Review

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Emerging roles for non-selenium containing ER-resident glutathione peroxidases in cell signaling and disease

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Abstract: Maintenance of cellular redox control is pivotal for normal cellular functions and cell fate decisions including cell death. Among the key cellular redox systems in mammals, the glutathione peroxidase (GPX) family of proteins is the largest conferring multifaceted functions and affecting virtually all cellular processes. The endoplasmic reticulum (ER)-resident GPXs, designated as GPX7 and GPX8, are the most recently added members of this family of enzymes. Recent studies have provided exciting insights how both enzymes support critical processes of the ER including oxidative protein folding, maintenance of ER redox control by eliminating H_2O_2, and preventing palmitic acid-induced lipotoxicity. Consequently, numerous pathological conditions, such as neurodegeneration, cancer and metabolic diseases have been linked with altered GPX7 and GPX8 expression. Studies in mice have demonstrated that loss of GPX7 leads to increased differentiation of preadipocytes, increased tumorigenesis and shortened lifespan. By contrast, GPX8 deficiency in mice results in enhanced caspase-4/11 activation and increased endotoxic shock in colitis model. With the increasing recognition that both types of enzymes are dysregulated in various tumor entities in man, we deem a review of the emerging roles played by GPX7 and GPX8 in health and disease development timely and appropriate.

Keywords: Ca^{2+} signaling; ER stress; GPX; oxidative protein folding; oxidative stress.

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Introduction

Maintenance of redox homeostasis is essential for preventing the oxidative damage of biological molecules such as proteins, lipids and DNA (Poli et al. 2004). The insufficient elimination of reactive oxygen species (ROS) may trigger cell death and as such might be a detrimental factor to human health and lifespan (Navarro-Yepes et al. 2014; Valko et al. 2007). Up until now, a number of diseases have been directly linked with dysregulated redox homeostasis, including cancer, neurological disorders, cardiovascular diseases, obesity and metabolic diseases, as well as aging (Sayre et al. 2001; Valko et al. 2006, 2007). To counterbalance the level of ROS, mammals have evolved a complex ROS scavenging system consisting of diverse enzymes, such as catalase (CAT), superoxide dismutase (SOD), thioredoxin reductases (TXNRD) and glutathione peroxidases (GPX) (Trachootham et al. 2008).

The enzymatic activity of GPXs is referred to the reaction of hydroperoxides with reduced glutathione (GSH). This reaction was first described by Gordon C. Mills in 1957, who discovered the presence of an enzyme in erythrocytes preventing the oxidative breakdown of hemoglobin using GSH. Therefore, he named this enzyme as GPX (Mills 1957). Over the years, members of this class of enzyme have been discovered across the three domains of life, i.e., bacteria, archaea and eukaryotes. So far, eight members of the GPX family of enzymes have been identified in mammals each encoded by its distinct gene (Toppo et al. 2008). However, despite the common phylogenic origin of mammalian GPX7 and GPX8 with canonical GPXs, their name might be a misnomer as they use more efficiently thiols for instance in protein disulfide isomerase (PDI), rather than actually GSH (Bosello-Travain et al. 2013; Nguyen et al. 2011).

Antioxidant systems including glutathione peroxidases

GPXs belong to one of the major class of enzymes in preventing cells from oxidative damage induced by so-called

ROS. ROS is an umbrella term comprising oxygen containing free radicals and non-radical compounds, such as hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radical, lipid radicals and (phospho)lipid hydroperoxides (Sies et al. 2017). Typically, GPXs are known to catalyze the reduction of H_2O_2 and organic hydroperoxides to water and to their corresponding alcohols, respectively (Ursini et al. 1995). The vast majority of cellular ROS are generated as a by-product of incomplete reduction of oxygen during mitochondrial respiration and oxidative protein folding in the ER (Malhotra and Kaufman 2007).

For many years, ROS had been considered to be only toxic molecules but to date it has become clear that several forms of ROS, for instance H_2O_2, are in fact deliberately generated and regulate several cellular processes for instance upon a viral infection, inflammation and receptor tyrosine kinase (RTK) signaling (Conrad et al. 2010; Gonzalez-Dosal et al. 2011; Morgan and Liu 2011). Similarly, insulin signaling (Goldstein et al. 2005) and the production of pro-inflammatory cytokines also require the presence of H_2O_2 (Ali et al. 1999). However, the levels of ROS need to be tightly kept in check in order to prevent their potentially deleterious effects towards cellular constituents like DNA, proteins and lipids. This is accomplished by either hydrophilic antioxidants, such as ascorbate, urea and flavonoids, or lipophilic antioxidants like tocopherols, carotenoids and ubiquinol, or as mentioned before, by enzymes including CAT, SOD, TXNRD, as well as certain GPXs (previously reviewed in (He et al. 2017; Ratnam et al. 2006)).

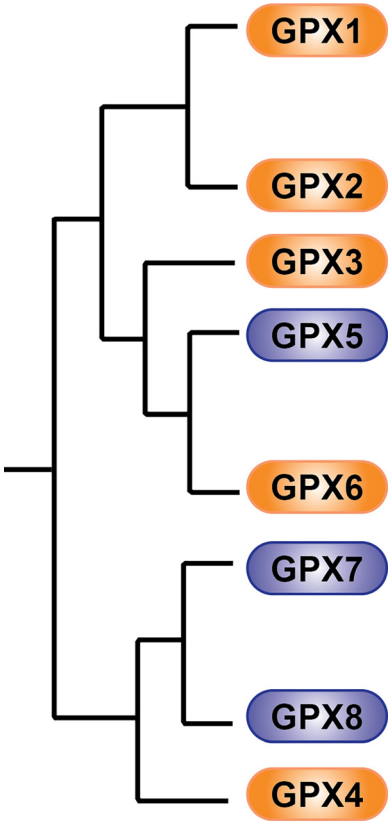

Figure 1: Phylogenetic map of human GPX family members. The phylogenic map of mammalian GPX family members represents the three closely related groups, i.e. GPX1/GPX2, GPX3/GPX5/GPX6 and GPX4/GPX7/GPX8. In humans, five out of eight family members contain Sec in their catalytic center (indicated in orange), while Sec is substituted to Cys as catalytic residue of other members (indicated in violet) (Mariotti et al. 2012).

Structural and mechanistic considerations of GPX

In vertebrates, the GPX family of proteins is the largest group containing selenoproteins. In human, out of the eight members five of them incorporate the 21st amino acid selenocysteine (Sec) instead of the functional analog cysteine (Cys) in their catalytic site. All members of this family show high sequence homology (Figure 1). Based on phylogeny, three groups can be distinguished evolving from a Cys-containing ancestor; GPX1/GPX2, GPX3/GPX5/GPX6 and GPX4/GPX7/GPX8. A tandem duplication of GPX3 led to the emergence of GPX5 and GPX6, while GPX7 and GPX8 evolved from a common GPX4 ancestor, which presumably occurred prior to the separation of mammals and fish (Mariotti et al. 2012).

Sec (U) in the catalytic center of GPX is located in an NVAxxU motif and the typical catalytic cycle of SecGPXs entails several steps (Orian et al. 2015). In the first step, the selenolate (Se^-) reacts with the hydroperoxide yielding selenenic acid (SeOH). Subsequently, SeOH reacts with GSH whereby a mixed selenyldisulfide bond is formed. In order to resolve the selenyldisulfide bond, a second electron is required coming from another GSH molecule, whereby GSSG is formed with Sec being regenerated. Deprotonation of the selenothiol closes the catalytic cycle (Conrad 2009) (Figure 2). Kinetic studies on GPX1, and later on GPX3 and GPX4, revealed that SecGPXs have an extremely high rate constant for the reaction with the hydroperoxide (Flohe et al. 1972; Takebe et al. 2002).

In general, the catalytic activities of non-vertebrate CysGPXs are similar to the Sec containing isoforms. In the reaction with the hydroperoxide, the peroxidatic Cys (C_P) in the catalytic site is first oxidized yielding sulfenic acid (Maiorino et al. 2007). In order to complete the reaction, the second resolving Cys (C_R) forms an intramolecular

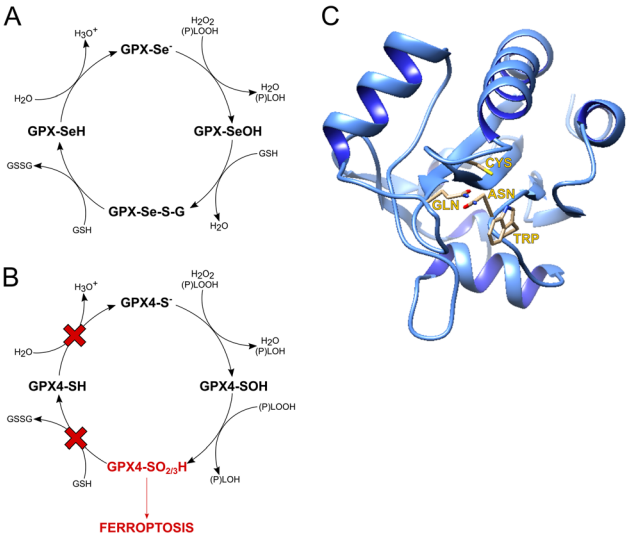

Figure 2: Catalytic cycle of GPXs. (A) The catalytic cycle of selenocysteine containing GPXs is shown. GPXs reduce H_2O_2 or organic hydroperoxides, whereby a selenenic acid (SeOH) is formed. For regeneration of the enzyme, SeOH forms a mixed disulfide with GSH. Subsequently, a second GSH molecule is required to fully reduce the enzyme thereby forming a glutathione-disulfide (GSSG), which is recycled by glutathione-disulfide reductase. To complete the catalytic cycle, selenothiol is deprotonated to selenolate anion (Se^-). (B) A site-directed replacement of Sec to Cys in the catalytic center of GPX4 renders the mutant enzyme highly vulnerable to peroxide-mediated overoxidation. In the first step, the reaction with hydroperoxide leads to the formation of a sulfenic acid. If the sulfenic acid is not immediately reduced by GSH, reaction of the sulfenic acid with another molecule of hydroperoxide will result in the formation of irreversibly overoxidized forms of cysteine (i.e., sulfinic/sulfonic acid), which no longer can be reduced by GSH. Thereby, the enzyme becomes inactive allowing the accumulation of lipid hydroperoxides causing ferroptotic cell death (Figure adjusted from (Ingold et al. 2018)). (C) Scheme illustrating the three-dimensional structure of monomeric GPX7; the amino acid residues (Cys, Asn, Trp, Gln) of the catalytic tetrad are highlighted. These amino acid residues are conserved among the members of the enzyme family. However, GPX1-4 and GPX6 in humans contain Sec instead of the Cys residue and the Gln is replaced by Ser residue in GPX8.

disulfide bridge, which is subsequently reduced by other thiol containing proteins, typically thioredoxin (Trx) (Flohe et al. 2011). Based on the structural analysis of GPXs, four amino acid have been identified determining the catalytic tetrad of the members of this enzyme family, Sec/Cys, glutamine, tryptophan and asparagine (Epp et al. 1983; Tosatto et al. 2008). Interestingly, mammalian GPX8 is an exception with glutamine being substituted by serine (Toppo et al. 2008).

The incorporation of selenocysteine (Sec) or cysteine (Cys) in the catalytic center of GPXs determines their reducing substrate specificity (Toppo et al. 2009). The presence of Sec affords a swift reactivation of the oxidized selenenic acid usually by two molecules of GSH in case of GPX1-4 and GPX6, although GPX4 is a highly promiscuous enzyme toward both its oxidizing and reducing substrates (Maiorino et al. 1995). A replacement of Sec to Cys in the active site of GPX4 was shown to render the mutant enzyme highly susceptible to H_2O_2 mediated irreversible overoxidation and inactivation of the enzyme (Ingold et al. 2018). Thereby it was shown that H_2O_2 oxidizes Cys46 in the catalytic site of mutant GPX4 yielding sulfinic and sulfonic acid ($SO_{2/3}H$) (Figure 2).

GPX1-3, GPX5 and GPX6 are homotetramers, while GPX4, GPX7 and GPX8 are considered to be monomeric due to the lack of an oligomerization interface (Figure 3) (Maiorino et al. 2015). The monomeric nature of these variants is considered to allow (i) the reaction with more complex hydroperoxides, such as contained in phospholipids and cholesterolesters, and (ii) a broader substrate specificity with the reducing substrate beyond GSH.

GPX7 and GPX8 were first annotated as secreted GPXs, but later studies revealed that both enzymes localize to the ER (Utomo et al. 2004). Human GPX7 consists of 187 amino acid residues, and contains a 19 amino acid long cleavable signal peptide at its N-terminus. Due to the closer homology to GPX4, GPX7 was first described and named as a non-selenocysteine containing phospholipid hydroperoxide (NPGPx). Upon cleavage of the C-terminal REDL retention signal, GPX7 translocates from the ER lumen to the Golgi apparatus (Raykhel et al. 2007). ER-anchored human GPX8 is 209 amino acid in length and a type two transmembrane protein (Figure 3) (Nguyen et al. 2011). It has a short, 18 amino acid long cytosolic loop, followed by the single transmembrane domain, while the remaining part of the protein faces to the ER lumen (Figure 4). Both GPX7 and GPX8 are CysGPX, and in humans they show 32 and 28% of sequence identity to GPX4, and around 31 and 25% to GPX1, respectively (Figure 3). Cys_{57} of GPX7 and Cys_{79} GPX8 are the conserved catalytic residues, respectively, and required for their putative peroxidase activity (Wang et al. 2014).

Mammalian GPX7 and GPX8 do not contain a canonical resolving cysteine (C_R) within the conserved cysteine block catalyzing the thioredoxin-coupled reduction of H_2O_2 and other hydroperoxides in other GPXs, including enzymes from plants (Flohe et al. 2011). The position of the noncanonical C_R (Cys_{86}) in reduced GPX7 is 11.58 Å away from the catalytic Cys. In principle, this distance is far too long for a disulfide bond, indicating major conformational changes upon oxidation in order to afford the formation of an intramolecular disulfide bond (Nguyen et al. 2011; Sanchez et al. 2008; Wang et al. 2014).

Figure 3: Sequence homology of mouse GPX family members. The alignment of murine GPX family members shows high sequence homology. The highly conserved catalytic residue is marked with an arrow, while the amino acid residues of the catalytic tetrad are highlighted in green. GPX1-4 are Sec (U) containing enzymes, while GPX5-8 are Cys (C) containing isoforms. As illustrated here, GPX4, GPX7 and GPX8 lack the oligomerization interface, therefore they are considered as being monomeric, while GPX1-3 and GPX5-6 contain the dimerization and tetramerization interface required for oligomerization. Blue refers to the mitochondrial leader sequence of GPX4 which is only present in the mitochondrial isoform of the protein (Arai et al. 1999), while pink is the second conserved cysteine residue among the family members. Moreover, both GPX7 and GPX8 contain an unusual endoplasmic reticulum (ER) retrieval signal at the C-terminus, marked in purple. The transmembrane domain of GPX8 is labeled with orange, while yellow indicates the signal peptide of GPX3, 5, 6 and 7 (Bosello-Travain et al. 2015).

Relevance of non ER-resident GPX family members in (patho) physiological contexts

GPXs isoenzymes show distinct subcellular localization and substrate specificity (Figure 5). GPX1 was the first identified member of the GPX family, with a localization in the cytosol and mitochondrial intermembrane space (IMS) (Flohe et al. 1973; Rotruck et al. 1973). Distinct allelic variants have been reported for GPX1, which impinge on its subcellular distribution. A single nucleotide polymorphism results in either the expression of leucine or a proline residue at position 198 at the 5′ end of GPX1. The other allelic variant is characterized by the number of alanines in the first exon (Moscow et al. 1994). Later studies indicated that GPX1 with a leucine in the position 198 and with seven alanine repeats is more

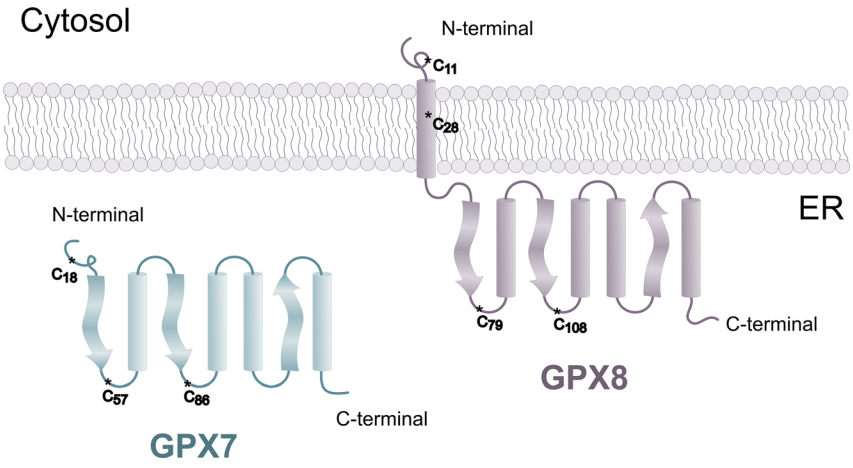

Figure 4: Topology of GPX7 and GPX8. GPX7 is located in the ER lumen, while GPX8 is a type II transmembrane protein. GPX8 has a short N-terminal cytosolic loop, followed by a single transmembrane domain, whereby the catalytically active cysteine is located in the ER lumen. The NS3-4A HCV protease targeted C_{11} is located in the cytosol.

likely to localize in the cytoplasm, than other allelic variants (Bera et al. 2014).

GPX1 is strongly expressed in the liver and kidney, where it plays an important role in cellular defense against oxidative stress. *In vivo* studies revealed that lack of the protein is compatible with life (de Haan et al. 1998; Ho et al. 1997), albeit *Gpx1*$^{-/-}$ animals exhibit morphological alterations in cardiac mitochondria and myocytes (Thu et al. 2010). In particular, acute oxidative stress leads to the death of *Gpx1*$^{-/-}$ mice, indicating the importance of the antioxidant function of the protein (Cheng et al. 1998). GPX1 was also reported to support cancer cell survival and to increase the incidence of metastasis (Okubo et al. 2013), although the regulation of GPX1 in cancer cell proliferation and survival seems to be dependent on the state of cancer development (Brigelius-Flohe and Kipp 2009).

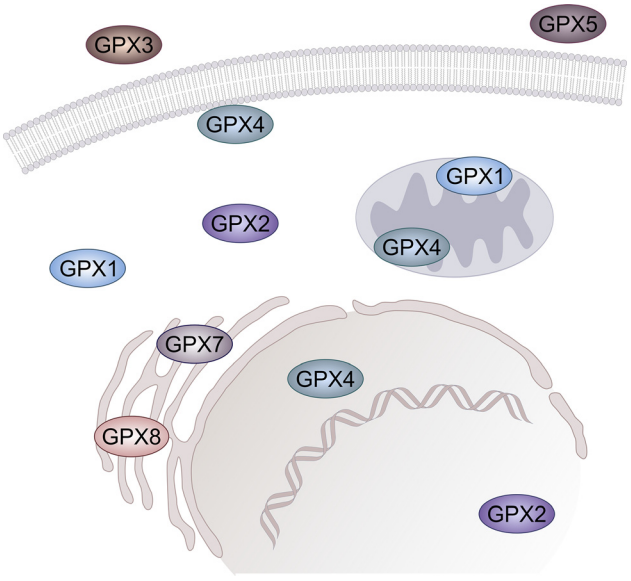

Figure 5: Subcellular localization of mammalian GPXs. GPX1 was reported to be present in both cytosol and mitochondrial intermembrane space (Esworthy et al. 1997), while GPX2 (GI-GPX) was found in cytosol and nucleus (Chu et al. 1997; Komatsu et al. 2001). GPX3 is present in the extracellular matrix and plasma (Avissar et al. 1994; Whitin et al. 2002). GPX4 has three isoforms, among them, only the cytosolic form is expressed in somatic cells, while the mitochondrial and nuclear isoforms are expressed in testis and are critical for male fertility (Conrad et al. 2005). GPX5 is expressed in the lumen of caput and cauda epididymis (Williams et al. 1998). Finally, GPX7 localizes to ER lumen, while GPX8 is found in the ER membrane (Nguyen et al. 2011).

GPX2 is closely related to GPX1 with highest expression levels in gastrointestinal tract and liver (Chu et al. 1993). *Gpx2*$^{-/-}$ mice do not show any obvious phenotype (Esworthy et al. 2000), though the protein is considered to be relevant for inflammation and cancer development (Banning et al. 2008; Liu et al. 2017; Naiki et al. 2018; Te Velde et al. 2008). In particular, the role of GPX2 in tumorigenesis has been extensively studied. While *Gpx2*$^{-/-}$ mice exhibit increased tumorigenesis, knockout mice show smaller tumor size (Krehl et al. 2012). Conclusively, similar to GPX1, GPX2 can be pro- or anti-carcinogenic, depending on the stage of carcinogenesis (reviewed in Brigelius-Flohe and Kipp 2009).

GPX3 has been characterized as an extracellular protein, which enters the ER for post-translational modification, but due to the lack of an ER retention signal, it is secreted into the blood stream mainly by kidney proximal tubular cells (Avissar et al. 1994; Whitin et al. 2002). GPX3 is one of the most abundant selenoproteins in plasma (Olson et al. 2010; Takahashi et al. 1987). Besides this, it is also present in other body fluids including amniotic fluid (Kingsley et al. 1998), thyroid colloid lumen (Kohrle 2005; Schomburg and Kohrle 2008), and as basement membrane binding form in kidney (Olson et al. 2010), bronchi, epididymis (Burk et al. 2011), placenta (Mistry et al. 2010) and in adipocytes (Maeda et al. 1997). For the latter, the

expression level of GPX3 increases during adipogenesis (Lee et al. 2008). GPX3 expression has been recently shown to be associated with insulin receptor (IR) expression of white adipose tissue of insulin-resistant and obese patients (Hauffe et al. 2020). Interestingly, non-stressed GPX3 null mice do not show any obvious phenotype (Olson et al. 2010). On the other hand, previous studies have indicated a tumor suppressor function for GPX3 (An et al. 2018; Falck et al. 2010; He et al. 2011; Lee et al. 2005; Yu et al. 2007; Zhu et al. 2018), while more recent studies, however, question a potentially antitumorigenic effect of GPX3 (Worley et al. 2019).

Meanwhile, GPX4 has become the most studied member of the enzyme family due to the recognition that GPX4 is nowadays considered as the key regulator of a quite recently described form of non-apoptotic cell death, known as ferroptosis (Dixon et al. 2012; Friedmann Angeli et al. 2014; Seiler et al. 2008; Yang et al. 2014). Whole body deletion of *Gpx4* in mice leads to early embryonic lethality around gastrulation (Yant et al. 2003). GPX4 comes in three different isoforms: a cytosolic, a mitochondrial and a nuclear one and different mechanisms account for the dual/different subcellular localization of the different GPX4 isoforms. Usage of the most upstream promoter generates the "long isoform" of *Gpx4* that is, however, specific to testicular cells (Pushpa-Rekha et al. 1995). The long mRNA transcript allows protein translation from the first AUG start codon, producing a protein which contains a canonical mitochondrial leader sequence (MLS) (Arai et al. 1996). The shortest transcript of *Gpx4*, predominantly expressed in somatic cells, is initiated from a more downstream promoter and translation proceeds from the second AUG codon, generating a protein lacking the MLS sequence (Knopp et al. 1999). This protein localizes to the cytosol, plasma membrane, nucleus and, remarkably, the mitochondrial intermembrane space, although the mechanism accounting for the latter remains unknown (Liang et al. 2009). In addition, the transcription of "sperm nuclei-specific" *Gpx4* is initiated from an alternative exon located in the first intron of the *Gpx4* gene (Moreno et al. 2003) and produces a protein that localizes to nuclei and that binds to sperm DNA.

Studies using constitutive knockout mouse model of the nuclear and mitochondrial isoforms of GPX4 revealed perturbed sperm chromatin condensation (Conrad et al. 2005) and male infertility (Schneider et al. 2009), respectively, indicating that the mitochondrial and cytosolic isoforms are only important for male fertility. Tissue specific knockout mouse models provided conclusive evidence that GPX4 is involved in neuroprotection in various neuronal subpopulations (Chen et al. 2015; Seiler et al. 2008; Wirth et al. 2014; Wirth et al. 2010), kidney tubular cells (Friedmann Angeli et al. 2014), $CD8^+$ T cell mediated immunity (Matsushita et al. 2015), hematopoiesis (Altamura et al. 2020; Canli et al. 2015), and liver protection (Carlson et al. 2016). *In vitro* studies further corroborated that this broad range of phenotypes is attributed to the unique relevance of GPX4 in suppressing ferroptosis, which is defined by the detrimental accumulation of phospholipid hydroperoxides (Dixon et al. 2012).

GPX5 is a selenium-independent GPX, which is abundantly expressed in epithelial cells and lumen of epididymis (Rejraji et al. 2002). It was also found to be present in the acrosome of spermatozoa, where it prevents premature acrosome reaction, thereby maintaining sperm fertility in the epididymis (Okamura et al. 1997). $Gpx5^{-/-}$ mice do not exhibit any obvious phenotype. Despite its fundamental role in detoxification of hydroperoxides, fertility of young males is not affected by the genetic ablation of *Gpx5*. This can be explained by a possible compensatory mechanism of other GPXs, including GPX1, GPX3, and GPX4. Only males older than one year exhibited impaired sperm DNA integrity (Chabory et al. 2009). Lately, high expression of GPX5 was found to correlate with worsening overall survival, while high expression of GPX3 mRNA improved the prognosis of patients with non-small cell lung cancer (Liu et al. 2018).

Intriguingly, GPX6 is a selenoprotein in humans, whereas in other species it is present as a Cys-containing enzyme (Kryukov et al. 2003). Although still little is known about GPX6 functions and substrate specificity, it is robustly expressed in the olfactory bulb, striatum, and frontal cerebral cortex in an age-dependent manner. Overexpression of GPX6 in striatum was found to protect against mutant Huntingtin-induced neurotoxicity, and accordingly GPX6 alleviates disease progression in a model of Huntington´s disease in mice (Shema et al. 2015). Further *in vivo* studies demonstrated a link between GPX6 expression and age-related hearing loss in mice (Tadros et al. 2014). Due to the lack of knockout animal studies, our understanding of the *in vivo* relevance of GPX6 remains still limited.

Cellular functions of the ER-resident GPX7 and GPX8

GPX7 and GPX8 promote oxidative protein folding

The first cell-based studies suggested that both GPX7 and GPX8 have a low GSH peroxidase activity, which is attributed to the lack of a GSH interface (Nguyen et al. 2011). Indeed, both enzymes were implicated to react with PDI

more readily than GSH. Moreover, based on a bimolecular fluorescence complementation assay, GPX7 and GPX8 were demonstrated to localize in close proximity to endoplasmic reticulum oxidoreductase 1 (ERO1α), arguing for a potential functional interaction (Nguyen et al. 2011; Ramming et al. 2016). Both GPX7 and GPX8 are indeed capable of increasing the PDI oxidizing activity of ERO1α (Nguyen et al. 2011; Ramming et al. 2016). Further studies confirmed that GPX7 facilitates oxidative protein folding (Wang et al. 2014). Upon oxidation by H_2O_2, the catalytic Cys (C_{57}) of GPX7 is first oxidized, which rapidly reacts with the non-canonical C_R (C_{86}), forming an intramolecular disulfide bond. Irrespective of whether GPX7 is present in its sulfenic acid or disulfide form, both were shown to be capable of interacting with PDI. In addition, treatment with H_2O_2 results in oxidation of GPX7, which in turn triggers covalent binding to GRP78 (alias heat shock protein family A (Hsp70) member 5) via an intermolecular disulfide bond (Wei et al. 2012). This interaction facilitates the formation of an intramolecular disulfide bond between Cys_{41} and Cys_{420} of GRP78, thereby increasing the binding ability of GRP78 to misfolded/unfolded proteins. Consequently, GPX7 promotes the refolding of misfolded proteins, and thereby attenuates ER and oxidative stress. In this context, loss of GPX7 leads to impaired chaperone activity of GRP78 and to the accumulation of unfolded proteins, which in turn results in elevated oxidative stress. Taken together, GPX7 emerges to be essential for the chaperone function of GPR78 and for the elimination of misfolded proteins.

The relevance of GPX8 in the detoxification of H_2O_2 produced by deregulated ERO1 was demonstrated in cells, corroborating the concept of the contribution of GPX8 to oxidative protein folding (Ramming et al. 2014). More recent studies, however, challenge a possible involvement of GPX7 and GPX8 in oxidative protein folding (Mehmeti et al. 2017). By studying insulin secretion in INS-1E cells, ectopic expression of either GPX7 or GPX8 failed to increase insulin and pro-insulin content in rat β-cells. Additionally, none of the ER-resident GPXs was able to bolster the oxidizing capacity of PDI in contrast to peroxiredoxin 4 (PRX4). Therefore, this study failed to prove the contribution of GPX7 and GPX8 to oxidative protein folding in the ER (Mehmeti et al. 2017).

GPX7 and GPX8 attenuate ER stress by eliminating H_2O_2

Protein misfolding causes H_2O_2 accumulation and induces the unfolded protein response (UPR), a cellular adaptation mechanism to cope with elevated stress (Hansen et al. 2012). However, sustained or severe ER stress can induce cell death (Hetz 2012). On the other hand, increasing evidence implicates that ROS, in particular H_2O_2 can also act upstream of the UPR (Buytaert et al. 2006; Hansen et al. 2012; Moserova and Kralova 2012; Santos et al. 2009). Notably, loss of GPX8 induces ER stress and cell death due to the leakage of H_2O_2 from the ER into the cytosol (Ramming et al. 2014). Consistent with this, *Gpx8* mRNA expression was found to be upregulated in response to ER stress (Ramming et al. 2014), and both GPX7 and GPX8 attenuate palmitic acid-induced ER stress and suppress H_2O_2 toxicity (Mehmeti et al. 2017). GPX7 was further described to act as an H_2O_2 scavenger, as it can efficiently reduce ERO1α-derived H_2O_2 (Wang et al. 2014), in a manner similar to GPX8 (Ramming et al. 2014). MEFs knockout for *Gpx7* are indeed more susceptible to oxidative stress and both the Cys_{57} and Cys_{86} residues seem to be critical for this process (Wei et al. 2012). Several other studies also implicated the role of GPX7 in attenuating oxidative stress by eliminating H_2O_2 induced by UV radiation (Hwang and Shim 2018), or bile acid in oesophageal epithelia cells (Peng et al. 2012). Bile acid has been proposed to induce oxidative DNA damage, which may results in cell death (Clemons et al. 2007; Zhang et al. 2009), and GPX7 was able to prevent oesophageal cells from DNA double strand breaks (Peng et al. 2012). H_2O_2 not only induces oxidative DNA damage, but also functions as a signaling molecule activating downstream molecules such as c-Jun N-terminal kinases (JNK) and P38 mitogen-activated protein kinases (p38). Both kinases belong to the class of mitogen-activated protein kinases (MAPK) involved in the cellular response to oxidative stress and DNA damage (Rezatabar et al. 2019). GPX7 was shown to be able to suppress the activation of both JNK and p38 presumably by eliminating H_2O_2 (Peng et al. 2012). Finally, in human umbilical vein endothelial cells (HUVECs), GPX7 alleviated ER stress induced by homocysteine (Wu et al. 2019). Homocysteine induces ERO1 expression through hypoxia inducing factor 1 alpha (HIF1α), which in turn increases H_2O_2 generation in the ER. In parallel, homocysteine stimulates GPX7 expression by activating nuclear factor E2-related factor 2 (Nrf2), and this event is crucial for attenuating ER stress.

A link between oxidative protein folding, H_2O_2 and calcium (Ca^{2+}) homeostasis in the ER has been recently established in which GPX8 was found to play a key role (Granatiero et al. 2019). H_2O_2, generated during oxidative protein folding, diffuses to the cytosol, where it activates Nrf2 via oxidation and ubiquitination of its binding partner Kelch-like ECH-associated protein 1 (Keap1) (Nguyen et al. 2005). Nrf2 is a transcription factor, regulating the expression of a series of genes which are crucial for an

adaptive stress response upon oxidative stress (Tonelli et al. 2018). Interestingly, GPX8 was found to be one of the target genes of Nrf2 in HeLa cells (Granatiero et al. 2019). In the same study, it was also demonstrated that GPX8 overexpression decreases both Ca^{2+} storage in the ER and histamine induced Ca^{2+} release. This is consistent with previous findings in HeLa cells, where it has been reported that GPX8 is enriched in the mitochondria associated membrane (MAM), and that the overexpression of the protein leads to decreased ER Ca^{2+} levels (Yoboue et al. 2017). Also, silencing of GPX8 increases histamine-induced Ca^{2+} release from the ER to mitochondria and cytosol. This phenomenon was attributed to the regulatory effect of GPX8 on the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity and on the passive Ca^{2+} release via inositol 1,4,5-triphosphate receptor (IP3R). Interestingly, the transmembrane domain (TMD) of GPX8 plays a critical role in the regulation of Ca^{2+} signaling, with the peroxidatic cysteine (C_{79}) also being important for this process (Yoboue et al. 2017). Hence, these data illustrate that the ER-resident GPXs not only prevent ER-stress and oxidative stress, but also regulate stress-related Ca^{2+} signaling.

GPX7 and GPX8 prevents lipotoxicity

Lipotoxicity is referred to a type of rather poorly defined cell death modality that is induced by saturated fatty acids (SFA). In general, accumulation of long chain SFA tends to induce ER stress, H_2O_2 production (Gehrmann et al. 2015), and, if cells fail to cope with stress, cell death (Cao and Kaufman 2014; Eizirik et al. 2008; Fonseca et al. 2011). Previously, it was reported that both GPX7 and GPX8 prevent palmitic-acid induced cell death in INS-E1 β-cells by eliminating H_2O_2 level, derived from palmitic acid oxidation (Mehmeti et al. 2017). Accordingly, ectopic expression of either GPX7 or GPX8 in β-cells attenuated palmitic acid induced ER stress.

More recently, Principal Component Analysis (PCA) and Partial Least Square Discriminant analysis (PLS-DA) of microsomal lipidome of HeLa cells revealed major remodeling of membrane lipid composition upon GPX8 depletion (Bosello Travain et al. 2020). The natural glycosphingolipid content was found to be mainly increased, while the ceramide content was slightly decreased upon genetic loss of GPX8. Not only the lipid composition, but also the fatty acid composition of membranes was found to be changed. Deprivation of GPX8 thus causes a decrease of polyunsaturated fatty acid (PUFA) content, and inversely increases monounsaturated and SFA content of all lipid species. Consistent with this, activation of hypoxia-inducible factor 2 alpha (HIF2α) renders cells competent to increase their PUFA content in membranes (Zou et al. 2019). Since GPX8 is a direct target gene of HIF2α (Bosello-Travain et al. 2015), GPX8 might be the link between HIF2α transcriptional regulation and changes in membrane fatty acid composition (Bosello Travain et al. 2020).

Contribution of GPX7 and GPX8 to disease development

The link between oxidative dyshomeostasis, ER stress and disease has been extensively studied in recent years. To date, numerous investigations have provided compelling evidence of the relevance of oxidative stress and ER stress in the development of cancer, metabolic diseases, neurodegeneration and cardiovascular diseases (Garcia-Sanchez et al. 2020; Kubra et al. 2020; Li et al. 2020; Liguori et al. 2018; Uddin et al. 2020b). According to the WHO, all of them belong to the leading causes of death in particular in developed countries. As described in the foregoing, cellular studies on GPX7 and GPX8 have hinted towards an important role in the prevention of oxidative stress, raising the question whether they might be involved in the progress of various pathological conditions (Figure 6).

Cancer

The first observation suggesting a possible link between GPX7 and carcinogenesis was made about a decade ago, when it was observed that GPX7 expression levels, along with other GPXs are frequently decreased in Barrett´s adenocarcinoma (Peng et al. 2009). Studies with mice deficient in *Gpx7* indeed revealed oxidative stress-related abnormalities including oxidative DNA damage and consequently, shortened lifespan, high risk for carcinogenesis and kidney failure (Wei et al. 2012). Although a downregulation of GPX7, along with GPX1, GPX4 and GPX5 was noticed also in breast cancer cell lines (Rusolo et al. 2017), other studies found higher levels of GPX7, along with other oxidative stress defense mechanism-related genes in liver cancer cell lines including HepG2 and Huh7 (Guariniello et al. 2015).

Hepatocellular carcinoma (HCC) is the most common and highly malignant type of primary liver cancers (Guerriero et al. 2015). The development of HCC is often caused by viral infection with either hepatitis B (HBV) or C virus (HCV) (see also further below), or by alcohol induced liver disease or non-alcoholic fatty liver disease (El-Serag and

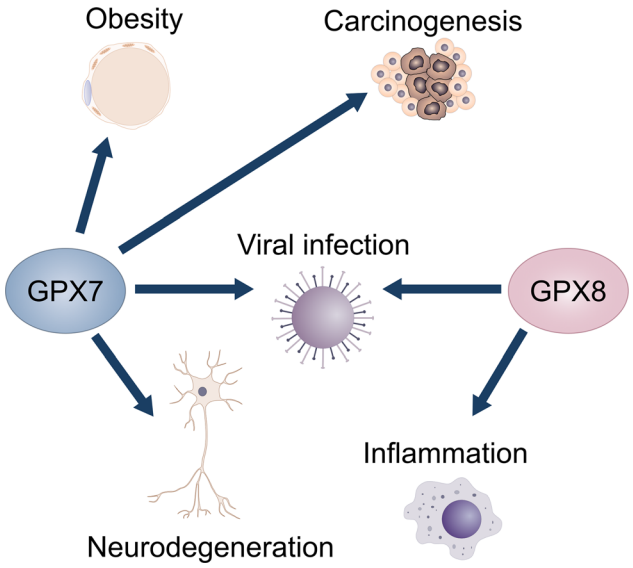

Figure 6: Pathological conditions reported to involve GPX7 and GPX8. Based on either *in vitro* or *in vivo* studies shown are diseases in which GPX7 or GPX8 or both have been proposed to be implicated in.

Rudolph 2007). Previous studies revealed that both GPX7 and GPX4 expression are significantly increased in grade III but not in grade I or II HCC, although the tumor size does not correlate with the expression of GPX4 or GPX7 (Guerriero et al. 2015).

Another evidence supporting that GPX7 confers anti-inflammatory and tumor suppressor function is that it dampens bile-salt induced nuclear factor-kappa B (NF-κβ) activation, thereby lowering cytokine and chemokine production (Peng et al. 2014b). A more in-depth analysis elucidated a possible role of GPX7 in Barrett's carcinogenesis. Barrett's esophagus dysplasia adenocarcinoma belongs to the inflammation-associated type of cancers, which commonly upregulates NF-κβ signaling via tumor necrosis factor-alpha (TNFα) (Abdel-Latif et al. 2004; Karin 2006; Li and Verma 2002; Tselepis et al. 2002). In this respect, GPX7 has been demonstrated as a key regulator of Barrett's carcinogenesis, by mitigating TNFα induced NF-κβ signaling (Peng et al. 2014c). This modulation is attributed to the ability of GPX7 to enhance protein degradation of both TNFα receptor (TNFR1) and TNF receptor associated factor 2 (TRAF2).

GPX7 was further suggested to act as a tumor suppressor, both in oesophageal adenocarcinoma cells and in tumor bearing mice (Peng et al. 2014a). Ectopic expression of GPX7 is sufficient to suppress cell proliferation by deregulating cell cycle of tumor cells, while GPX7 knockdown had the opposite effect. Mechanistic studies showed that GPX7 is frequently downregulated in gastric cancers due to hypermethylation of the Cp island in its promoter (Chen et al. 2017). Interestingly, genetic association studies on patients suffering from primary lung cancer uncovered a single nucleotide polymorphism (SNPs) present in *Gpx7*, which likely correlates with the risk for developing chemotherapy-induced peripheral neuropathy (CIPN) upon treatment with platinum alone or in combination with taxane (Johnson et al. 2015). Therefore, it might be worth in the future to consider genetic risk factors that are known to be associated with drug resistance, when treating patients with such chemotherapeutic regimens.

Unlike GPX7, by far less studies have been performed addressing a possible link between GPX8 and carcinogenesis. A genome wide transcriptome analysis of melanoma cells SK-MEL-3 exposed to the histone deacetylase (HDACs) inhibitor tricostatin A, known to prevent drug resistance in malignant melanoma, highlighted GPX8 to be one of the most downregulated genes (Mazzio and Soliman 2018). Gene signal-net analysis recently identified GPX8, along with other genes, including GPX2, Ras-related protein R-Ras (RRAS), GTPase HRase (HRAS), tumor protein p53 (TP53), and myc proto-oncogene protein (MYC), as an important players involved in *Aristolochia manshuriensis* induced malignant or benign gastric tumorigenesis (Wang et al. 2020). In addition, bioinformatics studies showed that high GPX8 expression level is correlated with higher clinical stage of gastric cancer (GC) along with decreased overall survival (Zhang et al. 2 2020).

In general, an emerging picture arises indicating that high GPX8 expression correlates with poorer prognosis in GC patient, and that GPX8 expression might be used, both as a prognostic biomarker and a potential therapeutic target for patients with GC. Here, it is worth mentioning that globoside 3 (Gb3) is increased in response to GPX8 depletion, with Gb3 being a major contributor to cell invasiveness (Kovbasnjuk et al. 2005). Hence, these findings may propose a link between GPX8 and epithelial-mesenchymal-transition (Bosello Travain et al. 2020), a process highly relevant for cancer metastasis (Campbell 2018).

Obesity and metabolic disorders

Emerging evidences established that increased oxidative stress is closely associated with fat accumulation leading to obesity, which has become an epidemic worldwide (Festa et al. 2001; Keaney et al. 2003; Urakawa et al. 2003). Since H_2O_2 levels are elevated during adipogenesis (Tormos et al. 2011), it was concluded that enzymes involved in oxidative stress defense might be relevant for the

pathology of obesity and obesity-related metabolic diseases. GPX7 is known to be highly expressed in wild type preadipocytes (Chang et al. 2013), and in fact GPX7--deficient preadipocytes tend to differentiate into adipocytes due to a higher ROS load, in particular H_2O_2, and consequently increased activation of CCAAT/enhancer-binding protein beta (C/EBPβ), a key regulator of adipogenesis. Not only increased adipocyte proliferation, but also adipocyte hypertrophy causes elevated body weight of GPX7 null mice, when kept on high fat diet (Chang et al. 2013). In humans, a genetic variant has been described located upstream of GPX7 gene leading to decreased expression of GPX7, which has been correlated with higher BMI (Chang et al. 2013). In line with this, metformin, a widely used antidiabetic drug, was shown to increase GPX7 expression in human diploid fibroblast (HDF) due to activation of Nrf2 (Fang et al. 2018). The GPX7 promoter contains an antioxidant response element (ARE), where activated Nrf2 binds and induces GPX7 transcription in order to protect against oxidative stress, and thereby eventually increases lifespan. It thus can be speculated that GPX7 could serve as a link between the molecular basis of mechanism of action of chronic low-dose metformin supplementation and improved health conditions (Bannister et al. 2014), and even lifespan extension (Fang et al. 2018).

Up until now, no *in vivo* data is available regarding the relevance of GPX8 and obesity or other metabolic diseases. As described above, GPX8 appears to play a protective role against lipotoxicity and in the regulation of fatty acid composition of membranes (Bosello Travain et al. 2020). In particular, insulin resistance in metabolic syndrome has been implicated to be a consequence of alteration of membrane composition (Perona 2017). Lipotoxicity is also known to exert a unique role in β-cell dysfunction and consequently in insulin resistance (Yazici and Sezer 2017). Therefore, future studies are warranted to investigate the potential link between GPX8 and metabolic diseases.

Neurodegeneration

Neurodegenerative diseases (NDs) are characterized by neuronal cell death or loss of function or structure of neurons. Oxidative stress was proposed as one of the leading causes in ND (Uddin et al. 2020a). Consequently, ferroptosis, a form of cell death marked by lipid peroxidation, has been considered to contribute to neuronal cell death in ND (Ren et al. 2020; Seiler et al. 2008; Wirth et al. 2010). Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease are the most common and best known forms of NDs. *In vivo* studies revealed that loss of GPX7 leads to an ALS-like phenotype in aged mice (Hsieh et al. 2019). Mechanistically, GPX7 interacts with O-linked b-N-acetylglucosaminidas (O-GlycNAcase or OGA) via disulfide bonding. O-GlycNAc is a form of posttranslational modification implicated to be critical for cellular stress response, and regulated by the activity of O-GlcNAc transferase (OGT) and OGA (Groves et al. 2013; Hart et al. 2011). Increased levels of O-GlycNAc have been demonstrated upon oxidative stress (Champattanachai et al. 2008). The interaction between GPX7 and OGA was shown to mediate oxidative stress by reducing the enzymatic activity of oxidized form of OGA as a negative feedback mechanism (Hsieh et al. 2019). Loss of GPX7 in mice results in dysregulation of O-GlcAcylation activity and consequently impaired cellular redox homeostasis. More importantly, GPX7 expression was found to be decreased in human patients with ALS. Therefore, GPX7 perhaps plays a critical role in neuroprotection, as loss of GPX7 may increase the risk for developing ALS.

A recent study has implicated GPX8 to protect motor neurons from abrogated oxidative protein folding induced stress through maintaining Ca^{2+} homeostasis (Granatiero et al. 2019). Elevated Ca^{2+} release from the ER abrogates ATP release by astrocytes, which has been demonstrated to increase astrocyte toxicity toward motor neurons (Kawamata et al. 2014), thus hinting toward an important role for GPX8 in neuroprotection (Granatiero et al. 2019).

Viral infection and inflammation

Whole blood and liver tissue analysis of patients with chronic hepatitis C virus recently revealed that expression of GPX7, along with other genes involved in cellular redox control, such as GPX2, GPX3 and arachidonate 12-lipoxygenase (ALOX12), are upregulated compared to healthy controls (Shahid et al. 2020). Furthermore, the expression level was correlated with viral load, liver fibrosis and the liver injury marker alanine-aminotransferase (ALT) (Shahid et al. 2020). Hence, this reflects the possibility for using GPX7 as a new non-invasive blood-based marker for chronic HCV infection (Shahid et al. 2020).

The first knockout mouse model for *Gpx8* has been published just recently (Hsu et al. 2020). Since these mice do not show any obvious phenotype under normal housing conditions, dextran sulfate sodium-induced colitis was applied. Mice lacking GPX8 were more susceptible to endotoxic shock and exhibited exacerbated colitis. This was attributed to the covalent binding of GPX8 to caspase 4/11 through a specific disulfide bond, by which GPX8 was shown to be able to suppress caspase 4/11 activity. In the

absence of GPX8, caspase 4/11 activation was enhanced eventually resulting in augmented pyroptotic cell death (Hsu et al. 2020). Patients with ulcerative colitis exhibit lower expression of GPX8 which was correlated with higher expression of caspase four when compared to healthy individuals. These data indicate an implication of GPX8 in the noncanonical inflammatory response.

Additionally, GPX8 was identified as a cellular target of hepatitis C virus (HCV) protease NS3-4A in cells and also in liver biopsies from patients suffering from chronic HCV (Morikawa et al. 2014). NS3-4A protease cleaves GPX8 at C_{11}, thereby removing the cytosolic loop of the protein (Figure 4). In addition, GPX8 was found to contribute to viral particle production, therefore this raises the question, why GPX8 is targeted by the viral protease? Speculatively, one possible reason is to maintain activity and stability of GPX8.

Concluding remarks

In the last few years, it has become evident that enzymes of the GPX family of proteins confer functions beyond their classically viewed role by acting just like "true glutathione peroxidases" scavenging H_2O_2 and other organic hydroperoxides. This has been first realized for selenium-containing GPX4, which, besides preventing ferroptotic cell death (Seibt et al. 2019), was shown to be essential for maintaining male fertility by acting as a so-called thiol peroxidase introducing disulfide bridges into sperm proteins due to the virtual absence of GSH in developing sperm (Conrad et al. 2005; Schneider et al. 2009; Ursini et al. 1999). Perhaps in analogy to GPX4, cysteine-containing, monomeric GPX7 and GPX8, which predominantly localize to the ER, fulfill multiple roles beyond scavenging ERO1--derived H_2O_2 including oxidative protein folding. Unlike GPX4, knockout of either GPX7 or GPX8 in mice is surprisingly well-tolerated indicating that both enzymes play more specific roles as increasingly being appreciated in tumor development, viral infection, lifespan and inflammatory processes. Therefore, targeting them in the future may allow to discover new therapeutic options for patients suffering from related pathologies.

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