

Lipid Peroxidation-Dependent Cell Death Regulated by GPx4 and Ferroptosis

Hiroataka Imai, Masaki Matsuoka, Takeshi Kumagai, Taro Sakamoto and Tomoko Koumura

Abstract Glutathione peroxidase 4 (Phospholipid hydroperoxide glutathione peroxidase, PHGPx) can directly reduce phospholipid hydroperoxide. Depletion of GPx4 induces lipid peroxidation-dependent cell death in embryo, testis, brain, liver, heart, and photoreceptor cells of mice. Administration of vitamin E in tissue specific GPx4 KO mice restored tissue damage in testis, liver, and heart. These results indicate that suppression of phospholipid peroxidation is essential for cell survival in normal tissues in mice. Ferroptosis is an iron-dependent non-apoptotic cell death that can be elicited by pharmacological inhibiting the cystine/glutamate antiporter, system Xc⁻ (type I) or directly binding and loss of activity of GPx4 (Type II) in cancer cells with high level RAS-RAF-MEK pathway activity or p53 expression, but not in normal cells. Ferroptosis by Erastin (Type I) and RSL3 (RAS-selective lethal 3, Type II) treatment was suppressed by an iron chelator, vitamin E and Ferrostatin-1, antioxidant compound. GPx4 can regulate ferroptosis by suppression of phospholipid peroxidation in erastin and RSL3-induced ferroptosis. Recent works have identified several regulatory factors of erastin and RSL3-induced ferroptosis. In our established GPx4-deficient MEF cells, depletion of GPx4 induce iron and 15LOX-independent lipid peroxidation at 26 h and caspase-independent cell death at 72 h, whereas erastin and RSL3 treatment resulted in iron-dependent ferroptosis by 12 h. These results indicated the possibility that the mechanism of GPx4-depleted cell death might be different from that of ferroptosis induced by erastin and RSL3.

Abbreviations

GPx4 Glutathione peroxidase
mGPx4 Mitochondrial GPx4
cGPx4 Non-mitochondrial GPx4
nGPx4 Nucleolar GPx4

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tBid	Truncated Bid
CL	Cardiolipin
TG	Transgenic
MEF	Mouse embryonic fibroblast
RSL	Ras-Selective Lethal
Tam	Tamoxifen
GSH	Glutathione
LOX	Lipoxygenase
DFO	Deferoxamine
Fer-1	Ferrostatin-1
LOX	Lipoxygenase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
ACSL4	Acyl-CoA synthetase long-chain family member 4
AA	Arachidonic acid

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1 Introduction

Ferroptosis is an iron- and lipid peroxidation-dependent and caspase-independent novel form of regulated cell death (RCD), which was recently named in 2012 by Dr. Brent R. Stockwell (Dixon et al. 2012). Ferroptosis is distinct from other types of cell death such as apoptosis, autophagic cell death, and necroptosis (Dixon et al. 2012; Yang and Stockwell 2008). Ferroptosis inducer, Ras-selective lethal small

molecule (RSL) including erastin and RSL-3 were found by high-throughput small molecule-screening that selectively killed the mutant Ras oncogene transformed human foreskin fibroblasts (BJeJR), but not their isogenic primary counterparts and normal cells in a non-apoptotic manner (Yang and Stockwell 2008; Yagoda et al. 2007). Multiple inhibitors of apoptosis, necrosis, and autophagy (e.g., Z-VAD-FMK, Boc-D-FMK, wortmannin, and necrostatin-1) cannot rescue ferroptosis by erastin and RSL3 treatment. In contrast, antioxidants [e.g., vitamin E and butylated hydroxytoluene (BHT)] and iron chelator (deferoxamine mesylate) inhibit RSLs induced cell death. These results indicated that ferroptosis refers to an iron-dependent, non-apoptotic cell death.

Glutathione peroxidase 4 (GPx4, Phospholipid hydroperoxide glutathione peroxidase PHGPx) is a unique intracellular antioxidant enzyme that directly reduces peroxidized phospholipids that have been produced in cell membrane (Imai and Nakagawa 2003). GPx4 knockout mice displayed early embryonic lethal in mice at 7.5 dpc (Imai et al. 2003a) and cell death in several tissues of conditional knockout mice (Imai 2010; Seiler et al. 2008). Before reports about ferroptosis, ablation of GPx4 induces lipid peroxidation-dependent, caspase-independent cell death in embryo and MEF cells (Imai et al. 2009; Seiler et al. 2008). GPx4 recently reported to be a regulator of ferroptosis by RSL3 and erastin, since RSL3 and erastin decreased the activity of GPx4 by direct binding to GPx4 and indirectly loss of glutathione respectively (Yang et al. 2014; Dixon et al. 2012).

On the other hand, mitochondrial GPx4 previously reported to be a suppressor of apoptosis by mitochondrial death pathway, since mitochondrial GPx4 inhibits the release of cytochrome c from mitochondria by reduction of cardiolipin hydroperoxide in apoptosis (Nomura et al. 1999, 2000).

In this review, we summarize recent studies on the lipid peroxidation-dependent cell death such as apoptosis and ferroptosis regulated by organelle-specific GPx4 from lessons of analysis of GPx4 overexpressed cells, knockout cells, and mice.

2 Structure and Expression of Three Types of Organelle-Specific GPx4

The GPxs family consists of various members, including GPx1–8 (Imai 2010; Imai and Nakagawa 2003). GPx1, 2, 3, 4, and 6 are selenoproteins that have seleno-cysteine at active site in human, except cysteine in GPx6 in mice. GPx5, 7 and 8 have very low glutathione peroxidase activity and thioredoxin-like activity because they have cysteine at active site. GPx3 exists in plasma, and GPx2 is expressed in gastrointestinal tract.

GPx1 and GPx4 are generally expressed in normal tissues. GPx1 can reduce hydrogen peroxide and fatty acid hydroperoxide in cytosol, but not phospholipid hydroperoxide in membrane using glutathione. GPx4 can effectively reduce

phospholipid hydroperoxide, fatty acid hydroperoxide, cholesterol hydroperoxide, and thymine hydroperoxide, but ineffectively hydrogen peroxide using glutathione.

Three types of GPx4 are transcribed by different transcriptional start codons and different exons (exon1a and exon1b) from one gene (Imai and Nakagawa 2003; Imai et al. 2006). Only N-terminal sequence including signal sequence for transport to different organelle such as mitochondria, nucleoli, cytosol, and nuclei is different among three types of GPx4, whereas the remaining amino acid sequence on the C-terminal side including the enzymatic active site is exactly the same. Thus, GPx4 contain three types of GPx4, mGPx4 that transported into mitochondria (Arai et al. 1996), nGPx4 that localized in nucleoli (Nakamura et al. 2003) and cGPx4 that distributed in cytosolic and nuclei (Imai et al. 1995). cGPx4 might also strongly associate with membrane in cytosolic sites of organelle (Arai et al. 1996) (Fig. 1).

The expression of cGPx4 mRNA was relatively high in somatic cells while that of nGPx4 mRNA was extremely low. In somatic tissues and cultured cells, the amounts of cGPx4 mRNA were approximately 2.5–12, 600–9000 times higher than those of mGPx4 mRNA and nGPx4 mRNA as determined by TaqMan assay. The expression of mGPx4 mRNA and nGPx4 mRNA was significantly higher only in testis than in other tissues. Expression of mGPx4 mRNA and nGPx4 mRNA is induced significantly in testis during spermatogenesis (Imai et al. 2006).

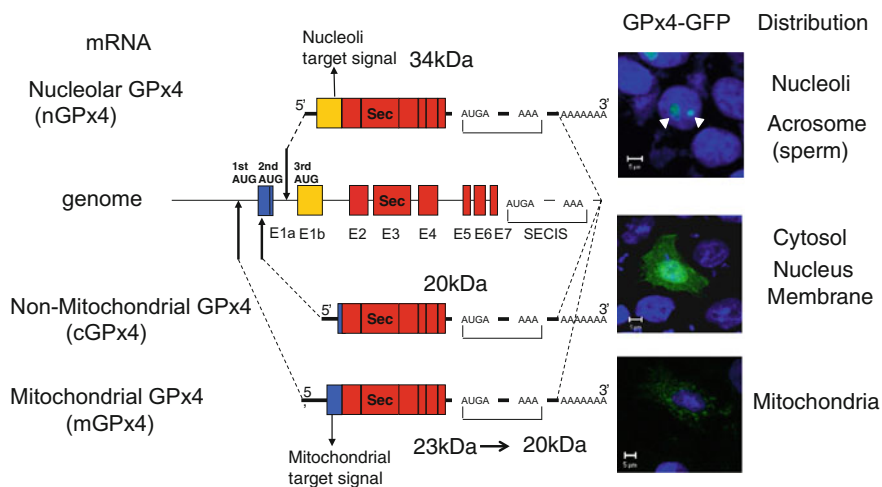


Fig. 1 Structure and distribution of three types of GPx4. Three types of GPx4, nucleolar, mitochondrial and non-mitochondrial isoforms, are transcribed from one gene by alternative transcription. GPx4 proteins contain nucleolar (34 kDa), non-mitochondrial (20 kDa) and mitochondrial GPx4 (23 kDa) with targeting signal (exon 1b and 1a) at the N-terminal of protein. The parts of C-terminal of three types of GPx4 including selenocysteine (Sec) at the enzymatic active site are the same. GPx4 is one of the selenoproteins including selenocysteine that is encoded by stop codon AUG. Selenocysteine insertion sequence (SECIS) is required for the incorporation of selenocysteine into AUG codon of the GPx4 protein. *Right panels* showed the distribution of each GPx4-GFP fusion protein in rat basophile leukemia (RBL2H3) cells. *Green* GFP fluorescence, *Blue* DAPI staining of nucleus

3 Function of Three Types of Organelle-Specific GPx4 in Cells

Analysis of overexpression of three types of GPx4 in rat basophile leukemia (RBL2H3) cells revealed that three types of GPx4 play organelle-specific independent roles in the modulation of inflammation, signal transduction, and cell death (Imai and Nakagawa 2003; Imai 2010) (Fig. 2).

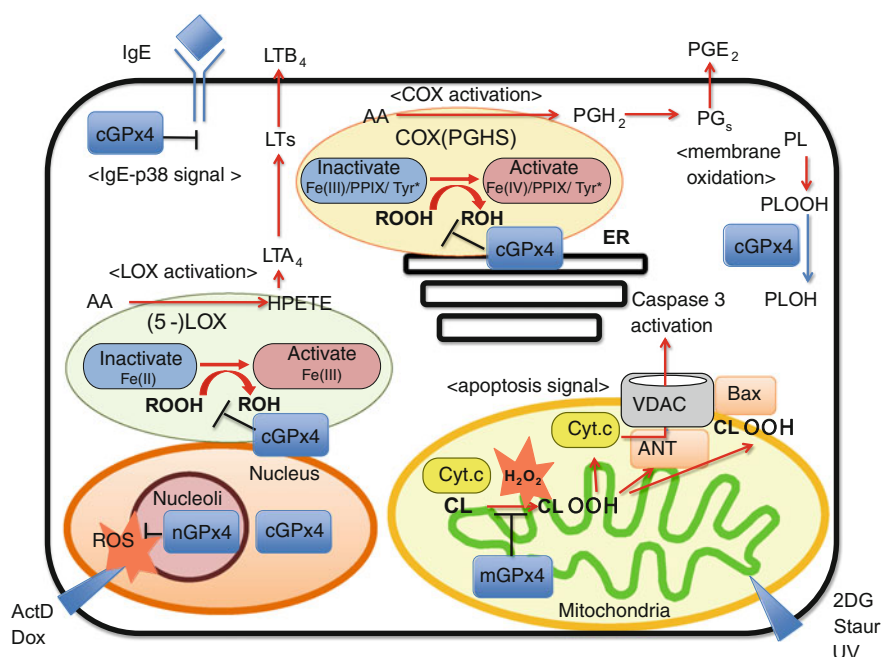


Fig. 2 Functions of three types of GPx4 in rat basophile leukemia cells. Functional analyses of stable transformants overexpressing of three types of GPx4 such as cGPx4 (L9 cells), mGPx4 (M15 cells) and nGPx4 (N63 cells) in rat basophile leukemia cells (RBL2H3 cells) were summarized. GPx4 could directly reduce phospholipid hydroperoxide (PLOOH) to hydroxyl lipid (PLOH) using glutathione (GSH). cGPx4 in the nucleus inhibited the activation of 5-lipoxygenase (5-LOX) by reduction of lipid hydroperoxide (ROOH) to hydroxyl lipid (ROH) as the activator of 5-LOX that is oxidized from inactive Fe (II) to active Fe (III), resulting in the suppression of production of leukotriene B_4 (LTB_4). cGPx4 in the endoplasmic reticulum (ER) inhibit the activation of cyclooxygenase (COX), prostaglandin H2 synthase (PGHS) by reduction of lipid hydroperoxide as the activator of COX that is oxidized from inactive heme Fe (III) to active heme Fe (IV), resulting in the suppression of prostaglandin E_2 (PGE_2). cGPx4 also suppress the activation of the IgE signaling via p38, resulting in the inhibition of production of platelet-activating factor (PAF). mGPx4 could suppress the release of cytochrome c (cyt.c), the activator of caspase 3 from mitochondria in apoptosis induced by mitochondrial death pathway such as 2-deoxyglucose (2DG), UV and staurosporine (Staur). mGPx4 inhibited the detachment of cytochrome c (cyt.c) from cardiophilin (CL), mitochondrial specific phospholipid and the conformational change of adenine nucleotide transporter (ANT) regulating the opening of permeability transition pore such as voltage-dependent anionic channel (VDAC)-Bax complex by reducing of cardiophilin hydroperoxide (CLOOH). nGPx4 could suppress the oxidative damage of nucleoli induced by doxorubicin (Dox) and actinomycin D (ActD). Three type of organelle-specific GPx4 played an independent and important regulator of local lipid hydroperoxide as a signal molecule. AA arachidonic acid, *HPETE* hydroperoxyeicosapentanoic acid, *PL* phospholipid

Overexpression of cGPx4 in the cytosol and nucleus could suppress the production of leukotriene and prostaglandin at the nucleus and endoplasmic reticulum in response to several stimuli, indicating that cGPx4 could suppress the activation of lipoxygenase and cyclooxygenase at the nucleus and endoplasmic reticulum by reducing fatty acid hydroperoxide as activators of lipoxygenase and cyclooxygenase (Imai et al. 1998; Sakamoto et al. 2000).

Overexpression of cGPx4 could suppress the production of platelet-activating factor (PAF) by IgE-antigen stimulation. cGPx4 could suppress the phosphorylation of p38 by lipid hydroperoxide signaling pathway (Sakamoto et al. 2002). However, cGPx4 could not inhibit suppress apoptosis induced by the mitochondrial death pathway (Imai et al. 1996; Arai et al. 1999; Nomura et al. 1999).

On the other hand, overexpression of mGPx4 in the mitochondria could suppress the apoptosis induced by staurosporine, 2-deoxyglucose and UV, whereas mGPx4 could not inhibit the production of leukotriene and prostaglandin (Arai et al. 1999; Nomura et al. 1999). mGPx4 could suppress the release of cytochrome c from mitochondria by inhibition of generation of mitochondria specific phospholipid, cardiolipin hydroperoxide during apoptosis induced by mitochondria death pathway (Nomura et al. 2000).

Overexpression of nGPx4 in the nucleoli could inhibit nucleoli-damaged cell death induced by doxorubicin and actinomycin D, but not staurosporine, 2-deoxyglucose, and UV (Nakamura et al. 2003).

These results demonstrated that three types of organelle-specific GPx4 played an independent and important regulator of local lipid hydroperoxide as a signal molecule, indicating that lipid hydroperoxide generated in the local area of each organelle might function as a signal molecule in inflammation and cell death (Imai 2010).

4 Mitochondrial GPx4 Inhibit the Release of cyt.c from Mitochondria by Cardiolipin Hydroperoxide in Apoptosis by Mitochondrial Death Pathway

Our mGPx4 transformant studies revealed that mGPx4 inhibits apoptosis induced by 2-deoxyglucose, staurosporine, actinomycin D, and UV, but not A23187 and anti-Fas antibody stimulation and mGPx4 could suppress the release of cytochrome c from mitochondria, resulting in inhibition of caspase-3 activation and PS externalization (Arai et al. 1999; Nomura et al. 1999) (Fig. 2).

Cardiolipin, that is located primarily in the mitochondrial inner membrane, has a unique structure containing three glycerol moieties, two phosphate residues, and four fatty acyl chains in the same molecule. Cardiolipin (CL) is critical for maintenance of cristae structure as well as stabilizing mitochondrial electron transport complexes, carrier proteins, and phosphokinases (Imai and Nakagawa 2003; Maguire et al. 2017).

Oxidative stress induced peroxidation of CL because CL is rich in polyunsaturated fatty acids, especially linoleic acid. We found that during apoptosis by mitochondrial pathway, oxidation of CL in the mitochondria occurs early events before the release of cytochrome c from mitochondria. Cytochrome c strongly associates with CL, whereas oxidation of CL is a required step for the dissociation of cytochrome c from CL in the inner membrane, since oxidized CL exhibits a reduced binding affinity for cytochrome c over CL. mGPx4 could suppress the peroxidation of cardiolipin in mitochondria and inhibit the dissociation of cytochrome c from mitochondrial inner membrane in apoptosis (Nomura et al. 2000). And mGPx4 could inhibit the change of conformation by loss of activity of adenine nucleotide translocator (ANT) that could regulate the opening of permeability transition pore by cardiolipin hydroperoxide (Imai et al. 2003b). We proposed “cardiolipin hydroperoxide cascade” for the release of cytochrome c from mitochondria in apoptosis in 2003 (Imai and Nakagawa 2003).

Kagan’s group found that pro-apoptotic stimuli induced H₂O₂ dependent peroxidation of CL by cytochrome c (Kagan et al. 2005). Recent works indicated that oxidation of CL results in migration of CL from inner membrane to the outer mitochondrial membrane (Maguire et al. 2017; Li et al. 2015). Oxidized CL recruits and interacts with Bax to initiate formation of mitochondrial transition pore (Korytowski et al. 2011).

Recently our data showed that mGPx4 could suppress the release of cytochrome c from mitochondria by different mechanisms of Bax- and tBid-induced apoptosis (Imai, unpublished). These data demonstrate that mGPx4 is a regulator of apoptosis by inhibition of CL peroxidation-dependent release of cytochrome c from mitochondria.

5 Suppression of Peroxidation of Phospholipid by Non-mitochondrial GPx4 and Vitamin E Is Essential for the Survival of Cells and Mice

Ablation of all GPx4 gene in mice induced early embryonic lethal at 7.5 dpc (Imai et al. 2003a). GPx4-null embryo could not develop into Inner Mass Cell (ICM), whereas vitamin E could rescue formation of ICM in GPx4-null embryo. Transfection of cDNA for cGPx4 also could recover the formation of ICM in GPx4-null embryo. These results indicated that suppression of generation of lipid hydroperoxide by GPx4 and vitamin E is required for embryo development.

We also succeeded that transgenic complementary rescue method using an all GPx4-loxP transgene rescued embryonic lethality in endogenous all GPx4 KO mice (Imai 2010; Imai et al. 2009). mGPx4 starts codon mutation transgene and nGPx4 start codon mutation transgene could rescue the embryonic lethality of all GPx4 KO mice, whereas cGPx start codon mutation transgene could not rescue the embryonic lethality. Double GPx4 mutation transgene containing double mutation of start codons for mGPx4 and nGPx4 could rescue the lethal phenotype, indicating that cGPx4 is essential for embryo development and normal growth in mice (Imai 2011).

In fact, mGPx4 KO mice and nGPx4 KO mice display normal development except sperm maturation (Schneider et al. 2009; Conrad et al. 2005). These results demonstrated that anti-apoptotic function of mGPx4 is not required for embryonic normal development and programmed cell death such as apoptosis in mice and cGPx4 is important role for embryo development.

mGPx4 KO mice showed male infertility by structural damage of mitochondria of spermatozoa, but showed normal production of the number of spermatozoa in testis (Schneider et al. 2009; Imai 2011). On the other hand, spermatocyte-specific all GPx4 KO mice showed severe defect of spermatogenic cells in testis, the significant low level of the number of spermatozoa, a hairpin-like flagella and abnormal structure of mitochondria of spermatozoa, resulting in male infertility (Imai et al. 2009; Fujii and Imai 2014). Administration of vitamin E with spermatocyte-specific all GPx4 KO mice could rescue the defect of spermatogenesis in seminiferous tubules in mice, leading to the recover of production of the number of spermatozoa.

Docosahexaenoic acid (DHA) is a long-chain omega-3 polyunsaturated fatty acid that is a critical component of lipid structure. DHA plays important roles throughout the body and is essential for maintaining the structure and function of the brain and eye. In the rod cells of retinal photoreceptors for example, DHA within membrane facilitates the conformational change triggered by a light signal. However, DHA is easy for oxidation, since it is polyunsaturated fatty acid. Although photoreceptor cells normally developed and differentiated into rod and cone cells by P12 in photoreceptor cell specific GPx4 KO mice, they rapidly underwent drastic degeneration and completely disappeared by P21. Photoreceptor cell death induced by loss of GPx4 was TUNEL positive, lipid oxidation dependent, and caspase-independent cell death. GPx4 is a critical antioxidant enzyme for the maturation and survival of photoreceptor cells (Ueta et al. 2012). Using in vivo wound repair model by application of *n*-heptanol on the cornea or laser-induced choroidal neovascularization mice model, we clarified that GPx4 plays important role for oxidative homeostasis and cell survival in the corneal epithelial cells and the retinal pigment epithelium (RPE)/choroid tissue (Sakai et al. 2016a; Roggia et al. 2014).

Tamoxifen inducible all GPx4 KO mice induced acute renal failure with ferroptotic cell death (Angeli et al. 2014).

Heart specific all GPx4 KO mice showed embryonic death by cardio sudden death. However heart specific all GPx4 KO mice could normally grow by administration with vitamin E diet. Heart specific all GPx4 KO mice rescued by vitamin E diet show cardio sudden death by exchange to the normal diet (Imai unpublished).

Liver specific all GPx4 KO mice also showed neonatal lethal, however liver specific all GPx4 KO mice normally grow by administration of vitamin E. And change of vitamin E diets to vitamin E deficient diet induces sudden death of Liver specific all GPx4 KO mice (Imai 2011; Carlson et al. 2016).

Endothelium-specific deletion of GPx4 had no obvious impact on normal vascular homeostasis in mice maintained on a normal diet. However when mice were fed a vitamin E depleted diet for 6 weeks before endothelial deletion of GPx4 was induced by tamoxifen, 80% of endothelium GPx4 knockout mice died with detachment of endothelial cells from the basement membrane (Wortmann et al. 2013).

These results demonstrated that suppression of phospholipid peroxidation by cGPx4 and vitamin E is essential for survival in certain cells and mice and imbalance of suppression of phospholipid peroxidation might cause the several diseases (Fig. 3).

Cell death by genetic depletion of all GPx4 in each tissue of mice is dependent of loss of cGPx4, since mGPx4 and nGPx4 double KO mice normally survive.

In human, a defect of expression of GPx4 in spermatozoa was found in human infertile male with oligoasthenozoospermia that have the low number and low motility of spermatozoa (Imai et al. 2001). This phenotype in human GPx4 deficient

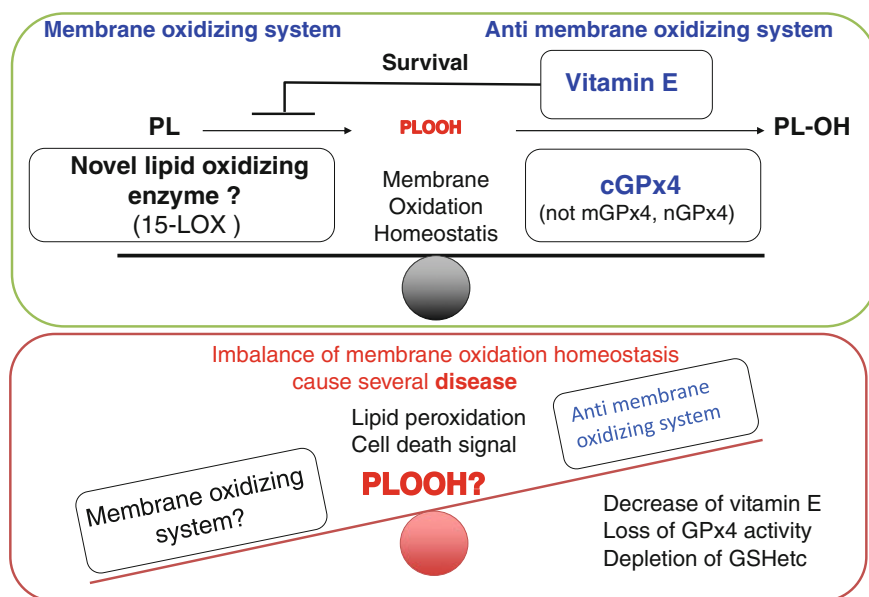


Fig. 3 Imbalance of membrane oxidation homeostasis cause several diseases in cGPx4 knockout mice and cGPx4 depleted cells. GPx4 knockout mice is early embryonic lethal at 7.5 dpc. GPx4 KO embryo could not form Inner cell Mass (ICM), but addition of vitamin E could rescue it. cGPx4 is required for the survival for the normal embryogenesis, since mGPx4 and nGPx4 KO mice normally grow. Testis, Liver specific GPx4 KO mice display the cell death of spermatogenic cells and hepatocyte, but administration of vitamin E diet could rescue the cell death in tissues of mice. 15-Lipoxygenase (15-LOX) directly could oxidize phospholipid. However, GPx4 depletion in 15-LOX KO mice induce acute renal injury and GPx4 depletion in 15-LOX null-MEF cells induce the lipid peroxidation-dependent cell death, indicating that 15-LOX is not essential for lipid peroxidation-dependent cell death in mice. 15-LOX might be one of the candidate for lipid peroxidation-dependent cell death in 15-LOX expressing cells. Thus, imbalance of membrane oxidation homeostasis (membrane oxidizing system vs. anti membrane oxidizing system) cause the oxidative stress related diseases in animal and human. PLOOH phospholipid hydroperoxide

oligoasthenozoospermia were consistent with that of spermatocyte-specific all GPx4 KO mice, indicating that low production of spermatozoa in human infertile patient is due to the defect of spermatogenesis by the deficiency of cGPx4 in testis (Imai et al. 2009).

Sedaghatian-type spondylometaphyseal dysplasia (SSMD) is a neonatal lethal form of spondylometaphyseal dysplasia characterized by severe metaphyseal chondrodysplasia with limb shortening, platyspondyly, cardiac conduction defects, and central nervous system abnormalities. By whole exome sequencing of a child affected with SSMD and her unaffected parents, two rare variants of GPx4 that the mutation results in a frameshift and premature truncation of GPx4 were identified (Smith et al. 2014).

These results indicate that truncating mutation in GPx4 in two families affected with SSMD supports the pathogenic role of mutated GPx4 in this very rare disease.

GPx4 is one of the selenoproteins in human. Selenocysteine insertion sequence-binding protein 2 (SBP2) is essential for the biosynthesis of selenoproteins including GPx4. Subjects with mutations in the SBP2 gene have decreased levels of many selenoproteins, resulting in a several phenotype with high lipid peroxidation in blood since they have low levels of antioxidant activity such as GPx1 and GPx4. Treatment of the vitamin E for 2 years to the subjects with SBP2 mutation reduced lipid peroxidation product levels to the control subjects, indicating that vitamin E treatment effectively inhibits the generation of lipid peroxidation products in human (Saito et al. 2015).

To clarify the mechanism of cell death by depletion of GPx4, we established GPx4-LoP TG/KO MEF cells (TK cells) from GPx4-loxP TG/KO mice and tamoxifen inducible GPx4 depleted TK cells (ETK cells) by infection of retrovirus of estrogen receptor binding Cre (CreERT2) (Imai et al. 2009; Imai 2010). Conrad's group already established the tamoxifen inducible GPx4 depleted MEF cells (Pfa1 cells) from GPx4 flox/flox mice (Seiler et al. 2008).

Retrovirus-mediated depletion of GPx4-LoxP TG transgene in EK cells resulted in the loss of all GPx4 protein 2 days and cell death 4 days after infection of Cre-expressing retrovirus. In tamoxifen inducible all GPx4 depleted TK cells and Pfa1 cells, addition of tamoxifen induce loss of all GPx4 24 h and cell death 3 days after tamoxifen treatment. As characteristics of GPx4-deficient cell death in MEF cells and mice, the time required for lethality is very long. Addition of Trolox, vitamin E derivative, or vitamin E could rescue GPx4 depleted cell death by retrovirus transfection in TK cells and addition of tamoxifen in ETK cells and Pfa1 cells. Vitamin E rescued GPx4 null-MEF cells could normally grow.

Retrovirus-infection of mGPx4, cGPx4, nGPx, cGPx4 (cys) and other antioxidants such as GPx1, SOD1, and SOD2 demonstrated that cGPx4 most effectively could rescue the all GPx4 depleted cell death, but not cGPx4 (cys) that mutated an enzymatic active site selenocysteine to cysteine, mGPx4, nGPx4, GPx1, SOD1, and SOD2 (Imai et al. 2009; Imai 2010).

These results demonstrated that the suppression of phospholipid peroxidation by cGPx4 and vitamin E is required for the growth and survival in MEF cells as the same as in mice. GPx4 depletion by genetic system induces lipid peroxidation-

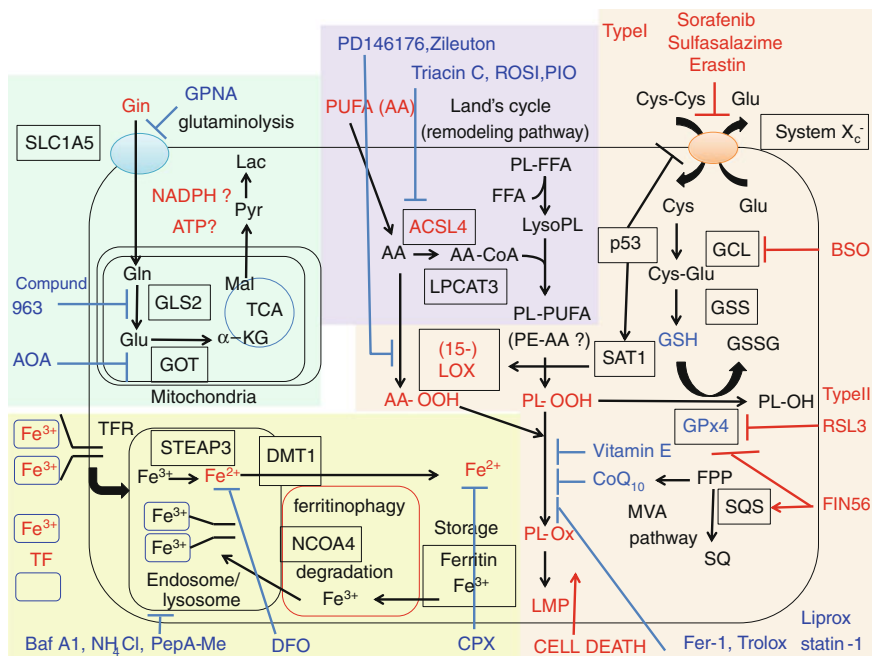
dependent, and caspase 3 independent novel cell death, since broad caspase-inhibitor, Z-Bad-FMK could not suppress GPx4 depleted cell death.

Lipid peroxidation by loss of GPx4 was generated in the cytosol sites of organelle, but not in mitochondria, as the signal for novel non-apoptotic cell death (Imai 2010, 2011).

6 Ferroptosis by Oncogenic Mutated Ras-Selective Lethal Compounds

The RAS family of small GTPase (HRAS, NRAS, and KRAS) is commonly mutated in cancer and several groups have searched for small molecules that are selectively lethal to cells expressing oncogenic mutated RAS proteins. Stockwell's group isolated two classes of novel oncogenic RAS-Selective Lethal (RSL) small molecules named erastin (of RAS and ST (erastin) (Yagoda et al. 2007) and RAS-selective Lethal 3 ((1S, 3R)-RSL3) (Yang and Stockwell 2008). Both compounds killed engineered human tumor cells expressing oncogenic HRAS^{V12} at lower concentrations than isogenic cells expressing wild-type HRAS. Erastin and RSL3 treatment could induce cell death very quickly for 8–12 h in RAS-mutated cancer cells. Erastin and RSL3 treatments do not trigger morphological changes or biochemical processes consistent with apoptosis such as chromatin condensation, DNA ladder formation, and caspase 3 activation. Erastin and RSL3 induced cell death is not inhibited by caspase-inhibitor (Z-VAD-FMK), by a necroptosis inhibitor (necrostatin-1) and by an inhibitor of autophagy (chloroquine, 3-methyladenine). Neither mitochondrial ROS production nor Ca²⁺ influx is necessary for cell death. Erastin treatment resulted in a unique “dysmorphic” mitochondrial phenotype observable by transmission electron microscopy. Erastin and RSL3 induced cell death is effectively suppressed by the iron chelators, DFO (deferrioxamine), and ciclopirox (CPX) as well as by the lipophilic antioxidants, Trolox (a soluble vitamin E analog), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), liproxstatin-1, and ferrostatin-1 (Fer-1). These results indicated that erastin- or RSL3-induced cell death is the iron-dependent oxidative novel non-apoptotic cell death, named “ferroptosis” (Dixon et al. 2012). Ferrostatin-1 (Fer-1) antioxidants was isolated as a ferroptosis specific inhibitor by Stockwell's group. Ferrostatin-1 could suppress the ferroptosis by erastin and RSL3, but not apoptosis and necroptosis (Dixon et al. 2012).

The elucidation of target molecule for Erastin and RSL3 provides that ferroptosis inducer can be divided into two classes of small molecule substrates. Class 1 ferroptosis inducers include erastin, sulfasalazine (SAS), artemisinin and its derivatives, which can inhibit system X_c⁻ that transport cystine into cells (Dixon et al. 2012). Class 2 ferroptosis inducers include Ras-selective lethal 3 compound (RSL3), ML162 (DPI17), DPI20, DP112, DP113, etc., which can directly inhibit glutathione peroxidase 4 (GPx4) activity and ultimately lead to an accumulation of lipid peroxides without the decrease of GSH (Yang et al. 2014) (Fig. 4).



6.1 Inhibition of System X_c^- Leads to Ferroptosis (Type I)

System X_c^- is a membrane Na^+ -dependent cysteine-glutamate exchange transporter, which is a disulfide-linked heterodimer composed of a light-chain subunit (xCT, SLC7A11) and a heavy-chain subunit (CD98hc, SLC3A2) (Sato et al. 1999). While system X_c^- transports intracellular glutamate to the extracellular space, it transports extracellular cystine into cells, which is the transformed into cysteine for glutathione (GSH) synthesis. Erastin acts as a direct inhibitor of system X_c^- function (Dixon et al. 2012) (Fig. 3). Erastin treatment leads to significant depletion of intracellular glutathione by inhibition of cellular uptake of cystine, since cystine is a key molecule for glutathione (GSH) synthesis. This effect could be inhibited by β -mercaptoethanol, because β -ME can enhance cysteine uptake through other pathways. Glutamate–cysteine ligase (γ -glutamylcysteine synthetase, GCL) is the rate-limiting first enzyme in the two step synthesis of glutathione (GSH). Blockage of GCL by buthionine-(S,R)-sulfoximine (BSO) can induce depletion of GSH, leading to ferroptosis that is prevented by vitamin E, Ferrostatin-1, and DFO. These results indicated that inhibition of GSH synthesis is required to trigger ferroptosis in some cells (Seiler et al. 2008; Angeli et al. 2014). GPx4 can reduce direct toxic lipid peroxide (PLOOH) to nontoxic lipid alcohols (PL-OH) using GSH as a cofactor. Depletion of intracellular glutathione by erastin induced accumulation of lipid peroxidation detected by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) and BODIPY-C11. Therefore, inhibition of system X_c^- by erastin suppresses GPx4

◀**Fig. 4** Regulation of ferroptosis by oncogenic mutated Ras-Selective Lethal compounds. Ferroptosis is an iron-dependent form of cell death involving the generation of phospholipid peroxidation (PL-Ox) in Ras-mutated cancer cells induced by oncogenic mutated Ras-selective lethal compounds (Type I and Type II RSL) as shown in pink zone. In cancer cells, cystine (Cys-Cys) uptake for maintaining of intracellular glutathione (GSH) via system X_c⁻ (pink zone), Iron uptake for production of iron containing enzymes, mitochondrial enzymes, p450 and lipoxygenase (LOX) via transferrin (TF) receptor (TFR) (yellow zone), and Glutamine (Gln) uptake for glutaminolysis in metabolic changes for energy production via SLC1A5 (green zone) were enhanced as compared to normal cells. Glutathione peroxidase 4 (GPx4) could reduce phospholipid hydroperoxide (PLOOH) to hydroxyl phospholipid (PLOH), oxidizing GSH to glutathione disulfide (GSSG) in the process. GPx4 is a key regulator of ferroptosis, since loss of GPx4 activity accumulates ferrous iron (Fe²⁺)-dependent oxidized phospholipid (PL-Ox), leading to the loss of membrane permeability (LMP) and cell death. Type I ferroptosis inducer, erastin, sulfasalazine and sorafenib, inhibit the cystine transporter, system X_c⁻, resulting in the decrease of GSH, loss of GPx4 activity to ferroptosis. Type II ferroptosis inducer, RSL3 and FIN56 directly bound to GPx4 or induce the degradation of GPx4, resulting in iron-dependent phospholipid peroxidation to ferroptosis without the decrease of GSH. Ferroptosis was inhibited by iron-chelator DFO (deferrioxamine) and CPX (ciclopirox), and anti-lipid peroxidation compounds such as vitamin E, coenzyme Q10 (coQ10), Trolox and ferroptosis specific inhibitor, ferrostatin-1 (Fer-1) and liproxstatin-1 (pink zone). Intracellular Fe²⁺ availability was regulated by degradation of extracellular transferrin (TF) bound Fe³⁺ incorporated by transferrin receptor (TFR) and intracellular ferritin bound Fe³⁺ in endosome / lysosome containing ferritinophagy (yellow zone). Sensitivity of phospholipid peroxidation in membrane is regulated by the quality of lipid membrane (Lipoquality) that means the change of the content of polyunsaturated fatty acid (PUFA) such as arachidonic acid (AA) in phospholipid membrane (violet zone). Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) could modulate the content of AA in phospholipid by remodeling pathway (Lands' pathway). The decrease of PL-PUFA is correlated to the sensitivity of lipid peroxidation and ferroptosis. Ferroptosis is required for transferrin and glutamine (Gln) in the culture media. Glutamine produces NADPH and ATP during glutaminolysis pathway, but this requirement of glutaminolysis remained to be resolved (green zone). p53 suppress the transcription of system X_c⁻ transporter and induction of spermidine/spermine *N*-acetyltransferase 1 (SAT1), up-regulator of 15-lipoxygenase (15-LOX). Other small molecule inducers of ferroptosis are indicated in red, while suppressors of ferroptosis are in blue. GCL Glutamate cysteine ligase, GSS glutathione synthetase, Cys, cysteine, Glu glutamate, Gln glutamine, α-KG alpha-ketoglutarate, GPNA l-g-glutamyl-p-nitroanilide, AOA amino-oxyacetate, GLS2 glutaminase, GOT glutamine oxaloacetate aminotransferase, TCA tricarboxylic acid, Mal Malic acid, Pyr pyruvic acid, Lac lactic acid, DFO deferrioxamine, CPX ciclopirox. Baf.A1 Bafriomycin A, PepA-Me pepstatin A-methyl ester, STEAP3 six-transmembrane epithelial antigen of the protease 3, DMT1 divalent metal transporter 1, NCOA4 cargo receptor for ferritinophagy, ROSI rosiglitazone, PIO pioglitazone, BSO buthione-(S,R)-sulfoximine, MVA Mevalonate, FPP Farnesyl diphosphate, SQS squalene synthase, SQ squalene

activity to cause accumulation of lethal lipid peroxides and to initiate the execution of ferroptosis. Erastin, sulfasalazine (Gout et al. 2001; Skouta et al. 2014) and sorafenib (Louandre et al. 2013; Lachaier et al. 2014) are ferroptosis inducers through this mechanism.

On the other hand, erastin is also reported to bind to voltage-dependent anion channels (VDAC2 and VDAC3) on the mitochondria to alter membrane permeability and the ion selectivity of the channels, leading to the mitochondrial dysfunction and oxidant release in ferroptosis (Yagoda et al. 2007). Erastin also leads

to activation of an endoplasmic reticulum stress response and up-regulation of CHAC1 (cation transporter homolog 1) during ferroptosis (Dioxn et al. 2014). However, this relationship between these events in mitochondria and ER and ferroptosis remained to be elucidated.

The p53 protein has been well characterized for its response to various cellular stresses, including of growth arrest, senescence and apoptosis. p53 inhibits cystine uptake and sensitizes cells to ferroptosis by repressing expression of SLC7A11, a key component of the system X_c^- transporter with oxidants. p53 binds to the SLC7A11 locus at a specific p53 response element within the 5' untranslated region (Jiang et al. 2015). p53 (3KR, R117, R161, and R162), an acetylation-defective mutant that fails to induce cell-cycle arrest, senescence and apoptosis, fully retains the ability to regulate SLC7A11 expression and induce ferroptosis upon reactive oxygen species (ROS)-induced stress. Acetylation of K98 of p53 is required for repression of transcription of SLC7A11 and induction of ferroptosis (Wang et al. 2016). p53 (4KR, R98, R117, R161, R162) could not induce tumor suppression. These results involve a new mode of tumor suppression based on p53 regulation of cystine metabolism, ROS responses and ferroptosis.

Addition of high concentration of Glu in neuronal cell lines induces the inactivation of system X_c^- and inhibition of uptake of cystine, leading to the GSH depletion and oxidative cell death (oxidative glutamate toxicity) named "Oxytosis" (Tan et al. 2001). Oxidative glutamate toxicity can be blocked by Ferrostatin-1, indicating that this cell death is ferroptosis (Dixon et al. 2012; Liu et al. 2015; Kang et al. 2014). GPx4 can suppress the glutamate-induced oxytosis in the retina (Sakai et al. 2015a). However, several reports indicated that mechanism of downstream of ROS accumulation in oxidative glutamate toxicity was different from ferroptosis (Henke et al. 2013; Tobaben et al. 2011). For example, extracellular Ca^{2+} influx, BH3 interacting domain death agonist (Bid) mediated mitochondrial damage and nuclear translocation of apoptosis inducing factor (AIF) are required for oxidative glutamate toxicity, but not required for ferroptosis. These differences remain to be resolved.

6.2 Inhibition of Chemical Inactivation of GPx4 Leads to Ferroptosis (Type II)

Type II inhibitor of ferroptosis such as (1S,3R)-RSL3 could inhibit ferroptosis without the decrease of GSH, whereas Ferrostatin-1, vitamin E, and DFO suppressed RSL3 induced ferroptosis (Yang and Stockwell 2008) (Fig. 4). GPx4 was identified as a major target protein of (1S,3R)-RSL3 by affinity purification with RSL3 and identification of sequence of amino acids. (1S,3R)-RSL3 contains an electrophilic chloroacetamide and covalently interacts with the active site "selenocysteine" of GPx4 to inhibit its enzymatic activity (Yang et al. 2014, 2016). Treatment of RSL3 induced the peroxidation of phospholipid that is prevented by

vitamin E, ferrostatin-1, and DFO. GPx4 overexpression suppresses the RSL3-induced ferroptosis, whereas GPx4 knockdown enhances the sensitivity for RSL3-induced ferroptosis, suggesting that inhibition of GPx4 activity is a major contributor to ferroptosis. Thus, GPx4 is currently believed to be a key regulator in ferroptosis induced by erastin and RSL3.

Another screening of caspase-independent lethal compounds revealed new six specific ferroptosis inducers (Shimada et al. 2016). FIN56, one of six ferroptosis inducer, induced cell death was prevented by vitamin E, DFO, and U0126, MEK inhibitor in Ras-mutated cancer cell lines (BJeIR). FIN56 treatment induced cell death for 48 h slowly that RSL3 at 8 h. But FIN56 treatment produces lipid peroxidation 3 h, whereas RSL3 produces it 1 h. FIN56 could not bind to GPx4. But FIN56 promoted degradation of GPx4, whereas a proteasome inhibitor MG132 did not inhibit FIN56-induced cell death. 5-(tetradecyloxy)-2-furoic Acid (TOFA), an inhibitor of acetyl-CoA carboxylase (ACC), inhibited the degradation of GPx4. TOFA also suppresses FIN56-induced cell death and lipid peroxidation. ACC is an enzyme involved in fatty acid synthesis. But ACC itself was not direct target of FIN56. Chemoproteomics analysis revealed that FIN56 binds and activates squalene synthase (SQS), an enzyme involved in the cholesterol synthesis, in a manner independent of GPx4 degradation. Squalene synthase inhibitors blocked FIN56-induced ferroptosis and increased the mevalonate metabolite such as farnesyl pyrophosphate (FPP) and coenzyme Q₁₀ (CoQ₁₀), an electron carrier in the mitochondrial respiratory chain and endogenous antioxidant. Supplementation of FPP or CoQ₁₀ suppresses the lethality of FIN56 and RSL3. These results indicate that mechanism of cell death by FIN56 involves two distinct pathways, GPx4 degradation pathway that requires the activity of ACC, and activation of SQS, that leads to CoQ₁₀ depletion as antioxidants. GPx4 degradation was also observed in RSL3-induced ferroptosis in MEF cells, indicating that RSL3 might involve another target protein except for GPx4 like FIN56 (Kagan et al. 2016; Shimada et al. 2016) (Fig. 4).

6.3 *The Role of Iron Homeostasis in Ferroptosis*

Iron is essential for the execution of ferroptosis. Erastin- and RSL3-induced ferroptosis can be inhibited by iron chelator of membrane impermeable (DFO) and membrane permeable (CPX) (Dixon et al. 2012; Yang et al. 2014).

Nutrient starvation in growth medium containing glucose but lacking both amino acids and serum, induces apoptosis in mouse embryonic fibroblasts (MEFs), whereas nutrient starvation medium in the presence of serum change from the apoptotic cell death to ferroptosis. Cystine deficiency of medium in the presence of serum causes ferroptosis in MEF cells, leading to the decrease of intracellular glutathione. This is interesting system that can change apoptosis to ferroptosis with or without serum (Gao et al. 2015). Two serum factors, the iron-carrier protein transferrin and amino acid glutamine, were identified as the inducer of ferroptosis

by cystine deficiency and erastin. Ferroptosis by erastin or cystine deficiency is inhibited by genetic silence of transferrin receptor that required for the uptake of transferrin-iron complexes into cells (Gao et al. 2015; Yang and Stockwell 2008). Supplementing the medium with iron-bound transferrin or a bioavailable form of iron (ferric ammonium citrate) accelerates erastin-induced ferroptosis (Gao et al. 2015; Dixon et al. 2012) and GPx4-depleted cell death (Sakai et al. 2016a). These results confirmed that the requirement for iron in ferroptosis (Fig. 4).

L-glutamine (L-Gln) is the most abundant amino acid in the body. Proliferating cells use L-Gln both as a nitrogen source for the biosynthesis of nucleotides, amino acids, and hexamine and as an important carbon source for the tricarboxylic acid (TCA) cycle. Pharmacological inhibition of SLC1A5, L-Gln receptor component by L-g-glutamyl-p-nitroanilide (GPNA) or RNAi knockdown of these receptor prevented ferroptosis induced by cystine deficiency. Gln is converted into glutamate (Glu) by glutaminases (GLS). An inhibitor of GLS, Compound 968, and RNAi knockdown of GLS2 but not GLS1 blocked ferroptosis induced by erastin or cystine deficiency. Downstream of glutaminolysis, glutamate can be further converted into α -ketoglutarate (α -KG) either transaminase-mediated transamination or by glutamate dehydrogenase (GLUD1)-mediated glutamate deamination. The amino-oxyacetate (AOA), a pan inhibitor of transaminases and RNAi of transaminase GOT1 inhibited ferroptosis induced by erastin and cystine deficiency, but not GLUD1 RNAi could not suppress. The glutamine-fueled intracellular metabolic pathway, glutaminolysis, involved important roles in ferroptosis in cancer cells. Inhibition of glutaminolysis can reduce heart injury triggered by ischemia/reperfusion. These results indicated that glutaminolysis is essential for ferroptosis. NADPH and ATP, which are produced by glutaminolysis might be related to the execution of ferroptosis in cancer cells (Fig. 4).

The Ras-mutated cancer cells accumulate iron by modulating expression levels of ferritin and the transferrin receptor (Yagoda et al. 2007). Iron that is taken up by the transferrin receptor bound to transferrin-Fe(III)₂ is routed into the endosome/lysosome pathway. The ferric ion, Fe(III), that is released from transferrin is reduced by an endosomal reductase activity (e.g., six-transmembrane epithelial antigen of the prostate 3) prior to export of ferrous ion, Fe(II), by a transporter such as divalent metal transporter1 (DMT1). Most cellular iron is stored as a component of ferritin in the Fe (III) form (Hentze et al. 2010). Recent studies showed that selective autophagic degradation of ferritin by the cargo receptor NCOA4 that recognized ferritin promotes iron release within lysosomes, and this ferritinophagy is important for cellular iron homeostasis for the increase of free iron (Mancias et al. 2014; Dowdle et al. 2014; Bellelli et al. 2016). The cellular labile iron pool might be used to control intracellular iron homeostasis through its own redox activity or to provide iron constituents for functional proteins such as heme enzymes including P450, cyclooxygenase, NADPH oxidases (NOXs), and non-heme enzyme including lipoxygenase.

Bafiromycin A1 (Baf A1), an inhibitor of vacuolar H⁺-ATPase, the lysosomal aspartic protease inhibitor pepstatin A-methyl ester (PepA-Me), ammonium chloride (NH₄Cl) that acts to neutralize acidic organelle such as lysosome blocked

erastin and RSL3-induced ferroptosis and ROS production. PepA-Me caused both an increase in ferritin protein levels and a decrease in iron content, indicating this may block ferroptosis by preventing autophagic degradation of ferritin, whereas Baf A1 and NH_4Cl inhibit the iron uptake by transferrin (Torii et al. 2016).

Inhibition of ferritinophagy by prevention of autophagy such as ATG13 and ATG3 or knockdown of cargo receptor NCOA4 for ferritinophagy, abrogated the accumulation of ferroptosis-associated cellular labile iron and ROS as well as ferroptosis. These results indicated endosomes/lysosomes and NCOA4 mediated ferritinophagy contribute to ferroptosis by modulating cellular iron homeostasis and ROS production (Gao et al. 2016; Hou et al. 2016) (Fig. 4).

Screening of shRNA suppressors of erastin in U2-OS cells revealed that PHKG2, the catalytic subunit of the PHK (phosphorylase kinase) complex, that activates glycogen phosphorylase (GP) to release glucose-1-phosphate from glycogen. PHK inhibitor, K252a inhibit erastin-induced ferroptosis, but not two glycogen phosphorylase inhibitors could not prevent, indicating that the metabolic pathway of glycogen breakdown is not responsible for the suppression of erastin-induced ferroptosis. Knockdown of PHKG2 suppressed the production of ROS and the availability of ferrous iron, indicating that novel iron regulatory function of PHKG2 is responsible for modulating sensitivity to erastin (Yang et al. 2016). Thus, iron availability to lipoxygenase by PHKG2 and lysosomal ferritin degradation is essential for erastin-induced ferroptosis.

Other pathways regulating the iron availability are also involved in the modulation of ferroptosis. Heat shock protein HSPB1 inhibition or heme oxygenase-1 (HO-1) induction enhanced the sensitivity of erastin-induced ferroptosis by the increase of intracellular free iron (Sun et al. 2015; Kwon et al. 2015).

6.4 Regulation of Phospholipid Peroxidation Modulate the Sensitivity of Ferroptosis

Lipid peroxidation occurs through the mechanism of both an enzymatic lipoxygenase reaction and a non-enzymatic free-radical chain reaction by Fenton reactions. Fenton reaction is defined as the oxidation of organic substrates by a mixture of hydrogen peroxide and ferrous iron. The Fenton reaction is a key reaction in oxidation of phospholipids and no specificity for classes of phospholipids. Singlet molecular oxygen also participates in a non-enzymatic reaction, which yields lipid hydroperoxide (LOOHs) from cholesterol and esterified lipids such as phospholipids and cholesteryl esters (Hauck and Bernlohr 2016). Ferroptosis is an iron-dependent cell death, accumulating lipid peroxidation. GPx4 that could reduce phospholipid hydroperoxide is a regulator of ferroptosis. Vitamin E or Ferrostatin-1 could suppress peroxidation of phospholipid and cell death in ferroptosis. The current proposal model is that after GPx4 activity is lost, lipid hydroperoxide was generated in membrane and attacked with ferrous iron, resulting in formation of

lipid radicals and enhancement of lipid peroxidation to the lethal damage of membrane. Erastin and RSL3 treatment resulted in accumulation of several classes of phospholipid hydroperoxide such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and lysophospholipids and depletion of several polyunsaturated fatty acid (PUFA) such as arachidonic acid (AA, 20:4n-6), indicating that specific oxidized PUFA are cleaved from the glycerophospholipids by phospholipase A₂ and subsequently degraded or destroyed in plasma membrane permeability (Angeli et al. 2014; Dixon et al. 2015; Yang et al. 2016) (Fig. 4).

Recent reports demonstrated that depletion of acyl-CoA synthetase long-chain family member 4 (ACSL4) or lysophosphatidylcholine acyltransferase 3 (LPCAT3) that encode enzymes required for the reacylation of membrane lysophospholipid such as lyso PC (lysophosphatidylcholine) and lysoPE (Lysophosphatidylethanolamine) with arachidonic acid and other PUFAs (Hashidate-Yoshida et al. 2015), inhibits ferroptosis induced by RSL3 and erastin (Dixon et al. 2015; Doll et al. 2016; Kagan et al. 2016; Yuan et al. 2016). Cell membrane contains several classes of glycerophospholipids, which have numerous structural and functional roles in the cells. Polyunsaturated fatty acids, including arachidonic acid, eicosapentanoic acid, docosahexanoic acid, are located at the sn-2 (but not sn-1)-position of glycerophospholipids in an asymmetrical manner. Using acyl-CoAs as donors, glycerophospholipids are formed by a de novo pathway (Kennedy pathway) and modified by a remodeling pathway (Lands' pathway) to generate membrane asymmetry and diversity (Sindou and Shimizu 2009). ACSL4 has a marked preference for long-chain PUFAs such as arachidonic acid (AA) (Yan et al. 2015; Cho et al. 2001). The content of PUFA in biomembrane such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were complicatedly regulated by lysophosphatidylcholine acyltransferase (LPCAT)1-4 and lysophosphatidylethanolamine acyltransferase (LPEAT)1-2, remodeling enzymes and phospholipase A₂. The remodeling of arachidonic acids in PC, PE, and PI were controlled by acyl-CoA synthetase long-chain family member 4 (ACSL4) and LPCAT2, LPEAT 2, LPCAT3 and LPIAT (Fig. 4).

Conrad' group showed that ACSL4 KO cells are significantly resistant for RSL3 induced ferroptosis in Pfa1 cells (MEF cells), whereas only a mild protective effect was observed in LPCAT3 KO cells. Loss of ACSL4 gene inhibited RSL3 induced and tamoxifen inducible GPx4 depleted induced ferroptosis and peroxidation of phospholipid. Decrease of arachidonic acid (AA: 20:4) and adrenoyl acid (AdA: 22:4) containing PE (phosphatidylethanolamine) and PI (phosphatidylinositol) were markedly less abundant in ACSL4 KO cells than in WT cells, whereas no decrease was found in phosphatidylcholine and phosphatidylserine (Doll et al. 2016). Kagan' group showed that loss of ACSL4 suppressed the formation of double and triple-oxidized AA- and AdA containing PE species (15-OOH-AA-PE, 15-OOH-8OH-AA-PE, 15-OOH-9OH-AA-PE and 15-OOH-12OH-PE) in RSL3-induced ferroptosis, whereas the elevation of four oxidized PE was enhanced in RSL3 induced WT cells (Pfa1 cells) and kidneys of GPx4KO mice. Lipoygenase inhibitor (NCTT-956, PD146176, ML351, and Zileuton) could suppress the RSL3

induced ferroptosis in Pf1a cells, but not COX inhibitor (Piroxicam) and P450 inhibitor (MSPPOH) could not. Liperfluor fluorescence intensity that was detected for phospholipid hydroperoxide was elevated in endoplasmic reticulum in RSL3-induced ferroptosis. His group demonstrated that oxidation of AA or AdA containing PE by 15-lipoxygenase (15-LOX) in ER is required for the execution for RSL3-induced ferroptosis in Pf1a cells as a ferroptotic death signal (Kagan et al.). Lipidomics analysis by other groups showed that phosphatidylcholine with PUFA was depleted, whereas the levels of ceramide and lysoPC (lysophosphatidylcholine) accumulated during erastin-induced ferroptosis in HT-1080 fibrosarcoma cells. In G401 cells and HT1080 cells, 15-LOX knockdown could suppress the erastin-induced ferroptosis, but not RSL3-induced ferroptosis. Also lipoxygenase inhibitor, Zileuton, Baicalein, and PD146176 could suppress the erastin-induced ferroptosis in G401 cells, but not cyclooxygenase (COX) inhibitor indomethacin (Dixon et al. 2015). ACSL4 knockdown could suppress erastin induced ferroptosis in HepG2 and HL60 cells. ACSL4 overexpression could recover erastin-induced ferroptosis in no ACSL4 expressing erastin-resistant cell lines, LNCaP and K562 cells. Addition of 5-lipoxygenase inhibitor, Zileuton could suppress the erastin-induced ferroptosis in LNCaP cells (Yuan et al. 2016). In these cell lines, 5-lipoxygenase is important for erastin-induced ferroptosis. GPx4 and vitamin E directly could suppress the initial activation of lipoxygenase by lipid hydroperoxide as an activator (Imai et al. 1998). These results indicated that 15-LOX is one of the candidate for initial oxidation of phospholipid in RSL3 and erastin-induced ferroptosis (Fig. 4).

The emerging role of p53 in ferroptosis has been a topic of great interest. But it is unclear how p53 orchestrates its activities in multiple metabolic pathways into tumor suppressive effects (Jiang et al. 2015; Wang et al. 2016). Spermidine/spermine *N*¹-acetyltransferase 1 (SAT1) gene was identified as a transcription target molecule of p53. SAT1 is a rate-limiting enzyme in polyamine catabolism. Interestingly induction of SAT1 mRNA results in the lipid peroxidation and ferroptosis upon reactive oxygen species induced stress. SAT1 induction was correlated with induction of 15-LOX mRNA. SAT1-induced ferroptosis significantly suppressed in the presence of PD146176, specific inhibitor of 15-LOX. Thus, 15-LOX is the key regulator of ferroptosis induced by SAT1, p53 up-regulator molecule (Ou et al. 2016).

On the other hand, in 15-LOX knockout MEF cells, GPx4 depletion and erastin also could induce ferroptosis. Expression of 15-LOX is especially localized in inflammatory cells and cancer cells. GPx4 depletion in 15-LOX deficient MEF cells also could induce the lipid peroxidation-dependent cell death (Angeli et al. 2014).

These evidences demonstrated that 15-LOX is not essential for the execution for ferroptosis and other oxidation system might be required for lipid oxidation during ferroptosis in 15-LOX deficient cells.

ACSL4 was preferentially expressed in a panel of basal-like breast cancer cell lines and its expression appeared to be strongly correlated with sensitivity to ferroptosis induction by RSL3. GPx4-Acsl4 double knockout cells showed marked resistance to ferroptosis, however RSL3 induced ferroptosis in GPx4-Acsl4 double

knockout cells by supplementation of arachidonic acid. Interestingly GPx4-Acs14 double knockout MEF cells were viable and proliferated normally in cell culture. But we showed that GPx4 knockout MEF cells also was viable and proliferated normally in cell culture with vitamin E. From these results, we proposed that antioxidant balance of lipid peroxidation in phospholipid such as elevation of vitamin E and decrease of PUFA in phospholipid might regulate the fate of cells (Fig. 3). And disruption of imbalance of oxidation of phospholipid might cause lipid peroxidation-dependent cell death such as ferroptosis.

7 Lipid Peroxidation-Dependent Cell Death by GPx4 Gene Disruption

GPx4 depletion by Cre-LoxP system or knockdown strategy induced lipid peroxidation-dependent cell death in many cells such as T cells (Matsushita et al. 2015), corneal endothelial cells (Uchida et al. 2016), conjunctival epithelial cells (Sakai et al. 2015b), vascular endothelial cells (Sakai et al. 2016b) and keratinocytes (Sengupta et al. 2013). These cell death without anti-cancer drug such as erastin and RSL3 was also rescued by vitamin E or ferrostatin-1, anti-lipid peroxidation compounds.

Mechanism of cell death by depletion of GPx4 gene were mainly reported using tamoxifen inducible GPx4 depleted MEF cells (Pfa1 cells) from GPx4 flox/flox mice established by Conrad' group (Seiler et al. 2008; Angeli et al. 2014; Doll et al. 2016; Kagan et al. 2016). Pfa1 cells have the expression of 15-lipoxygenase (15-LOX). Tamoxifen inducible GPx4 depleted cell death in Pfa1 cells was inhibited by 15-LOX inhibitor, iron-chelator deferoxamine, ferrostatin-1 and vitamin E. RSL3, a direct inhibitor of GPx4, induced ferroptosis in Pfa1 cells also were inhibited by 15-LOX inhibitor, Baicalen, PD146176, and ACSL4 inhibitor, Triacsin C, pioglitazone, iron-chelator deferoxamine, ferrostatin-1 and vitamin E. Active site of 15-LOX contain ferrous irons and GPx4 inhibit the activation of 15-LOX. From these results, Kagan et al. demonstrated that GPx4 depletion in Pfa1 cells initiate 15-Lipoxygenase activation dependent phospholipid peroxidation and induced ferroptosis (Kagan et al. 2016; Doll et al. 2016).

Until now, the mechanism of ferroptosis by both RSL-induced and GPx4 depleted cell death by GPx4 gene disruption in MEF cells are considered to be the same cell death mechanism. However, our established tamoxifen inducible GPx4 depleted MEF cells (ETK cells) did not express 15-LOX, 12-LOX, and 5-LOX (Imai et al. 2009; Imai 2010). In ETK cells, GPx4 depleted cell death by tamoxifen treatment is not inhibited by apoptosis inhibitor Z-BAD-FMK, by knockdown of autophagy regulating protein ATG5 and necroptosis regulator RIP kinase 1, indicating that GPx4 depleted cell death is caspase-independent non-apoptotic cell death. In ETK cells, erastin and RSL3 also induced ferroptosis as reported previously, since these cell deaths could inhibit by iron chelator, ferrostatin-1 and

vitamin E. However, GPx4 depleted cell death by tamoxifen treatment in ETK cells could not be inhibited by 15-LOX inhibitor, Baicalen and ACSL4 inhibitor, Triacsin C and pioglitazone, iron-chelator deferoxamine, whereas ferrostatin-1 and vitamin E effectively suppressed the cell death. In ETK cells, erastin and RSL3 induce ferroptosis by 12 h, but tamoxifen treatment induced GPx4 depleted cell death 72–96 h after treatment although GPx4 expression could not be detected by 24 h. Lipid peroxidation was detected 6h after treatment of erastin in ETK cells and suppressed by iron chelator. However, lipid peroxidation by tamoxifen treatment was observed at 26 h early time before cell death at 72 h. Lipid peroxidation 26 h after tamoxifen treatment was suppressed by vitamin E, but not by iron chelator. Addition of scavengers for superoxide, hydrogen peroxide, and overexpression of antioxidant enzyme such as SOD1, SOD2, and GPx1 could not rescue GPx4-depleted cell death in ETK cells (Imai 2010). When vitamin E is added by 26 h after treatment of tamoxifen, GPx4-depleted cell death can be effectively inhibited, but the lethality can not be suppressed after 26 h, indicating iron-independent lipid peroxidation by 26 h is required for GPx4 cell death in ETK cells. Short hairpin RNA (shRNA) mediated knockdown of GPx4 in 15-LOX null-MEF cells is sufficient to induce cell death, and vitamin E also inhibited GPx4 deleted cell death in 15-LOX null-MEF cells. These results indicated that 15-LOX, independent and iron-independent lipid peroxidation is necessary for GPx4-depleted cell death in ETK cells.

Although RSL3 and erastin quickly induced cell death 12 h in ETK cells, GPx4 depletion by tamoxifen slowly induced cell death 72–96 h in ETK cells. One possibility of the differences of time for cell death between GPx4 gene disruption and RSL-compounds is that the existence of another accelerate pathway in ferroptosis by RSL except for GPx4 inactivation, since erastin can bind to VDAC in mitochondria and induce ER stress and RSL can bind to the several proteins except for GPx4. Recent works demonstrated that RSL3 could degrade GPx4 in MEF cells (Pfal cells) like FIN56, however whether protein degradation by RSL3 is specific for GPx4 or not remained to be solved (Kagan et al. 2016; Shimada et al. 2016). The other possibility is that the mechanism of GPx4 depleted cell death by GPx4 gene disruption is different from the mechanism of ferroptosis by erastin and RSL3.

In mouse erythroid precursor cells, inactivation of GPx4 induced to the accumulation of toxic lipid intermediates that covalently modify caspase-8 and trigger necroptosis in the absence of death receptor stimulation such as TNF α (Canli et al. 2016). GPx4 inhibition can sensitize cancer cells to apoptosis induced by second mitochondrial-derived activator of caspase (SMAC) mimetics, connecting ferroptosis to apoptotic cell death pathway (Dächert et al. 2016). These results demonstrated that differences of lipid peroxidation producing system such as specific enzyme and random oxidation by Fenton reaction, and producing local site in organelle in cells might execute different cell death signaling, such as apoptosis, ferroptosis, necroptosis, and novel lipid peroxidation cell death.

Thus, GPx4 could regulate several cell death pathways by suppression of phospholipid peroxidation in specific site or local site of organelle (Fig. 2).

8 Ferroptosis in Disease Model

Analysis of role of ferroptosis in pathological cell death has been enabled by the ferroptosis specific small molecule, Ferrostatin-1 (Fer-1) (Dixon et al. 2012), Liproxstatin-1 (Angeli et al. 2014) and vitamin E.

Erastin enhanced chemotherapy drug such as temozolomide, cisplatin, cytarabine/ara-C, and doxorubicin/Adriamycin in certain cancer cells (Yu et al. 2015; Chen et al. 2015; Yamaguchi et al. 2013). In vivo, erastin, piperazine erastin, and RSL3 inhibited tumor growth in a xenograft model (Yang et al. 2014; Sun et al. 2015). In a rat organotypic hippocampal slice culture model, glutamate-induced neurotoxicity was prevented by Fer-1 (Dixon et al. 2012). Fms-like tyrosine kinase3 (FLT-3, also termed CD135) is a cytokine receptor, that is important for the normal development of hematopoietic stem cells and progenitor cells. Inhibitors for Fms-like tyrosine kinase3 (FLT-3) and its downstream signaling molecule phosphoinositide 3-kinase α can suppress lipid peroxidation to inhibit ferroptosis in neuron (Kang et al. 2014).

In a Huntington's disease model, Fer-1 restored the number of healthy neurons by inhibition of ferroptosis (Skouta et al. 2014). Fer-1 significantly protected the death of developing oligodendrocytes from cystine deprivation (Skouta et al. 2014).

Fer-1 prevented lethality in a model of acute injury of freshly isolated renal tubules, implicating ferroptosis-mediated cell death by acute kidney failure (Skouta et al. 2014). Ferrostatin analog (SRS 16–86) inhibits acute ischemia-reperfusion injury and oxalate nephropathy related acute kidney failure (Linkermann et al. 2014). Inducible knockout of GPx4 in the kidney leads to ferroptosis, which contributes to acute kidney failure in mice (Angeli et al. 2014).

High dose of acetaminophen frequently cause acute liver failure. Fer-1 can inhibit acetaminophen induced ferroptotic cell death (Lorincz et al. 2015). Ischemia/reperfusion-induced liver injury can be prevented in mice by liproxstatin-1 (Angeli et al. 2014). Prevention of glutaminolysis and ferroptosis by compound 968, DFO or Fer-1 inhibits ischemia/reperfusion-induced heart injury ex vivo (Gao et al. 2015).

9 Conclusion and Prospective

Ferroptosis by erastin and RSL3 is an iron-dependent lipid peroxidation induced non-apoptotic cell death in RAS-mutated cancer cells. Erastin inhibits cystine transporter activity and induces the decrease of glutathione and GPx4 activity, resulting in iron or 15-LOX dependent lipid peroxidation induced cell death. 15-LOX is one of the candidates for initial lipid peroxidation in ferroptosis. Fenton reaction by ferrous iron enhances the propagation of phospholipid oxidation and degradation of membrane lipid. Ferroptosis inducer is important for therapy of

cancer. Identification of the downstream signaling pathway or executors of 15-LOX independent lipid peroxidation in ferroptosis remained to be solved.

GPx4 could directly reduce phospholipid hydroperoxide in specific organelle and regulate several signal transductions by analysis of GPx4 overexpressing cells.

Overexpression of mGPx4 inhibited apoptosis induced by mitochondrial death pathway. Overexpression of cGPx4 also suppressed the iron-dependent lipid peroxidation in membrane induced by erastin and RSL3, resulting in inhibition of ferroptosis. Where and how lipid peroxidation in organelle such as ER, Golgi, and plasma membrane is generated in ferroptosis by erastin and RSL3 remained to be elucidated.

Indeed, GPx4 is a regulator of ferroptosis by erastin and RSL3. But cell death by GPx4 gene disruption progresses extremely slower than ferroptosis induced by erastin and RSL3. It may be possible that the mechanism of cell death is different between ferroptosis by erastin and RSL3 and GPx4 depleted cell death by GPx4 gene disruption. Differences of site and enzymes of phospholipid oxidation in organelle might show differences of its downstream signal transduction of cell death between ferroptosis by erastin and RSL3 and GPx4 depleted cell death.

The phenotype of tissue specific GPx4 KO mice and cells is recovered with treatment of vitamin E. These results demonstrated that imbalance between lipid oxidation system and lipid peroxidation suppression system such as GPx4 and vitamin E causes several diseases in mice and human.

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