Review

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GLS and GLS2 Glutaminase Isoenzymes in the Antioxidant System of Cancer Cells

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Abstract: A pathway frequently altered in cancer is glutaminolysis where glutaminase (GA) catalyses the main step: the deamidation of glutamine to form glutamate and ammonium. There are two types of GA isozymes, named GLS and GLS2, which differ considerably in their expression patterns and can even perform opposing roles in cancer. GLS correlates with tumor growth and proliferation, while GLS2 may function as a context-dependent tumor suppressor. However, both isoenzymes have been described as essential molecules handling oxidant stress because of their involvement in glutathione production. We have reviewed the literature to highlight the critical roles of GLS and GLS2 to restrain ROS and regulate both cellular signaling and metabolic stress due to their function as indirect antioxidant enzymes and also by modulating both reductive carboxylation and ferroptosis. Blocking GA activity appears to be a potential strategy to dual activate ferroptosis and inhibit cancer cell growth in a ROS-mediated mechanism.

Keywords: cancer; ferroptosis; glutaminase; glutaminolysis; glutathione; metabolic reprogramming; oxidative stress; ROS

1. Introduction

Glutaminase (GA, EC 3.5.1.2) is the enzyme responsible for catalysing the conversion of glutamine (Gln) to glutamate (Glu) and represents the first step in glutaminolysis [1]. Gln has essential functions in cancer, providing amino acids, lipids, nucleotides, hexosamines, and polyamines, but also rendering metabolic energy in the form of adenosine triphosphate (ATP) or being used as a pleiotropic cell signaling molecule [2]. In addition, Gln is indispensable for synthesizing reduced glutathione (GSH), the most abundant intracellular antioxidant molecule and the main player of the GSH-antioxidant system, formed by GSH, oxidized glutathione (GSSG), glutathione reductase (GR) and glutathione peroxidases (GPXs) [3]. While Glu affords resources for GSH synthesis, limited oxidation of Gln through glutaminolysis restricts oxidative phosphorylation (OXPHOS) and mitigates reactive oxygen species (ROS) levels that otherwise are harmful to cells [2]. Nonetheless, ROS can cause damage to cancer cells when levels are excessive but are indeed a needed signaling mechanism for tumor homeostasis [4]; hence, ROS levels are precisely regulated by a set of antioxidant enzymes, including indirectly the function of GA isoenzymes [1]. The key role of antioxidant systems and ROS' balance can be exploited for the design of novel strategies to target cancer [2,3] and will be discussed in this review.

2. Glutaminase Isoenzymes in the Control of Cancer Redox Homeostasis

Metabolic alterations observed in cancer are a consequence of the dysregulation of the expression of metabolic enzymes such as GA isoenzymes [5]. In mammals, including humans, the

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GLS gene encodes two isoforms, the largest kidney-type glutaminase (KGA) and glutaminase C (GAC), the latter usually being the variant most frequently overexpressed in cancers. Both isoforms can be collectively called GLS isoenzymes [6]. On the other hand, the *GLS2* gene encodes two more isoforms, glutaminase B (GAB) and the shorter liver-type glutaminase (LGA), while both can be referred to as GLS2 isoenzymes [7]. Secondary functions of these metabolic enzymes have been shown to be linked with several molecular mechanisms affecting cancer, being sharply different for GLS and GLS2. Therefore, GAs have been related to both cancer progression or antitumoral effects by altering the signaling, regulation, and the oxidative status of cancer cells (Tables 1 and 2).

Table 1. GLS in the control of redox status in cancer.

Molecular mechanism(s)/Impact in cancer	Cancer type(s)	*In vitro* model(s)	*In vivo* model(s)	Reference
NRF2[1] caused tumor growth through a mechanism that includes GLS overexpression	Breast	BT474, SKBR3	Nude mice in orthotopic models	[8]
Gln-independent cells lowered GLS, increased oxidative stress and enhanced resistance to drugs undergoing EMT[2]	Breast (TNBC[3])	MDA-MB-231, SUM149, 4T1	Female athymicnude xenografted mice	[9]
GLS inhibition by CB-839 synergistically works with the inhibition of ERR◎◎ blocking NADPH[5] synthesis and decreasing tumor growth	Breast (TNBC[3])	MDA436	None	[10]
Iron oxidative nanoparticles coupled to GLS inhibitor CB-839 both increased ROS and decreased GSH, boosting DNA oxidative damage and cancer cell death	Breast (TNBC[3])	MDA-MB-231	Mice injected with tumor cells and iron-CB-839 nanoparticles	[11]
AMPK[6] activated NRF2[1] and its target proteins to allow tumor cells to grow and to maintain their redox status through GLS, supporting anchorage-independent cancer cell survival	TNBC[3], Liver, Pancreas, Skin	MDA-MB-231, HepG2, BxPC-3, HT-1080, HaCaT	None	[12]
HYL001, a new drug with low IC_{50} against cancer cells and minimal toxicity towards normal cells and healthy mice, repressed GLS, reduced GSH, enhanced ROS and blunted TCA[7] cycle and OXPHOS[8]	TNBC[3], Liver (HCC[9])	4T1, H22	4T1 metastatic, and orthotopic models, in BALB/c mice	[13]
Induction of ARHI[10] resulted in oxidative stress, which was augmented following GLS inhibition by BPTES	Ovarium	SKOv3	Xenografted mice bearing SKOv3 cells	[14]
BPTES prevents the interaction between NQO2[11] and caveolin-1 of cancer cells that induce their metastatic activity	Prostate	LNCaP, C4, C4-2	None	[15]
Inhibition of GLS increased DNA oxidative damage and boosted susceptibility to ionizing radiation	Prostate	DU145, LNCaP	PC3 injected into nude NSG[12] mice	[16]
Mutant G6PD[13] melanoma cells increased glutaminolysis, which correlated with higher ROS levels, decreased NADPH, and lower GSH/GSSG ratios	Skin (melanoma)	M481, M214, A375	Melanoma cell lines injected in nude NSG[11] mice	[17]
GLS inhibition by CB-839 increased mitochondrial ROS, lowered the GSH/GSSG ratio, enhanced apoptosis, and diminished cancer growth *in vitro* and *in vivo*	Colon (CRC[14])	HCT116, C26	CRC[14] in BALB/c mice, and CRC[14] from patients in NSG[12] mice	[18]

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KRAS-mutant cells increased sensitivity to GLS inhibition by CB-839 through NRF2[1]	Pancreas	BxPC3, Panc-1, MiaPaC2	None	[19]
Oxidative stress increased glutaminolysis and the production of NADPH[5] and GSH	Pancreas (PDAC[15])	SW1990	Nude BALB/c mice	[20]
GLS succinylation is essential to maintain redox homeostasis measured as NADPH and GSH levels as well as ROS formation	Pancreas (PDAC[15])	SW1990	Male athymic nude BALB/c mice	[21]
Lactate imported by MCT1[16] maintained redox homeostasis via NRF2[1] and thereby cell viability following GLS inhibition by CB-839, which shortened GSH and increased ROS	Pancreas (PDAC[15])	T3M4, A818-6	PDAC[15] patients with a tumor disease staged T3N1M0	[22]
Following GLS inhibition by BPTES or CB-839, cancer cells showed decreased survival and more apoptosis associated with a lowered GSH/GSSG ratio, increased NRF2[1], and higher oxidative DNA damage	Kidney	SN12, 786-O	Mice with orthotopic injections and treated with CB-839	[23]
HSP60 silencing activated the MEK/ERK/c-MYC axis to evoke Gln addiction while increasing susceptibility to oxidative stress and GLS inhibition by BPTES	Kidney	786-O, 769-P	None	[24]
More aggressive tumors showed higher GLS activity, increased ROS levels, enhanced GSSG/GSH ratios, and accumulation of NAD^+ and $NADP^+$	Thyroid	B-CPAP, K1, TPC-1	None	[25]
GLS inhibition by CB-839 induced oxidative stress, i.e.: lowering GSH/GSSG, enhancing TrxR1[17], and diminishing tumor growth	Uterine, Cervix	CaSki, SiHa	Nude mice were xenografted with SiHa cells	[26]
CB-839 in combination with radiation increased oxidative stress and boosted DNA oxidative damage	HNSCC[18]	CAL-27, FaDu, HN5	Nude mice xenografted with CAL-27/HN5	[27]
Keap1-mutant cells displayed a robust sensitivity to GLS inhibition by BPTES and CB-839 through NRF2[1], increasing survival ratios	Lung	Human adenocarcinomas	Mice xenografted, treated with CB-839	[28]
Oxidative stress, by NRF2[1] malfunction, depleted Glu, which was lowered by GLS inhibition by CB-839, blocking cancer growth	Lung	LKR10/13	Mice subcutaneously injected with tumor cells	[29]
Selenite impaired GLS expression and increased the GSSG/GSH ratio	NSCLC[19]	A549	*Ex vivo* NSCLC[18]	[30]
TGF◎◎◎ induced EMT[2] that evoked sensitivity to BPTES, which reduced citrate levels and OXPHOS[8], lowering the cells' antioxidant capacity	NSCLC[19]	A427, NCI-H358	None	[31]
Lower levels of NADH, GSH and GSSG were concomitants to longer survival after using a combination of an HDAC6[21] inhibitor + CB-839 in *in vitro* and *in vivo* KRAS/LKB1[22] models (displaying high GLS activity)	NSCLC[19]	H23, H358	C57BL/6 mice inoculated with KRAS/TP53 or KRAS/LKB1 cancer cells	[32]
Activation of SAT1[23] increased GLS activity and GSH synthesis, ameliorating oxidative stress to support lung cancer cell proliferation, which was blocked by inhibiting GLS, resulting in ROS accumulation	NSCLC[19]	A549, PC9, H1650, H1792, H358, H1944	PC9 cells were subcutaneously injected into BALB/c nude mice	[33]

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GAC isoform was highly expressed, *versus* KGA isoform, in GBM[24] and more malignant astrocytomas	Brain	U87MG	Astrocytomas of different malignancy	[34]

[1]NRF2, nuclear factor erythroid 2-like 2; [2]EMT, epithelial to mesenchymal transition; [3]TNBC, triple negative breast cancer; [4]ERRα, estrogen-related receptor alpha; [5]NADPH, nicotinamide adenine dinucleotide phosphate; [6]AMPK, adenosine monophosphate-activated protein kinase; [7]TCA, tricarboxylic acid; [8]OXPHOS, oxidative phosphorylation; [9]HCC, hepatocellular carcinoma; [10]ARHI, Aplasia Ras homolog member I (also named DIRAS3); [11]NQO2, nicotinamide riboside:quinone oxidoreductase; [12]NSG, NOD-scid IL2Rgammanull; [13]G6PD, glucose 6-phosphate dehydrogenase; [14]CRC, colorectal cancer; [15]PDAC, pancreatic ductal adenocarcinoma; [16]MCT1, monocarboxylate transporter-1; [17]TrxR1, thioredoxin reductase 1; [18]HNSCC, head and neck cancer squamous cell carcinoma; [19]NSCLC, non-small-cell lung carcinoma; [20]TGFβ, transforming growth factor beta; [21]HDAC6, histone deacetylase 6; [22]LKB1, liver kinase B1; [23]SAT1, spermidine/spermine N1-acetyltransferase 1; [24]GBM, glioblastoma.

Table 2. GLS2 in the control of redox status in cancer.

Molecular mechanism(s)/Impact in cancer	Cancer type(s)	*In vitro* model(s)	*In vivo* model(s)	Reference
Inhibition of GA by compound 968 in apigenin-treated cells decreased NADPH and increased intracellular ROS levels, boosting apoptosis	Lung	H1299, H660	None	[35]
GLS silencing and *GLS2* overexpression induced oxidative stress, increased apoptosis and decreased cell migration	Brain (GBM[1])	SFxL, LN229, T98G	None	[36]
Silencing of *GLS* or overexpression of *GLS2* decreased oxidative status and boosted antioxidant enzymes	Brain (GBM[1])	LN229, T98G	None	[37]
GLS2 reduced the TMZ[2]-resistance of GBM[1] *in vitro* and *in vivo* through the long non-coding RNA ATXN8OS, which mediated ferroptosis and increased oxidative damage to lipids	Brain (GBM[1])	U251, U251TR	U251+GLS2 transfected cells were injected into the brains of nude mice	[38]
p73 transcriptionally activated GLS2, increasing serine and diminishing oxidative stress	NSCLC[3], Osteosarcoma	H1299, SaOs-2	None	[39]
GLS2 overexpression shortened oxidative stress by a GSH-independent mechanism	NSCLC[3]	CL1-0	None	[40]
Knock out of GLS2, a tumor suppressor in this study, reduced the GSH/GSSG ratio and increased oxidative damage to lipids during ferroptosis	Liver (HCC[4])	HepG2, HepG3, SKHep1	Injections of SKHep1 cells on the flanks of NSG[5] mice	[41]

[1]GBM, glioblastoma; [2]TMZ, temozolomide; [3]NSCLC, non-small-cell lung carcinoma; [4]HCC, hepatocellular carcinoma; [5]NSG, NOD-scid IL2Rgammanull.

GLS2 has been related to both tumor suppression properties and prooncogenic effects.Thus, its role in cancer is considered to be context-dependent and has been thoroughly reviewed recently [42]. However, its function as an oncogenic factor or a tumor suppressor has been related, at least partially, to its ability of modulating antioxidant capacity [1]. A mechanism implying its tumor suppressor ability is related to p53 and was first characterized in 2010 [43,44]. GLS2 expression was found to be induced by p53, and evoked oxygen consumption, mitochondrial respiration and ATP generation, increasing Glu, GSH and nicotinamide adenine dinucleotide (NADH) levels, and reducing ROS' amounts to protect cells from H_2O_2-induced apoptosis [43]. GLS2 protected cells from DNA oxidation by preventing the generation of 8-hydroxy-2'-deoxyguanosine, the main source of oxidation-associated mutagenesis [44]. In various cell models, GLS2 knockout works as an anticancer factor

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correlating with increased levels of ROS in several cancer types, i.e.: neuroblastoma [45], cervical cancer [46], and triple-negative breast cancer (TNBC) [47]. Although GLS2 increased GSH levels in neuroblastoma, its expression correlated with the proliferation and aggressiveness of neuroblastoma by an N-MYC-dependent mechanism [45]. In cervical cancer, following radiotherapy, tissues of radiosensitive patients showed diminished GLS2 levels and their lower GLS2 amounts were concomitant to decreased values of GSH, NADH and nicotinamide adenine dinucleotide phosphate (NADPH), as well as higher oxidative stress. Hence, GLS2 was associated with radioresistance of cervical cancer [46]. Of note, when GLS was either pharmacologically inhibited by CB-839 or genetically silenced, GLS2 mimicked its metabolic function as well as its impact on redox balance in a long number of TNBC cell lines [47]. Remarkably, after analyzing data from breast cancer tissues available from The Cancer Genome Atlas (TCGA) these authors found a correlation between higher GLS2 expression, increased epithelial to mesenchymal transition (EMT), and worse prognosis and mortality [47]. However, in a deeper study including 1075 patients samples from TCGA database, higher GLS/GLS2 ratios were correlated with increased EMT in breast cancer patients and worse survival [48].

3. Mitochondrial Metabolism of Glutamine in Cancer: Redox Balance

Metabolic reprogramming is one of the hallmarks of cancer [49]. Metabolic adaptations are essential for providing the hugely increased bioenergetics and biosynthetic demands to sustain tumor growth [50], but also for maintaining an adequate oxidative-reductive equilibrium for cancer cells' survival and proliferation [51]. Maximizing OXPHOS is among the metabolic traits most commonly altered in tumor cells [52]. In this metabolic shuffling, Gln is one of the most valuable playing cards [53]. Although Gln is not an essential amino acid, cancer cells usually promote its import and/or additional biosynthesis from other sources [54] through several oncogenes that activate glutaminolysis, i.e: c-MYC [55], KRAS [56], hypoxia-inducible factor 1 (HIF1) [57], isocitrate dehydrogenase 1 and 2 (IDH1/2) [58], epidermal growth factor receptor (EGFR) [59] or even tumor suppressor p53 [43]. Gln is the most abundant amino acid in circulating blood and in muscle [60]. Tumor cells can use Gln for biosynthetic purposes through the tricarboxylic acid cycle (TCA cycle) [61], allowing cancer cells to sustain TCA cycle activity and produce reductive equivalents as NADPH during proliferation [62]. Additionally, it may be used to produce NADH, flavin adenine dinucleotide ($FADH_2$), and ATP [63]. Oxidation of Gln equally sustains redox homeostasis by reduction of $NADP^+$ to NADPH by malic enzyme (ME1) due to the transformation of Gln-derived metabolites to pyruvate [53]. On the other hand, Gln is required for the synthesis of the main non-enzymatic antioxidant in the cell, the tripeptide GSH [64], that is synthetized from Glu, cysteine (Cys) and glycine (Gly) [5]. Glutamate production from glutamine is essential for GSH biosynthesis since it not only provides one of the three amino acids needed but because produced glutamate may also serve to import cysteine to the cell through the xCT transport system, and thus GLS and GLS2 become key enzymes in cancer [3], contributing to maintain redox homeostasis and balance ROS levels [65]. Additionally, TCA cycle rewiring causes changes in redox homeostasis, connecting cancer metabolism of amino acids and the redox biology of tumor cells [66].

From a strict metabolic point of view, the catabolism of Gln enables cells to sustain enough anaplerotic flux to utilize a wide amount of TCA cycle intermediates as precursors for macromolecular biosynthesis [67]. Of note, Gln's main role in many routes of intermediary metabolism that produce Glu and alpha-ketoglutarate (AKG) makes this amino acid an essential source of carbon for the TCA cycle [68]. Gln can be transformed to Glu by mitochondrial GA, which delivers Gln's amide group as cationic-free ammonium (Figure 1). Glu can be turned to AKG by Glu dehydrogenase (GDH), which releases the amino group as a free ammonium ion, or by mitochondrial aminotransferarses such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which transfers the amino group of Glu to pyruvate or to oxaloacetate to form alanine or aspartate, respectively [5]. AKG enters the TCA cycle and through sequential reactions generates oxaloacetate (OAA) [67]. Then, Gln can be a substrate for the synthesis of both OAA or, subsequently, lactate, through glutaminolysis, which accumulates reductive capacity as NADH and NADPH [68].

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Glutaminases are required by many cells to achieve maximal growth both *in vitro* and *in vivo* [69]. However, even Gln-addicted cells can reprogram their carbon metabolism from glucose to synthesize building blocks normally supplied by Gln [70]. The enzyme responsible for this form of glucose-dependent anaplerosis is pyruvate carboxylase (PC), fundamental for cell survival and proliferation of cells adapted to growth in low-Gln concentrations [57]. PC replenish OAA and generates citrate through reductive carboxylation, facilitating an alternative anaplerotic pathway in Gln-independent tumor cells [71]. Silencing GLS in tumor cells increases PC activity to compensate for reduced anaplerosis from Gln, affecting cancer growth [72]. Gln-dependent anaplerosis is energetically advantageous as PC requires ATP to generate OAA, while GLS is not ATP-dependent [70]. Gln catabolism through the TCA cycle forms reducing equivalents (NADH) for OXPHOS [72]. Anyhow, cancer cells can utilize bidirectional metabolism from Gln-derived AKG to synthesize citrate and generate reducing equivalents, which is advantageous for adapting to nutrient availability [71].

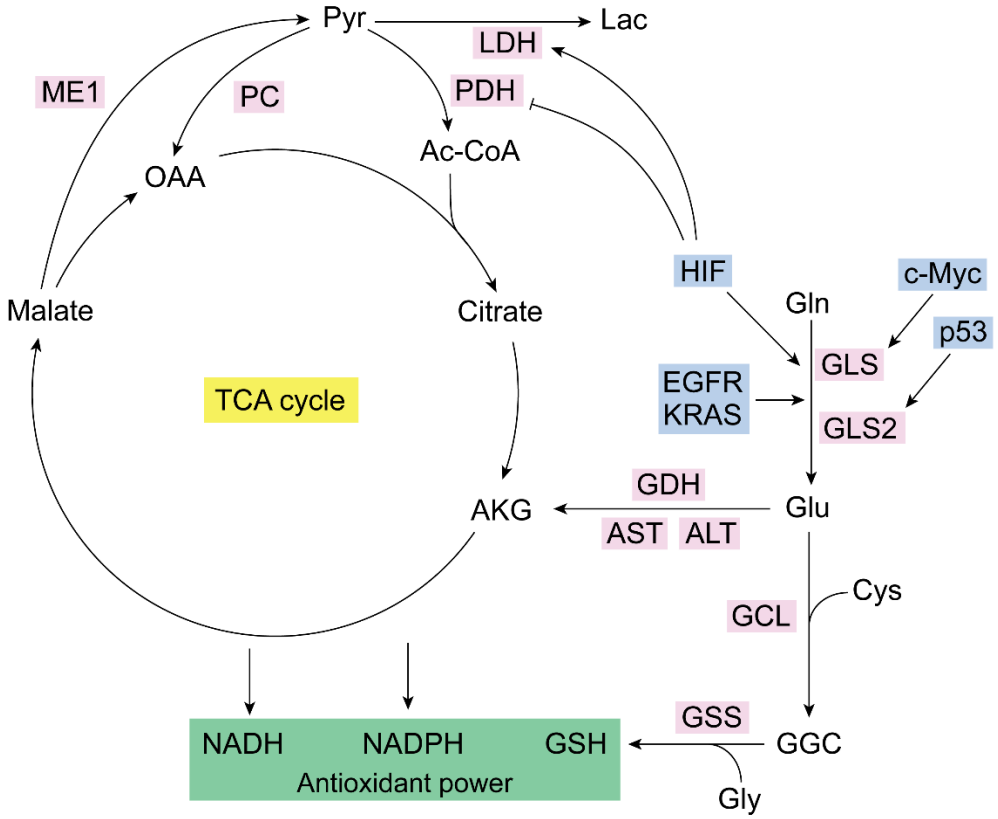

Figure 1. Glutaminolysis and antioxidant capacity. Glutaminase isoenzymes generate Glu from Gln. GLS is regulated by oncogene c-Myc and GLS2 is modulated by tumor suppressor p53. Glu together with Cys (via GCL), and Gly (via GSS) form, respectively, GGC, and GSH (the most important antioxidant molecule in the cell). Glutaminolysis is accurately regulated. IDH, KRAS, EGFR and HIF are some of its key modulators. HIF also controls LDH and PDH. NADPH generation does not occur in the TCA cycle, but in the IDH1/2 reaction that converts isocitrate to AKG. Ac-CoA, acetyl coenzyme A; AKG, alpha-ketoglutarate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cys, cysteine; EGFR, epidermal growth factor receptor; GCL: glutamate-cysteine ligase; GDH, glutamate dehydrogenase; GGC: *gamma*-glutamylcysteine synthetase; Gln, glutamine; GLS, glutaminase isoenzyme 1; GLS2. Glutaminase isoenzyme 2; Glu, glutamate; Gly, glycine; GSH, glutathione; GSS, glutathione synthetase; HIF, hypoxia-inducible factor; Lac, lactate; LDH, lactate dehydrogenase; ME1, malic enzyme 1; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; OAA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase.

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3.1. Glutaminase Can Trigger Reductive Carboxylation in Cancer

IDH1 isoform is located in the cytoplasm whereas IDH2 and IDH3 isoforms are placed in mitochondria. IDH1 and 2 catalyze the decarboxylation of isocitrate into AKG rendering NADPH, whilst IDH3 forms AKG, NADH and CO_2 from isocitrate [73]. The non-canonical process of formation of isocitrate from AKG and CO_2 is named reductive carboxylation, and it is fundamental in hypoxia conditions or when high synthesis of lipids is required [74]. Reductive carboxylation is catalyzed by IDH1/2 through the consumption of NADPH. Of note, NADPH and $NADP^+$ cannot cross the mitochondrial membrane, a fact that gives rise to the implication of IDH1/2 in balancing oxidative status in the cytosol and the mitochondria [63,73,74,75]. IDH1 has been related to the scavenging of ROS in the cytoplasm by generating NADPH in this cellular compartment [76]. Besides, cytosolic ROS' generation mediates the induction of reductive carboxylation through IDH1by negatively regulating a protein tyrosine phosphatase, which results in increased phosphorylation of IDH1 and, hence, its activation [75].

There are tumor-associated IDH1/2 mutant versions that consume NADPH to transform AKG to the (*R*) enantiomer of 2-hydroxyglutarate, (*R*)-2HG, which accrues in cancer cells [73]. These (*R*)-2HG-producing mutants of IDH1/2 are commonly found in a subtype of gliomas [77]. (*R*)-2HG is considered an oncometabolite capable of inhibiting the branched-chain amino acid aminotransferase 1 and 2 (BCAT1/2), which use AKG and branched-chain amino acids as substrates to produce Glu and the corresponding branched-chain alpha-ketoacid, in a reversible amino group transfer reaction [78]. Hence, in this molecular context, the generation of Glu by transamination is compromised and becomes limiting for the biosynthesis of GSH [79], therefore impacting redox homeostasis (Figure 2). The activation of reductive carboxylation reprograms Gln metabolism, increasing GSH biosynthesis in a global compensatory rewiring [66]. This mechanism follows TCA cycle inhibition or impairment of the mitochondrial thioredoxin reductase (TrxR) antioxidant system [68]. Thus, to normalize antioxidant power, reductive carboxylation of Gln-derived AKG takes place [80]. The exchanging of glucose to Gln in the TCA cycle toward reductive Gln metabolism is conducted by HIFs in a mechanism regulated by the Warburg effect, also known as fermentative glycolysis [81]. For example, in normoxic 3D spheroids lung cancer cells, reductive citrate, produced by IDH1 in the cytosol from AKG derived from Gln is used to maintain redox homeostasis. The citrate generated in the cytosol was subsequently imported into the mitochondria where an oxidative metabolism occurs via IDH2, forming NADPH, which can be used by various antioxidant systems to detoxify mitochondrial ROS [82]. Similarly, in H460 lung cancer cells, AKG's reductive carboxylation allowed a citrate flow from the cytosol to the mitochondria, resulting in oxidative stress mitigation and faster growing rates [80]. Eventually, increased consumption of Gln by reductive carboxylation of Gln-derived AKG supports anabolic reactions, such as lipid synthesis, to activate cell proliferation [83]. Reductive carboxylation of Gln-derived AKG was equally found in hepatoma cells and mouse xenograft models to support *de novo* lipogenesis and the cellular antioxidant system by increasing the levels of NADPH and GSH [84]. On the other hand, Gln-derived metabolites in the TCA cycle may serve for synthesizing aspartate, an essential metabolite for *de novo* nucleotide biosynthesis. In renal cell carcinoma, GLS inhibitor BPTES impaired the reductive transformation of Gln-derived carbons to aspartate and subsequently minimized the *de novo* synthesis of pyrimidines [85, 86]. Accordingly, in three GBM cell lines treated with the more potent GLS inhibitor CB-839 was found a sharp drop of aspartate together with a diminishing in both oxidative metabolism in the TCA cycle and reductive carboxylation of Gln-derived AKG. These findings correlated with lower levels of metabolites from *de novo* purine and pyrimidine biosynthesis pathways [87]. In NSCLC, reductive carboxylation of AKG increased cytosolic ROS by a mechanism that includes upregulation of NADPH oxidase 2 (NOX2) [75]. On the other hand, GLS inhibition by BPTES also evoked higher intracellular ROS levels by affecting GSH formation [85].

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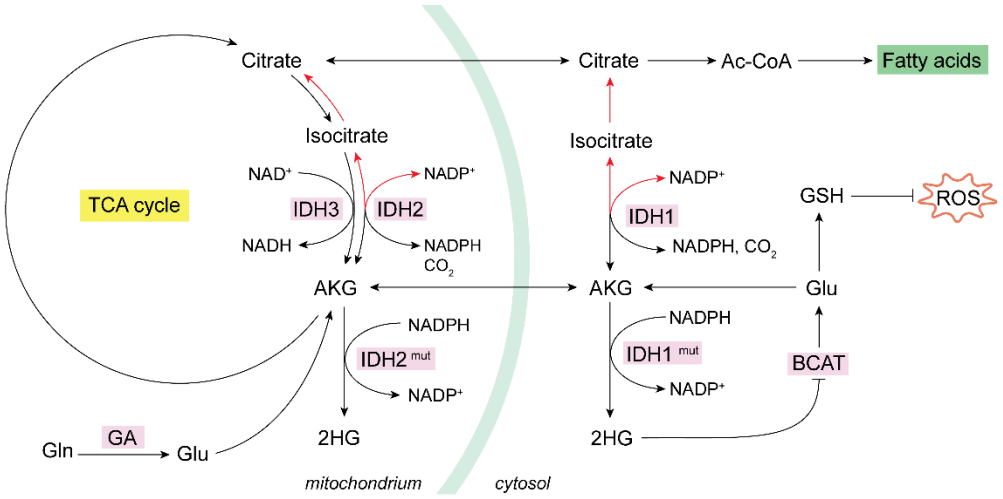

Figure 2. Mitochondrial oxidative and reductive metabolism of Gln**.** IDH isoenzymes modulate the oxidative or reductive metabolism of Gln in NADP-dependent reactions. In cancer cells, the increase of glycolysis and glutaminolysis boost ROS levels. In some cancer types mutant IDHs produce oncometabolite 2HG, which inhibits BCAT isoenzymes and lower GSH amounts. Many types of tumor cells activate reductive metabolism (red arrows) of AKG to generate Ac-CoA for *de novo* lipogenesis as a response to their biosynthetic needs for rapid proliferation. Reductive carboxylation by IDH1 in the cytosol consumes NADPH to generate isocitrate and then citrate; subsequently, the citrate is imported into the mitochondria and there follows oxidative metabolism and decarboxylation via IDH2 to generate mitochondrial NADPH. Ac-CoA, acetyl coenzyme A; AKG, alpha-ketoglutarate; BCAT, branched-chain amino acid aminotransferase; GA, glutaminase; GDH, glutamate dehydrogenase; GLS, glutaminase isoenzyme 1; GLS2. Glutaminase isoenzyme 2; Gln, glutamine; Glu, glutamate; GSH, glutathione; HG, hydroxyglutarate; HIF, hypoxia-inducible factor; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; OAA, oxaloacetate; ROS, reactive oxygen species.

4. Glutaminases, Ferroptosis and ROS

Ferroptosis is an iron-dependent specific and regulated type of cell death. The mechanism is mainly characterized by high levels of intracellular iron, which through the Fenton reaction leads to the production of hydroxyl radicals subsequently provoking polyunsaturated fatty acid peroxidation, lately resulting in membrane disruption and cell death [88]. Glutathione peroxidase 4 (GPX4) may act as an oncogene as it works as the major inhibitor of ferroptosis, being also related to the regulation of the electron transfer chain (ETC), OXPHOS and other metabolic pathways in cancer [89]. Noteworthy, erastin, an inhibitor of the xCT cystine-Glu antiporter, is another of the fundamental regulators of ferroptosis (Figure 3). Induction of ferroptosis by erastin evokes GSH depletion by limiting cysteine supply [90]. Erastin-dependent induction of ferroptosis is impacted by the mitochondrial metabolism of Gln, as it has been found that reductive carboxylation of Gln-derived metabolites favors resistance in NSCLC cells via a redox mechanism involving the GSH antioxidant system [91]. GA isoenzymes have a profound link with ferroptosis since they are fundamental enzymes for achieving a high glutaminolytic flux in cancer cells [81]. Hence, ferroptosis is modulated by the TrxR system upon erastin action as well as by glutaminolysis [90]. Thus, it has been demonstrated a connection of Gln metabolism to ferroptosis via the GLS2-p53 axis [92,93] Mechanistically, p53 inhibited the transcription of the cystine/Glu antiporter SLC7A11 (system xCT) [94]. Additionally, in ccRCC GLS2 was upregulated during ferroptosis induced by erastin or RSL3. GLS2 was related to higher GSH amounts in the cell and was considered as a potential ferroptosis suppressor in ccRCC [95]. Ferroptosis was equally activated in human TNBC cells in a mechanism dependent on the action of the Activating Transcription Factor 4 (ATF4) and ChaC glutathione-

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specific gamma-glutamylcyclotransferase 1 (CHAC1), which degrades GSH and induces cystine deprivation [96]. Mechanistically, this might comprise the relocation of reducing equivalents from cystine to thioredoxin (via TrxR1) and whereby avoid ROS augmentation [97]. Although GSH prevents the interaction of iron, oxygen and polyunsaturated fatty acids from triggering ferroptosis, the exact role of total GSH (tGSH) in ferroptosis and if there exist differences between cytosolic and mitochondrial GSH for the induction of ferroptosis is still to be clarified [98].

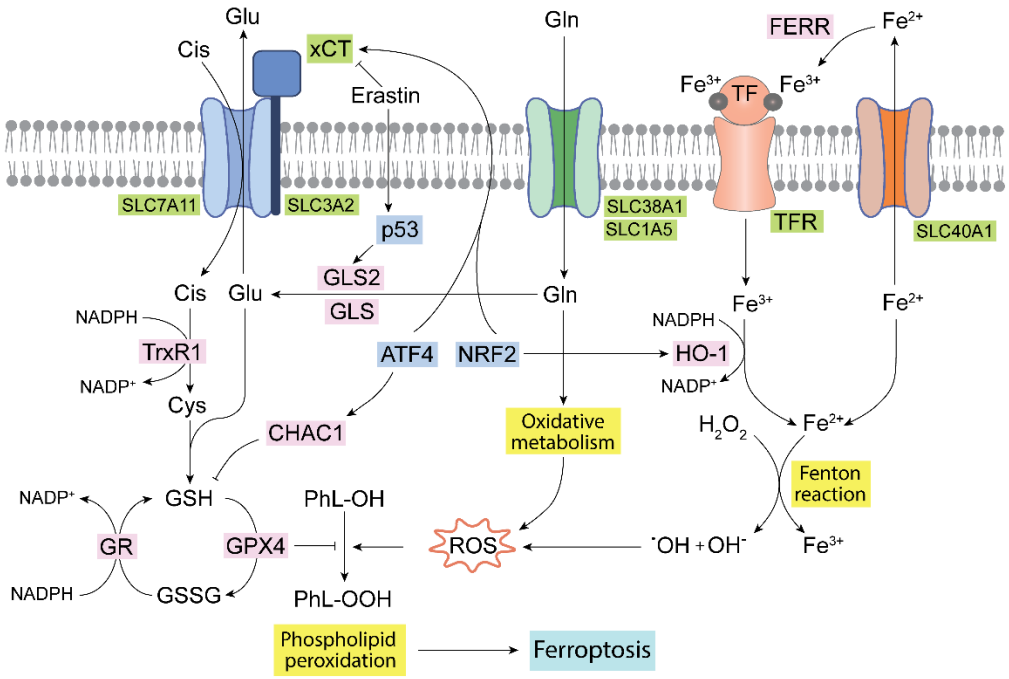

Figure 3. Ferroptosis and Gln homeostasis. Iron metabolism in the mitochondria generates oxidative molecules and radicals that together with the oxidative metabolism of Gln produce a large amount of ROS, leading to lipid peroxidation and ferroptosis. The glutathione antioxidant system formed by GR, GPX and TrxR isoenzymes, and by GSH, is the main antioxidant network to detoxify ROS. Some transcription factors such as ATF4 and NRF2 regulate and connect the GSH antioxidant system, Glu transport and iron metabolism. ATF4, activating transcription factor 4**;** CHAC1, ChaC glutathione specific gamma-glutamylcyclotransferase 1; Cis, cystine; Cys, cysteine; FERR, ferritin; GLS, glutaminase isoenzyme 1; GLS2. Glutaminase isoenzyme 2; Gln, glutamine; Glu, glutamate; GPX4, glutathione peroxidase 4; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase 1; NADPH, nicotinamide adenine dinucleotide phosphate; NRF2, Nuclear erythroid factor 2-related factor 2; PhL-OOH, peroxidized phospholipids; PhL-OH, phospholipids; ROS, reactive oxygen species; TF, transferrin; TFR, transferrin receptor; TrxR1, thioredoxin 1; xCT, cystine/glutamate antiporter.

Anyhow, ferroptosis modulation is essential in the chemoresistance event in TNBC [99], where tumor cells are characterized for commonly being Gln-dependent via glutaminolysis, which favors drug resistance [100]. To defeat these cancer cells a dual combination strategy has been suggested by targeting both GLS and ferroptosis [99]. Gallbladder cancer cells, which also need GLS for growth and proliferation, triggered ferroptosis following GLS inhibition and a drop in antioxidant capacity (GSH- and NADPH-dependent) that evoked oxidative stress [101]. Ovarian cancer cells are enriched in iron and rewire their Gln metabolism through HIF-mediated metabolic reprogramming [102]. However, ovarian cancer cells are resistant to ferroptosis through NRF2-mediated activation of additional antioxidant mechanisms, as those elicited by ferritin and heme oxygenase-1 (HO-1) that decrease free iron amounts, modulating iron metabolism and ROS levels [103]. In these conditions, therapeutic strategies that either block the xCT system or target specific metabolic vulnerabilities may be an option to overcome ferroptosis resistance [102]. In several types of aggressive and metastatic

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cancers a combination treatment including the natural product beta-elemene, in a codelivery strategy, was an efficacious drug for tumor patients reversing multidrug resistance and activating ferroptosis [104]. In KRAS mutant CRC cells combination of beta-elemene and cetuximab was successful, both *in vitro* and *in vivo*, and resulted in induction of ferroptosis, via downregulation of GLS, xCT, GPX4, ferritin and ferroportin (SLC40A1), which exports intracellular iron out of the cell [105].

5. Future Perspectives

As has been reported above, GLS and GLS2 isoenzymes are metabolic enzymes [42,53,60,63] whose roles may imply key antioxidant functions [64,65,91,95] that might lead to therapeutic implications for cancer therapy [15,16,26,35]. Of interest, several GLS inhibitors can block cancer progression by promoting oxidative stress [106]. Thus, a novel derivative of lonidamine, named HYL001, greatly inhibited cancer stem cells (CSCs) by hampering Gln metabolism, with 380-fold and 340-fold lower IC_{50} against breast CSCs and hepatocellular carcinoma (HCC) stem cells, respectively, as compared to lonidamine, while having minimum toxic effects towards non-tumor cells and immune-competent mice [13]. Although lonidamine increased ROS and promoted cell death by reduction of the pentose phosphate pathway (which lowered NADPH and GSH formation) [107], it is not approved in the clinic due to its hepatotoxicity, which is caused by its low solubility and pharmacokinetic characteristics [108]. However, HYL001 constitutes a promising derivative, confirmed to be a good candidate for clinical trials as reduced cancer cell proliferation on fresh tumor tissues from HCC patients by diminishing GSH, increasing ROS levels and inducing apoptosis [13]. Similarly, Gln metabolism and, particularly GLS activity, was found to be essential for androgen receptor antagonist-resistant prostate cancer cells, xenografts, patient-derived organoids, patient-derived explants, and tumor samples, by increasing antioxidant capacity [106].

Cancer is extremely adaptive with compensatory metabolic pathways that inevitably ease resistance to Gln shortage, specifically with monotherapy [109]. Blocking GLS by CB-839 or genetic silencing made resistant cancer cells vulnerable in an antioxidant-dependent mechanism which included GSH action [106]. However, because of the plasticity of tumors and heterogeneity in cancer it is not simple to target their vulnerabilities, as normal and malignant cells share many essential enzymes, metabolic network pathways and antioxidant systems [51]. Additional efforts are vital to delimitate how to modulate the redox status against every type of cancer and optimize combinations for synergistic therapy, hopefully to achieve personalized successful treatments. For example, because poly(ADP-ribose) polymerase (PARP) inhibitors synergize with GLS ones [85], a phase II clinical trial was opened to explore the consequences of the association of CB-839 with a PARP inhibitor in metastatic cancer prostate cells (NCT04824937). Ferredoxin 1 (FDX1) is another targetable antioxidant system because of its key function in the mitochondrial membrane of tumor cells with an impaired ETC [110]. Resistant pancreas cancer cells were also sensitive to an FDX1-selective drug which increased ROS levels and decreased cell viability [106]. A compensatory dual strategy targeting GLS-associated antioxidant program might be reinforced by a ferredoxin inhibition and/or a ferroptosis activation to provide novel therapeutic tools [91]. Thus, targeting the xCT system, ferroptosis and GLS in a combination therapy, consisting of erastin + doxorubicin + CB-839, has reached promising results in TNBC, tumor cells very sensitive to CB-839 treatment, because its high reliance on Gln and elevated GLS expression [100]. Other efforts to synergistically cooperate with both GLS silencing and ROS enhancement might span MYC, and the PTEN/PI3K/mTOR axis [109], as well as xCT, GPX and glucose transporters (GLUTs) [102]. Interestingly, disrupting both cellular redox balance and Gln availability by erastin and CB-839 has produced a positive synergistic effect in cisplatin therapy, sensitizing chemo-resistant TNBC cells [100]. These findings offer promising clinical relevance and deserve additional in-depth studies.

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Abbreviations

AKG, alpha-ketoglutarate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATF4, activating **t**ranscription factor 4; ATP, adenosine triphosphate; BCAT1/2, branched chain amino acid aminotransferase 1 and 2; ccRCC, clear cell renal cell carcinoma; CHAC1, ChaC glutathione specific gamma-glutamylcyclotransferase 1; CSC, cancer stem cell; Cys, cysteine; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ESCC, oesophageal squamous cell carcinoma; ETC, electron transfer chain; $FADH_2$, flavin adenine dinucleotide; FDX1, ferredoxin 1; GA, glutaminase; GAB, longer GLS2 transcript and isoform; GAC, shorter GLS transcript and isoform; GBM, glioblastoma; GDH, glutamate dehydrogenase; Gln, glutamine; GLS, glutaminase isoenzyme; GLS2, glutaminase isoenzyme 2; Glu, glutamate; GLUT, glucose transporter; Gly, glycine; GR, glutathione reductase; GSH, reduced glutathione; GSSG; oxidized glutathione; GPX4, glutathione peroxidase 4; HCC, hepatocellular carcinoma; HIF1, hypoxia-inducible factor 1; HNSCC, head and neck cancer squamous cell carcinoma; HO-1, heme oxygenase-1; IDH1, isocitrate dehydrogenase 1; KGA, longer GLS transcript and isoform; LGA, shorter GLS2 transcript and isoform; mTOR, mammalian target of rapamycin; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NOX2, NADPH oxidase 2; NRF2, Nuclear erythroid factor 2-related factor 2; NSCLC, non-small-cell lung carcinoma; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PARP, poly(ADP-ribose) polymerase; PC, pyruvate carboxylase; PDAC, pancreatic ductal adenocarcinoma; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; TCA, tricarboxylic acid; TCGA, The Cancer Genome Atlas; tGSH, total glutathione; TNBC, triple negative breast cancer.

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