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GPx4: From Prevention of Lipid Peroxidation to Spermatogenesis and Back

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From Prevention of Lipid Peroxidation to Spermatogenesis and Back

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7.1   FOR INTRODUCTION: THE HISTORY OF PHGPX AND LIPID PEROXIDATION

Lipid peroxidation is a transition metal-dependent, oxidative degradation of polyunsaturated fatty acids of membrane phospholipids. Comprehensive available reviews (Cheng and Li, 2007; Yin et al., 2011; Davies and Guo, 2014) underscore the notion that phospholipid hydroperoxides (PLOOH) are both the primary product of lipid peroxidation and the most prominent initiating species. Minute amounts of PLOOH, necessary to start lipid peroxidation, are continuously generated in a biological environment. The methylene bond between two nonconjugated double bonds in the fatty acid chain esterified in a phospholipid is, in fact, prone to the abstraction of a hydrogen atom, thus forming a carbon-centered radical. Free radicals competent for this reaction, the hydroperoxyl ($HOO^•$) and hydroxyl ($HO^•$) radicals, are continuously produced by metabolic oxygen activation. The reversible oxygen addition to the carbon-centered radical in an unsaturated chain of a phospholipid produces a phospholipid hydroperoxyl radical ($PLOO^•$), and a hydrogen transfer finally stabilizes this species forming a PLOOH. Available evidence on lipid peroxidation supports the notion that propagation is rather limited, since $PLOO^•$ is prone to undergo a termination reaction. However, from PLOOH, in the presence of

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a redox-active metal such as iron, the highly reactive phospholipid alkoxy radical (PLO•) is produced, which primes new oxidations. The most relevant initiator of iron-dependent lipid peroxidation is therefore a PLOOH. The final products of iron-dependent lipid peroxidation are a large series of electrophiles generated from decomposition of PLOOH. It is therefore not surprising that it is the reduction of PLOOH that accounts for the most efficient antiperoxidant mechanism known, irrespective of the mechanism of PLOOH generation.

In 1960, the occurrence of lipid peroxidation was observed in tissues of vitamin E-deficient animals (Zalkin and Tappel, 1960) and in subcellular fractions incubated in the presence of oxygen and hematin (Tappel and Zalkin, 1960), ferrous ions (Ottolenghi, 1959; Hunter et al., 1963), or ascorbate (Ottolenghi, 1959; Thiele and Huff, 1960). Shortly later, an enzymatic form of lipid peroxidation was described, sparked by the activity of microsomal rat liver NADPH P_{450} oxidoreductase, reduced pyridine nucleotide, and Fe^{3+} chelates of ADP or ATP (Hochstein and Ernster, 1963). The observation that iron-dependent lipid peroxidation is primed by physiological compounds at physiological concentrations contributed to the perception that the process could have a physiological relevance, envisaging biomedical implications (Hochstein and Ernster, 1964; Ernster et al., 1982). This motivated the search of the antioxidant defense system(s) and expanded the field of free radicals in biology and medicine, on the assumption that all these processes must proceed exclusively by free radical reactions. This came out not to be true.

In 1976, McCay first demonstrated that reduced glutathione (GSH), when incubated with dialyzed rat liver cytosol, prevents NADPH-induced microsomal lipid peroxidation. This effect was attributed to a "thermolabile factor," tentatively identified as glutathione peroxidase (McCay et al., 1976). Glutathione peroxidase (known today as the tetrameric GPx1, E.C. 1.11.1.9) was already known for almost 20 years as the enzyme that, by catalyzing the reduction of H_2O_2 by GSH, protects hemoglobin from oxidative breakdown (Mills, 1957). Yet, the real nature of the "thermolabile factor" remained controversial. Burk proposed that protection could be due, at least in part, to a glutathione transferase (E.C. 2.5.1.18) (Burk et al., 1980), while Gibson showed that enzymatic lipid peroxidation was not prevented by an enriched preparation of glutathione transferase. These authors argued that, instead, a still unidentified protein acting on intact PLOOH could be the actual antiperoxidant factor (Gibson et al., 1980).

In the meanwhile, in our laboratory at the "Istituto di Chimica Biologica" in Padova, while studying the regulation of HMG-CoA reductase using microsomes as the enzyme source, we had to deal with the problem of lipid peroxidation. Thus, we resorted to learn more about the mechanism of the antioxidant capacity of GSH and the "thermolabile factor" by purifying it. Looking for a simple and quick lipid peroxidation test for assaying the protective activity present in the chromatographic fractions, we came across the experiments of Kaschnitz and Hatefi (1975), showing that lipid peroxidation could be induced by hematin in a suspension of phospholipids containing polyunsaturated fatty acids and traces of PLOOH. These could be easily obtained by spontaneous autoxidation, i.e., leaving the phospholipid suspension at +4°C for some days. However, GSH alone inhibited hematin-driven peroxidation (Kaschnitz and Hatefi, 1975), and thus this compound

was not suitable for assaying the activity of the antiperoxidant factor. Exploring other iron complexes inducing lipid peroxidation regardless of the presence of GSH, we identified Fe^{3+}-triethylenetetramine (TETA), a complex previously described by Wang (1955) as a catalase mimic. Fe-TETA induced a fast lipid peroxidation in autoxidized phospholipid suspensions by a mechanism that was assumed to be PLOOH-dependent, similar to that of hematin, with the advantage that GSH was not inhibitory in the absence of the "thermolabile factor" (Maiorino et al., 1980).

This approach was pivotal to its purification. This revealed a monomeric protein, which was devoid of any GSH transferase activity and accounted for the activity of the cytosol in the McCay protection assay (Ursini et al., 1982). We showed that the purified enzyme catalyzed a GSH oxidation coupled to a 2:1 stoichiometric reduction of the PLOOH contained in the phospholipid suspension. We also provided an mass spectrometry (MS) evidence that phospholipid alcohols (PLOH) were produced in the enzymatic reaction (Ursini et al., 1982; Daolio et al., 1983). The molecular weight of the protein, as detected by size exclusion chromatography and polyacrylamide gel electrophoresis, was approximately 20 kDa, quite different from the previously known homo-tetrameric GPx1. Finally, by proton induced X-ray fluorescence, we demonstrated the presence of selenium. The second glutathione peroxidase came to light and, due to the peroxidase activity on PLOOH, we named it "phospholipid hydroperoxide glutathione peroxidase" (PHGPx) (E.C. 1.11.1.12) (Ursini et al., 1985). Today, PHGPx, also known as GPx4, is the product of the *GPx4* gene (Gladyshev et al., 2016). Enzymological and structural characterization followed in a span of a decade.

7.2 PHGPX: KINETIC CHARACTERIZATION AND SUBSTRATE SPECIFICITY

Steady-state kinetic analysis of the reaction indicated that PHGPx adopts a uni–ter ping-pong mechanism, similar to that of GPx1 (Ursini et al., 1985; Maiorino et al., 1990; Ursini et al., 1995). The reaction was assumed encompassing two ordered independent chemical events: the catalytic selenol (–SeH) is first oxidized by the hydroperoxide, and then reduced back in two steps by two molecules of GSH. Kinetic data were compatible with a mechanism where the –SeH of catalytic selenocysteine residue is oxidized by the hydroperoxide to selenenic acid (–SeOH) while the corresponding alcohol and water are produced, then glutathionylated by the first molecule of GSH (-Se-SG), and eventually reduced back by the second molecule of GSH, whereby oxidized glutathione (GSSG) is released and the ground state enzyme is regenerated.

As already observed for GPx1 (Flohé et al., 1972), in the reaction catalyzed by PHGPx, the values of V_{max} and K_m are infinite, since the redox transitions are faster than the formation of the enzyme–substrate complexes. In other words, the kinetics do not comply with the Michaelis–Menten model. PHGPx acts according to the concept of "Zwischenstoff-Katalyse" (catalysis by intermediate formation) (Flohé, 2011) and does not display any saturation pattern. Therefore, any variation in substrate concentration invariably results in a corresponding variation in the enzymatic rate, and thus, the Michaelis–Menten parameters K_m and V_{max} are meaningless.

The rate constants used for describing kinetic features of PHGPx were deduced by applying a simplified version of the Dalziel equation [see Ursini et al. (1995) and Toppo et al. (2009) for a review]. In pig heart PHGPx, the rate constant for the oxidizing step (k_{+1}), measured in the presence of PLOOH substrate in mixed micellar form with Triton X-100 is 1.4×10^7 M^{-1} s^{-1}, which is among the fastest ones measured for bimolecular enzymatic reactions. A recent computational approach by Density Functional Theory Quantum Mechanics (DFT-QM) [(Orian et al., 2015), reviewed in Chapter 3] indicated that such a fast rate is obtained by the formation of a charge-separated intermediate. This represents the clue of the catalysis of PHGPx and of the other selenium-dependent glutathione peroxidases, where the selenol undergoes deprotonation via long-range proton transfer involving the selenol proton, two water molecules, and the indole group of the Trp present in the catalytic tetrad. Upon binding of the peroxide substrate, the products of the oxidative part of the catalytic cycle (i.e., the –SeOH, PLOH, and H_2O) are generated in a barrierless reaction, since the proton, when shifting back, hits the hydroperoxide group. This primes the nucleophilic substitution, whereby the hydroperoxide is reduced and the selenium is oxidized (see also Chapter 3).

Since the reductive part of the catalytic cycle is comparatively slow ($k'_{+2} = 1.2 \times 10^5$ $M^{-1}s^{-1}$ for the pig heart PHGPx) and the regeneration of reduced glutathione is typically even slower, the availability of GSH at the catalytic center emerges as a major controller of PHGPx turnover. This, therefore, is expected to play a physiological role in respect to either activation of lipid peroxidation or oxidation of alternative thiol substrates.

Unlike GPx1, PHGPx has a broad specificity for both the hydroperoxide and the reducing substrates. All of the hydroperoxides so far assayed are reduced by PHGPx, from H_2O_2 and free fatty acid hydroperoxides, which are also the substrates for GPx1, to the hydroperoxide derivatives of phospholipids, cholesterol, or cholesterol esters inserted in membranes or lipoproteins, which are reduced only by PHGPx (Thomas et al., 1990a,b; Maiorino et al., 1991). This lack of specificity is apparently due to the fact that only the monomeric structure allows the access of large substrates to the active site (Ursini et al., 1995; Maiorino et al., 2015). As for the reducing substrate, PHGPx accepts different thiols such as the monothiol mercaptoethanol or dithiols such as dithiothreitol or dithioerythritol, which indeed react even faster than GSH (Roveri et al., 1994). In a physiological context, this is comparable with the reactivity of PHGPx on adjacent cysteine residues in specific proteins (Maiorino et al., 2005) (see below).

The reduction of hydroperoxides in membranes by PHGPx is a multistage process (Cozza et al., 2017). First, PHGPx binds to the lipid monolayer by specific electrostatic interactions between a cationic area adjacent to the redox center and the phospholipid polar head. This addresses the esterified fatty acid hydroperoxide, which floats and is stabilized on the surface of the membrane, to the active site of the enzyme. The redox reaction between the –SeH and the PLOOH facilitates the access of GSH, which reacts with the –SeOH forming –Se–SG. The second GSH interacts with the catalytic center competing with the same amino acids docking the enzyme to the membrane and brings the catalytic cycle to completion. Consequently, the reduced enzyme is therefore released from the membrane, ready for the next catalytic cycle (see also Chapter 3).

7.3   PHGPX: SEQUENCING, CLONING, AND MRNA EXPRESSION

The issue of the primary structure of PHGPx was initially attempted on the enzyme purified from pig heart by the laborious Edman degradation, the best technique available at the time. Although this approach yielded no more than partial information on the structure, it allowed to conceive degenerated primers that were used successfully to fish, by polymerase chain reaction, the corresponding mRNA from a pig heart cDNA library. The combination of these two techniques provided an almost complete information on the sequence, while just a small stretch at the N-terminal was missing (Schuckelt et al., 1991). Interestingly, the peptides containing the selenium could not be identified by direct sequencing, while the presence of selenocysteine was just suggested by a tiny peak in the amino acid analysis, compatible with the presence of carboxy-methyl-selenocysteine. The presence of selenocysteine was eventually proven by the identification of the in-frame TGA codon (Schuckelt et al., 1991). Some years before indeed, it had been discovered, first in GPx1 (Chambers et al., 1986) and later in other selenocysteine-containing proteins of mammalian (Takahashi et al., 1990) and bacterial origin (Stadtman, 1987; Leinfelder et al., 1988), that selenocysteine is inserted co-translationally by an in-frame TGA, previously known as a termination codon only. The complete coding region of PHGPx was obtained 3 years later, when the gene structure was elucidated. These results established for PHGPx a maximum length of 170 amino acids (Brigelius-Flohé et al., 1994).

Not surprisingly, some parts of the PHGPx primary structure proved to be similar to GPx1. The most homologous part involved sequences building up the active site, adding strong support to the notion that the catalytic mechanism of PHGPx is identical to that of GPx1 (see earlier and Chapter 3). Divergent strings of amino acids included large gaps in the PHGPx sequence, in regions corresponding to the subunit interaction sites in bovine GPx1, thus giving an account for the monomeric nature of PHGPx (Brigelius-Flohé et al., 1994). Primary structure elucidation allowed the modeling of PHGPx on the scaffold of the known crystal structure of the homo-tetrameric GPx1. This yielded the first model of three-dimensional structure of PHGPx (Ursini et al., 1995) that was largely confirmed by the PHGPx crystal structure resolved years later on a Cys variant of PHGPx (Scheerer et al., 2007).

These studies established that the residues involved in GSH binding in GPx1 are mutated or deleted in PHGPx, shading doubts on whether PHGPx could be strictly considered a *glutathione* peroxidase (Brigelius-Flohé et al., 1994; Ursini et al., 1995). It was later shown that GSH actually docks to PHGPx by different amino acids that, worth of note, are the same involved also in electrostatic interaction with the polar head of membrane phospholipids (Flohé et al., 2011; Bosello-Travain et al., 2013; Cozza et al., 2017).

The overall homology of pig PHGPx with GPx1 of different species is approximately 30%, indicating that the two peroxidases are phylogenetically related (Schuckelt et al., 1991; Brigelius-Flohé et al., 1994; Toppo et al., 2008). To date, mammalian glutathione peroxidase homologs encompass eight phylogenetically related proteins (Brigelius-Flohé and Maiorino, 2013).

The gene encoding PHGPx, *GPx4*, has been initially described as a complex 7 exons-containing gene, yielding a product of 170 amino acids (Brigelius-Flohé et al., 1994). Later it emerged that *GPx4* produces different transcripts mainly expressed in a tissue-dependent manner. Rat testis contains a cDNA encoding for the additional 27 amino acids at the N terminus of the mitochondrial localization sequence (Pushpa-Rekha et al., 1995). This longer transcript, usually referred to as the mitochondrial *GPx4* (m-*GPx4*), also carries an upstream translational start. The smaller transcript of somatic tissues, therefore, was identified as the "nonmitochondrial" *GPx4* transcript (Pushpa-Rekha et al., 1995), i.e., the cytosolic *GPx4* (c-*GPx4*) transcript yielding the product previously isolated, purified, and sequenced (Schuckelt et al., 1991; Brigelius-Flohé et al., 1994). Few years later, a third mRNA product arising from an alternative exon inside the first intron of *GPx4* was discovered. This yields a 32-kDa protein containing an N-terminal nuclear localization sequence (referred to as nuclear PHGPx, the product of the n-*GPx4* transcript). This protein is predominantly expressed in the testis (Pfeifer et al., 2001), although all the three transcripts coexist, in different proportions, in somatic and germ cell (Maiorino et al., 2003b; Liang et al., 2009). In the mature protein, the mitochondrial but not the nuclear targeting sequence is cleaved off, so that both the m- and the c-*GPx4* transcripts yield the identical mature PHGPx (Arai et al., 1996).

7.4   PHGPX AND SPERMATOGENESIS

In 1992, we observed a large PHGPx activity in rat testis, which, differently from other tissues, was higher in the nuclei and in the mitochondrial fraction than in the cytosol (Roveri et al., 1992). Immunohistochemical examination of the testicular tissue revealed that the majority of PHGPx is located in maturating spermatogenic cells, where the enzyme is located in the peripheral part of the cytoplasm, while a relatively larger portion is associated with the membranes of the nuclei and the mitochondria. These results established, for the first time, that male germ cells are a preferred site of PHGPx expression, with a distribution pattern quite different from somatic tissues. A high level of PHGPx expression in the male germ cells was also observed by *in situ* hybridization (Maiorino et al., 1998). Yet, the signaling pathway leading to high transcriptional activation is still unknown, while just the direct role of testosterone was ruled out. Testosterone, in fact, induces testicular spermatogenesis and thus stimulates the proliferation of the germ layer where PHGPx is located, mainly in spermatocytes and spermatids (Maiorino et al., 1998; Haraguchi et al., 2003; Puglisi et al., 2003).

At that time, a critical role of selenium in male fertility had been known for almost 20 years. From selenium deprivation studies, indeed, it was known that second-generation selenium deficiency results in reduced fertility in mouse and loss of fertility in rat (McCoy and Weswig, 1969). The seminiferous tubule from the Se-deficient animals contains fewer elongating spermatids and spermatozoa. Furthermore, when spermatogenesis proceeds, microscopic lesions at the level of the mid piece of maturating spermatozoa become apparent. An abnormal shape and arrangement of the mitochondria in the mid piece sheath, resulted in bends between the principal piece and the mid piece (i.e., a "kink morphology") (McCoy and Weswig, 1969;

Wu et al., 1979; Wallace et al., 1983). The discovery of PHGPx in testis germ cells paved the way to the understanding of a major molecular mechanism underlying the phenotype produced by Se deficiency.

The evidence that PHGPx is a major protein of mitochondrial capsule of spermatozoa, i.e., the outermost layer of the external mitochondrial membrane (Ursini et al., 1999), was a milestone achievement. However, the enzyme was inactive and only high concentrations of thiols (i.e., 0.1 M of 2-mercaptoethanol or dithiotreitol), in the presence of guanidine, could regenerate the activity. Interestingly, the increase of PHGPx activity by this solubilizing procedure (herein comprehensively referred to as "rescuing") was not observed in spermatogenic cells from testicular tubules, showing a high activity without any "rescuing" procedure. These results suggested a functional shift of PHGPx during spermatogenesis. This selenoenzyme, in fact, is transformed from a soluble, active peroxidase in immature germ cells to an enzymatically inactive protein in spermatozoa, where, apparently, it gains a structural role. We proposed that this moonlighting is triggered by a shortage of GSH taking place in late spermatogenesis, when also a massive protein disulfide formation takes place (Shalgi et al., 1989; Bauché et al., 1994; Fisher and Aitken, 1997). As a consequence of GSH depletion, PHGPx, due to its broad thiol specificity (Roveri et al., 1994), can use the capsular protein thiols as alternative substrate and produces protein disulfides and mixed Se-disulfides instead of GSSG. In doing this, the enzyme remains self-incorporated in the capsule. This functional shift occurs in step 19 spermatids, when PHGPx migrates from the matrix of the mitochondria to the outermost membrane region (Haraguchi et al., 2003). The PHGPx enzymatic activity is actually "rescued" *in vitro* when these mixed disulfides and Se-disulfides are reduced. Altogether, these observations point for the specific functional role for the production of a hydroperoxide in maturating germ cells and epididymal spermatozoa (Fisher and Aitken, 1997), which results in massive GSH depletion.

In spite of this remarkable increase of knowledge, the fine structure of the sperm mitochondrial capsules still remains largely unknown. Some studies attempted to define the protein substrate(s) interacting with PHGPx indicated as a likely candidate the "sperm mitochondrion associated cysteine-rich protein (SMCP)," the protein that was initially erroneously declared to be the selenoprotein in the capsule (Pallini and Bacci, 1979). Indeed, peptides containing the typical motifs of SMCP, i.e., adjacent cysteine residues (Cys-Cys), are oxidized by PHGPx. Since a disulfide between Cys-Cys generates a large conformational redox switch, it has been speculated that the cystine residues produced by PHGPx on SMCP are a transient intermediate that, by producing the conformational switch, primes the complex oxidative polymerization involving forthcoming thiol-disulfide exchanges (Maiorino et al., 2005).

In these studies, however, the individual role of the products of the three *GPx4* transcripts was not specifically addressed. Moreover, after it had become evident that deletion of *GPx4* yields a lethal phenotype (Imai et al., 2003; Yant et al., 2003), it became a real challenge to unravel which of the three *GPx4* transcripts is endowed of the vital function. This question had been elegantly answered by inverse genetic approaches that remarkably disclosed the multifaceted physiological roles of the PHGPx reaction.

At first, the expression of either n-*GPx4* or m-*GPx4* mRNA was disrupted in the mice by whole body knocked out (KO) (Conrad et al., 2005; Schneider et al., 2009). From these experiments, it has been concluded that PHGPx from n-*GPx4* is neither vital nor crucial to male fertility. It just stabilizes nuclear chromatin in maturating spermatozoa; n-*GPx4*$^{-/-}$ mice exhibiting defective chromatin condensation only in the sperm isolated from the caput epididymis (Conrad et al., 2005). Consistently, PHGPx in the nuclei had been identified as a chromatin-bound enzyme (Godeas et al., 1996) where it exhibits a protamine thiol peroxidase activity (Godeas et al., 1997). PHGPx expressed from the mitochondrial transcript is not vital either. However, the male offspring is infertile, and sperm shows the typical kink morphology observed under severe Se deficiency. Notably, despite the abnormal spermatozoa causing infertility in m-*GPx4*$^{-/-}$, the male germinal epithelium is not morphologically altered (Schneider et al., 2009).

Eventually, the experiments of Liang et al. clearly established that PHGPx from c-*GPx4* accounts for survival, while ~~conforming~~ that the deficiency of PHGPx from m-*GPx4* just results in the kink morphology of spermatozoa. *GPx4*$^{-/-}$ mice, indeed, could be rescued by PHGPx from the c-*GPx4* but not by the m-*GPx4* transcript, indicating that embryogenesis is supported only by the "cytosolic" form of enzyme (Liang et al., 2009). As expected, the male offspring of mice expressing c-*GPx4* over a *GPx4*$^{-/-}$ background, and thus lacking the product from the m-*GPx4* transcript, is infertile. The spermatozoa exhibits the typical kink morphology also described in m-*GPx4*$^{-/-}$ mice (Schneider et al., 2009), while testis and epididymis do not show any relevant histological alteration (Liang et al., 2009). All together, these studies show that it is the PHGPx from the m-*GPx4* transcript that plays the structural role in sperm, while the PHGPx from the c-*GPx4* one supports survival of the whole embryo and normal cell growth in testis germinal epithelium.

The double, vital and structural, role of PHGPx in testicular cells was further confirmed by Imai (Imai et al., 2009), showing that targeted deletion of the entire *GPx4* in spermatocytes but not in spermatogonia (i.e., in the haploid but not diploid stem cells of the testis) or somatic cells induces death of the haploid spermatocytes where *GPx4* is knocked out. Furthermore, the few spermatozoa derived from these spermatocytes show the typical kink morphology reported for *GPx4*$^{-/-}$ male mice expressing c-*GPx4* only (Liang et al., 2009) or for m-*GPx4* whole-body KO mice (Schneider et al., 2009).

The burst of PHGPx expression during spermatogenesis in rodents was also described in humans (Maiorino et al., 1998; Imai et al., 2001). As in rodents, PHGPx in human sperm is largely inactive if not previously "rescued" (Foresta et al., 2002). A lower "rescued" PHGPx activity in human spermatozoa is associated with infertility, irrespective of the etiology. PHGPx content/activity in spermatozoa is positively related to typical parameters of sperm quality, such as motility, morphology, and viability, although the correlation with the latter is less straightforward. Thus, PHGPx appears as a global marker of human fertility (Imai et al., 2001; Foresta et al., 2002). On the other hand, although screened on a limited number of infertile males, gene analysis failed to demonstrate that *GPx-4* polymorphism may account for the described correlation between PHGPx activity and the sperm and parameters of fertility (Maiorino et al., 2003a).

In summary, PHGPx has a well-documented role in male fertility, and a decreased activity gives an account for the lesions observed in the germinal epithelium and spermatozoa of rodents under severe Se deficiency (McCoy and Weswig, 1969; Wu et al., 1979; Wallace et al., 1983).

7.5   BACK TO LIPID PEROXIDATION

Using inducible deletion of PHGPx in mouse embryonic fibroblasts (mouse embryonic fibroblast, MEFs), and C11 BODIPY, a probe for detecting membrane peroxidation in living cells, it has been shown that cell death due to *GPx4* disappearance is preceded by an increased C11 BODIPY fluorescence, indicating membrane peroxidation (Seiler et al., 2008). This evidence that the control of lipid peroxidation is a critical element for cell survival, revived the whole field, which before had largely been confined to toxicology and experimental pathology (Plaa and Witschi, 1976).

In the meanwhile, while searching for new anticancer drugs by high-throughput screening, two nonstructurally related compounds, namely, erastin and RSL3, have been identified as selectively lethal to cell lines containing an oncogenic RAS mutant. In the studied cells, the RAS-RAF-MEK signaling pathway was constitutively activated and, for this reason, the compounds were named "RAS-selective lethal (RSL)" (Dolma et al., 2003; Yang and Stockwell, 2008).

The cell death induced by erastin was associated with an increase of oxidants as detected by dichlorofluorescein and BODIPY fluorescence. This suggested that lipid peroxidation could be involved in the mechanism of erastin-mediated cell death. This type of cell death was prevented by iron chelation and genetic inhibition of cellular iron uptake (Yang and Stockwell, 2008; Dixon et al., 2012) and, at least partially, by the NOX inhibitor diphenylene iodonium (Dixon et al., 2012). Moreover, RAS-mutated tumor cells contained both (1) an increased iron content (Yang and Stockwell, 2008) and (2) upregulated superoxide-producing NADPH oxidases (NOX1–5, DUOX1,2) (Shinohara et al., 2010). Taken together, these observations suggested that NOX-derived superoxide and iron are involved in erastin-mediated cell death.

Notably, the nuclei of erastin-treated cells did not display the typical morphological pattern of apoptosis. Major changes in mitochondrial morphology were instead observed, pointing to a dysfunction of mitochondria as a critical element of this form of regulated cell death. Mitochondrial dysfunction, on the other hand, is not detectable when cell death is primed by agonists known to specifically induce apoptosis, necrosis, or autophagy (Yagoda et al., 2007; Dixon et al., 2012). Further, classic features of apoptosis, such as mitochondrial cytochrome *c* release, caspase activation, and chromatin fragmentation, were not detected (Dolma et al., 2003; Yagoda et al., 2007; Yang and Stockwell, 2008). Thus, in order to describe the phenotype of regulated cell death (RCD) by the RSL compounds, which depends on the presence of iron, the term "ferroptosis" was introduced in 2012 (Dixon et al., 2012).

Direct targets of erastin were first identified as the voltage-dependent anion channels 2 and 3 (VDAC2/3), but overexpression studies demonstrated that these channels are necessary but not sufficient to induce cell death by this compound (Yagoda et al., 2007). Later, the system x_c^- emerged as the most relevant erastin

target (Dixon et al., 2012). The system x_c^- is a cystine/glutamate antiporter composed of a light-chain subunit (xCT, SLC7A11) and a heavy-chain subunit (CD98hc, SLC3A2). The system imports cystine to cells, where it is reduced to cysteine and feeds GSH biosynthesis (Lo et al., 2008). Thus, erastin apparently induces a deep decrease of cellular GSH, and this primes the particular form of iron-dependent and lipid peroxidation-mediated cell death.

Repression of expression of the x_c^- subunit, SLC7A11, is one of the mechanisms by which p53 suppresses tumorigenesis (Jiang et al., 2015). This link expands the concept of ferroptosis to the physiological control of cell survival. Notably, also the toxicity of glutamate to neuronal cells was interpreted as the outcome of a functional x_c^- inhibition (Tan et al., 2001).

RSL3, the other RSL compound identified, similar to erastin induces ferroptosis, with a downstream phenotype impinging on mitochondria. Differently from erastin, however, RSL3 action appears not to be confined to cells expressing mutant RAS and has an effect neither on VDACs nor on the x_c^- system (Yang and Stockwell, 2008). Instead, RSL3 inactivated PHGPx (Yang et al., 2014) by targeting the selenocysteine at the active site (Yang et al., 2016).

Thus, the investigation of the RSL compounds disclosed the common cell death pattern of ferroptosis: decreased PHGPx turnover due to GSH shortage or direct PHGPx inhibition. The two distinct mechanisms converge on iron-dependent lipid peroxidation, which executes ferroptotic cell death. Accordingly, the Nomenclature Committee on Cell Death, 2015 filed ferroptosis as an "iron-dependent form of regulated cell death, under the control of glutathione peroxidase 4" (Galluzzi et al., 2014).

PHGPx is therefore considered as a promising target for induction of ferroptosis in cancer chemotherapy. The concept has been validated by the observation that *GPx4* expression is critical for survival of chemoresistant mesenchymal epithelial tumor cells (Viswanathan et al., 2017) and that drug-tolerant persister cancer cells are vulnerable to PHGPx inhibition (Hangauer et al., 2017). Despite the obvious relevance of the issue of PHGPx activity in supporting the life of cancer cells, the function of this enzyme as regulators of cell life and death is also been discussed for seemingly unrelated pathophysiological conditions. Indeed, in the brain from Alzheimer's patient, *GPx4* expression is downregulated (Yoo et al., 2010) and neuron-specific PHGPx depletion causes neurodegeneration *in vivo* and *ex vivo* (Seiler et al., 2008). Furthermore, inducible total body inactivation of *GPx4* in adult mice activates ferroptosis in kidney tubular cells (Friedmann Angeli et al., 2014), and conditional silencing in the immune system prevents immunity (Matsushita et al., 2015). All this evidence converges to the notion that iron-dependent lipid peroxidation is a physiological controller of cell death, and PHGPx is an appealing molecular target for diverse medical intervention strategies. It is to be inhibited for cancer treatment, where the goal is cell death, and its activity should be nutritionally optimized in degenerative diseases, where the goal is cell survival.

Apart from the key players dampening ferroptosis, PHGPx and GSH, also the nature of the fatty acids in the membranes play an important role. This has been elegantly confirmed in cells while searching for agonists rescuing the death phenotype of *GPx4* silencing. In this study, an acyl-CoA synthetase long-chain

family member 4 (ACSL4), which quite specifically activates polyunsaturated fatty acids for the insertion into phospholipids, was identified. Cells carrying the double KO *GPx4/ACSL4* were found to be resistant to ferroptosis (Doll et al., 2017). In agreement, α6β4 integrin promotes resistance to ferroptosis by activating Src and STAT3 which suppresses the expression of ACSL4 and, in consequence, decreases the content of pro-ferroptotic fatty acids esterified in the membrane phospholipids (Brown et al., 2017).

In summary, we learned from *in vitro* and *in vivo* studies that missing PHGPx activity yields iron-dependent lipid peroxidation of membrane containing polyunsaturated fatty acids, and this evolves into a novel form of RCD, the ferroptosis. The remarkable feature of ferroptosis is that, unlike the other RCD pathways, it does not require any specific agonist, such as FAS ligand or TNF. It is sufficient that the critical mechanism for the inhibition of lipid peroxidation becomes limiting. Inversely, the PHGPx/GSH system minimizes cell death and allows proliferation. This general concept elegantly fits the recent observation that ATF4, a mediator of the metabolic and oxidative homeostasis, promotes glioma cell growth by transcriptional targeting of the xCT system, thus increasing cellular GSH content (Chen et al., 2017).

Yet, many crucial questions remain still unresolved:

Vitamin E and free radical scavengers such as ferrostatin and liproxstatin were reported to inhibit ferroptosis in the absence of PHGPx activity. This has been attributed to their peroxidation chain breaking activity (Dixon et al., 2012; Friedmann Angeli et al., 2014). However, the reduction of PLOO$^•$ yields PLOOH, which continuously generates new chain reactions in the presence of iron. Thus, scavenging of lipid hydroperoxyl radical cannot likely be the mechanism of inhibition of ferroptosis. An interaction of vitamin E with the species distinct from the PLOO$^•$ should be investigated.

How is PHGPx turnover regulated *in vivo*?

How is the PHGPx substrate PLOOH formed in cells? Is superoxide produced by mitochondria or NADPH oxidases the chain-initiating species or do we have to consider an initial involvement of lipoxygenases? Indeed, a lipoxygenase could act on intact phospholipids. In favor of this, it is known for years that vitamin E, which rescues cell death by GPx4 depletion, dampens lipoxygenase activity (Panganamala and Cornwell, 1982), although the *in vivo* relevance of this vitamin E action remains controversial. Reverse genetic experiments so far failed to provide a nonambiguous answer about the effect of the hottest candidate for ferroptosis induction, the product of 12/15 lipoxygenase. While in cells 12/15 LOX deletion rescued the lethal phenotype of *GPx4* KO (Seiler et al., 2008), this was not confirmed in whole mice (Friedmann Angeli et al., 2014; Brutsch et al., 2015). Is any other lipoxygenase involved?

Are only, or just mainly, the mitochondrial membranes the target of lipid peroxidation in ferroptosis? Indeed the high affinity of PHGPx for cardiolipin polar heads (Cozza et al., 2017) supports the direct involvement of mitochondrial membranes in ferroptosis. Also the association of PHGPx with the inner mitochondrial membranes in wild-type mice or in mice

overexpressing c-*GPx4* over a *GPx4*$^{-/-}$ background (Liang et al., 2009) points into this direction.

How is the availability of iron of the labile cytosolic pool modulated?

Is there any specific physiological species competent for the inactivation of PHGPx acting similar to the candidate drug RSL3?

We are confident that answers to these questions will contribute to the description of an intriguing scenario where oxygen, GSH, PHGPx, and vitamin E are the pivotal regulators of both life and death of aerobic cells. Needless to say, biomedicine and pharmacology is expecting a remarkable gain of information, which will pave the way to innovative therapeutic approaches.

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