	-0,035	0,966	-0,144	0,469
IARS2	isoleucyl-tRNA synthetase 2, mito	-0,161	0,354	-0,24	0,0571	-0,174	0,326	-0,103	0,683
LARS2	leucyl-tRNA synthetase 2, mito	0,137	0,765	-0,019	1	0,07	0,949	0,287	0,371
MARS2	methionyl-tRNA synthetase 2, mito	-0,058	0,927	-0,055	0,953	0,052	1	-0,333	0,101
NARS2	aspl-tRNA synthetase 2, mito	0,096	0,786	0,175	0,264	0,196	0,26	0,17	0,619
RARS2	arginyl-tRNA synthetase 2, mito	-0,195	0,402	-0,102	0,732	-0,093	0,872	-0,127	0,842
VARS2	valyl-tRNA synthetase 2, mito	-0,046	0,945	0,185	0,179	-0,004	1	0,079	0,951
WARS2	tryp tRNA synthetase 2, mito	0,293	0,126	0,1	0,697	-0,013	1	0,182	0,651
YARS2	tyr-tRNA synthetase 2, mito	-0,548	0,000323	-0,418	0,00453	-0,313	0,0789	-0,293	0,178
MTFMT	met-tRNA formyltransferase, mito	-0,479	0,0135	-0,486	0,00781	-0,487	0,0125	-0,469	0,0283
GARSP1	glycyl-tRNA synthetase pseudo 1	0,118	1	-0,906	0,515	-0,751	0,704	-0,606	1
KARSP1	lysyl-tRNA synthetase pseudo 1	-0,114	1	-0,37	0,881	0,786	0,456	-0,417	1
KARSP2	lysyl-tRNA synthetase pseudo 2	0,746	0,647	0,087	1	0,956	0,584	0,578	0,643
KARSP3	lysyl-tRNA synthetase pseudo 3	-0,247	1	-0,064	1	0,05	1	-1,067	0,624
cut-off: p-value = 0.0001									
deregulated tRNA-synthetases (% of 22 transcripts)		1 (5%)		3 (14%)		0 (0%)		0 (0%)	
deregulated overall (% of all 21893 transcripts)		2010 (9%)		2568 (12%)		1675 (8%)		1675 (8%)	
significance tRNA synthetases vs overall		p=0.88		p=0.49		p=1***		p=1***	
significance DPH1+2+4+5 (diphthamide) effect		p=0.77							

(continued on next page)

Table 1 (continued)

B	selenoprotein	DPH1 ko		DPH2 ko		DPH4 ko		DPH5 ko	
		log ratio	p-value	log ratio	p-value	log ratio	p-value	log ratio	p-value
DIO1	deiodinase, iodothyronine, type I	-1,82	0,00491	-1,492	0,0394	-2,677	0,00000265	-3,711	5,89E-15
DIO2	deiodinase, iodothyronine, type II	-3,011	1,54E-28	-2,341	4,79E-13	-0,153	1	-2,37	7,64E-14
SELO	selenoprotein O	-0,375	0,0384	-0,331	0,0368	-0,356	0,0535	-0,371	0,0366
SEPW1	selenoprotein W 1	-0,326	0,0186	-0,441	0,000115	-0,297	0,0352	-0,163	0,453
TXNRD1	thioredoxin reductase 1	-1,271	7,62E-30	-1,047	2,63E-26	0,037	0,997	-0,727	5,81E-08
MSRB1	methionine sulfoxide reductase B1	-0,602	0,0000635	-0,359	0,0241	-0,03	1	-0,068	0,878
SEPP1	selenoprotein P	0,422	0,441	0,255	0,697	-2,146	2,11E-27	-0,817	0,00914
GPX3	glutathione peroxidase 3	0,5	0,0243	-0,035	1	1,856	2,27E-21	0,889	0,0001
GPX4	glutathione peroxidase 4	0,111	0,64	0,229	0,0518	0,395	0,000973	0,388	0,017
SEPHS2	selenophosphate synthetase 2	0,2	0,258	0,307	0,00606	0,38	0,00189	0,454	0,00361
SELM	selenoprotein M	-0,432	0,455	-0,682	0,123	0,679	0,173	-1,249	0,0000507
SEPN1	selenoprotein N	0,18	0,513	0,13	0,582	0,086	0,872	-0,36	0,0103
SELT	selenoprotein T	-0,064	0,87	-0,328	0,0075	0	1	-0,163	0,521
TXNRD2	thioredoxin reductase 2	0,343	0,0357	0,261	0,101	-0,067	0,872	-0,509	0,000337
C11orf31	chromosome 11 ORF 31	0,379	0,0233	0,118	0,694	-0,047	1	-0,072	0,893
GPX1	glutathione peroxidase 1	0,254	0,945	-0,623	0,697	0,293	1	0,432	0,864
GPX2	glutathione peroxidase 2	-0,76	0,345	-1,202	0,0475	-0,257	0,949	-0,148	0,968
SELK	selenoprotein K	-0,2	0,462	-0,224	0,286	0,236	0,316	-0,219	0,478
DIO3	deiodinase, iodothyronine, type III	-0,537	0,844	0,062	1	0,206	1	-0,199	1
EPT1	ethanolaminephosphotransferase 1	0,118	0,669	-0,152	0,342	0,104	0,716	-0,027	0,969
SELV	selenoprotein V	-0,08	1	-0,536	0,697	-0,054	1	0,067	1
SEP15	15 kDa selenoprotein	0,013	1	-0,133	0,512	0,01	1	-0,307	0,0781
VIMP	VCP-interacting membrane protein	-0,076	0,855	0,136	0,431	0,122	0,679	-0,144	0,595
cut-off: log ratio -0.3; p-value 0.05									
induced selenoproteins (% of 23 transcripts)		6 (26%)		7 (30%)		4 (17%)		8 (35%)	
induced overall (% of all 21893 transcripts)		2835 (13%)		2391 (11%)		2282 (10%)		2926 (13%)	
significance selenoprotein vs overall*		p=0.067		p=0.009		p=0.213		p=0.008	
significance DPH1+2+4+5 (diphthamide) effect**		p=0.0001							
cut-off: log ratio -1.0; p-value 0.005									
induced selenoproteins (% of 23 transcripts)		3 (13%)		2 (9%)		2 (9%)		3 (13%)	
induced overall (% of all 21893 transcripts)		522 (2%)		462 (2%)		480 (2%)		748 (3%)	
significance selenoprotein vs overall*		p=0.017		p=0.084		p=0.089		p=0.042	
significance DPH1+2+4+5 (diphthamide) effect**		p=0.00028							

length SELENOP and oligoHis containing fragments from cell culture supernatants by NiNTA capture [30,31]. Fig. 3 shows the results of Western blot analyses of proteins that were NiNTA-affinity purified from culture supernatants. SELENOP specific polyclonal antibodies detect proteins with apparent MW of 62 kDa, 55 kDa, 50 kDa and 30 kDa in supernatants of MCF7 cells and of DPHko derivatives. The 62 kDa band appears in a comparable size range as previously described for full length NiNTA-purified SELENOP [31]. Smaller proteins may reflect prematurely terminated SELENOP fragments. Comparison of the ratios between 62 kDa SELENOP and proteins of reduced size reveal different distribution patterns between parent MCF7 and DPHko cells. Parent MCF7 cells harbor relatively higher amounts of large 62 kDa SELENOP. The ratio is switched to increased amounts of prematurely terminated SELENOP fragments in cells with inactivated DPH1 or DPH2 or DPH4 or DPH5 genes. Thus, generation of 62 kDa SELENOP protein which requires read through of the SeCys codons is more pronounced in parent MCF7 cells than in diphthamide deficient DPHko cells.

2.3. Diphthamide deficient cells contain high basal ROS levels and are hypersensitive towards induced oxidative stress

Selenoproteins maintain the cellular redox balances and ameliorate or detoxify oxidative stress. Alterations of selenoprotein synthesis may thus affect susceptibility towards oxidative stress. We therefore assessed the sensitivity towards oxidative stress of MCF7 wildtype cells and diphthamide deficient MCF7 derivatives. Live cells were labeled with a ROS sensor (CellRox green reagent) that visualizes oxidative stress via generation of fluorescence signals.

Analyses of MCF7 by FACS (Fig. 4) and confocal microscopy (Fig. 5A) revealed low ROS levels when cells were propagated under normal growth conditions. Induction of oxidative stress by exposure to 400 μ M TBHP (tert-butylhydroperoxide) increases the signals, indicating induction of oxidative stress. Diphthamide deficient DPHko cells revealed significant ROS associated signals even under normal growth conditions without a TBHP trigger. Stress-signal levels without TBHP were similar (or in some instances even higher) as those observed in TBHP-treated wildtype MCF7. Addition of TBHP to diphthamide deficient MCF7 aggravated the CellRox signals to levels that exceeded

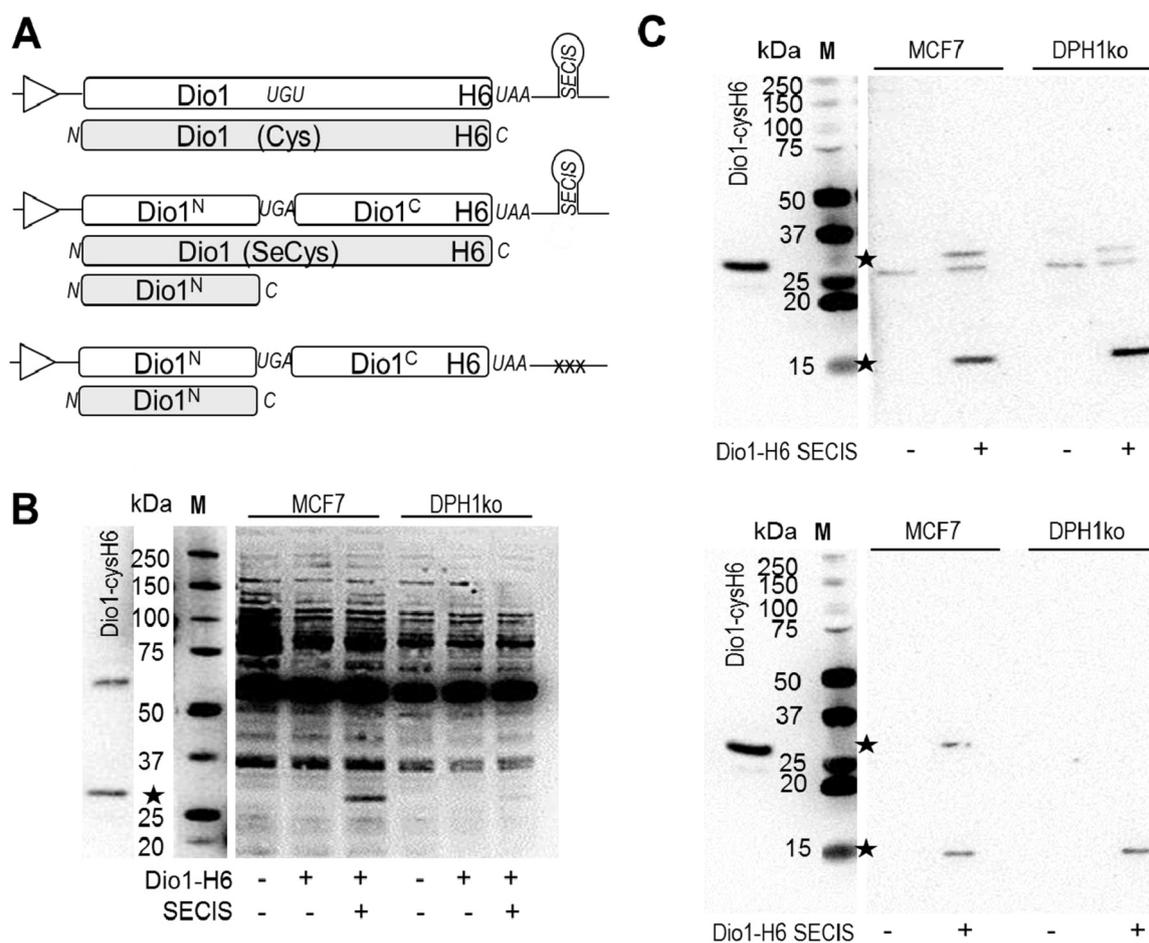


Fig. 1. Expression of Dio1 protein in MCF7 cells. (A) Expression cassettes for Dio1 with attached H6 (His6)-tag and encoded proteins. top: TGT(Cys)-containing control; middle: TGA(SeCys) encoding Dio1-His6 with 3' SECIS; bottom: TGA(SeCys) encoding Dio1-His6 without 3' SECIS. (B) The anti-His5 antibody (Qiagen 34660 1:1500, anti-mouse IgG-HRP 1:1500 Dako P0447, blocking & ab-incubation in 5% BSA) detects the C-terminal His tag of full length Dio1 protein. (*) Dio1-H6 specific signal. Presence of the UGA codon as well as SECIS is required to generate full length Dio1. UGA without SECIS or UAA with SECIS do not generate Dio1-H6 protein. Dio1-CysH6 is a control protein that contains a UGU codon for Cys incorporation in place of UGA (SeCys). (C) Western analyses with polyclonal rabbit Dio1-antibodies that detect truncated as well as full length Dio1 protein: upper panel – Sigma SAB1304992 1:120, lower panel – Abcam 175973 1:120. Blocking & ab-incubation in 5% BSA, detection with anti-rabbit IgG-HRP 1:1500 Dako P0217. The polyclonal antibody from Sigma generated stronger Dio1-specific signals as well as a one nonspecific background signal (present also in mock-transfected cells). The Abcam reagent generated no background but resulted also in weaker signal intensities for Dio1 proteins.

those observed in wildtype cells. High basal levels of oxidative stress and hypersensitivity towards external triggers were observed for all diphthamide deficient MCF7 derivatives (DPH1/2/4/5ko), independent of which DPH gene was inactivated. Thus, diphthamide deficiency renders cells hypersensitive towards oxidative stress.

Oxidative stress activates NFκB and induction of NFκB pathway genes was previously observed as a common phenotype of diphthamide deficiency in MCF7 cells [17]. Because NFκB translocation into the nucleus is a prerequisite for induction, nuclear localization serves as a marker for initiation of NFκB pathway responses. Fig. 5B shows the subcellular distribution of NFκB in MCF7 cells and in diphthamide deficient MCF7 (DPH2ko) derivatives under normal growth conditions, and upon exposure of MCF7wt cells to TNF (as positive control). In unstressed wildtype MCF7 cells, NFκB resides in the cytoplasm without significant nuclear signals and translocates to the nucleus upon TNF treatment. Exposure to TBHP or TNF also triggers translocation of NFκB into the nucleus, representing induction of the pathway response as consequence of oxidative stress. In contrast, diphthamide deficient MCF7 (DPH2ko) derivatives display significant levels of nuclear NFκB under normal growth conditions without an external trigger. Nuclear NFκB levels increase further upon induction of oxidative stress, reaching levels that far exceed those observed in wildtype MCF7 cells.

2.4. Diphthamide deficient cells tolerate high concentrations of selenite

Selenoprotein synthesis requires intracellular selenium. Selenite supplementation is therefore used to enable selenoprotein detection or recombinant expression of SelenoMabs [28,29,32]. Selenium is necessary for cell growth but also becomes toxic above tolerated levels [33,34]. To define a safe concentration for supplementation in our studies, we assessed the Selenite sensitivity of parent MCF7 and DPHko cells. Fig. 6 shows that MCF7 tolerate 1–2 μM Selenite, higher concentrations are toxic. DPH1ko cells tolerated higher concentrations up to 5 μM. Increased tolerance was also observed for cells that lack diphthamide due to inactivation of DPH2 or DPH4 genes (Fig. 6). DPH5ko cells which also lack diphthamide but differ because they harbor a diphthamide precursor on eEF2 [17] tolerated even higher concentrations (> 20 μM, Fig. 6). Thus diphthamide deficiency reduces selenite sensitivity.

3. Discussion

Influence of diphthamide on the biosynthesis of selenocysteine containing proteins was deduced from observations that diphthamide deficiencies (i) reduce translation of recombinant full length selenoproteins, (ii)

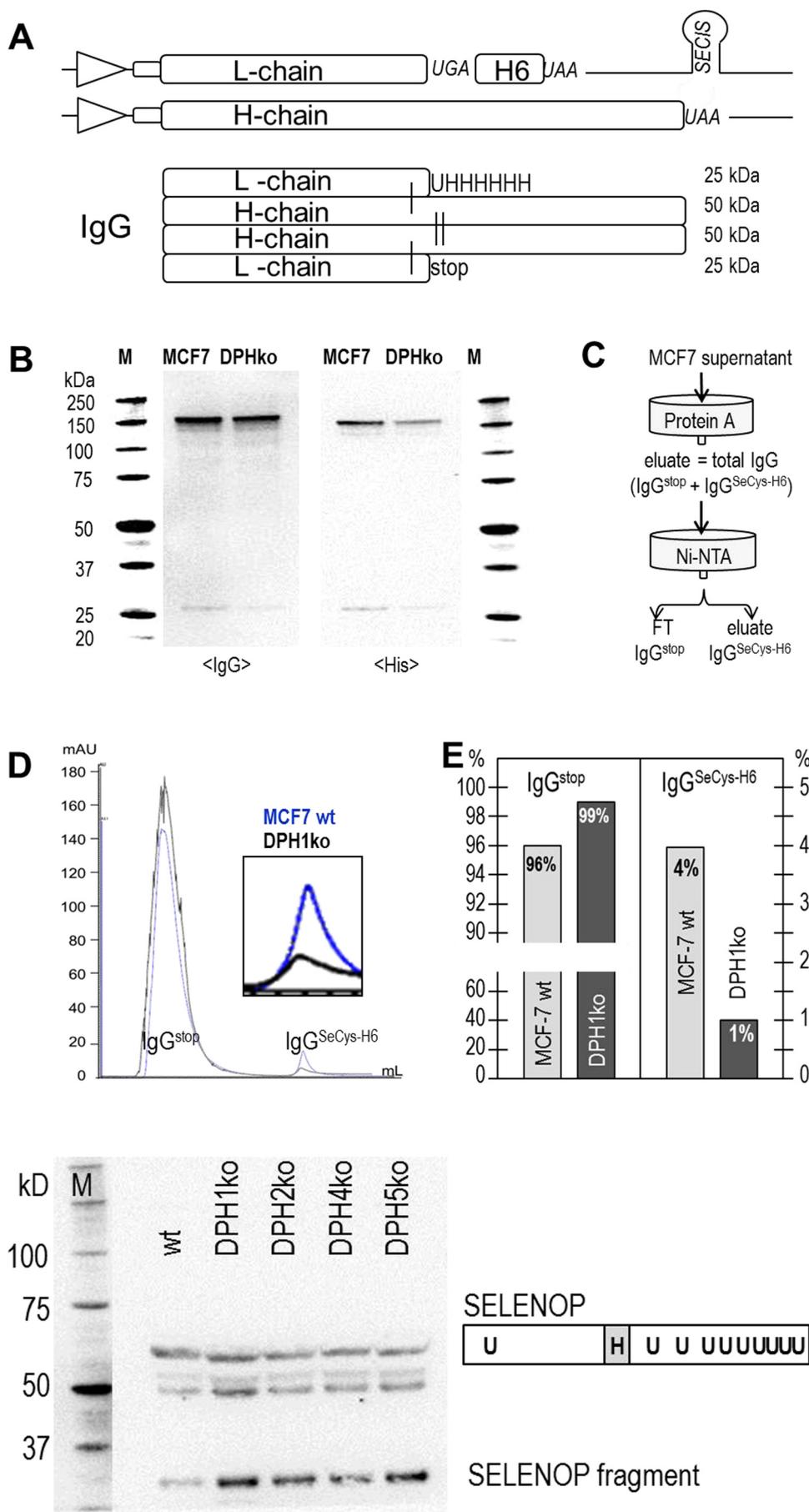


Fig. 2. Expression of a SelenoMab in MCF7 cells. (A) Expression cassettes (top panel) encoding H- and L-chains of a SelenoMab with SeCys-UGA codon followed by H6-tag on the C-terminus of the L-chain. Bottom panel: expressed protein products encoded by the SelenoMab expression cassette contain either a H6-extended or unextended L-chains. (B) Anti-IgG and anti-His5 antibodies detect overall IgG or His tagged SelenoMab fraction, respectively. (C) Purification of IgG from cell culture supernatants followed by separation via NiNTA chromatography differentiates stop vs SeCys reading of the UGA codon. (D&E) Chromatography profile (D) and quantification (E) of IgG-stop and IgG-SeCys-H6 fractions of antibodies produced in MCF7wt and DPH1ko cells.

Fig. 3. Analysis of SELENOP protein in supernatants of parent MCF7 cells and DPHko derivatives. Oligo-His containing proteins present in culture supernatants of parent MCF7 cells and DPHko derivatives were enriched by NiNTA absorption as described by Tujebajeva et al. [30] and Turanov et al. [31]. Equal amounts of NiNTA eluate were subsequently subjected to Western Blot analyses with a SELENOP specific antibody to detect full length SELENOP and oligo-His containing SELENOP fragments.

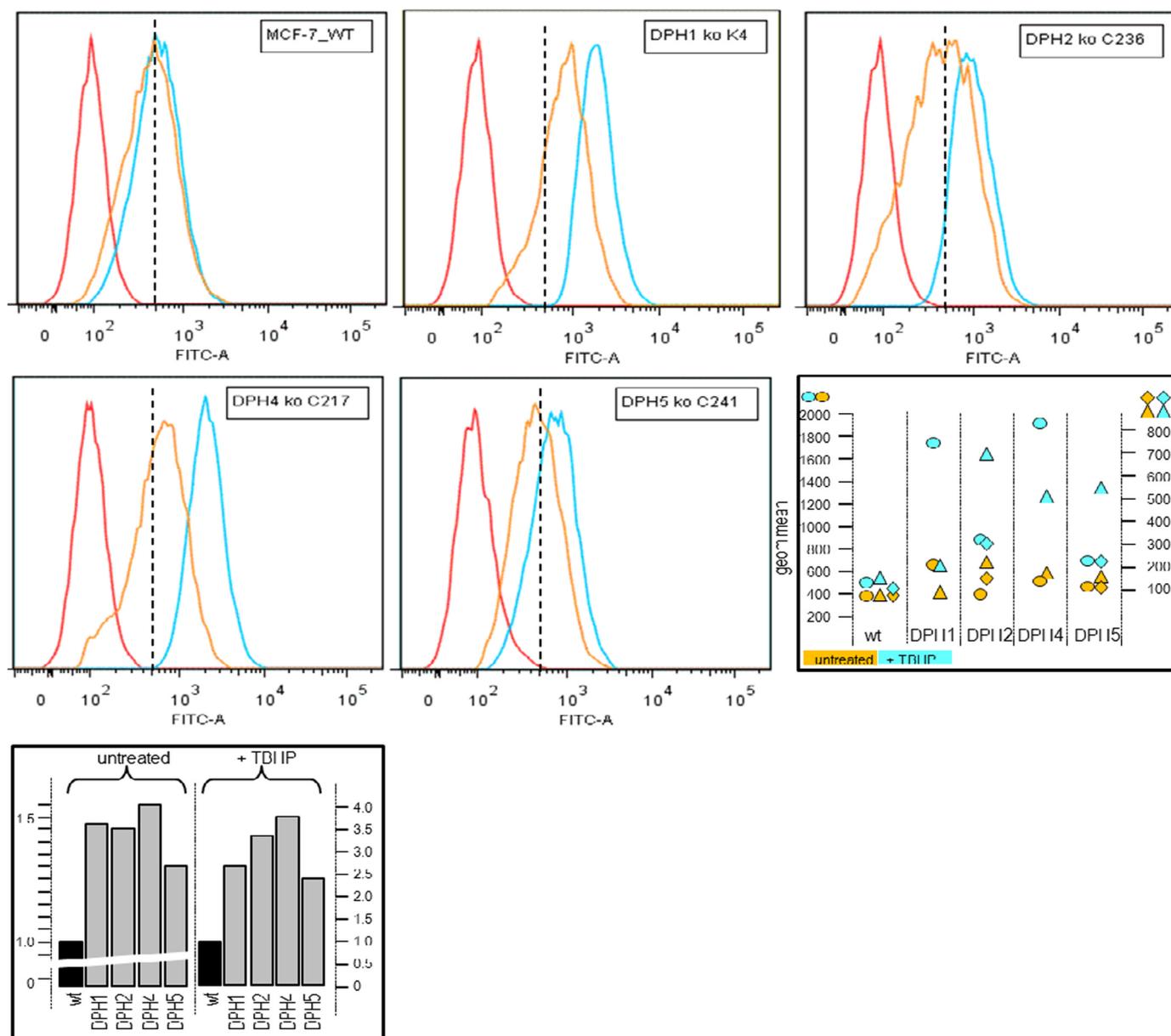


Fig. 4. Comparison of oxidative stress levels in MCF7 cells and DPHko derivatives. Cells were grown under non-stressed conditions in duplicate samples, one of which subsequently treated with TBHP (tert-butylhydroperoxide) at 37 °C to induce oxidative stress. The cells were subsequently exposed to CellRox Green Reagent (C10444) as ROS biosensor and subjected to FACS to assess fluorescence signals that reflect their ox-stress status. Upper and middle panels: FACS of MCF7 cells and DPHko derivatives. Middle-right: Geom.mean values of untreated and TBHP treated cells observed in independent experiments. Lower panel: Signals of DPHko cells relative to MCF7 wildtype cells (set to 1).

affect the transcription of selenoproteins, (iii) render cells hypersensitive to oxidative stress and (iv) increase tolerance towards selenite. The basic principle of selenocysteine incorporation at UGA codons accompanied by SECIS elements is conserved in all organisms [20–22]. Incorporation processes, however, differ between eukaryotes and archaeobacteria on one side, and eubacteria on the other side. Eubacteria utilize SECIS signals proximal to selenocysteine encoding UGA. In contrast, eukaryotic and archaeal selenoprotein transcripts harbor SECIS elements distant from the incorporation site in 3′ untranslated regions. This requires additional factors to transmit the selenocysteine-vs-stop information to the incorporation site. Diphthamide on translation elongation factor (in eukaryotes and archaea, not in eubacteria) correlates or co-incides with the more complex selenocysteine incorporation process in eukaryotes and archaea [20–23].

EEF2 is necessary for translation elongation and it is thus possible that diphthamide affects the ‘stop-vs-continuation’ decision point

during selenoprotein synthesis. Translational pauses that occur upon encountering UGA are either resolved by selenocysteine incorporation and continuation of translation to generate functional selenoproteins. Alternatively, mis-sense incorporation (cysteine instead of selenocysteine) can occur at these positions [35–37], or the translational pauses are resolved by termination and release (release/separation factor acquisition). A role of diphthamide at the termination-vs-continuation point may also explain the effect of lack of diphthamide on translation fidelity [13,14]; stalled translation might either continue via potential frameshifts or misincorporations, or be resolved via termination.

Our work provides evidence for the relevance of diphthamide at the ‘stop-vs-continuation’ (incl. potential mis-incorporation or frameshift) decision point on ribosomes at UGA-SECIS positions. However, our analyses do not imply that loss of diphthamide completely abrogates selenocysteine incorporation. We observe that selenoproteins are still generated in diphthamide deficient cells with residual protein of correct

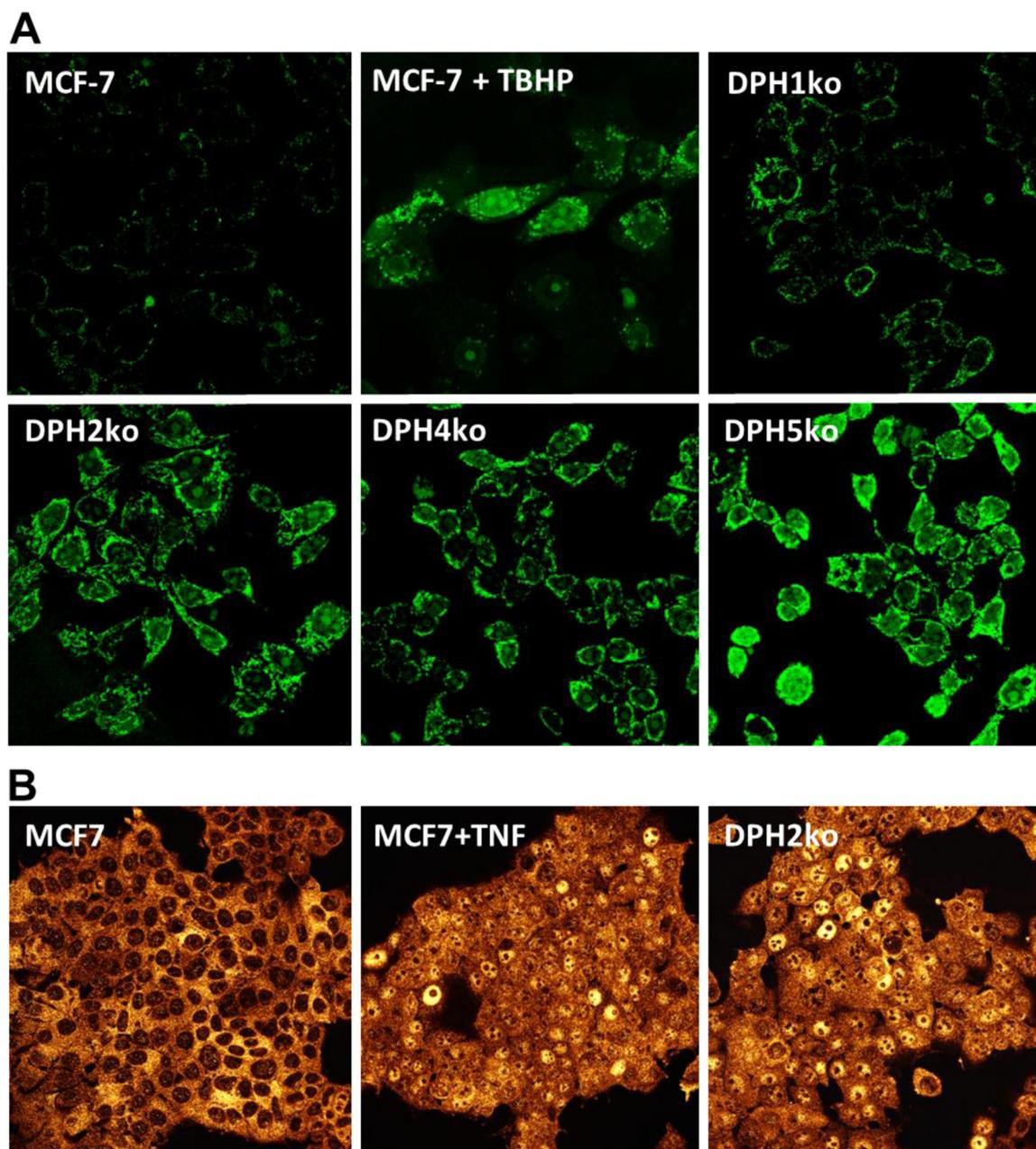


Fig. 5. Oxidative stress and nuclear translocation of NFκB in MCF7 cells and DPHko derivatives. (A) Cells were grown on coverslips under non-stressed conditions, duplicate samples of MCF7 cells subsequently treated for 1 h to 400 μM TBHP at 37 °C to induce oxidative stress. The cells were then exposed to 5 μM of CellRox Green Reagent as ROS biosensor, washed with PBS and immediately imaged w/o fixation by confocal microscopy on a Leica SP5x, 100x lens, Pinhole @ 1 AU. (B) To visualize intracellular distribution and nuclear translocation of NFκB, cells were grown on coverslips under non-stressed conditions, one MCF7wt duplicate samples subsequently treated with TNF (as NFκB translocation control). Intracellular distribution of NFκB was assessed by antibody-detection of NFκB on fixed cells.

size (detectable by Western blot analyses, Figs. 1–3) still being present. Also activity of the cytoplasmatic selenoprotein glutathione peroxidase can still be detected in diphthamide deficient cells (Suppl. Data S2). This indicates that diphthamide is important for selenoprotein synthesis, but it appears not to be absolutely required for selenocysteine incorporation.

Diphthamide could affect selenoprotein translation as a structural component of eEF2 and/or be involved in translational regulation of selenoprotein synthesis. As selenoproteins contribute to maintaining the redox balance, it is reasonable to assume that selenocysteine incorporation could be regulated in a redox-dependent manner. It is important to consider that eEF2-diphthamide is located in the elongating ribosome at the ‘pseudo-stalled’ SeCys-vs-stop decision point in proximity to the binding site for release factor ABCE1 (Fig. 7). eEF2 and

ABCE1 bind the ribosome in a mutually exclusive manner, either contributing to nascent chain elongation (eEF2) or to termination and ribosome recycling ABCE1, [38–43] on stalled ribosomes. ABCE1 exists like diphthamide only in eukaryotes and archaea, and it is ‘unusual’ as it contains Fe-S clusters with so far unexplained function [38–44]. Thus, diphthamide affects the synthesis of redox-modulating selenoproteins and it is located in the ribosome at the same position as a redox-reactive termination/release factor.

Altered availability of functional selenoproteins as consequence of diphthamide deficiency can explain high basal oxidative stress levels and hypersensitivity towards oxidative stress. Absence of diphthamide affects selenoprotein synthesis on a translational level as well as their mRNA levels. Because selenoproteins are involved in maintaining the cellular redox status, diphthamide deficiency deregulates the cellular

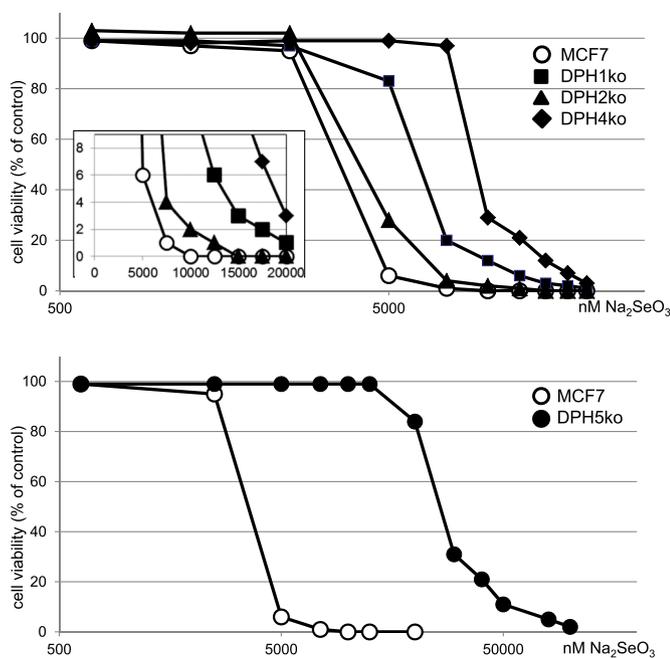


Fig. 6. Selenite tolerance of diphthamide deficient cells. MCF7wt and DPHko derivatives were treated with Na_2SeO_3 for 48 h followed by determination of cell viability (CTG assay). All cell lines tolerated 2.5 μM , 5 μM reduced the viability of MCF7 cells but diphthamide deficient derivatives were not affected (DPH1,4,5) or only to some degree (DPH2). 20 μM kills MCF7 cells and affects the viability of DPH1ko, DPH2ko and DPH4ko cells (albeit to a lesser degree than parent cells) but is still tolerated by DPH5ko cells.

redox balance and generates oxidative stress even without an external trigger. This link between selenoprotein function and oxidative stress also explains NF κ B pre-activation phenotypes of diphthamide deficient cells [17], because oxidative stress induces NF κ B [24–26]. It is interesting to note that the selenocysteine-dependent activity of cytosolic thioredoxin reductase system (TrxR1/TXNRD1) is not only required for the cytotoxicity of diphtheria toxin intracellular release, [45] but also modulates the activity of NF κ B [46,47]. Furthermore, loss of thioredoxin reductase (which acts as repressor of NF κ B activation) not only affects NF κ B but has also been shown to increase sensitivity towards TNF [48]. That –in turn– is another phenotype of diphthamide deficient cells [17]. Thus, reduced activity in particular of TrxR1/TXNRD1 may contribute to many of the phenotypes of diphthamide deficient cells.

Altered selenoprotein availability may also explain decreased selenite-sensitivity of diphthamide deficient cells. Sensitivity to selenite may be caused by interference of (too much) selenium or selenoproteins

with the cellular redox balance [23,31,32]. It was initially surprising to observe increased selenite tolerance in cells that are simultaneously hypersensitive to oxidative stress. That paradox can be resolved considering that selenium availability may translate to selenoprotein content [31,49], and that maintaining the redox balance requires neither too little nor too much selenoprotein. High selenium levels may translate to unfavorably high levels of selenoproteins which interfere with the intracellular redox balance. Reduced sensitivity of diphthamide deficient cells could thus be due to reduced selenoprotein translation which may counteract selenium hyperavailability.

While a functional diphthamide synthesis machinery sufficient to modify all eEF2 represents the normal set-up in mammalian cells [17], altered diphthamide synthesis genes or DPH gene expression occurs in various diseases. Some ovarian tumors display loss of heterozygosity of the diphthamide synthesis gene DPH1 DPH1 = tumor suppressor gene OVCA1; [50] and mutations in diphthamide synthesis genes that affect their expression are present in other tumors [51]. Additionally, several independent studies link mutations in DPH1 with inherited developmental/neuronal disorders [52–56]. The observation of developmental deficiencies in patients carrying mutations in diphthamide synthesis genes may reflect the relevant roles of NF κ B in development and correlate with developmental defects of DPHko mice [14,57,58]. Interestingly or co-incidentally, phenotypes that include tumor development and developmental/neuronal aberrations may also be associated with alterations in selenium status (incl. dietary contributions) and/or aberrant selenoprotein metabolism [59–68].

4. Methods

Diphthamide deficient MCF7 derivatives with inactivated DPH1/2/4/5 genes were generated by Zinc finger nuclease (ZFN) -mediated gene editing [17]. Unless noted otherwise (Selenite supplementation with Na_2SeO_3 , (Sigma-Aldrich, Cat. No. S5261), cells were grown in RPMI1640/10%FCS + 2mM L-glutamine at 37 °C in humidified 5% CO_2 .

Transcriptome analyses of MCF7 and diphthamide deficient derivatives base on mRNAseq [64] data as input [17]. In contrast to NF κ B– and other pathway transcripts, tRNA-synthetase and selenoprotein clusters are not pre-set parameters in analysis programs and were therefore manually defined. Significances of transcript alterations between parent cells and diphthamide deficient derivatives (p-values) were computed for the single experiments using the hypergeometric test with the total population sizes and events as specified. Combined p-values were computed using Stouffers z-Score method [65] and the R package metap, sumz function.

Expression of recombinant selenoproteins required plasmids that harbor CMV-promoter driven expression cassettes with SECIS elements in 3'UTRs. Dio1 expression plasmids (incl. C-terminal His6, Fig. 1A)

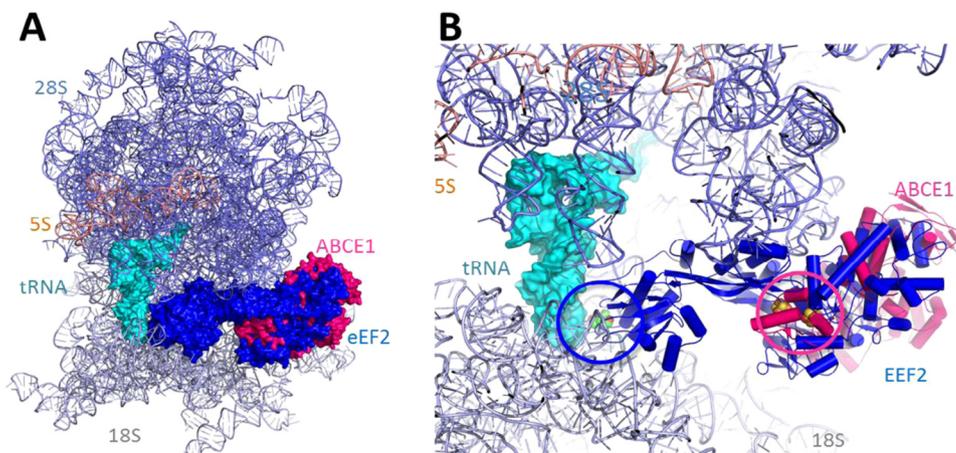


Fig. 7. Mutually exclusive ribosomal positioning of eEF2-diphthamide and ABCE1 at the termination vs continuation decision point for selenocysteine incorporation. (A) overview, (B) zoomed-in: shown is a mammalian ribosome-tRNA-ABCE1 complex with the 28 S subunit in slate, 18 S subunit in grey, 5 S subunit in orange and ribosomal proteins omitted for clarity, tRNA is shown as cyan surface and ABCE1 in pink (rabbit, PDB 3JAI). eEF2 (blue surface) from PDB structure 5JUJ (yeast) was superimposed by structural alignment of the large ribosome subunit RNA (P-atoms, rmsd = 1.116 Å). The iron-sulfur clusters in ABCE1 and the diphthamide of eEF2 are highlighted and circled.

were transfected into MCF7 or -derivatives grown in medium supplemented with 100 nM Na₂SeO₃. Extracts were prepared 48 hrs after transfection by washing cells twice with PBS followed by RIPA/protease inhibitor lysis on ice. Proteins were subjected to reducing SDS-PAGE, blotted and analyzed in Western Blots with an anti-His5 antibody or with polyclonal anti-Dio1 antibodies. For SelenoMab expression, one plasmid encoded the L-chain as C-terminal His6-tagged mRNA transcribed via CMV promoter (Fig. 2A) in the same manner as described for Dio1. Another plasmid encoded the H-chain, both were co-transfected. Antibodies were secreted into culture supernatants from which they were purified via protein A chromatography. His6-containing UGA-SECIS read-through products were differentiated from UGA-terminated IgGs by binding to NiNTA.

Analysis of SELENOP protein that becomes secreted into cell culture supernatants was performed as described by Tujebajeva et al. [30] and Turanov et al. [31]. SELENOP harbors oligoHis stretches that enable NiNTA capture of full length SELENOP and oligoHis containing N-terminal fragments. Supernatants of 4 × 10⁶ cells seeded in T175 flasks containing 25 ml medium and cultured for 4 days were subjected to NiNTA columns (Roche). After washing with phosphate/salt buffer (50 mM Na₂HPO₄, 200 mM NaCl, pH7.2), oligoHis proteins were eluted with imidazole buffer. SELENOP protein in NiNTA eluates was subsequently subjected to Western blot analyses with polyclonal antibodies that detect human SELENOP (ab155185, Abcam, 1:120 diluted). We noted that ‘normal’ sample processing (5 min boiling in reducing SDS sample buffer (Invitrogen NuPage NP0007 + reducing agent NP0009)) was insufficient to completely dissolve high molecular weight SELENOP materials. Therefore, samples were incubated for 30 min at 72 C in NuPage sample buffer containing twice the amount of reducing agent prior to loading on SDS gels (Fig. 3). Differences in SELENOP signals between MCF7 parent and DPHko cells could also be observed on blots containing non-reduced or partially reduced samples (Suppl. Data S1). The first SeCys codon of SELENOP locates close to the N-terminus and precedes oligoHis encoding sequences. SELENOP fragments terminated at this position can therefore not be detected in NiNTA-enriched samples. All other SeCys codons are C-terminal of oligoHis. Fragments terminated at those positions may hence be present and detectable with SELENOP specific antibodies in NiNTA enriched samples.

Selenite sensitivity of MCF7 cells and DPH deficient derivatives was assessed by quantifying viability (ATP content, Cell-Titer-Glo, CTG, Promega G7572) after selenite exposure for 48hrs.

ROS levels and oxidative stress were detected in and compared between MCF7 and DPHko cells. Therefore, cells were initially grown without applying external stress in duplicate samples. One set of those was subsequently treated with 400 μM TBHP (tert-butylhydroperoxide) to induce oxidative stress. The cells were subsequently exposed to CellRox Green Reagent (C10444) as ROS biosensor and subjected to FACS. The resulting fluorescence reflects the levels of ROS within cells.

Visualization of oxidative stress and NFκB translocation by confocal microscopy was achieved by growing cells on coverslips under non-stressed condition in duplicates, subsequently treating one of those for 1 h with 400 μM TBHP. The cells were then exposed to 5 μM of CellRox Green Reagent (C10444), washed with PBS and immediately imaged. To detect intracellular distribution of NFκB, cells were grown on coverslips under non-stressed conditions, duplicate samples subsequently treated with TNF (NFκB translocation control) or 400 μM TBHP. NFκB localization was assessed on fixed cells with an anti-NFκB antibody and confocal imaging (Leica SP5x, 100x lens).

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Conflict of interest statement

The authors are employed by Roche Pharma Research & Early Development. Roche is interested in identifying novel targets and approaches for disease diagnosis and therapy.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.09.015.

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