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Discoveries

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# Regulatory Phenomena in the Glutathione Peroxidase Superfamily

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## Abstract

***Significance:*** The selenium-containing Glutathione peroxidases (GPxs)1–4 protect against oxidative challenge, inhibit inflammation and oxidant-induced regulated cell death.
***Recent Advances:*** GPx1 and GPx4 dampen phosphorylation cascades predominantly *via* prevention of inactivation of phosphatases by $H_2O_2$ or lipid hydroperoxides. GPx2 regulates the balance between regeneration and apoptotic cell shedding in the intestine. It inhibits inflammation-induced carcinogenesis in the gut but promotes growth of established cancers. GPx3 deficiency facilitates platelet aggregation likely *via* disinhibition of thromboxane biosynthesis. It is also considered a tumor suppressor. GPx4 is expressed in three different forms. The cytosolic form proved to inhibit interleukin-1-driven nuclear factor $\kappa$B activation and leukotriene biosynthesis. Moreover, it is a key regulator of ferroptosis, because it reduces hydroperoxy groups of complex lipids and silences lipoxygenases. By alternate substrate use, the nuclear form contributes to chromatin compaction. Mitochondrial GPx4 forms the mitochondrial sheath of spermatozoa and, thus, guarantees male fertility. Out of the less characterized GPxs, the cysteine-containing GPx7 and GPx8 are unique in contributing to oxidative protein folding in the endoplasmic reticulum by reacting with protein isomerase as an alternate substrate. A yeast 2-Cysteine glutathione peroxidase equipped with CP and CR was reported to sense $H_2O_2$ for inducing an adaptive response.
***Critical Issues:*** Most of the findings compiled are derived from tissue culture and/or animal studies only. Their impact on human physiology is sometimes questionable.
***Future Directions:*** The expression of individual GPxs and GPx-dependent regulatory phenomena are to be further investigated, in particular in respect to human health. *Antioxid. Redox Signal.* 33, 498–516.

**Keywords:** glutathione peroxidases, alternate substrate reaction, silencing of lipoxygenases, apoptosis, ferroptosis, inflammation-induced carcinogenesis, transcriptional regulation

## Introduction

THE ACTION OF GLUTATHIONE PEROXIDASE (GPxs) as ''an enzyme that protects hemoglobin from oxidative breakdown'' (124) provided the link between the research on the trace element selenium and its role in biochemistry. Rotruck *et al.* (148) observed an hemoglobin oxidation in the hemolysate of selenium-deficient rats that was not associated with glutathione (GSH) loss and could not be reversed by glucose, as in normal hemolysates. The authors, therefore, concluded that the use of GSH for the reduction of $H_2O_2$ was selenium dependent. After a precise stoichiometry of one selenium atom per subunit of isolated bovine GPx (now GPx1) had been measured (56), the selenoprotein nature of the enzyme was unequivocally established.

Also, the second mammalian selenoprotein discovered a decade later (182), the phospholipid hydroperoxide GPx (now GPx4), proved to be involved in the prevention of oxidative damage. In view of this background, it cannot be a matter of surprise that selenium is widely considered a ''biological antioxidant.'' However, later discoveries of selenoproteins that are unrelated to balancing oxidant challenge

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(53) rate this label as an oversimplification, and even the GPxs do not always act as antioxidant enzymes (21, 55, 181).

Meantime, eight distinct GPxs have been detected in humans, with only five of them, GPx1–4 and 6, being selenoproteins. Their active site contains a selenocysteine residue (Sec), which, together with a glutamine (Gln), tryptophan, and asparagine constitutes a conserved tetrad (177). In GPx5,7, and 8, in rodents and also in GPx6, the active site Sec is replaced by cysteine. Sec-containing GPxs are found not only in vertebrates but also sporadically in lower animals (55).

The majority of GPxs, however, contain a cysteine in their active site (peroxidatic cysteine [$C_P$]) and an additional cysteine residue in a flexible loop, which on oxidation forms an internal disulfide with $C_P$, thus mimicking the reaction scheme of 2-Cysteine (2-Cys)-peroxiredoxins. Similar to the peroxiredoxins, these GPx-type enzymes prefer redoxins, that is proteins characterized by a cysteine-xx-cysteine motif characteristic for redoxins (CxxC motif), as reducing substrates (55, 120).

The common denominator of the different GPxs is their ability to catalyze the reduction of hydroperoxides by thiols.

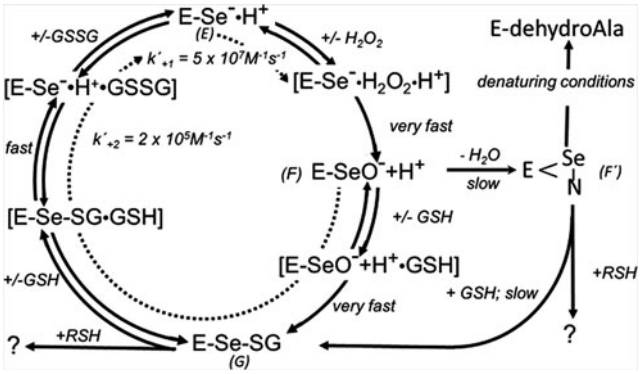



**FIG. 1. Reaction scheme of SecGPxs.** The catalysis comprises a sequence of bimolecular reactions. In the ground state enzyme (*E*), the seleno-cysteine residue is dissociated, but its proton is kept within the catalytic tetrad in a strategic position, preferentially at the ring nitrogen of Trp. The $E.H_2O_2$ adduct decays without any activation energy, which complies with the extreme velocity of the oxidative step of the catalysis. In each case, the formation of complexes (in *brackets*) is slower than the reaction within complexes, which explains the lack of saturation kinetics. The shown bimolecular apparent rate constants are those of bovine GPx1 (57). $k'_{+1}$ describes the net forward reaction of the zwitterionic *E* with $H_2O_2$; $k'_{+2}$ approximates the slowest rate constant of the reductive part of the catalysis. For rate constants of other GPxs, see Toppo *et al.* (176). In the absence of GSH, the selenenic acid of the oxidized intermediate, oxidized enzyme (*F*) forms an Se–N bond with an amide nitrogen of the backbone (*F′*; verified for GPx1 and GPx4). *F′* can be re-integrated into the canonical cycle by GSH at the level of the semi-reduced form, semi-reduced enzyme (*G*). Each of the intermediates of the cycle has to be considered as a separate chemical entity that can react with substrates that are distinct from $H_2O_2$ or GSH, respectively, thus allowing multiple reactions (133). GPx(s), glutathione peroxidase(s); GPx1, mammalian glutathione peroxidase type 1; GPx4, mammalian glutathione peroxidase type 4; GSH, glutathione; SecGPx, glutathione peroxidase containing a catalytic selenocysteine; Trp, tryptophan.

All GPxs display ping-pong kinetics, which reveals that the enzymes are oxidized by the hydroperoxide and then stepwise reduced by thiols. Substrate specificity, however, varies substantially between the subfamilies. The catalytic efficiency of GPxs is remarkable irrespective of their Sec or Cys content. The oxidation of their active site Sec (or Cys) by organic hydroperoxide (ROOH) exceeds that of low molecular mass thiols or selenols by four to seven orders of magnitude. This extraordinary catalytic power can be attributed to a concerted attack on the hydroperoxide bound to the reaction center, an electrophilic attack by a dislocated proton, and a nucleophilic one by the selenolate or thiolate, respectively (133) (Fig. 1).

In general, peroxidases can interfere with regulatory processes in different ways: (i) They can compete with redox-regulated proteins for hydroperoxides used as signaling molecules. (ii) They can silence enzymes that require hydroperoxides for being activated. (iii) They may undergo transient inactivation, thereby saving hydroperoxides for signaling purposes. (iv) They could use hydroperoxides to build Se-thioylated homo- or heterodimers or polymers. (v) They may sense $H_2O_2$ or ROOH and specifically transduce the oxidation equivalents to target proteins, thereby lending efficiency and specificity to the promiscuous low-molecular-weight oxidant $H_2O_2$.

The scope of this review is to summarize the physiological roles of the various GPxs beyond their mere antioxidant function. To this end, the regulation of their biosynthesis and their impact on metabolic regulation will be compiled. The focus will be on mammalian Sec-containing GPxs, whereas the regulatory roles of some CysGPxs will only be mentioned to exemplify the regulatory potential of the entire GPx superfamily.

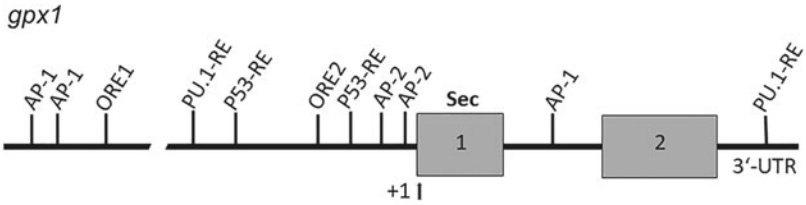
## Mammalian GPxs

### *GPx1*

GPx1 is the most abundant and ubiquitously expressed GPx. The gene is located on chromosome 3 at band 3p21.31 (104) and consists of two exons. Its transcriptional regulation has been reviewed by Lubos *et al.* (116). Transcription factor binding sites that have been validated for activity are shown in Figure 2.

The p53 sites appear to be of particular importance to understand GPx1 function. The p53 transcription factor belongs to tumor suppressors. It is activated by stress conditions, DNA-damaging compounds, or hypoxia. By activating the expression of proteins contributing to DNA repair, cell cycle arrest, and apoptosis, p53 helps to maintain the genomic integrity and, thus, prevents tumorigenesis.

On the other hand, p53 regulates the expression of anti-apoptotic proteins [reviewed by Jänicke *et al.* (94)]. Thus, there appears to be a dark side of p53 activation. p53 is postulated to activate apoptosis *via* reactive oxygen species production (52). Induction or overexpression of GPx1, by removing $H_2O_2$ or other hydroperoxides, will protect cancer cells from being destroyed by apoptosis. This way GPx1 acts in an antiapoptotic manner and, in consequence, supports tumor cell survival. Moreover, a correlation between high GPx1 expression (and a high p53 level) with a higher recurrence and incidence of metastases has recently been observed in patients with giant cell tumor of bone (131). p53-dependent regulation may thus be pro- or anticarcinogenic and appears to depend on the state of cancer development. At




| | |
|---|---|
| ORE-1 | 1282-ctgtcatcctcaaagaaagtgtattg-1257 |
| PU.1-RE | 864-ctttctaaacTTCCTgtatcctgt-841 |
| P53-RE | 770-aggtttgttg-760-(*8bp*)-751-ggactagctt-742 |
| ORE-2 | 337-caggaacctctgagaaaaacggagg-312 |
| P53-RE | 257-GGGCCAGACC-AGACATGCCT-238 |
| AP-2 | 172-CTGAGG-167 |
| AP-2 | 115-CCCGGA-110 |
| AP-1s | 5′-tgactaca-3′ in the region -1500 to -2000 in the 5′UTR and in the intron |
| PU.1-RE | in the 3′-UTR, 5′-tatgagggtgtTTCCTctaaacct-3′ |

**FIG. 2. Gene structure of human GPx1.** The *gpx1* gene consists of two exons (*gray boxes*). Transcription factor binding sites validated for transcriptional activity are listed. The promoter region contains two binding sites for P53, which comply with the consensus sequence consisting of two copies of the 10 bp motif 5′-PuPuPuC(**A/T**)(T/A)GPyPyPy-3′ separated by 0–13 bp (46, 95, 171). One starts 257 bp, the other one 770 bp upstream of the translation start (+↓) (87). The 5′-UTR further contains two OREs, ORE1 and a less responding ORE2 (37). PU.1 sites are present in the 5′- and 3′-flanking region (174). Functionally active AP-1 and AP2 sites were identified in the 5′-flanking region (97). Element sequences (consensus in *upper case letters*) and positions are given in the *insert*. AP-1, activator protein-1 (transcription factor); AP-2, activator protein-2 (transcription factor); ORE, oxygen-responsive element; P53, P53-protein (transcription factor; tumor suppressor); PU.1, transcription factor PU.1 (or 31 kDa-transforming protein); UTR, untranslated region.



early states, GPx1 removes $H_2O_2$ and prevents DNA damage; at later states, it prevents cancer cell removal by $H_2O_2$-driven apoptosis (19).

As a member of the ETS-1 family (141), transcription factor PU.1 (or 31kDa-transforming protein) (PU.1) *inter alia* regulates the maturation of neutrophils, macrophages, B cells, and dendritic cells. Out of these options, regulation of GPx1 expression by PU.1 was only verified for neutrophils. By induction of GPx1, PU.1, thus, might control the expression of a gene that protects neutrophils against the products they produce to kill pathogens (174). Binding of transcription factor C for AP2 to AP2 augmented *gpx1* expression in some breast cancer cells and was discussed to explain its estrogen dependence (108), which has been known since 1968 (140). The regulation of *gpx1* expression by activator protein (transcription factor) 1 and 2 although documented in principle (61, 97, 108, 206), appears to depend on cell type and species and needs further clarification. The physiological role of the oxygen-responsive elements remains enigmatic, since $gpx1^{-/-}$ mice tolerated exposure to hyperbaric oxygen (85). Epigenetically, *gpx1* transcription is downregulated by methylation of DNA methylation site (CpG) islets of the second exon (129).

At the translational level, GPx1 biosynthesis is inhibited by homocysteine (73). The position of GPx1 within the hierarchy of selenoproteins is low (170). Its expression readily declines in moderate selenium deficiency and even its messenger RNA is degraded under these conditions; GPx1, thus, readily responds to the availability of selenium. The low position in the hierarchy implies that health effects seen in marginal to moderate selenium deficiency have to be considered to primarily result from a decreased activity of GPx1.

At the protein level, GPx1 is regulated by the selenium-binding protein 1 (SBP1). Overexpression of SBP1 in a variety of cellular systems resulted in a decrease of GPx1 activity associated with unchanged GPx1 protein levels. Inversely, overexpression of GPx1 led to decreased levels of SBP1. Intriguingly, SBP1 is progressively lost during the development of many clinical tumors, which may enhance GPx1 activity (48). This observation further complicates the role of GPx1 in different stages of carcinogenesis.

GPx1 is widely considered an antioxidant enzyme (21, 71, 116). Its main function appears to be the reduction of $H_2O_2$ and other soluble hydroperoxides at the expense of GSH. This view is consistent with the lack of any obvious phenotype in unstressed $gpx1^{-/-}$ mice, but with a significantly increased susceptibility to oxidant challenges such as exposure to paraquat, bacterial lipopolysaccharides, other inflammatory stimuli, viral infections, or reperfusion injury [reviewed in refs. (55, 71, 116)].

However, for some decades already, $H_2O_2$ is recognized to be an important signaling molecule (51, 54), and even peroxynitrite, which is also a GPx substrate, has recently been shown to be a signal for proliferation in schwannomas (137). Therefore, a regulatory role of GPx1 can no longer be disregarded.

The first related observation came from Arrigo's group. Kretz-Remy *et al.* (107) observed a downregulation of tumor necrosis factor alpha (TNFα)-driven activation of the transcription factor nuclear factor κB (NF-κB) on overexpression of *gpx1* and this effect could be attributed to an impaired degradation of inhibitor of NF-κB (IκB-α), which depends on the state of its phosphorylation. Evidently, GPx1 here competed with a phosphatase for $H_2O_2$ or another hydroperoxide (see section ''GPx4''), a phenomenon now considered the major role of GPx1 in regulating signaling cascades. A simplified scheme of TNFα-triggered NF-κB activation

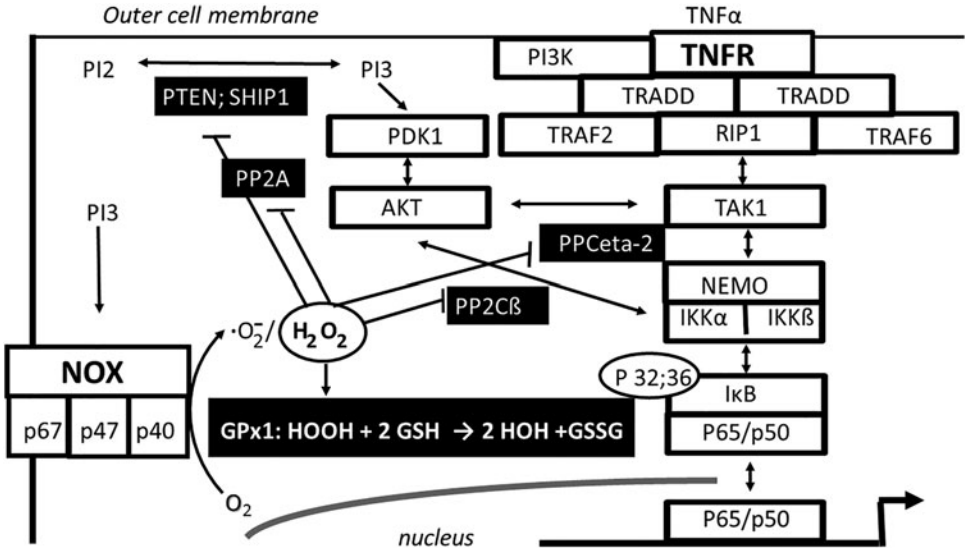



**FIG. 3. Interplay between canonical TNFα signaling, NOX-dependent $H_2O_2$ production, and GPx1**. On binding of TNFα to its receptor and auto-phosphorylation, PIK3 is recruited to the receptor complex and activated. The product of PIK3 (P3) activates NOX1 (by enabling the association of the phox subunits p40, p47, and p67 phos to the central flavoprotein) and recruits PDK1 and AKT (49, 98). The latter phosphorylates TAK1 (80), which, in turn, activates IKK$\beta$ at the IKK-NEMO complex (41, 93, 192). Next, the inhibitor of NF-$\kappa$B, I$\kappa$B, is phosphorylated at the positions 32 and 36, which primes it for degradation. Finally, NF-$\kappa$B (P65 and P50) moves to the nucleus to trigger gene expression. The phosphorylation of PI3 is counteracted by PTEN and SHIP1, that of AKT by PP2A, and that of IKK$\beta$ by PP2C$\beta$ and PPCeta-2, respectively. *Double-headed arrows* stand for phosphorylation (names of kinases in *empty fields*) counteracted by phosphatases (*black fields* with *white letters*). Each of the phosphatases listed is susceptible to oxidative inhibition by $H_2O_2$, which here is provided by NOX1. GPx1 protects phosphatases from inactivation and, thus, dampens activation by protein kinases. AKT, protein kinase B (serine/threonine protein kinase); I$\kappa$B, inhibitor of NF-$\kappa$B; IKK($\beta$), I-kappa-B-kinase (b)eta; NEMO, NF-kappa-B essential modulator; NOX1, NADPH oxidase type 1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PIK3, phosphoinositol-3-kinase; PP2A, heterotrimeric protein phosphatase; PP2C$\beta$, protein phosphatase type 2C-beta; PP2Ceta-2, protein phosphatase type 2C (isoform eta-2); PTEN, phosphatase and tensin homolog; SHIP1, phosphatidylinositol phosphatease; TAK1, TGF-$\beta$-activated kinase 1; TNFα, tumor necrosis factor alpha (transcription factor).



(there are many different ones dependent on cell type and triggering compound!) is shown in Figure 3.

The critical features are a co-activation of an NADPH oxidase (here NOX1), an activating phosphorylation cascade, and the possibility to counteract each phosphorylation step by distinct phosphatases that are overwhelmingly inactivated under oxidative conditions. These characteristics build the basis of the hyper-phosphorylation, which is observed whenever the metabolism of hydroperoxides is impaired. An activation of protein kinases due to oxidative modification is also discussed (17), but it appears to be less common.

The scheme outlined for TNFα-driven NF-$\kappa$B activation appears, in essence, to be relevant to many phosphorylation cascades, even if triggered by different stimuli or activating different genes. It applies to interleukin-1- or pathogen-associated molecular patterns-dependent NF-$\kappa$B activation and inflammation (71), growth factor-dependent signaling, caspase activation in $H_2O_2$-mediated apoptosis, apoptosis due to apoptosis-inducing factor (AIF)-transmigration or apoptosis signaling kinase (ASK)1 activation, and insulin signaling (165). In sporadic cases, $H_2O_2$ was identified as the target of GPx1, as the latter could be substituted by overexpression of the more specific catalase, as, for example, in epidermal growth factor-driven increased phosphorylation of Akt in *gpx1*$^{-/-}$ mice (72). In general, however, every substrate of GPx1 has to be considered a potential inactivator of phosphatases.

In this context, it appears worth mentioning that a direct oxidative inactivation of phosphatases by $H_2O_2$ or other hydroperoxides has been questioned in view of the rather low rate constants of such reactions (17). Enzymatic support in the oxidative modification of signaling proteins by *S*-transferases (173) or peroxiredoxins (161, 166) is, therefore, gaining interest. Phosphatases that can be inhibited by oxidation of their redox-sensitive cysteine are numerous. Most often implicated in the context of GPx1-dependent regulation are PTP1B, which, for example, counteracts insulin receptor phosphorylation, heterotrimeric protein phosphatase, which inhibits Akt signaling, phosphatase and tensin homolog (PTEN), and SHIP-1, which reverse the phosphorylation of phosphatidylinositol (3,4)-bisphosphate by phosphoinositol-3-kinase, and protein phosphatase type 2C-beta and protein phosphatase type 2C (isoform eta-2), which reverse IKK phosphorylation (21, 71, 116).

Phosphatases, however, are not the only enzymes that are regulated by GPxs. As early as 1971, Lands *et al.* (109) observed that cyclooxygenase, the key enzyme of prostaglandin biosynthesis, could be completely inhibited by addition of GSH and GPx1 and reactivated by an organic hydroperoxide. The authors postulated that cyclooxygenase is under the control of the ''peroxide tone.'' The concept was later expanded to other lipoxygenases, to 5-lipoxygenase, the key enzyme of leukotriene biosynthesis (78), to 12-lipoxygenase of platelets (83), and (with GPx4) to the 12,15-lipoxygenase (154).

This inhibition of lipoxygenases, however, appears not to be the primary domain of GPx1. GPx4 (see section ''cGPx4'') and possibly GPx3 (see section ''GPx3'') appear to be more important physiologically, yet any other hydroperoxide-metabolizing enzyme could substitute for GPx1 (126).

The oxidation of thioredoxin (Trx) is implicated in GPx1-regulated apoptosis. In its reduced state, Trx binds to an aptosis signaling kinase (ASK1) upstream of the stress-activated kinase c-Jun N-terminal kinase (JNK). Oxidized Trx dissociates and thereby activates ASK1, which leads to apoptosis *via* JNK activation (162).

Similarly, nucleoredoxin (Nrx) has been implicated in signaling. In the Wnt pathway, reduced Nrx binds and inactivates ''dishevelled'' (Dvl). Oxidized Nrx dissociates from Dvl and binds to the $\beta$-catenin degradation complex. $\beta$-Catenin finally moves to the nucleus and induces a proliferative response (64). By an analogous mechanism, Nrx redox-regulates toll-like receptor-mediated signaling *via* redox-dependent association with myeloid differentiation primary response 88 (79). Again, it may be doubted that increased $H_2O_2$ levels are sufficient to account for enforced Trx or Nrx oxidation in GPx1 deficiency. Without any doubt, the redoxins with their CxxC motif are redox-sensitive proteins, yet they are typically oxidized by peroxiredoxins.

It appears difficult to discriminate between prevention of oxidative damage and regulatory phenomena due to altered GPx1 levels, because its substrates are both toxic compounds and messengers. This Janus-faced nature of hydroperoxides often leads to unexpected findings, when their metabolism is altered.

- Epidemiological studies had suggested an increased cancer risk in selenium deficiency (158), which likely reflects GPx1 deficiency. The mutagenic potential of hydroperoxides made this hypothesis highly attractive, yet controlled clinical studies remained largely ambiguous (35), and in squamous cell carcinoma of the larynx *gpx1* expression positively correlated with increased metastases and an overall poor prognosis (200). Possibly, the anti-apoptotic role of GPx1 or the activation of pro-survival signaling cascades overrules its anticarcinogenic potential in some types of cells.
- Pancreatic islets contain a variety of $H_2O_2$-metabolizing enzymes, be they selenium dependent or not, and $H_2O_2$ is required for the glucose-induced insulin secretion. Nevertheless, GPx1 appears pivotal in protecting the islets against oxidative damage. In $gpx^{-/-}$ mice, a mild pancreatitis was observed; the $\beta$ cell mass was diminished; and a slight impairment of glucose-induced insulin secretion and a decrease of the fasting insulin level were detected. GPx1 may, therefore, be rated as antidiabetogenic [extensively reviewed in refs. (165, 204)]. On the other hand, overexpression of GPx1 (122) yielded obese mice with hyperinsulinemia, hyperglycemia, and insulin resistance, that is, typical signs of type II diabetes. Thus, an optimal antioxidant environment in the pancreas guarantees normal insulin production, whereas enhanced $H_2O_2$ removal by overexpressed GPx1 alters insulin signaling by inhibiting PTEN and/or other phosphatases (165, 204).
- Numerous animal studies with $gpx1^{-/-}$ mice suggest an important role of the enzyme in preventing endothelial and cardiac dysfunction (116), and observational clinical studies pointed to beneficial effects of an adequate selenium status. Certainly, the eradication of Keshan disease, which is a cardiomyopathy, by selenium supplementation in China is a highlight of translational selenium research. Nevertheless, prospective controlled studies in cardiovascular diseases remained ambiguous (21, 71).

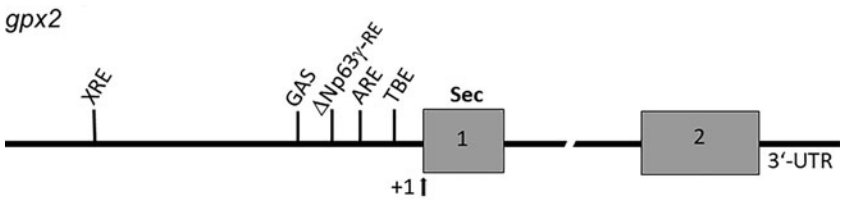
The discrepancies between experimental and clinical studies can often be attributed to inadequate dosing. In most of the cancer prevention and cardiovascular studies, selenium was administered to patients with a normal selenium status and at dosages beyond the requirement of 55 $\mu$g/day (for United States of America). Selenium, however, is not a drug whose efficiency increases with dosage according to mass law. It is a part of selenoproteins and, thus, optimizes their expression only up to a fixed concentration. Everything beyond this threshold causes oxidative trouble, likely by autoxidation of selenols with formation of superoxide radicals (26, 163). It should be mentioned that even clinical trials in cardiovascular and other diseases comply with the often-reported U-shaped response to selenium (146, 147). Although observational studies revealed a 24% reduction in cardiac disease by selenium supplementation (58), serum selenium levels beyond 135 ng/mL positively correlated with all kinds of cardiovascular risk factors (13).

### *GPx2*

In 1993, Chu *et al*. detected GPx2 as a GPx restricted to the gastro-intestinal tract and, therefore, called it ''GSHPx-GI'' (31). By now, the enzyme has been seen in many endothelial cells, in particular in malignant tissues and pluripotent stem cells (102, 103). In the hierarchy of selenoprotein biosynthesis, it adopts a position opposite to that of GPx1: Its expression only responds to severe selenium deficiency and its messenger RNA is increased under selenium restriction (188).

The GPx2 gene is located on chromosome 14 and contains one intron (99). It harbors binding sites for nuclear factor erythroid 2-related factor 2 (transcription factor) (Nrf2) (6), $\beta$-catenin (101), transformation-related protein 63, isoform delta N gamma (transcription factor) ($\Delta$Np63$\gamma$) (193), xenobiotics (70), STAT family members, an interferon-$\gamma$-activated site (putative) [GAS, a binding site for STATs (84)] (Fig. 4), and a putative binding site for homeobox protein Nkx-3.1 (transcription factor) (Nkx-3.1) (134).

Upregulation by the Nrf2/Kelch-like ECH-associated protein, inhibitor of Nrf2 (Keap1) system classifies GPx2 as an antioxidant and anti-inflammatory enzyme. It also explains the induction of GPx2 by glucosinolates, which activate the Nrf2/Keap1 system. In this respect, the Nrf2/Keap1/antioxidant responsive element (ARE) pathway interferes with the aryl hydrocarbon receptor/xenobiotic response element pathway in an antagonistic way, which may explain the differential response to glucosinolates of different Brassicaceae (115). An anti-inflammatory role of GPx2 may also be deduced from the interaction of its gene with the STAT/GAS couple, which is typically activated by anti-inflammatory cytokines such as interleukin-22. Upregulation by Nkx-3.1, commonly considered an antioncogene, also favors an anticarcinogenic function. Other binding sites, in particular those for $\Delta$Np63$\gamma$ and $\beta$-catenin, might suggest peculiar roles of GPx2 in different scenarios such as maintenance of




| | |
|---|---|
| TBE | 121-ctCAAAG-115 |
| ARE | 141-GCTAAGTCA-133 |
| ΔNp63γ-RE | 153-′ggaCCTGtttt-(ttgctaag)-catCCTGggg-125 |
| GAS | 298-ttcctgtaa-289 |
| XRE | 1036-gcACGCgt-1029 present as 5′-agGCGTgc-3′ in the reverse direction |

**FIG. 4. Human GPx2 promoter with validated transcription factor sites.** The promoter contains a p63-binding site that specifically binds p63$\gamma$ and $\Delta$Np63$\gamma$ (193). Further upstream is a $\beta$-catenin/T cell factor binding element (101), an ARE binding Nrf2 (6), a GAS responding to STAT3 and STAT5 (84), and an XRE (70). Three NKx3.1 sites (not shown) (8) are still putative as they have not yet been investigated for activity in humans, but an NKx3.1 site proved to be active in the mouse (134). $\Delta$Np63$\gamma$, transformation-related protein 63, isoform delta N gamma (transcription factor); ARE, antioxidant responsive element; GAS, interferone-$\gamma$-activated site (putative); Nkx-3.1, homeobox protein Nkx-3.1 (transcription factor); Nrf2, nuclear factor erythroid 2-related factor 2 (transcription factor); STAT, family of transcription factors (''transducer and activator of transcription''); XRE, xenobiotic response element.



pluripotent stem cells, embryogenesis, differentiation, proliferation, and carcinogenesis (139, 193). However, upregulated GPx2, by its intrinsic antioxidant function, might equally just balance an oxidant challenge invoked by proliferative stimuli (193). The expression of *gpx2* was also reported to be upregulated by all-trans retinoic acid in the human breast cancer cell line MCF-7 (33) and human liver cells (125).

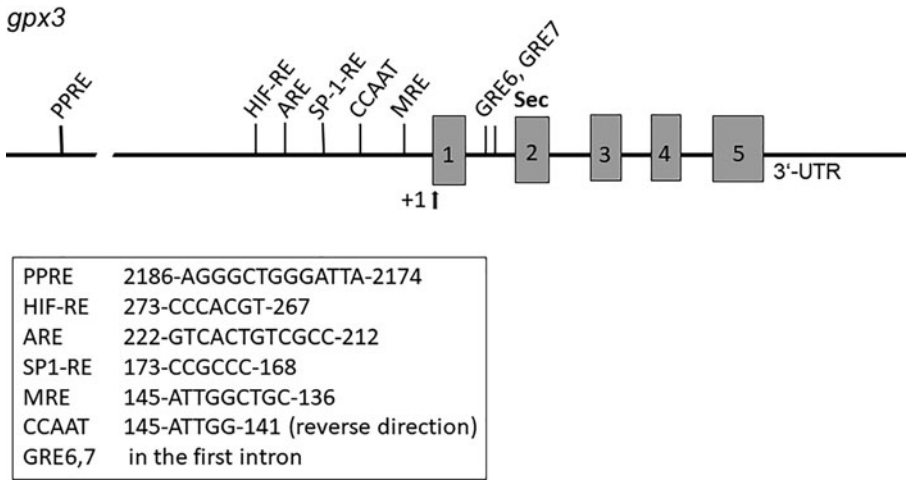
A high transcription of *gpx2* is seen in the brain, kidney, skin, inner ear, nasal conchae, gastrointestinal tract, liver, urinary bladder, and lung of embryos (5). Differential transcription of *gpx2* points to a critical role of the enzyme in nephrogenesis (82). During colon development, *gpx2* transcription parallels the development from an undifferentiated endoderm to a single-layered epithelium. Finally, GPx2 is considered pivotal for self-renewal, pluripotency, and survival of stem cells (102). Despite these persuasive hints for an important role of GPx2 in embryogenesis, $gpx2^{-/-}$ mice developed normally, did not display any obvious abnormalities, and had normal life expectancy (50). In fact, the only pathological finding observed in $gpx2^{-/-}$ mice under unstressed conditions was an increase of apoptotic cells in the crypts of the colon (59). However, the lungs of GPx2-deficient mice are more sensitive to inflammatory stimuli such as hyperoxia and antigen challenge (42). In this respect, the pathological findings largely mimic those seen in $nrf2^{-/-}$ mice (29, 106).

The prominent pathology of $gpx2^{-/-}$ mice is observed in the gastro-intestinal tract. A double knock-out (KO) of GPx2 and GPx1 causes ileo-colitis (50) and, months later, adenomas and malignant cancers (32). These findings reveal a co-operation of both GPxs in the prevention of inflammation and carcinogenesis of the gut. GPx2, however, dominates here, since the gut pathology is prevented by one allele of *gpx2*, but not of *gpx1* (50). Accordingly, the bowel pathology seen in $gpx2^{-/-}$/$gpx1^{-/-}$-double-KO mice can only be partially reproduced by a single KO of *gpx2* plus GPx1 deficiency induced by selenium restriction. Inflammatory scores after application of azoxymethane/dextran sodium sulfate (AOM/DSS; a model of inflammation-mediated colon carcinogenesis) were generally higher in $gpx2^{-/-}$ than in wild type (WT) mice and tended to become normalized with increasing selenium concentration (106) and associated increased GPx1 activity (127). However, carcinogenesis was not prevented by selenium (106).

Comparing the outcome of the AOM/DSS model and AOM application discloses that the anticarcinogenic effect of GPx2 is primarily due to its anti-inflammatory efficacy. Administration of AOM alone, which just alkylates DNA, does not induce more tumors, but it rather inhibits the AOM-induced carcinogenesis in $gpx2^{-/-}$ mice (127). This surprising observation has been attributed to inhibition of apoptosis, which allows cells with AOM-induced lesions to develop into malignant tumors (102).

GPx2 appears to be particularly efficient in preventing apoptosis. In the colon of $gpx2^{-/-}$ mice, *gpx1* is dramatically overexpressed at both the transcriptional and translational level (59). In fact, the total GPx activity in the gut of $gpx2^{-/-}$mice is significantly higher than in that of WT mice (127). In the colon of $gpx2^{-/-}$ mice, GPx1 even adopts the unusual tissue distribution of GPx2 (59). [GPx2 is high at the crypt ground and declines toward the luminal surface, a phenomenon that has been implicated in the steady renewal of the intestinal epithelium by protecting the proliferative crypt ground from oxidative challenge and allowing apoptotic shedding of cells at the luminal site (21, 102)]. Despite its overproduction, GPx1 cannot fully substitute for a loss of GPx2 (59). The mechanism of the compensatory overproduction of GPx1 in $gpx2^{-/-}$ mice is enigmatic, and the unique role of GPx2 in preventing ectopic apoptosis in the intestine remains equally unexplained.

GPx2 confronts us with some more carcinogenesis-related phenomena. The enzyme shares with other thiol peroxidases the ability to inhibit lipoxygenases, including cyclooxygenases (126). GPx2, however, appears also to regulate the biosynthesis of cyclooxygenase-2 and m-prostaglandin-E-synthase-1




| | |
|---|---|
| PPRE | 2186-AGGGCTGGGATTA-2174 |
| HIF-RE | 273-CCCACGT-267 |
| ARE | 222-GTCACTGTCGCC-212 |
| SP1-RE | 173-CCGCCC-168 |
| MRE | 145-ATTGGCTGC-136 |
| CCAAT | 145-ATTGG-141 (reverse direction) |
| GRE6,7 | in the first intron |

**FIG. 5. Human GPx3 gene with validated transcription factor binding sites.** Sequences and positions of binding sites, as far as available, are given in the *insert*. For further explanations and references see text.



and thereby the formation of the pro-inflammatory prostaglandin $E_2$ ($PGE_2$) (7). Knockdown of *gpx2* expression revealed that $PGE_2$-dependent migration and invasiveness of HT-29 adenocarcinoma cells are inhibited by GPx2, thus adding further anticarcinogenic functions to GPx2 (9). In the very same publication, however, the authors also describe that GPx2 facilitates anchorage-independent growth in soft agar and stimulates the growth of tumor xenographs in nude mice (9).

These seemingly contradictory results led to the hypothesis that the role of GPx2 depends on the stage of carcinogenesis (19, 21, 103). In short, in respect to carcinogenesis, GPx2 is Janus faced, a behavior that the enzyme shares with GPx1 (see above) and some other selenoproteins (24, 179).

### *GPx3*

The human GPx3 gene is located on chromosome 5, precisely at 5q33.1 (30). Its reading frame is composed of five exons. In 2004, Bierl *et al.* (11) found transcription factor binding sites and validated them by reporter gene constructs in Caki cells. Accordingly, *gpx3* contain hypoxia-responsive element, ARE, SP-1, metal-responsive element, and a CCAAT box in inverse direction (11) and a PPAR-responsive element, which binds peroxisome proliferator activated receptor (gamma) and is implicated in prevention of insulin restistance (34). An *et al.* (3) added 10 glucocorticoid response elements, of which the 2 in the intron near the regulatory region were validated by reporter gene assays (Fig. 5). Similar to *gpx2*, *gpx3* is also considered a retinoid target gene (47). Moreover, GPx3 expression is silenced by hypermethylation of CpG islets (27, 199). Its position in the hierarchy corresponds to that of GPx1.

GPx3 is an extracellular glycoprotein with specificities similar to those of GPx1. Apart from GSH, it accepts Trx and glutaredoxin (Grx) (12). However, GSH, Trx, and Grx are present in the extracellular space only in the low micromolar range and overwhelmingly as oxidized molecules. For the extracellular space, any systems regenerating the reduced substrates are unknown. GPx3 may, therefore, be rated as an antioxidant device that, although efficient, has to work with an extremely limited capacity. To rate the extracellular GPx system as the primary defense line against oxidative stress sounds naive. Possible exceptions are the epididymis and the thyroid follicles. Yet also in the cauda epididymis, the GSH content is as low as 16 $\mu M$ (157), and KO of GPx3 did not reveal any morphological or functional alteration of the thyroid (155).

GPx3 was found to be associated with basement membranes in kidneys (132) and elsewhere (23). Supply of secreted GSH is believed to be improved in this location. High concentrations of GPx3 prevail in white and brown adipose tissue, where GPx3 has been implicated in adipocyte differentiation, suppression of inflammation, and insulin-regulated lipid metabolism (105, 112).Nevertheless, $gpx3^{-/-}$ mice are largely asymptomatic (132). The so far only pathological symptom reported for $gpx3^{-/-}$ mice is increased platelet aggregation (96), which complies with the clinical observation that GPx3 deficiency might be a risk factor for stroke or embolism (189). Possibly, the limited capacity of the GSH/GPx3 system is high enough to silence cyclooxygenase-mediated formation of thromboxane $A_2$ in platelets (126, 132).

More recently, GPx3 has been claimed to act as a tumor suppressor, as it *inter alia* inhibits proliferation and invasiveness of hepatocarcinoma and lung cancer cells (3, 144). Mechanistically, GPx3 protected MAP (mitogen-activated protein) kinase phosphatase, an extracellular-signal regulated kinase (ERK)-specific phosphatase, against oxidative degradation. Thereby, GPx3 inhibits the ERK-NF-$\kappa$B pathway (2). This hypothesis is just another example of the prevention of oxidative phosphatase inactivation outlined earlier under the section ''GPx1.'' It may appear irritating that an enzyme that is designed for the extracellular space should have specialized for the regulation of an intracellular phosphorylation cascade. However, loss of GPx3, mostly due to hypermethylation, has, indeed, been observed in many tumors, for example, of prostate (199), stomach (136, 201), head and neck (27), esophagus (81), thyroid (202), skin (28), and bone marrow (205). Loss of GPx3 was throughout associated with poor prognosis. Intriguingly, methylation of the GPx3 promoter region increased the sensitivity to platinum drugs in colorectal cancer (135), as did GPx3 knockdown by RNA interference in adenoma cell lines (150). However, the inverse relationship between GPx3 expression and platinum sensitivity was not consistently observed (27).

### *GPx4*

The human gene of GPx4 is located on chromosome 19, precisely at band 19p13.3 (100). Deletion of the entire *gpx4*

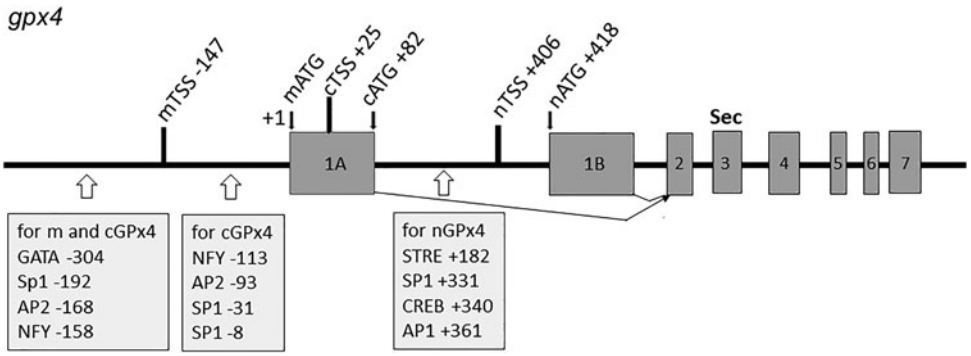



**FIG. 6. Gene structure of mouse GPx4.** The first exon exists in three different forms depending on the expression form. For cGPx4, the first exon consists just of the translation start ATG (''cATG''). mGPx is encoded by the alternative exon 1A, and nGPx is encoded by 1B. The three distinct clusters of binding sites regulating the different expression forms are given in the *inserts*. Position numbers are those reported by Imai *et al.* (90). mTSS, cTSS, and nTSS are the transcription starts for mGPx, cGPx, and nGPx, respectively. mATG, cATG, and nATG designate the corresponding translation starts. For further explanations and references see section ''GPx4.'' ATG, translation start codon; cGPx4, cytosolic expression form of GPx4; mGPx4, mitochondrial expression form of GPx4; nGPx4, nuclear expression form of GPx4; TSS, transcription start.



proved to be embryonically lethal (88, 197).The mammalian GPx4 genes contain seven exons and are expressed in three different forms, each having their distinct transcription and translation starts. The translation converges at the second exon and from there onward (exon2–7) the sequences of all GPx4 forms become identical (138) (Fig. 6).

The mitochondrial expression form of GPx4 (mGPx4) uses the most upstream translation start codon (ATG) codon (+1; mATG). The exon 1A codes for a mitochondrial import sequence (142). After processing this sequence, the protein is identical with cytosolic expression form of GPx4 (cGPx4) (4). Translation of cGPx4 starts at +82 with an exon consisting just of one ATG. The use of an ATG at +418, that is, an ATG located in the first intron of *mgpx4* and *cgpx4* expression, creates the alternate exon 1B, which yields the nuclear expression form of GPx4 (nGPx4). It is characterized by a nuclear import sequence followed by a stretch of residues that differ from those of the other two expression forms.

A systematic analysis of transcription factor binding sites, considering all three expression forms, was performed by Imai *et al.* (90). The binding sites were validated in mouse fibroblasts, yet they were similarly detected in the pig and human genes, which implies that the analysis is likely valid for mammals in general.

The most upstream cluster comprises a GATA box and sites for SP1, AP2, and NFY. It regulates the expression of both the mitochondrial and the cytosolic form. In the human gene, the NFY site is followed by a sequence that combines a CCAAT/ enhancer binding protein (epsilon or alpha; transcription factor) (C/EBP) site with a cAMP-responsive element binding protein (transcription factor) (CREB) (86, 164). According to Hattori *et al.* (77), this region (−158 to −141 counted from the translation start of mGPx4) preferentially binds C/EBPε after stimulation of HL60 cells with TNFα, whereas Speckmann *et al.* (164) suggested preferential binding C/EBPα in differentiated Caco-2 cells. The second cluster, after the transcription start for the mitochondrial form, contains an NFY, an AP2, and two SP1 sites and only regulates the expression of the cytosolic form. The most downstream cluster is only relevant to the expression of *ngpx4*. It contains an stress-responsive element, sites for SP1 and AP1, and CREB, the latter one possibly related to the one that binds cAMP-responsive element modulator tau (transcription factor) in the rat (178). Otherwise, the investigation of Imai *et al.* (90) confirmed earlier reports (86) and critically discussed that by Ufer *et al.* (180).

Overexpression of *gpx4* suppressed cell death due to oxidative damage (91) and leukotriene formation (89) in rat basophilic leukemia cells. GPx4 is consistently upregulated by pro-inflammatory cytokines, for example, by TNFα in HL60 cells (77) or polymorphonuclear leukocytes (76) and by interleukin-1-β, docosahexaenoic acid, and low dosages of TNFα in endothelial cells of human umbilical veins (160). Interleukin1-driven NF-κB activation was completely abrogated by a mild overexpression of GPx4, whereas large variations of GPx1 only moderately affected this pathway (18).

Collectively, the data comply with an antioxidant and anti-inflammatory role of GPx4, which is similar to that of GPx1 with an important difference: In contrast to all other GPxs, GPx4 can also reduce peroxidized complex lipids that are firmly integrated in biomembranes. As recently proposed, the enzyme glides over the lipid bilayer and binds with a cluster of basic residues near the reaction center to the negatively charged polar heads of phospholipids in a way that preferred positions of hydroperoxy groups can be attacked by the enzyme's active site selenium (38).

At the translational level, GPx4 depends, of course, on selenium. Its rank in the hierarchy of selenoprotein biosynthesis is, however, much higher than that of GPx1 (187) and GPx3, but lower than that of GPx2 (188).

mGPx4. Similar to *gpx1*, *gpx4* is also expressed in a sex-specific manner. Estrogens downregulate GPx4 in the male reproductive tract (128), but they upregulate it in the oviduct (110). As early as 1992, Roveri *et al.* (149) detected a massive expression of GPx4 in rat testis, which is restricted to the post-puberal phase, and it is prevented by hypophysectomy and restored in the hypophysectomized animals by gonadotropin. In fact, the GPx4 concentration in testes of sexually mature mammals is the highest ever seen. The gonadotropin dependence was later shown to be indirect. Gonadotropin, *via* testosterone, increases the level of round spermatids, which

produce high amounts of GPx4 (121). Gene disruption technologies that selectively destroy the individual expression forms of *gpx4* revealed that it is the mitochondrial form that is indispensable for male fertility (152).

Its role in spermiogenesis has been partially clarified. Late in spermiogenesis, the cells' GSH becomes oxidized; oxidized glutathione (GSSG) is excreted and degraded by γ-glutamyl transpeptidase and a cysteinyl-glycine dipeptidase in the epididymis (157). The nature and source of the oxidant, as well as the mechanism of the unusually high mGPx4 formation in spermatids, is unknown (157). Deprived of its preferred co-substrate GSH, mGPx4 uses a cysteine residue of itself as an alternate substrate, whereby linear polymers are built *via* selena-disulfide bonds. These polymers, together with cross-linking cysteine-rich proteins, form the keratin-like material, the mitochondrial sheath, which fixes the helix of mitochondria in the mid piece of spermatozoa. In fact, more than 50% of the sheath material proved to be GPx4 by mass spectroscopy (181).

During passage through the epididymis, mGPx4, thus, changes from an active peroxidase to an enzymatically inactive structural protein (181). Candidates for cross-linking proteins are the outer dense fiber protein, the ''sperm mitochondria-associated cysteine-rich protein'' (SMCP, formerly erroneously called mitochondrial selenoprotein for ''mitochondrial capsule seleno-protein'') (181), and other keratin-like proteins with vicinal SH groups (117). The sheath material may be considered to represent a semi-reduced enzyme-like dead-end product resulting from using alternate substrates (Fig. 1). The observation is a striking example of how a thiol peroxidase forms polymers *via* selenylation of itself or other proteins and thereby initiates a differentiation process. It may also be stressed that this phenomenon is the opposite of an antioxidant function; mGPx4 uses peroxides to create a structure that is indispensable for sperm stability and motility and, thus, for fertilization.

Although ''the moonlighting of mGPx4'' is still a field with many unknowns, it explains the long-known role of selenium in male fertility (123, 169, 190). This process is evidently restricted to mammals (143), and here it appears to constitute the only essential function of mGPx4; *mgpx4*$^{-/-}$ mice do not display any pathology apart from infertility in the male gender (152).

nGPx4. The nuclear expression form of GPx4 (nGPx4) was originally detected in sperm (138) and, therefore, called sperm nuclei GPx4. Initially, this protein had been discussed to explain male infertility observed in selenium deficiency (138), which is now unequivocally attributed to mGPx4 (see above). Later, *ngpx4* transcripts could be identified in various tissues (119). The discovery of a nuclear expression form of GPx4 also raised the hope to finally explain the essentiality of this enzyme, which had remained unique in the GPx family. However, selective KO of *ngpx4* yielded mice that were vital and fertile. The only pathological finding in these mice was a delayed chromatin compaction in epididymal sperm (36), which complies with the observation of the Godeas group that sperm protamines are alternate substrates of GPx4 (68, 69).

Although the originally suspected impact of nGPx4 on fertility and viability could not be substantiated, it may still be speculated that this expression form contributes to the genetic stability of maturating sperm or nuclear DNA in general.

cGPx4. Surprisingly, the cytosolic expression form of GPx4 proved to be of vital importance (113). Oxidant-driven cell death had been repeatedly described to be inhibited by GPx4 overexpression and, in analogy to the capability of other GPxs, interpreted as suppression of apoptosis (15, 91); however, early studies paid little attention to the precise type of programmed cell death. Seiler *et al.* (156) proposed a special pathway that took into account the particular substrate specificity of GPx4. Signs of classical apoptosis such as caspase-3 activation and phosphatidylserine exposure were not seen in *gpx4*$^{-/-}$ mice. Instead, the authors showed the involvement of 12,15-lipoxygenase products by pharmacological intervention studies (but not of other lipoxygenases!) and of AIF in cell death due to *gpx4* KO. However, Brütsch *et al.* (22) could not salvage the lethal phenotype of mice bearing an inactive GPx4 by knocking out the 15-lipoxygenase gene.

A novel way of interference of GPx4 was detected in the course of a screening project for anticancer drugs (196). One of the compounds (erastin) inhibited the glutamate/cystine antiporter, which leads to a deprivation of intracellular cysteine and, thus, to loss of GSH. A second one (inhibitor of GPx4 [RSL3]) proved to inhibit GPx4 *in situ* (195), a phenomenon that has recently been shown to require the participation of the redox-regulated adaptor protein 14-3-3ε (185). The cell death caused by these compounds was also not associated with the typical pattern of apoptosis, but was accompanied by massive lipid peroxidation; was inhibited by lipophilic antioxidants, inhibitors of iron uptake and iron chelators (43, 196), therefore named ferroptosis (43); and in 2015 classified as an ''iron-dependent form of regulated cell death under the control of glutathione peroxidase 4'' (65).

Over the past few years, reviews on this topic (62, 63, 66, 92, 118, 167, 168, 191, 195) flooded the scientific scene, without, however, clarifying the concept. Some critical questions are listed in Gao and Jiang (66) and Maiorino *et al.* (118). Here, we will only address a few of them.

- It remains undefined which of the six lipoxygenases (in humans) dominates in ferroptosis. 15-Lipoxygenase, which is commonly considered to build the preferred substrate of GPx4, was knocked out without reversing the lethal phenotype in mice bearing an inactive GPx4 (22). Knockdown of all six lipoxygenase genes, however, increased the sensitivity to ferroptotic cell death (194).
- It is neither clear to what extent nonenzymatic lipid peroxidation contributes to ferroptosis.
- The mice with all six lipoxygenases knocked down were sensitive to an inhibitor of the glutamate/cysteine antiporter, which lowered GSH. However, they did not respond to the GPx4 inhibitor RSL3, which left the GSH levels unchanged (194). This observation points to a GPx4-independent protective role of GSH, which, however, remains elusive. However, recently, a coenzyme Q-reducing flavoprotein, the ferroptosis suppressor protein 1 (FSP1, formerly AIFM2), has been shown to inhibit ferroptosis in a GSH-independent way (10, 44).
- α-Tocopherol, which consistently inhibits ferroptosis, is discussed as acting as an antioxidant or an inhibitor of lipoxygenases. The usually discussed role of tocopherols in the context of lipid peroxidation is the reduction of lipid superoxide radicals to lipid hydroperoxides, the preferred substrates of GPx4. Accordingly, α-tocopherol

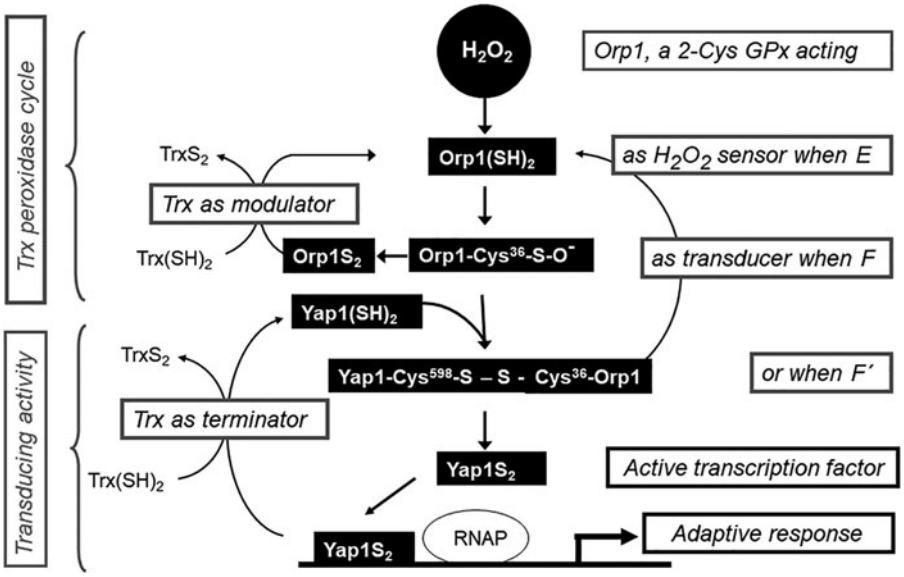



**FIG. 7. A glutathione peroxidase as sensor for $H_2O_2$ (40).** Orp1 (also called yeast GPx3) is a 2-Cys-GPx-type protein with Trx specificity. The *upper part* shows the conventional antioxidant catalysis. When reduced, Trx becomes limiting due to continued oxidant challenge; the oxidized peroxidatic Cys36 of Orp1 ($F$ = sulfenic form or $F'$ = disulfide form) reacts with the reduced (inactive) transcription factor Yap1 as an alternate substrate at Cys598, forming a mixed disulfide. Thiol/disulfide reshuffling then regenerates reduced Orp1 and the oxidized (active) transcription factor, which triggers an adaptive response. In this scheme, reduced Orp ($E$) is either the ground-state enzyme for the thioredoxin peroxidase cycle or the $H_2O_2$ sensor for the regulatory function, whereas oxidized Orp ($F$ or $F'$) is the transducer of the oxidant signal. Trx functions either as a co-substrate in the antioxidant cycle or as a terminator for the transcriptional activation. 2-Cys-GPx, 2-Cysteine glutathione peroxidase equipped with CP and CR; Orp1, 2-Cys-GPx of yeast (yeast GPx3); Trx, thioredoxin; Yap1, AP-1-like transcription factor of yeast.

should enhance ferroptosis this way. An alternative antioxidant function of tocopherols has been proposed based on density functional theory calculations: the reduction of alkoxy lipid radicals (1). This method, however, does not provide realistic rate constants. Available data suggest that the concentrations of α-tocopherol that inhibit lipoxygenases are higher than those inhibiting ferroptosis. In short, the role of α-tocopherol in ferroptosis is as unclear as its function in general (16).

Ferroptosis appears to result from a complex interplay between shortage of GSH, GPx4, and iron with the polyunsaturated lipids of biomembranes, which in detail is anything else but clear. Also, the therapeutic perspectives deserve a critical consideration. The envisaged treatment of special malignancies with GPx4 inhibitors (74, 75, 151, 153, 196) has to take into account the essentiality of this enzyme and the sensitivity of cells in the brain and kidney to ferroptotic stimuli (63, 167). The discussed inhibition of ferroptosis for prevention of neurodegenerative diseases (43) by increasing GPx4 levels has to face the relatively high ranking of the enzyme in the selenoprotein hierarchy and the nonavailability of specific inducers. However, the concept for the first time offers a plausible explanation for the essentiality of cGPx4.

### *Mammalian CysGPxs*

*GPx5* was detected as an androgen-regulated protein that is expressed preferentially in the caput epididymis and excreted into the epididymal lumen (67). It is a CysGPx and reportedly does not have any GPx activity, if measured with the conventional substrates, $H_2O_2$ and GSH (172). Alternate substrates have not yet been identified. It is, nevertheless, claimed to be an antioxidant enzyme and a marker of sperm quality (184). In cells (chinese hamster ovary, cell line) overexpressing GPx5, lipid peroxidation and DNA damage were reduced (172). *gpx5*$^{-/-}$ mice were fertile and their sperm appeared histologically normal, as long as the animals were young, but the rate of abortions and malformations in offsprings was increased. In *gpx5*$^{-/-}$ animals older than 1 year, DNA compaction in epididymal sperm was impaired and oxidative DNA damage was observed (25).

*GPx6 h*as been identified as cDNA that is only associated with genes encoding olfactory-specific biotransformation enzymes in the nasal mucosa (39). It is a glutathione peroxidase containing a catalytic selenocysteine (SecGPx) in man, but a CysGPx in rodents. Little else is known about this protein. More recently, *gpx6* has also been found to be expressed in the striatum and in the frontal cerebral cortex in an age-dependent manner, and its overexpression ameliorated motor defects and molecular parameters in a mouse model of Huntington's disease (159).

*GPx7* is worth mentioning, since it provides the first well-documented example of a complete change in donor substrate specificity. Its $k'_{+2}$ (Fig. 1) for GSH is just 12.6 $M^{-1}$ $s^{-1}$, which complies with nonenzymatic reactions of GSH with disulfides or sulfenic acids. Instead, the corresponding value for the SH groups of protein disulfide isomerase (PDI) is $>10^3$ $M^{-1}$ $s^{-1}$ (14). Thus, in functional terms, GPx7 is not a *GSH* peroxidase, but a *PDI* peroxidase. It is located in the lumen of the endoplasmic reticulum (ER), where it uses the $H_2O_2$ produced by Ero1α to oxidize PDI. Oxidized PDI then terminates the oxidative folding of secreted proteins (130,

145). GPx7 also reacts with SH groups of other proteins, for example, the glucose-regulated protein GRP78. Oxidation of GRP78, thus achieved, results in improved chaperone activity (186). Finally, *gpx7*$^{-/-}$mice suffer from multiple organ dysfunction, reduced life span, and increased cancer incidence (145). GPx7 is, therefore, considered an antioncogene.

*GPx8* is exceptional in having an altered catalytic center; the Gln in the tetrad is replaced by serine. The functional consequences of this fundamental change have not yet been investigated. GPx8, similar to GPx7, has marginal GPx activity (''<0.01% of the relative activity of bovine GPx''), is associated with the ER, interacts with Ero1$\alpha$, accepts PDI as substrate, and is implicated to be involved in oxidative protein folding (130). In contrast to GPx7, however, GPx8 is a transmembrane protein.

### GPxs as Hydroperoxide Sensors

Cytosolic GPx4, when reacting with lipid hydroperoxides to prevent programmed cell death (156), and GPx7, when oxidizing GRP78 (186), have been called peroxide sensors. In principle, all peroxidase functions beyond the detoxification of peroxides may be addressed as peroxide sensing. Originally, however, sensing of $H_2O_2$ or lipid hydroperoxides had a different meaning. It was to describe the induction of an adaptive response to increased oxidative challenge *via* activating oxidation of a transcription factor (175).

The prototype of an $H_2O_2$ sensor is the bacterial OxyR (203), which combines sensor and transcription factor activity in one molecule. As soon as a particular Cys residue (Cys199 in *Escherichia coli* OxyR) is oxidized, OxyR binds to DNA, where it regulates the expression of >30 proteins, which overwhelmingly protect against the oxidative challenge (45).

In eukaryotes, the SH groups of transcription factors are usually not reactive enough to interact directly with a hydroperoxide; they require the help of a thiol peroxidase that senses $H_2O_2$ and then transfers the oxidation equivalents *via* disulfide reshuffling to the transcription factor. The principle is becoming widely accepted for peroxiredoxins. It has been confirmed for yeast (*Saccharomyces pombe*), where a typical 2-Cys-peroxiredoxin reacts with, that is, senses, $H_2O_2$, and then oxidizes, thereby activating the transcription factor AP-1-like transcription factor of yeast (Yap1) (183). It also works in mammals, where the oxidized peroxiredoxin-2 activates the transcription factor STAT 3 (161). It is discussed for plants (114) and, more recently Stöcker *et al.* (166) provided compelling evidence that $H_2O_2$ sensing by peroxiredoxins (for whatever purposes) might be more common than hitherto anticipated; surprisingly, deficiency of peroxiredoxin-1 and 2 in human cells dramatically decreased the overall protein thiol oxidation.

However, despite the fashionable focus on peroxiredoxins in this context, it should not be forgotten that the very first observation of $H_2O_2$ sensing in eukaryotes was made with a GPx-type protein: 2-Cys-GPx of yeast (yeast GPx3) (Orp1) (40). Its basic function and sensing mechanism are recalled in Figure 7. Orp1 is a 2-Cys- glutathione peroxidase equipped with CP and CR of yeast (*S. cerevisiae*) with Trx specificity and, as Trx peroxidase, it fulfills its usual antioxidant function. However, the enzyme reacts with an alternate substrate, when reduced Trx becomes limiting due to an excessive peroxide challenge. It, thus, forms a heterodimer with the transcription factor Yap1 (linked by a disulfide bridge between Cys36 of Orp1 and Cys598 of Yap1). Cys303 of Yap1 then attacks the disulfide bridge that links the heterodimer, whereby the transcription factor is fully oxidized and reduced Orp1 is regenerated. The now activated Yap1 can initiate the adaptive response, which consists of the upregulation of at least 32 genes comprising *inter alia* those of the Trx and GSH systems, the pentose phosphate pathway, peroxiredoxin, cytochrome *c* peroxidase, and catalase (111). Once the Trx levels are recovered, the adaptive response is terminated by reduced Trx.

Hydroperoxide sensing for control of adaptive response has not yet been reported for any of the mammalian SecGPxs. But the chemistry of the alternate substrate reactions, for example, of the reaction of GPx7 with PDI or GRP78, the polymerization and crosslinking of mGPx4, and the chromatin compaction by nGPx4 in spermiogenesis, is analogous. Therefore, no theoretical reason argues against this kind of regulatory phenomenon to occur also with SecGPxs. In higher eukaryotes, however, adaptive (17) and hormetic (60) responses are usually achieved by the Keap1/Nrf2 system and Nrf2 activation is increased in moderate selenium deficiency (20), although a short-term feeding of a selenium-deficient diet may not be efficient enough to induce an Nrf2 response (198). These observations render a peroxide sensing role unlikely for GPxs that readily respond to the selenium status such as GPx1 or GPx3.

### Epilogue

Evolution is based on trial and error. Accordingly, useless remnants of evolution do exist, which makes a meaningful interpretation of biological findings risky. Moreover, despite the thousands of related publications, our knowledge remains fragmentary. In particular in complex organisms, generalizing deductions from regulatory element in genes in respect to function are difficult and only justified in exceptional cases. Their function is dictated by the level of transcription factors, pertinent regulatory cascades, and epigenetic modulation, which differ between cell types and vary with differentiation. In consequence, many burning questions are left unanswered.

However, we can safely state that nature has successfully experimented with the basic concept of GPx catalysis and thereby exploited almost every variation a thiol peroxidase can offer. It created an active site that reacts with hydroperoxides near limitation by diffusion and, thus, the most efficient antioxidant system. GPxs use this ability to regulate phosphorylation cascades *via* preventing oxidative inactivation of phosphatases or to silence lipid oxygenases. They can save hydroperoxides for signaling purposes *via* transient self-inactivation. Nature created specialists for the interaction with complex lipids with long chain fatty acids that decide on death or survival. It loosened the specificity for reducing thiols, thus enabling (co)polymerization reactions or multiple protein modifications *via* alternate substrate reactions. It finally modified the structures in a way to create a complete change of substrate specificity, which paved the way to unexpected functions such as oxidative protein folding.

From its beginnings, the research on GPxs surprised us with unexpected findings and one can with confidence look forward to future surprises.

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**Abbreviations Used**

ΔNp63γ = transformation-related protein 63, isoform delta N gamma (transcription factor)
2-Cys-GPx = 2-Cysteine glutathione peroxidase equipped with $C_P$ and $C_R$
AIF = apoptosis-inducing factor
AOM = azoxymethane
AP-1 = activator protein-1 (transcription factor)
AP-2 = activator protein-2 (transcription factor)
ARE = antioxidant responsive element
ASK1 = apoptosis signaling kinase
ATG = translation start codon
C/EBP(ε or α) = CCAAT/ enhancer binding protein (epsilon or alpha; transcription factor)
cGPx4 = cytosolic expression form of GPx4
$C_P$ = peroxidatic cysteine
CpG = DNA methylation site
CREB = cAMP-responsive element binding protein (transcription factor)
CxxC motif = cysteine-xx-cysteine motif characteristic for redoxins
DSS = dextran sodium sulfate
Dvl = dishevelled
*E* = ground state enzyme
ER = endoplasmic reticulum
ERK = extracellular-signal regulated kinase
*F* = oxidized enzyme
*G* = semi-reduced enzyme
GAS = interferon-γ-activated site (putative)
Gln = glutamine
GPxs = glutathione peroxidase(s)
GPx1 = mammalian glutathione peroxidase type 1
GPx2 = mammalian glutathione peroxidase type 2
GPx3 = mammalian glutathione peroxidase type 3
GPx4 = mammalian glutathione peroxidase type 4
GPx5 = mammalian glutathione peroxidase type 5
GPx6 = mammalian glutathione peroxidase type 6
GPx7 = mammalian glutathione peroxidase type 7
GPx8 = mammalian glutathione peroxidase type 8
Grx = glutaredoxin
GSH = glutathione
IκB = inhibitor of NF-κB
Keap1 = Kelch-like ECH-associated protein, inhibitor of Nrf2
KO = knock-out
mGPx4 = mitochondrial expression form of GPx4
NF-γ = CCAAT-binding heteromeric transcription factor NF-Y
NF-κB = nuclear factor κB (transcription factor)
nGPx4 = nuclear expression form of GPx4
Nkx-3.1 = homeobox protein Nkx-3.1 (transcription factor)
NOX1 = NADPH oxidase type 1
Nrf2 = nuclear factor erythroid 2-related factor 2 (transcription factor)
Nrx = nucleoredoxin
Orp1 = 2-Cys-GPx of yeast (yeast GPx3)
OxyR = bacterial $H_2O_2$ sensor
P53 = P53-protein (transcription factor; tumor suppressor)
PDI = protein disulfide isomerase
$PGE_2$ = prostaglandin $E_2$
$PI_2$ = phosphatidylinositol (3,4)-bisphosphate
PTEN = phosphatase and tensin homolog
PU.1 = transcription factor PU.1 (or 31kDa-transforming protein)
ROOH = organic hydroperoxide
RSL3 = inhibitor of GPx4
SBP1 = selenium-binding protein 1
Sec = selenocysteine residue
SecGPx = glutathione peroxidase containing a catalytic selenocysteine
TFAP2C = transcription factor C for AP2
TNFα = tumor necrosis factor alpha
Trx = thioredoxin
WT = wild type
Yap1 = AP-1-like transcription factor of yeast