

## **Glutathione compartmentalization and its role in glutathionylation and other regulatory processes of cellular pathways**

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## **Abstract**

Glutathione is considered the major non-protein low molecular weight modulator of redox processes and the most important thiols reducing agent of the cell. The biosynthesis of glutathione occurs in the cytosol from its constituent amino acids, but this tripeptide is also present in the most important cellular districts, such as mitochondria, nucleus and endoplasmic reticulum, thus playing a central role in several metabolic pathways and cytoprotection mechanisms. Indeed, glutathione is involved in the modulation of various cellular processes and, not by chance, it is a ubiquitous determinant for redox signaling, xenobiotic detoxification, and regulation of cell cycle and death programs. The balance between its concentration and redox state is due to a complex series of interactions between biosynthesis, utilization, degradation, and transport. All these factors are of great importance to understand the significance of cellular redox balance and its relationship with physiological responses and pathological conditions. The purpose of this review is to give an overview on glutathione cellular compartmentalization. Information on its subcellular distribution provides a deeper understanding of glutathione-dependent processes and reflects the importance of compartmentalization in the regulation of specific cellular pathways.

### **Abbreviations:**

GSH, reduced glutathione; GSSG, oxidized glutathione; GCL, glutamate cysteine ligase; GS, glutathione synthetase; GGT,  $\gamma$ -glutamyltranspeptidase; GST, glutathione-S-transferases; MG, methylglyoxal; AGE, advanced glycation endproduct; Grx, glutaredoxin; mtGSH, mitochondrial glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; PDI, protein disulfide isomerase; ROS, reactive oxygen species.

**Key words:** Glutathione; glutathionylation; redox signaling; post-translational modification (PMT); **subcellular compartments.**

## 1. Introduction

Glutathione is a tripeptide constituted from glutamic acid, cysteine and glycine. The sulfhydryl group (SH) of cysteine (Cys) is the functional group of the molecule, responsible for its biological activity. The thiol group of glutathione is a potent reducing agent, rendering GSH able to scavenge many dangerous endobiotic and xenobiotic electrophiles, both through direct or indirect enzymatic reactions (1, 2). This ubiquitous molecule is the main low-molecular-weight thiol found in living cells, also considered one of the major non-protein defenders against oxidative stress and the most important thiol-disulfide redox buffer of the cell. It is present in both reduced (GSH) and oxidized (GSSG) states, and the reduced one is maintained by glutathione reductase (GR), a cytosolic NADPH-dependent enzyme (3). To permit normal cell functions and a correct differentiation it is of great importance to maintain an optimal GSH:GSSG ratio (4). Indeed, under physiological conditions intracellular glutathione is mainly found as GSH at low millimolar concentrations (between 1 and 10 mM), while the content of oxidized species (oxidized GSH, GSSG and mixed disulfide, GSSR) does not exceed 1% of its total intracellular content (5). In its reduced form, consistent evidences have shown that GSH can help the body to repair oxidant damage caused by pollution, stress, infection, radiation, aging, drugs, unhealthy diet and burns (6-8). The importance of GSH is also shown by its involvement in many cellular processes that are connected with the control of the redox status of protein thiols (9). In this respect, the redox potential of the GSH/GSSG couple ( $E_h$ ) is at the same time a sensitive measure of the cellular oxidative state and an oxidative stress indicator (10), and a biochemical player of key cellular processes, such as the transition through cell cycle checkpoints that drive the cell from quiescence and differentiation to proliferation and death pathway activation with characteristic differences between the redox environments of terminally differentiated, pre-cancerous and cancerous cells (11, 12). These and many other pieces of evidence in the last years have led to figure out the existence of a “redox code” (the term redox code applies to the redox organization of cells, tissues, and organisms), where two major systems as the nicotinamide nucleotide (NAD and NADP) and thiol systems (protein thiols, glutathione and associated disulfides) are the key players (13). In this context, GSH and its influence on cellular thiols provide a sensing and coordination platform for the homeostatic response to oxygen-derived signaling molecules, such as hydrogen peroxide and superoxide anion (13). Glutathione also plays a critical role in protecting such redox platform under conditions of cellular oxidant stress. Accordingly, it has been highlighted that the control of such  $E_h$  is fundamental for maintaining both the activity of detoxification and redox signaling pathways (14, 15). In this scenario, the electrons of GSH can be utilized by several redox reactions within the cell

and the formed GSSG returns to the reduced state by the NADPH-dependent activity of glutathione reductase (GR). In turn, NADPH is rapidly regenerated from  $\text{NADP}^+$  using electrons derived from catabolism of substrate molecules such as glucose or isocitric and malic acid. Alternatively, the accumulating GSSG gets available for other reactions by cellular export and catabolism in other tissues, for instance in the liver (further discussed in Section 1.2). Also, GSSG that is not quickly reduced in the cytosol can be transported into the vacuole (10).

The multifaceted involvement of glutathione in these cellular processes also include a role as substrate for leukotriene biosynthesis (16), storage and transport of Cys (17), neutralization and transmembrane transport of harmful and lipophilic xenobiotics (18), substrate in redox cycling of Cys-containing enzymes such as peroxiredoxin 6 (Prxd-6) (19) or protein S-glutathionylation (20). Many other fundamental metabolic pathways like DNA and protein synthesis, enzyme activation or cell transport are affected by its fundamental protecting role (2, 15). Proper GSH concentrations are required for physiological functions, such as spermatogenesis, activation of T-cells and white blood cells, cytokines production, and also activation of immune responses (21-23). Glutathione is essential in mammalian and plants since its biosynthesis inhibition lead to embryonic lethality (24, 25) while in prokaryotes it has been shown to be fundamental during stress conditions (26).

## 1.1 GSH biosynthesis

The *de novo* biosynthesis of GSH occurs exclusively in the cytosol, from where it goes through many cellular compartments like endoplasmic reticulum (ER), mitochondria, nuclear matrix, and peroxisomes (14, 27) (Figure 1). GSH is synthesized from cysteine and glutamate, linked by an atypical peptide bond in a reaction catalyzed by the glutamate-cysteine ligase (GCL). Then, glutathione synthetase (GS) catalyzes the condensation of  $\gamma$ -glutamylcysteine and glycine (Gly) to produce GSH. Coupled ATP hydrolysis is required in both reactions. The first step is the rate-limiting one, and cysteine availability together with GCL activity regulates the GSH synthesis. Typically, Cys supply derives from the diet, protein catabolism and trans-sulfuration of methionine that is the way for this sulphur-containing amino acid to enter the biosynthesis of GSH via homocysteine (HCys) formation. Outside the cell, Cys is not stable and it quickly auto-oxidizes to cystine (Cyss), which is recruited by some cells and converted again to Cys intracellularly, that is the Cys-Cyss cycle (27). Cys and glutamate (Glu) are linked through the  $\gamma$ -carbonyl group of Glu instead of the typical  $\alpha$ -carboxyl group. This particular bond confers to glutathione a high stability since only very specific enzymes under particular conditions may operate on its degradation (28). One of these enzymes is  $\gamma$ -glutamyltranspeptidase (GGT) that catalyzes the hydrolysis of this unusual peptide bond, forming glutamate and Cys-Gly (29), and others enzymes capable of

degrading glutathione were discovered in the last years (30). Available evidence suggests that glutathione degradation is an important process that may take place in different cellular conditions and on distinct pools of this tripeptide to regulate cellular physiology. An excellent review on this topic was recently published by Bachhawat and Kaur (30).

Key biosynthesis genes, such as  $\gamma$ -GCS subunits and Cyss transporters, are regulated at the transcriptional levels by the activity of several transcription factors that also control GSH-dependent detoxification genes, such as glutathione peroxidase (GPx) and glutathione S-transferase (GST) (Section 1.2), and the thioredoxin (Trx) system (reviewed elsewhere (31-34)). Because the genes associated with the GSH and Trx systems serve multiple and fundamental functions, a complex of regulatory interactions are expected to influence their transcription mechanisms. For example, the promoter region of the same GSH-dependent gene may present consensus sequences and responsive elements for different transcriptional proteins, and the same transcription factor can control several GSH-related genes at the same time. However, some redox-sensitive transcription factors appear to have specific activity on certain groups of genes that include many innate and inducible antioxidant genes (35). These include the Cap 'n' Collar family of transcription factors, including the nuclear factor erythroid 2 (NFE2)-related factor (Nrf) transcription factors. In particular, the isoform 2 (Nrf2) is a cellular protein that once activated by a cellular electrophile or protein kinase-mediated phosphorylation migrates into the nucleus to dimerize with small Maf proteins and bind to a specific DNA sequence, identified as to antioxidant or electrophile response element (ARE or ERE, respectively). Such sequence is found in the promoters of many cytoprotection and oxidant stress responsive genes that include those responsible for GSH biosynthesis, redox restoration, conjugation and transport (34, 36).

## **1.2 GSH as cofactor and detoxification molecule**

GSH is the cofactor of the antioxidant enzymes GSH-peroxidase (GPx) and GSH S-transferase (GST) families of enzymes (Figure 2) (37-40).

Glutathione peroxidases (GPxs) belong to a family of phylogenetically related enzymes (38). Within this family, mammalian GPx1-4 are selenoproteins with a selenocysteine (Sec) in the catalytic centre (reviewed in (41) and references therein) with crucial role in flux regulation of, and protection from, hydrogen peroxide (such as by the isoenzyme GPx1) and lipid peroxides (the GPx4 isoform). The GPx-mediated reduction of the latter substrates shows some peculiarities and is essential to control the integrity of the cell membrane and lipid signalling functions, such as that of the 12/15-lipoxygenase-induced cell death and regulation of inflammatory responses (42, 43). Besides to GSH substrate, the GPx4-mediated reduction of lipid peroxides is indirectly assisted by

another redox player, that is vitamin E (as  $\alpha$ -tocopherol). This fat-soluble micronutrient vitamin is responsible for the H-atom (or proton) donation reaction (a  $1-e^-$  reaction step) that reduces a peroxy radical to the peroxide substrate of the GPx reaction (44). That said, an emerging view is that vitamin E and possibly few other H atom donors on the cell membranes, such as ubiquinol, operate in conjunction with GPx4 to inhibit lipid peroxidation and to control its signal transduction effects throughout different cellular pathways. Accordingly, a deficit of vitamin E or GPx4 activity, or both together, may lead to membrane damage and impaired lipid signaling. Emerging evidence has been provided on the fact that vitamin E supplementation can compensate, at least in part, for liver dysfunction and increased mortality of mice in which the liver expression of *GPx4* was manipulated (45). A possible mechanism for vitamin E to produce such effects is to prevent ferroptosis, an iron-induced and lipid peroxide-mediated cell death program associated with the degeneration of the liver and other tissues (43). Alternatively, this vitamin can influence other pathways of the ferroptotic signaling independent of GPx4 that include those sustained by the enzymatic peroxidation of arachidonic acid through 12/15 lipoxygenases.

According with the processes introduced in Section 1 and regardless of the lipid peroxide substrate involved, a post-reaction process is also essential to compensate for the GSH-consuming role of GPx, thus restoring the cellular GSH/GSSG redox. This occurs through the NADPH-dependent and glutathione reductase (GR)-mediated reduction of the reaction product GSSG (3).

The GSTs superfamily of Phase II detoxification enzymes, utilizes GSH as a cofactor to derivatize cellular electrophiles of different origin, such as xenobiotics and endogenous reactive metabolites, representing another important cellular defense mechanism of protection from reactive substances (37). After conjugation with electrophiles, the resulting GSH-adducts are actively excreted from the cell (46). The detoxification process continues by a series of enzymatic steps aimed to convert a water-soluble conjugate into a mercapturic acid (47). An increased GSH oxidation and conjugation activity is associated with a sustained efflux of GSSG and GSH-conjugates (reviewed elsewhere (48, 49)). Both passive and active (transcription-mediated) processes of transport cooperate to the efflux of these species. For example, cellular GSSG formation is associated with its extrusion by passive efflux, while the GSH-conjugates of anticancer drugs are typically actively secreted by a series of transporters that include the family of multidrug resistance proteins (MRP).

Furthermore, GSH and even more Cys are also actively secreted under conditions of cellular stress (50) and possibly as an active mechanism to commit and execute cell death programs (48). The same occurs in normal cells as a physiological process aimed at control the extracellular redox and the intercellular Cys metabolism through the cystine-cysteine cycle (recently reviewed in (49)).

Cumulatively, all these processes of transport may lead to deplete the intracellular pool of Cys and decreasing the levels of reduced glutathione. This happens for example in cells exposed to carcinogens and thiol peroxidase compounds (50, 51); when the drop of intracellular GSH is severe, cellular defenses and the redox of protein Cys can be severely impaired with effects on different cellular functions, such as the accumulation of protein damage products, ER stress pathway activation, and an increased signaling through programmed cell death pathways (52, 49).

*In vivo*, however, even conditions of severe oxidant stress and systemic toxicity, such as those observed in chronic kidney disease (CKD) and hyperbilirubinemia (53), do not appear to provoke any GSH depletion effects in peripheral blood cells, rather cellular GSH of CKD patients appears to increase compared to healthy control subjects. Worth of note, this happens in the presence of a significant decrease of the redox couples GSH/GSSG and NADPH/NADP<sup>+</sup> measured in the red blood cells of these patients (54, 55) and with more than two-fold up-regulated expression and activity of cellular GST (56), which should lead to a higher consumption rate of GSH (53).

Hence, such *in vivo* data demonstrate the existence of compensatory mechanisms that restore GSH homeostasis under conditions of oxidant stress. These mechanisms may depend on both local and systemic gene responses. At the local (cellular) level, electrophiles (endogenous metabolites, toxins, drugs and xenobiotics, etc.) are expected to sustain the induction of GSH metabolism genes, for example the genes that mediate the uptake of Cys and the *de novo* biosynthesis and reduction of GSH. Electrophiles promote this gene response by the activation of transcription factors that bind to specific consensus sequences in the promoter region of these genes, such as the antioxidant/electrophile responsive element (ARE/ERE). This provides the cells with a converging mechanism of response that modulates these genes in a coordinated and even redundant manner through the activity of several transcription factors. The main ones include Nrf2 (34) and AP-1 transcription factor (57), but many others appear to bind to these response elements, such NFkB, PXR/CAR and PPARs, functionally interacting at different levels of cellular metabolism to increase the complexity and efficacy of the detoxification response (32). Interestingly enough, these interactions include gene repression and negative feedback effects that are aimed at avoid conditions of allostatic overload. For this reason, the transcriptional response of ARE/ERE-associated genes often produce a *U*-shaped transcriptional and metabolic response. This *U*-shaped effect depends on the electrophilic compound's relative concentration and thiol reactivity (58, 59), in a way that high cellular concentrations of the compound may lead to acute depletion of cellular glutathione and massive activation of cell death programs (48). Low concentrations of the electrophile, on the opposite, produce an acute, but slight, decrease of cellular GSH and PSH/PSSG levels that is followed by the transcriptional (ARE-mediated) up-regulation of different adaptive

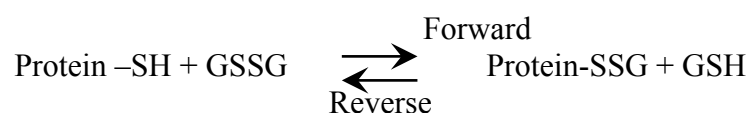
stress response genes, compatible with an increased resistance of the host to oxidative stress. This type of response is often identified as “positive hormesis” or “para-hormesis” response (60, 34). At the systemic level, an inter-organ metabolism of GSH can help compensating for the demand of GST, GPx and other GSH-consuming enzymes. This type of metabolism essentially provides a higher flux of substrates (mainly Cys and energy substrates to produce reducing equivalents) for the cellular biosynthesis and reduction of GSH of the different tissues. The liver plays a major role in coordinating this flux and its homeostatic effects (61).

Another important detoxification pathway in which GSH takes part as a cofactor is the glyoxalase system. It comprises two enzymes, namely glyoxalase I (Glx-I) and glyoxalase II (Glx-II), that sequentially catalyze the conversion of methylglyoxal (MG) to D-lactate via the formation of a S-D-lactoylglutathione intermediate (62, 63). Cells utilize the glyoxalase system to eliminate toxic  $\alpha$ -oxoaldehydes, especially methylglyoxal, thereby preventing  $\alpha$ -oxoaldehyde-mediated formation of advanced glycation end-products (AGEs) (64-66). When in the second step of this detoxification process Glx-II catalyzes the hydrolysis of the glutathione thioester S-D-lactoylglutathione to D-lactate, the GSH consumed in the first reaction is restored and, eventually, becomes available to glutathionylate specific proteins (67, 68).

Glutathione is also involved in the synthesis of eicosanoids like cysteinyl leukotrienes and prostaglandins (16). Enzymes like leukotriene C4 synthase, prostaglandin-endoperoxide synthase 1 or 2 and prostaglandin E synthase, require glutathione as a cofactor for their activity. These enzymes are part of the superfamily of large, membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), sharing structural evolutionary relationship with GSTs (69).

### 1.3 GSH and thiol status of proteins

One more function of GSH concerns the redox control of protein thiols. In cell metabolism, this redox control mechanism consists of the ability of protein cysteinyl residues to reversibly change their redox state with consequent modifications in conformational, catalytic or regulatory functions of that protein. Sulfhydryl groups of protein Cys residues can be present as thiols, disulfides or mixed disulfides. The latter, for example, can form by protein S-glutathionylation (P-SSG) when Cys residues are conjugated through a disulfide bond with GSH. This is a reversible post-translational modification the dynamics of which results in a thiol-disulfide exchange process:





The direction of this reversible reaction is determined by the redox state, or nucleophilic tone (60) of the cell that is, in turn, closely related to the concentrations of GSH and GSSG (proportional to the log of  $[GSH]_2/[GSSG]$ ) (70). The formation of protein mixed disulfides often occurs under oxidative and nitrosative stress (71) (further discussed in Section 1.4), and it is believed to represent a mechanism of prevention for the irreversible oxidation of protein thiols. However, it also operates as a biological redox switch in the regulation of signal transduction and metabolic pathways (72, 20). Indeed, S-glutathionylation occurs on protein kinases, checkpoints of cell cycle regulation and cell death pathways, as well as on proteins involved in glycolysis/energy metabolism, antioxidant enzymes, and on cytoskeletal and chaperone proteins (73-78, 20, 67).

The forward reaction is stimulated under conditions of oxidative and nitrosative stress (further discussed in Section 1.4) (71) and appears to include both spontaneous and enzyme-mediated processes.

Glutathione S-transferases (GSTs), and in particular the isoform P (GSTP), are believed to represent the main player in the enzymatic S-glutathionylation of cellular proteins, with glutaredoxins (Grx) playing a secondary role. Accordingly, GSTP gene manipulation results in a major lack of S-glutathionylated proteins in cells exposure to redox stressors and glutathionylating agents, such as the Se-organic molecule PhSeZnCl (51).

The former substrate of the GSTP-dependent S-glutathionylation function to be identified was peroxiredoxin 6 (Prdx6) (reviewed in (79)), a 1-Cys peroxidase with reducing activity towards membrane hydroperoxides and also hydrogen peroxide. Such activity is dependent on a GSTP-mediated and GSH-dependent redox restoration of the catalytic Cys of Prdx6; the role of GSTP in this redox-cycling process consists in the direct (physical or binary) interaction with this Prdx, in the transferring of the GSH molecule to its 1-Cys and then on a thiolase step of reaction to complete the redox restoration process of this Cys along with its own. Many other binary interactions of GSTP have been identified to occur with cellular proteins in the recent years (now identified as the GSTP interactome (40)) and at least some of these are expected to have the same, or similar, redox regulation function. In keeping with this, some of us (49) recently proposed a role for this GSH-dependent detoxification enzyme as a redox chaperonine and functional metronome of signal transduction proteins strategically distributed at the interface between inflammation, adaptive stress, cell senescence and death pathways. These interaction partners include for example the stress-activated kinase JNK, the TNF-alpha-activated kinase TRAF2 and ASK1; the latter kinase is also claimed to interact with another forms of GST, i.e. GSTM and possibly GSTPO (80), and to depend for its function from the redox co-factor thioredoxin. Early evidence was also obtained on GSTP and S-glutathionylation-dependent interactions with downstream inflammatory and redox-sensitive

transcription factors, such as NF $\kappa$ B via I $\kappa$ B modulation, and STAT3 (reviewed in (49)). Other recently identified interaction nodes of this interactome associated with partner S-glutathionylation include the estrogen receptors alpha (ER- $\alpha$ ) (81) and some endoplasmic reticulum stress proteins of the UPR pathway (82).

Recent work by some of us tentatively identified a binary interaction with the Nrf2 transcription factor, a key regulator of the oxidant-activated adaptive stress response (59). The GSTP-Nrf2 interaction was identified to occur under conditions of redox stress and apparently of GSTP protein oligomerization generated by the exposure to electrophilic compounds that reacted with its Cys residues (i.e. alkylating or thiol peroxidase agents). In these studies, the expression of GSTP gene was demonstrated to be essential in controlling Nrf2 activity with a downregulation effect that helps bringing the adaptive stress response to completion. The fact that the GSTP-Nrf2 regulatory interaction could also depend on a protein S-glutathionylation mechanism cannot be excluded. In effect, critical Cys residues of Keap1, the canonical inhibitory protein of Nrf2, are proposed to undergoes S-glutathionylation (83) pointing to a more complex interaction mechanism for the redox chaperonine GSTP with the Nrf2-Keap1 pathway. Recent studies in single and double knockout cells, clearly demonstrated that such functional interaction influences the same metabolism of cellular GSH. Indeed, the ablation of the GSTP gene was found to abnormally increase with a Nrf2-dependent mechanism the *de novo* biosynthesis and efflux in the extracellular medium of cellular GSH, ultimately interfering with protein S-glutathionylation and possibly with its signaling function (49). These aspects further highlight the importance of GSTP in the redox homeostasis process that the cell implements as an adaptive stress response through the Nrf2/GSH axis.

In this context it is worth noting that S-glutathionylation of protein thiols should be removed when its signal transduction or Cys protection function is over by the restoration of homeostatic conditions. Grx appear to play a major role in protein de-glutathionylation. GSH is the substrate for the catalytic mechanism of Grx (84, 85) that during the reaction with an S-glutathionylated protein, exposes the target disulfide to a nucleophilic attack for bond breaking and subsequent reduction of the Cys residue of the previously S-glutathionylated protein. The resulting glutathionylation of Grx (Grx-SSG) is then recycled to active reduced form by the oxidation of a GSH molecule and formation of glutathione disulfide (75, 86).

#### **1.4 Other functions of GSH relevant to NO metabolism and protein Cys redox**

Other functions of glutathione include the reaction with nitric oxide (NO) to produce S-nitrosoglutathione (GSNO), a physiological metabolite with important cellular roles. GSNO represents a storage form of this gaseous radical in tissues that is available for diffusion at the

extracellular level. The reaction with superoxide anion to form peroxynitrite (ONOO-) is a physiological process that can convey the GSNO-derived NO in the extracellular fluid than contributing to control the physiological levels of this bioactive gas and signaling molecule in tissues. For example, these properties may help to explain the *in vitro* and *in vivo* vasodilation properties of GSNO that are not observed in the case of other pharmacological and inorganic NO donors (87).

S-nitrosoglutathione can promote transnitrosylation reactions with critical Cys residues of proteins, that is a highly regulated post-translational modification of specific cellular proteins. For example, these include the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and some cytoskeletal proteins that however show very low apparent rate constants for the direct nitrosylation reaction (88). These reaction kinetics data may thus represent a major limit for a significant biological role of GSNO-mediated spontaneous transnitrosylations; however, specific protein interactions and the activity of the cellular GSNO reductase (GSNOR) could increase the biological relevance of this process modulating the local availability and reactivity of NO of some cellular microenvironments.

Accordingly, a dysregulation of GSNOR can lead to drastic changes in protein S-nitrosylation (89) thus producing other consequences downstream by a modified availability of NO and GSNO. For example a modification of their levels may influence smooth muscle relaxation (90, 91), cardiopulmonary regulation (92, 93), neuronal signaling (94), and in dozens of other intra/extracellular functions (95).

Besides to a physiological role in mediating protein nitrosylation, GSNO could have other, and so far poorly investigated, roles under conditions of oxidative and nitrosative stress that may simultaneously lead to different effects on protein Cys. For example, GSNO-mediated S-nitrosylation can compete with S-glutathionylation for the post-translational modification of specific Cys residues in mammalian proteins, and nitrosative stress and oxidative stress are both involved in the S-glutathionylation and functional regulation of certain target proteins (71). Chen et al. (96) showed that S-glutathionylation of endothelial nitric oxide synthetase (eNOS) reversibly decreased the enzyme activity leading to lower NO availability and relative gain of  $O_2^{\cdot-}$  levels, two effects that are both associated with impaired endothelium-dependent vasodilation and functional interaction between S-glutathionylation and secondary control of the NO available for S-nitrosylation of protein Cys residues.

Therefore, S-nitrosylation can compete with S-glutathionylation for protein Cys reaction, and these two modifications apparently exert opposing effects on protein function. This is the case, for instance, of Cys<sub>134</sub> on fast Troponin I that influences activity and Ca-sensitivity of skeletal muscle

fibers (97). Other candidate proteins for this redox competition on critical Cys have recently been investigated and include the transcriptional protein STAT3, that is a S-glutathionylation target under conditions of oxidative stress and selectively undergoes to GSNO-dependent nitrosylation on Cys<sub>259</sub> with functional implications on the inflammatory response of tissues (98), or the starch degradation enzyme of plant leaves  $\beta$ -Amylase 3 that is selectively nitrosylated and inhibited by GSNO on Cys<sub>433</sub> (99).

## **2. Subcellular compartmentalization**

### **2.1 Mitochondrial glutathione**

Mitochondria are organelles constituted by double membranes that hold two aqueous compartments: 1) the matrix, which is surrounded by the inner mitochondrial membrane (IMM), and 2) the intermembrane space (IMS), which is sandwiched between the IMM and the outer mitochondrial membrane (OMM). Mitochondria lack the enzymes required for GSH biosynthesis, therefore the mitochondrial GSH pool must be imported from the cytoplasm.

Glutathione is mainly present in mitochondria (mtGSH) in its reduced form. It represents 10-15% of the total cellular GSH, with a concentration range of 5–10 mM, similar to cytosolic concentrations, but it appears constitute an independent redox pool of the cell (27).

Glutathione is unable to pass freely across a lipid bilayer because it is negatively charged at physiological pH, so OMM and IMM must be equipped with transporters or channels to facilitate the entry of GSH. The OMM is rich in porins (in *S. cerevisiae* Por1, Por2) which form aqueous channels through the lipid bilayer and allow diffusion between the IMS and cytosol of molecules smaller than ~5 kDa, including glutathione. Kojer et al. (100) showed that glutathione pools of the IMS and the cytosol are connected by porins. These authors proposed a model for a dynamic glutathione exchange where cytosolic glutathione reductase system exerts the major influence on the composition of the IMS GSH pool. In this model, this IMS pool provides reducing equivalents to Mia40 oxidoreductase that could be involved in several redox functions of this compartment, such as oxidative protein folding. A slight shift in  $E_{\text{GSH}}[\text{IMS}]$  may have a central role in redox regulation by reversible disulfide bond formation at the levels of this and other redox sensitive proteins of IMS. Since the IMS does not seem to contain any GSSG-reducing machinery, GSSG could diffuse out of the mitochondrial membrane or be used to glutathionylate proteins (100). Conversely, the GSH pool in the matrix is maintained separately from that of IMS. In fact, GSH anionic properties at physiological pH and the highly negative potential of IMM, does not allow GSH to diffuse into the matrix. It was demonstrated that the IMM prevents the equilibration of the

GSH pool between IMS and matrix. In the matrix the recovery of  $E_{\text{GSH}}$  is entrusted to the efficient reduction of GSSG mediated by the activity of glutathione reductase, and not to the import of GSH from the IMS/cytosol that appears to be a poorly efficient process. In fact, in cells lacking glutathione reductase in the matrix, the  $E_{\text{GSH}}[\text{matrix}]$  does not recover sufficiently showing also that the export of GSSG from the matrix is not present in appreciable amounts (100) (Figure 1).

Among the various GSH carriers identified in the IMM, two of them appear to play a role in the passage of GSH from the IMS into the matrix, namely dicarboxylate, (DIC Slc25a10) and 2-oxoglutarate (OGC Slc25a11) (101). Nevertheless, in rat liver mitochondria the inhibition of dicarboxylate and 2-oxoglutarate carriers resulted in partial reduction of GSH uptake (between 45 and 50%), compatible with the involvement of other transporters in the mitochondrion uptake of GSH (102). On the other hand, a recent alternative way for supplying GSH to the mitochondrial pool has been described by our group. Our studies demonstrated that S-D-lactoylglutathione, a thioester of glutathione and an intermediate of the glyoxalase system, enters the mitochondria to undergo Glx-II mediated hydrolysis to D-lactate and GSH that is available to restore the mtGSH pool (103).

Mitochondria are considered the major source of highly reactive species of the cell, as a small amount of the consumed oxygen in these organelles is subjected to activation by the electron leakage of the electron transport chain (ETC). The superoxide anion is the main product of the reaction between these ETC-derived electrons and molecular oxygen in mitochondria. Indeed, the concentration of superoxide anion in the mitochondrial matrix has been estimated to be 5- to 10-fold higher than in the cytosol (104). The first line of defence against superoxide in the mitochondrial matrix is superoxide dismutase 2 (SOD2 or MnSOD) that catalyzes the dismutation of superoxide anion to  $\text{H}_2\text{O}_2$ , whereas the superoxide formed into the IMS is reduced by SOD1 (or Cu,Zn-SOD) (105).  $\text{H}_2\text{O}_2$  metabolism in mitochondria mainly occurs through GSH-dependent reactions catalyzed by the activity of some GPx and Prxd isoforms. Thioredoxins may also play a role in this metabolism. In fact, most cell types lack catalase, that is a major (GSH- and Trx-independent)  $\text{H}_2\text{O}_2$  scavenging enzyme of *S. Cerevisiae* mitochondria.

More in detail,  $\text{H}_2\text{O}_2$  reduction at the matrix level (106-108) appears to occur through the oxidation of the peroxidatic cysteine residue (Cys<sub>91</sub>) to the sulfenic acid form of the 1-Cys peroxiredoxin (Prxd1). A glutathionylation reaction of this Cys residue is required to restore the catalytic properties of Prxd1. As described earlier in this section, the GSSG formed in this and other  $\text{H}_2\text{O}_2$  scavenging reactions cannot be exported out of the matrix. NADPH-dependent mitochondrial oxidoreductases are responsible of its reduction and mtGSH pool restoration. Such reaction is important to prevent the effect of GSSG accumulation on spontaneous glutathionylation of

mitochondrial proteins (109, 110). In this respect, it has been calculated that if the GSH:GSSG ratio decreases to  $\sim 1$  and an oxidant environment is present, non-enzymatic glutathionylation occurs as a spontaneous reaction in order to protect the irreversible oxidation of proteins Cys (111, 112). This is a reversible S-glutathionylation mechanism that depends on mitochondrial GSH:GSSG fluctuations, but at the same it is non-specific and as such dangerous post-translational modification of mitochondrial proteins. In keeping with this, non-enzymatic S-glutathionylation of mitochondrial proteins is connected to oxidative damage and pathological situations (113). By contrast, enzymatic S-glutathionylation is specific and reversible process, that selectively modulates cellular processes (114). Different proteins are glutathionylated in mitochondria (115) with roles in  $H_2O_2$  metabolism and redox regulation of the mitochondrial environment, protein microenvironment function and signaling (109, 115, 110). As an example, Complex I, which is sensitive to the redox environment, is a possible S-glutathionylation target and the mitochondrial enzyme Grx2 is responsible for its reversible glutathionylation. The process is greatly influenced by the GSH:GSSG ratio, with a high GSH:GSSG ratio that promotes Complex I de-glutathionylation favoring its activation (116, 117). Generally, the whole ETC is sensitive both to changes in redox environment and to fluctuations in the GSH:GSSG ratio, as it has been observed that almost all of the components of ETC are subjected to S-glutathionylation (118-121). Moreover, also uncoupling proteins UCPs appear to be modulated by glutathionylation, consistent with a functional interconnection between the electrochemical proton gradient and the mitochondrial redox balance (122).

Redox regulation is critical for numerous mitochondrial functions. For example, GSH deficiency in mammalian cells can cause mitochondrial damage (123, 124). Yeast strains lacking GSH cannot grow by the defective respiration that derives from the oxidative damage to mitochondrial DNA (125). Again, oxidation of mitochondrial thioredoxin promotes cell death in yeast (126).

Mitochondria play a strategic role in the regulation of cell death programs (127). Exhaustion of mtGSH has been linked to apoptosis, either preparing for cell death (commitment phase of the apoptotic program) or by modulating the permeability of mitochondrion and activating apoptotic effector caspases (execution phase) (128-130). A sudden increase of the inner membrane permeability is a characteristic early event of the mitochondrial (or canonical) pathway of apoptosis. It is induced by the opening of mitochondrial permeability transition (MPT) pores that causes a loss of membrane potential, mitochondrial swelling, rupture of the outer membrane, and release of pro-apoptotic factors, such as cytochrome c (Cyt c), to the cytosol. The pore structure and the proteins involved in MPT during apoptosis have been subject of many studies (131-134). An MPT model proposes that the mitochondrial membrane-bound glutathione transferase (mtMGST1) is activated by oxidative stress through S-glutathionylation and formation of a disulfide linked

mtMGST1 dimer, which contributes to MPT pore opening and Cyt *c* release from the mitochondria (135). A critical step in mitochondrial apoptosis is the release of cytochrome *c* from IMS. Under physiological conditions a part of Cyt *c* seems to be associated to the membrane lipid cardiolipin (136). A mechanism proposed for the dissociation of Cyt *c* from this lipid involves its oxidation in that the oxidized form of cardiolipin has a much lower affinity for the cytochrome than the reduced form. In this respect, mGSH could represent an important modulator of this apoptotic pathway being involved in the control of mitochondrial ROS and indirectly of cardiolipin oxidation (137). On the other hand, cardiolipin peroxidation has also been involved in ferroptosis, a cell death program specifically activated by a defective cellular supply of Cys (and thus of GSH) and GPx4 activity, that leave uncontrolled the iron-mediated oxidation of cellular lipids. The latter are the actual ferroptotic mediators and cardiolipin appears to represent a characteristic target of this process that is associated with small mitochondria and a virtual absence of cristae, condensed membrane densities and sometimes evidence of membrane rupture (138, 139). According with an important role of GSH in preventing the oxidative changes of mitochondrial lipids, ferroptosis is negatively regulated by increased levels of cellular GSH, and activity of glutathione peroxidase 4 (Gpx-4), heat shock protein 27 (Hsp27), cysteine/glutamate antiporter and Nrf-2 (140).

Glutathione contributes also to the biosynthesis of iron-sulfur (Fe-S) clusters. These are co-factors of many proteins of crucial importance in metabolism, electron transport and regulation of gene expression. This biosynthesis process is accomplished by the so-called iron-sulfur-cluster assembly (ISC) machinery of mitochondria. ISC is responsible for the biogenesis of iron-sulfur proteins both within and outside the organelle (141, 142). In the model of Fe/S protein biogenesis in mitochondria proposed by Lill et al. (141), during the second step of biogenesis the monothiol glutaredoxin Grx5 helps to transfer the Fe/S cluster toward apoproteins, presumably via transient binding of the Fe/S cluster in a glutathione-containing complex. The ISC assembly machinery generates also a sulfur- and possibly glutathione-containing molecule (X-S) that is exported by the ABC transporter Atm1 and the sulfhydryl oxidase Erv1 to the cytosol to support cytosolic-nuclear Fe/S protein biogenesis (141). At this stage, the glutaredoxins Grx3-Grx4 are involved in the formation of the glutathione (G)-coordinated [2Fe-2S] cluster assembled with the help of the molecule X-S emanating from mitochondria (141).

In conclusion, the many functions of mtGSH pool and its maintenance are obviously important aspects of mitochondrial pathophysiology, and remain a fascinating subject of cellular biochemistry that is still open to investigation.

## **2.2 Endoplasmic reticulum glutathione**

The endoplasmic reticulum (ER) is the gateway of the secretory pathway, where proteins are *co*-translationally translocated and post-translationally modified. In the ER lumen proteins undergo oxidative folding process, which comprises disulfide bond formation. The involvement of GSH in this instance is a highly argued question with a full majority of authors that suggest it serves as reducing agent for disulfide cleavage. Then, we here discuss the glutathione redox of ER and its contribution to disulfide reduction and oxidative protein folding during normal and ER stress conditions.

Former, the ER reduction/oxidation (redox) environment influences the activity of enzymes responsible of disulfide bond formation. Such environment depends on numerous factors that include the glutathione redox state (143-145). For many years, GSSG has been considered the readily available oxidant for the protein thiol group, and more recently, also dehydroascorbate (146) and hydrogen peroxide (147) are indicated as possible oxidant molecules in the ER (148, 149).

GSH and GSSG transport and concentrations into the ER of high eukaryotes have been investigated by several groups, but remain not fully elucidated (145, 150, 151). In the periplasm of *E. coli*, the uptake of glutathione from cytosol into the ER might occur via a dedicated transporter, such as a homologue of CydDC. This complex is an ATP-binding cassette (ABC) transporter that moves glutathione and Cys (152). CydDC is similar to the cystic fibrosis transmembrane conductance regulator (CFTR), which is responsible for flux of GSH in lung airway epithelial cells (153). The active transport of GSH into ER, against a concentration gradient, has been hypothesized also by Montero et al. (154). Also, it was suggested that GSH is preferentially transported into the ER, compared to GSSG, and that this transport takes place in an energy-independent manner (155, 156). However, a recent study shows that transport of glutathione from cytosol to ER proceeds via facilitated diffusion through Sec61 in a coupling system with Ero1 and Bip (157).

Considering the importance of retaining an oxidizing environment, a few studies have tried to measure glutathione levels directly in the ER and to determine its redox state. Using a glyco-peptide probe targeted to the exocytic compartment, the global GSH:GSSG ratio in the ER was determined between 3:1 and 1:1, whereas it was identified to be between 3:1 and 4:1 by a later study performed with isolated rat liver microsomes (145). However, GSH redox status quantification in ER is affected by significant *ex vivo* oxidation that appears to occur during microsomal isolation. Taking such pre-analytical bias into consideration, ER GSH:GSSG ratio was calculated to range from 4.7:1 to 5.5:1 (158). These values however may not reflect the situation in the ER of living cells because GSH, but not GSSG, is transported in microsomes (155). Recently, the ER GSH redox potential was directly measured in HeLa cells, by employing a combination of a GSH-specific redox-sensitive variant of green fluorescent protein (159) and another specific fluorescent protein sensor



of the GSH:GSSG ratio (154). Based on these measurements, it was concluded that GSH:GSSG of ER is less than 7:1. Nevertheless, further and more accurate measurements of redox have recently been carried out in some studies in which fluorescent probes that rapidly detect glutathione were used, allowing to directly measure glutathione levels in the ER lumen of live cells (160, 161, 159). From these studies, a GSH:GSSG ratio of 35:1 was predicted (162). This high concentration of glutathione is widely believed to keep the disulfide isomerase family of proteins (PDIs) in the reduced form (163) while its contribution to oxidative protein folding remains debated.

The PDI family is the most important constituent of the oxidative folding machinery with the thiol oxidase Ero1. The Ero-PDI system is responsible for disulfide-bond exchange in client proteins and for isomerization of non-native disulfide bonds (Figure 3) (164-166). These reactions, essential in promoting proper folding and stability of polypeptides (167, 168), are mainly orchestrated by a family of oxidoreductases containing thioredoxin-like domains (169). These include protein disulphide isomerases (PDI), ERp57, ERp72, P5, and PDI-related protein (PDIR). The PDI, the main oxidoreductases involved in native disulfide bonds formation, is considered as a proper multifunctional redox chaperone. It is constituted by two catalytic sites, *a* and *a'*, that show broad substrate specificity and contain a characteristic CxxC active site separated by two non-catalytic domains, identified as *b* and *b'* (170, 171). The *a* and *a'* domains interact with the thiol group of a newly synthesized protein catalyzing thiol-disulphide exchange reactions while the redox-inactive *b'* domain has an important role in binding unstructured protein substrates. For mere isomerization reactions a catalytic domain and the *b'* domain can combine, while to isomerize a protein substrate that has undergone conformational changes all PDI domains are needed (172, 173). These tasks take place with cycles of reduction and oxidation of the PDI enzyme. When PDI is in the oxidized form, a disulphide bond in native proteins will be formed, while when catalytic cysteines of PDI are in the reduced form, isomerization of non-native disulphides in misfolding protein will be carried out (Figure 3) (174). In the first case, the reaction leads to reduced PDI, then PDI has to be oxidized again to complete the catalytic cycle. This last function is performed by a few proteins like Ero1 flavoenzyme or Prx4, as well as by oxidized glutathione and indirectly by glutathione (175-177, 166). Ero1 is a flavoenzyme that oxidizes PDI and transfers two electrons via flavin adenine dinucleotide (FAD) cofactor onto oxygen to produce H<sub>2</sub>O<sub>2</sub> (178). *In vitro* experiments have shown that PDI *a'* domain is the target of oxidation by Ero1 (179) while *a* domain is preferentially reduced by GSH (180, 181) (Figure 3). Cuozzo and Kaiser provided the first demonstration that glutathione is not the source of oxidizing thiol equivalents in the ER, rather it competes with Ero1 to regulate the PDI redox (Figure 3) (182). Reduced PDI has a high affinity to bind misfolded protein and to function as an important chaperone in the ER: Molteni et al. showed as GSH depletion can lead to

PDI oxidation that is reversed by GSH level restoration, thus demonstrating the important role of GSH in preserving reduced the PDI pool (183). Lyles and Gilbert showed that reduced GSH influences PDI's catalytic efficacy to renature reduced Rnase A (167) while Kulp and colleagues showed that the RNase A folding takes place in the absence of GSH and in presence of PDI-Ero1 systems (184). In addition, GSH was found to reduce ERp57, another ER oxidoreductase, *in vitro* (185). Based on these studies, glutathione seems to have a profound influence on PDI redox state.

In GSH depleted cells, the reoxidation of immunoglobulin J chain (JcM), used as model of oxidative folding, entails with misfolded proteins (183). On the contrary, in cells depleted of GSH the maturation of plasminogen activator (tPA), occurred with similar kinetics compared with non-modified cells (186). Reciprocally, excessive concentrations of GSH compromise the protein folding and also triggers ER stress through unregulated PDI recycling (9). In the same way, in specific conditions, when nitrosative stress is induced, abnormal post-translational modifications occur on PDI; increases of intracellular NO lead to the production of SNO-PDI and successively GSH may bind to the catalytic (*a*, *a'*) domains of PDI, resulting in S-glutathionylation of its cysteine residues (187). S-glutathionylation of PDI has been demonstrated to inhibit isomerase activity in human leukemia (H160) and ovarian cancer (SKOV3) cells after somministration of PABA/NO drug (187) and to abolish chaperone activity versus estrogen receptor alpha in breast cancer cells (188). Abnormal post-translational modification of PDI has been shown to be implicated in neurodegenerative diseases (189). Many other chaperone proteins have been shown to be regulated by S-glutathionylation in physiological and pathological conditions (190), these include the redox chaperonine GSTP that has recently been demonstrated to promote the S-glutathionylation of ER proteins associated with the unfolded protein response and ER stress signaling (82).

Also ryanodine receptors activity is influenced by S-glutathionylation. Ryanodine receptors (calcium-induced calcium release channels) mediate the release of calcium from the endoplasmic and sarcoplasmic reticulum into the cytosol converting different extracellular stimuli into intracellular calcium signals (191). RyR1, essential for skeletal muscle excitation–contraction coupling, is activated through S-glutathionylation, with an increase in calcium release (192). ROS, physiologically produced in the heart, trigger heart muscle contraction by promoting endogenous cardiac RyR2 S-glutathionylation. Cardiac cells utilize this redox modification to increase RyR2 activity under conditions like tachycardia and exercise, but it has also been observed during heart failure, causing calcium release abnormalities (193).

These results suggest that GSH is involved in maintaining ER oxidoreductases in the reduced form, allowing them to act in recovery of proteins containing non-native disulfides, and support their role

in preventing ER stress by improving protein secretion. However, the pioneer work of Tsunoda and colleagues challenge the importance of GSH in ER by an engineered system to degrade ER GSH (194). ER depletion of GSH by ectopic expression of Chac1 in the ER did not alter protein folding and the ER stress response as well. As elegantly discussed by Ponsoero and Toledano (195) the Chac1 degradation of GSH supplies the dipeptide that contains cysteine thus potentially supporting the reductive function of GSH. Therefore, their data did not conclusively exclude a contribution of GSH in oxidative protein folding. Other experiment should be carried out to address the role of GSH in this important aspect of ER redox.

The altered redox environment into ER produces a condition of “ER stress”, a situation whereby misfolded proteins accumulate inside the ER lumen, activating a regulated program termed as unfolded protein response (UPR). This program enhances the capacity of protein folding, attenuates the rate of de novo protein synthesis and increases the ability to degrade proteins localized in the ER. If homeostasis is not restored, a prolonged ER stress and the use of UPR entirely overtake the mechanisms of cellular protection, ultimately inducing apoptosis (196, 151).

The redox environment of ER (197, 198) plays a fundamental role in molecular mechanisms that control cellular processes such as proliferation, differentiation, and cell death (199) as well as in several pathophysiologic conditions (200, 201). An increasing number of diseases are now ascribed to the defection of the secretory protein folding. Moreover, misfolding and aggregation of some proteins into aberrant, toxic molecules is the cause of neurodegenerative diseases such as Alzheimer and Parkinson disease, as well as of other human diseases (202, 203).

### **2.3 Nuclear glutathione**

Glutathione plays an important role in many nuclear processes (204-207). It has been reported in various studies on animal and plant cells that the distribution of GSH between the nucleus and cytoplasm over the course of the cell-division cycle is dynamically regulated and may serve to control redox-sensitive checkpoints in this cycle (208-210). In fact, the amount of glutathione of the nuclear pool changes in correlation to various events of the cycle, playing a critical role for example in preserving the nuclear redox during the early phases of cell proliferation (12).

The mechanisms of nuclear glutathione transport and sequestration are still being debated (211). For sure, the synthesis of GSH does not take place in the nucleus because like mitochondria, it lacks  $\gamma$ -GCS and GS. Therefore, the GSH pool in the matrix necessarily derives from cytoplasmic glutathione, probably via nuclear pores. Indeed, it has been observed that during the early phase of cell proliferation the GSH nuclear:cytoplasm ratio reaches 4:1(12), suggesting the presence of some

transport mechanisms. Voehringer et al. first demonstrated that cancer cells over-expressing Bcl-2 showed increased glutathione levels in the nucleus (212), then Markovic et al. also observed that Bcl-2 proteins are more abundant in the nucleus of fibroblasts during the early stages of cell proliferation (12). In cultured epithelial cell lines it has been observed that Bcl-2 proteins are expressed in connection with the cell cycle (213). Their amount is huge during prophase and metaphase, as they even bond with chromosomes during mitosis. On the contrary, this association is reduced during telophase and falls to subtle levels shortly after mother and daughter cells are separated (213). Bcl-2 proteins own a BH-3 domain where GSH binds (214) and as their presence seems to be correlated to the increase of the GSH pool in the nucleus, it is possible that Bcl-2 proteins are involved in GSH translocation to the nucleus.

Combining information obtained from studies on mammalian (12, 209) and plant cells (215), Diaz Vivancos et al. proposed a model for the glutathione cycle in the nucleus. In this model GSH is recruited and directed into the nucleus in early G1 phase, so GSH increases in the nucleus while cytosolic GSH is depleted; the altered cytosolic redox environment promotes the synthesis of new GSH, so the overall glutathione pool significantly increases; the nuclear envelope dissolves so there is a rebalancing between cytosolic and nuclear GSH during G2 and M phase; during telophase the nuclear membrane is reassembled, the cell divides and the total GSH pool is allocated equally between daughter cells (216). In this contest, reduced conditions in the nucleus are required for the cell to proliferate while, in order to activate mitogenic pathways that allow entry into S-phase and activate DNA replication, an oxidized cytosolic state must occur during the G1 phase (217). To determine if the accumulation of GSH in the nucleus influences gene expression patterns, experiments on a comparison of transcript profiles were performed on *A. thaliana* cells (210). During the G1 phase, when GSH accumulates in the nucleus, a substantial decrease in various transcripts involved in oxidative signalling and stress tolerance was observed in combination with great increase of GSH1 and GSH2 transcripts. In particular, transcripts associated to cellular defense mechanism as heat shock proteins or cell death processes markedly decrease or are down-regulated (210). Therefore, there is evidence that GSH accumulation in the nucleus or GSH depletion in the cytosol regulate gene expression. The different distribution of GSH between nucleus and cytoplasm in relation to the cellular cycle not only impacts on gene expression, but also alters the ability of transcription factors to bind DNA. Changes of nuclear GSH redox may affect the transcriptional regulation of important elements, as hypothesized for NF- $\kappa$ B and AP-1 transcription factors or p53 and nucleophosmin (NPM1) (218, 69). NPM1 is a histone chaperone that under oxidative stress dissociates from nucleic acids and activates p53 to bind DNA and p53-dependent apoptosis (69). GSH levels in the nucleus may also influence the organization of

chromatin structure (219). Indeed, the physical organization of chromosomes depends on associations with the nuclear matrix (220), maintaining their integrity thanks to disulfide bonds and metal-protein interactions (221). Torres et al. demonstrated that GSH variations have an impact on gene expression, influencing chromatin remodeling complexes that result in an open structure conformation more accessible to transcription factors (222). In this work it was shown that after treatment with BSO, that inhibits the *de novo* synthesis of GSH, expression of the transcriptional regulator *c-myc* was altered by H3 histone hyperacetylation. Chromatin remodeling and altered pattern of gene expression are indeed correlated to epigenetic post-transcriptional modifications of HATs and HDACs (223). Also, increasing GSH inside the nucleus seems to influence the structure of chromatin and its condensation through regulation of epigenetic modifications that include methylation and S-glutathionylation (219, 224).

S-adenosyl methionine synthetase (MAT1A) is required to form S-adenosyl methionine (SAM). SAM is then utilized by DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) for DNA and histone methylation. Pajares et al. first showed that oxidized glutathione inhibits the activity of this enzyme (225) therefore suggesting that the nuclear GSH:GSSG ratio may affect epigenetic processes by modulating SAM levels. Interestingly enough, restoring nuclear GSH, would also restore MAT1A activity and the ability of DNA and histones to undergo methylation.

Histone modifications have been shown to be important for cell cycle progression (226) because they can influence the state of chromatin compaction (227, 228). Progression in S-phase depends on chromatin fibers loosening in front of the replication fork and assembly of nascent DNA strands with core histones. Recently, another post-translation modification of histones has been identified that changes chromatin organization, i.e. the S-glutathionylation of histone H3 (229, 230). Covalent binding of GSH to sensitive Cys residues of H3 destabilizes DNA-histone interaction and interferes with nucleosome stability, thus influencing chromatin assembly-disassembly as a critical step in the creation of the replication fork (230). GSTP, one of the main S-glutathionylation enzymes (discussed in Section 1.3), has been found to be present in the nucleus (59), and the levels of the nuclear form of this protein change during the exposure to electrophiles that significantly increase the the S-glutathionylation of cellular proteins (49). If this type of response influences the S-glutathionylation and thus the functions of specific nuclear proteins remains to be established.

### **3. Conclusions**

Glutathione is one of the most investigated redox-active molecules. The GSH:GSSG redox couple is maintained in a dynamic equilibrium that depends on the redox (or nucleophilic) tone of the cells, and its changes are known to influence many metabolic and regulatory processes like gene

expression, modulation of DNA synthesis, cell proliferation and apoptosis, protein S-glutathionylation and signal transduction, detoxification of endogenous substances and xenobiotics with electrophilic properties.

The lack of adequate restoration of the cellular glutathione redox is believed to represent a major cue and underlying event of oxidant (or redox) stress, sustaining, cell damage and premature aging of tissues. Its role in pathogenic processes of many human diseases has been proposed in an impressive number of studies. These include neurodegenerative diseases, liver disorders, cystic fibrosis, kidney disease, diabetes and cardiovascular disease. For each of these pathological conditions it is not always easy to establish if a depletion or dysfunctional redox of cellular GSH are a cause or a consequence of the pathogenic mechanism. In both these cases, it is desirable to maintain an optimal status (concentrations and redox) of this cellular tripeptide. As described in this review article, different pools of GSH are present in the cell and all with specific and important roles. All these pools should be considered within the concept of “optimal GSH status” and for a role in cytoprotection and anti-aging strategies based on the modulation of the glutathione gene system together with other batteries of redox-modulation, detoxification and stress response genes. Internalization and compartmentalization mechanisms of GSH into distinct subcellular pools thus represent fascinating subjects of cellular biochemistry that is still open to investigation. Several factors that influence the intracellular pools of GSH and their biological roles remain still elusive and their identification hold great potential to develop novel strategies of prevention and even therapies for many age-related diseases.

## Legends to figures

### Figure 1. Synthesis and localization of glutathione

GSH is synthesized in the cytoplasm through two sequential enzymatic ATP-dependent reactions: glutamate cysteine ligase (GCL) links the cysteine to glutamate and glutathione synthetase (GS) catalyzes the bond between glycine and  $\gamma$ -glutamyl-cysteine to produce GSH. After synthesis, cytosolic GSH is transported into subcellular compartments.

### Figure 2. Main glutathione -dependent antioxidant enzymes

Glutathione is the cofactor for many antioxidant enzymes, such as glutathione peroxidases (GPx) and glutathione S-transferases (GST). GSH is available for these enzymes by *de novo* biosynthesis or reduction of its oxidized form GSSG by the activity of glutathione reductase (GR). GSH is also used in the glyoxalase system: the first enzyme Glx-I uses GSH, while the second one Glx-II regenerates GSH. Reprinted from (39).

### Figure 3. Glutathione participation in disulphide bond formation in endoplasmic reticulum

(1) Cysteines residues reach the ER as nascent proteins. (2) These residues can form disulphide bonds after oxidation and then reduce PDI. Ero1 is able to oxidize again PDI, leading to the generation of ROS that can react with GSH increasing GSSG concentration. (3) Also GSSG can oxidize PDI, then increasing GSH concentration. (4) GSH reduces PDI, increasing concentration of GSSG. Modified from (164).

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