ANTIOXIDANTS & REDOX SIGNALING
Volume 27, Number 15, 2017

DOI: 10.1089/ars.2017.7121

Discoveries

FORUM REVIEW ARTICLE

Mitochondrial Glutathione: Regulation and Functions

Gaetano Calabrese,[1] Bruce Morgan,[2] and Jan Riemer[1]

Abstract

Significance: Mitochondrial glutathione fulfills crucial roles in a number of processes, including iron–sulfur cluster biosynthesis and peroxide detoxification.
Recent Advances: Genetically encoded fluorescent probes for the glutathione redox potential (E_{GSH}) have permitted extensive new insights into the regulation of mitochondrial glutathione redox homeostasis. These probes have revealed that the glutathione pools of the mitochondrial matrix and intermembrane space (IMS) are highly reduced, similar to the cytosolic glutathione pool. The glutathione pool of the IMS is in equilibrium with the cytosolic glutathione pool due to the presence of porins that allow free passage of reduced glutathione (GSH) and oxidized glutathione (GSSG) across the outer mitochondrial membrane. In contrast, limited transport of glutathione across the inner mitochondrial membrane ensures that the matrix glutathione pool is kinetically isolated from the cytosol and IMS.
Critical Issues: In contrast to the situation in the cytosol, there appears to be extensive crosstalk between the mitochondrial glutathione and thioredoxin systems. Further, both systems appear to be intimately involved in the removal of reactive oxygen species, particularly hydrogen peroxide (H_2O_2), produced in mitochondria. However, a detailed understanding of these interactions remains elusive.
Future Directions: We postulate that the application of genetically encoded sensors for glutathione in combination with novel H_2O_2 probes and conventional biochemical redox state assays will lead to fundamental new insights into mitochondrial redox regulation and reinvigorate research into the physiological relevance of mitochondrial redox changes. *Antioxid. Redox Signal.* 27, 1162–1177.

Keywords: glutathione, mitochondria, genetically encoded sensors

Introduction

GLUTATHIONE (γ-GLUTAMYLCYSTEINYL-GLYCINE) is a nucleophilic, thiol-containing tripeptide that is found at high concentrations ($\sim$1–10 m*M*) in all eukaryotes and many prokaryotic species (Fig. 1A). Glutathione can exist in a thiol-reduced state (GSH) or an oxidized state (GSSG), which consists of two GSH molecules that are linked together by a disulfide bond.

GSH serves many crucial roles in the cell, including iron–sulfur cluster biogenesis, the detoxification of certain reactive oxygen species (ROS), toxic electrophiles, and heavy metals, as well as the reduction of protein disulfide bonds (Fig. 1B). GSH may also form disulfide bonds with protein thiol groups, a post-translational modification known as protein S-glutathionylation that may act to protect thiols from hyperoxidation or to regulate protein activity. Glutathione can fulfill some of these roles alone but more frequently, in cooperation with dedicated enzyme catalysts (Fig. 2) such as glutaredoxins (Grx), glutathione-S-transferases (Gst), and glutathione peroxidases (Gpx). Serving in these reactions often results in the oxidation of GSH to GSSG, which can, subsequently, be reduced back to GSH by glutathione reductase (Glr), by using electrons supplied by NADPH.

In living cells and tissue, glutathione exists predominantly as GSH, with GSH and GSSG typically found in a $\sim$50:1 molar ratio in whole-cell and tissue extracts (40). Whole-cell or tissue GSH:GSSG measurements report the average of different glutathione pools from all subcellular compartments. In reality, different subcellular compartments harbor very different glutathione pools. For example, the cytosolic glutathione pool is highly reduced, with a GSH:GSSG ratio in the order of 10,000:1 to 50,000:1 and a redox potential of the GSH/GSSG couple (E_{GSH}) below −300 mV (26, 50, 68, 71,

[1]Institute of Biochemistry, University of Cologne, Cologne, Germany.
[2]Department of Cellular Biochemistry, University of Kaiserslautern, Kaiserslautern, Germany.

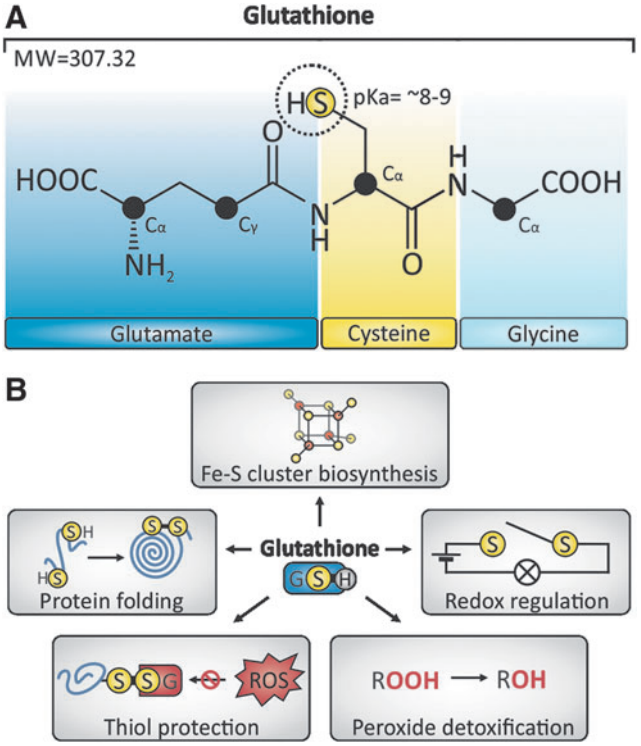

FIG. 1. Glutathione and its functions in mitochondria. **(A)** The chemical structure of the tripeptide glutathione. **(B)** Different functions of glutathione include roles in redox regulation, iron–sulfur cluster biogenesis, protection of protein thiols against oxidative stress, and protein folding.

Cytosol, other	IMS	Matrix
GSSG reductase		
Glr1 (cyto.)	-	**Glr1**
Glutaredoxin		
Grx1,2,8 [2C] (cyto.) **Grx3,4** [1C] (cyto.) **Grx6,7** [1C] (ER/secr.)	**Grx1,2** [2C]	**Grx2** [2C] **Grx5** [1C]
GSH peroxidase		
Hyr1/Gpx3 (cyto., nucl., perox.) **Gpx1** (perox.) **Gpx2** (cyto., nucl.)	**Hyr1/Gpx3** **Gpx1,2** (OMM)	**Gpx2**
Peroxiredoxin		
Tsa1,2 (cyto.) **Ahp1** (cyto., PM) **Dot5** (nucl.)	-	**Prx1**
Glutathione-S-transferase		
Gtt1 (PM, ER) **Gtt2,3** (cyto.) **Gto1,2,3** (cyto.)	**Gtt1** (OMM ?)	**Gtt1,2** (?)

FIG. 2. Glutathione-handling enzymes inside and outside mitochondria. Various enzymes use glutathione, including glutathione reductase, glutaredoxins, glutathione peroxidases, and glutathione-S-transferases. In the scheme, [1C] stands for monothiol glutaredoxins and [2C] stands for dithiol glutaredoxins. Protein localization was obtained from the *Saccharomyces cerevisiae* genome database. cyto., cytosol; ER, endoplasmic reticulum; secr., secretory pathway; nucl., nucleus; perox., peroxisome; OMM, outer mitochondrial membrane; PM, plasma membrane.

96, 97) (Fig. 3). Conversely, the glutathione pool in the endoplasmic reticulum (ER) is much more oxidized, with a GSH:GSSG ratio in the range of 1–15:1 (15, 87). These observations point toward the independent regulation of glutathione pools in different compartments and might suggest that the role and importance of glutathione may differ in different subcellular compartments. Mitochondria are no exception. They are double membrane-bound organelles, consisting of two aqueous compartments: the matrix, which is surrounded by the inner mitochondrial membrane (IMM), and the intermembrane space (IMS), which is sandwiched between the IMM and the outer mitochondrial membrane (OMM) (Fig. 3). The matrix and the IMS harbor different sets of glutathione-utilizing enzymes (Fig. 2) and employ different mechanisms to supply NADPH and to exchange small molecules, including glutathione, with other compartments.

In this review, focusing mainly on yeast for which there are extensive experimental data, we describe the different pathways that handle glutathione in mitochondria, highlight the dynamics of glutathione in the matrix and the IMS, and focus on open questions regarding the role of mitochondrial glutathione.

Glutathione Redox Homeostasis in Mitochondria

Glutathione fulfills a number of important roles in mitochondria, and, consequently, it is likely crucial that glutathione homeostasis is robustly maintained. However, it remains unclear as to what the important parameters of glutathione homeostasis actually are. For example, do cells seek to preserve a constant concentration of GSH or GSSG, or do they rather strive to maintain a specific GSH:GSSG ratio? Alternatively perhaps, cells seek to maintain a constant mitochondrial E_{GSH}, which would require regulation of both glutathione concentration and the GSH:GSSG ratio.

Technical limitations remain the major impediment to gaining a deeper understanding of glutathione redox homeostasis, and the measurement of either glutathione concentration or the GSH:GSSG ratio inside defined subcellular compartments remains impossible with current technology. Nonetheless, it is clear that many processes, including glutathione synthesis, degradation, import, export, oxidation, and reduction, will influence glutathione redox homeostasis in the IMS and matrix. Here, we discuss the current knowledge of each of these factors and their relevance for mitochondrial glutathione regulation.

Mitochondrial glutathione is synthesized in the cytosol

Mitochondria lack the enzymes to synthesize glutathione. In both yeast and human cells, glutathione synthesis is

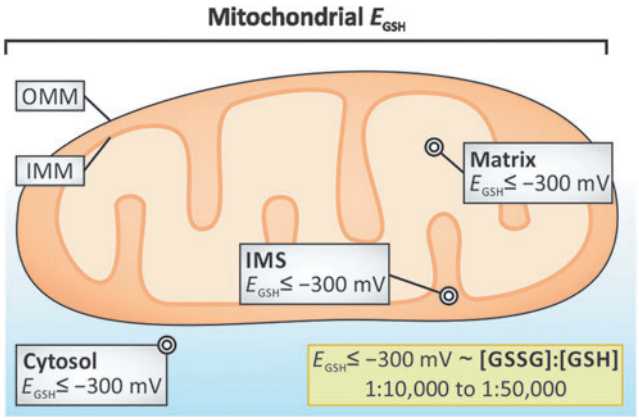

FIG. 3. E_{GSH} in different mitochondrial compartments. Measurements performed with genetically encoded fluorescent sensors of the glutathione redox potential (E_{GSH}) have revealed extremely GSH pools in the cytosol, IMS, and matrix. In all three compartments, E_{GSH} is below −300 mV. GSSG:GSH values have been calculated *via* the Nernst equation with pH 7, $T = 25°C$, and a total glutathione concentration of 10 m*M*. GSH, reduced glutathione; GSSG, oxidized glutathione; IMS, intermembrane space.

confined to the cytosol and glutathione must be imported into both the IMS and the matrix. The synthesis of GSH involves two separate ATP-consuming steps (Fig. 4). Initially, glutamate and cysteine react to form γ-glutamylcysteine, followed by a second step in which γ-glutamylcysteine forms a peptide bond with glycine to yield the tripeptide GSH (82). Interestingly, γ-glutamylcysteine can partially substitute for GSH when GSH synthesis is blocked (41, 135). The first step of glutathione synthesis is rate limiting and is catalyzed by

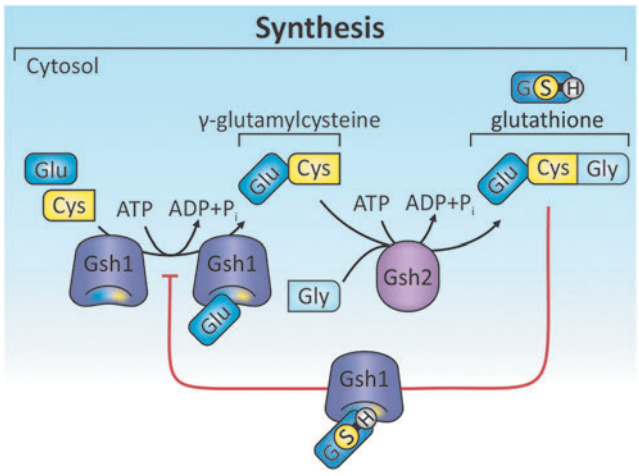

FIG. 4. Glutathione synthesis. Glutathione synthesis is exclusively restricted to the cytosol. Synthesis of glutathione involves two ATP-dependent steps. First, γ-glutamylcysteine synthetase catalyzes the formation of a peptide bond between the γ-carboxyl group of glutamate and the amino group of cysteines. In a second step, glutathione synthetase catalyzes the formation of a conventional peptide bond between glycine and γ-glutamylcysteine. γ-glutamylcysteine synthetase is non-allosterically inhibited by the product of the pathway, glutathione. Gsh1, γ-glutamylcysteine synthetase; Gsh2, glutathione synthetase.

the γ-glutamylcysteine synthetase (Gsh1 in yeast) (67, 95). γ-glutamylcysteine synthetase is composed of two subunits that are encoded by different genes in drosophila and humans, and as a single protein with two domains in bacteria and yeast. The catalytic domain/subunit is subject to non-allosteric feedback inhibition by GSH, which competes with the binding of glutamate (112) (Fig. 4). The interaction of the first with the second domain/subunit increases the K_i toward GSH and, thus, can increase activity of the first subunit in the presence of higher levels of GSH (51, 52). A second level of regulation of the formation of γ-glutamylcysteine is the concentration of available L-cysteine. The intracellular cysteine concentration is in the order of the enzyme K_M, whereas the intracellular glutamate concentration is about 10 times the K_M. This becomes relevant in situations of sulfur/cysteine starvation (76, 82).

In yeast, the *GSH1* gene is also subject to transcriptional regulation by the transcription factors Yap1 (positive regulation) and Skn7 (negative regulation) (92, 122, 123, 140). It has been shown that yeast cells lacking *GSH1* display an irreversible respiratory incompetency and mitochondrial DNA loss over several cell divisions (7, 73). Further, the transcription factor Met4, which regulates the sulfur amino acid pathway, plays a role in GSH biosynthesis (135). Thus, glutathione synthesis is integrated into the complex network of responses to oxidative stress, osmolarity, and starvation. GSH synthetase (Gsh2 in yeast) catalyzes the second step in GSH synthesis (55, 95). This enzyme is not limiting for GSH synthesis as overexpression of GSH synthetase does not result in increased GSH levels (41).

Degradation of GSH

Similar to the synthesis of glutathione, its degradation is a two-step process involving the enzymes γ-glutamyltranspeptidase and L-cysteinylglycine dipeptidase (Fig. 5), neither of which is present in mitochondria. γ-glutamyltranspeptidase is responsible for the removal of the γ-glutamyl moiety of any γ-glutamyl compound, including GSH, yielding an L-glutamate molecule and cysteinylglycine. The γ-glutamyltranspeptidase in yeast, Ecm38, is glycosylated and localized in the vacuole as a membrane-anchored protein, with the catalytic domain facing the vacuolar lumen (57, 81, 129). Ecm38 is involved in the detoxification of electrophilic xenobiotics, and its expression is mainly induced by nitrogen starvation (119, 129). There also exists a γ-glutamyl cyclotransferase, which cleaves the γ-glutamyl bond of glutathione to yield 5-oxoproline and cysteinylglycine. A GFP fusion of this enzyme localizes to the cytosol and the nucleus, and it is periodically expressed during the metabolic cycle (70). L-cysteinylglycine dipeptidase activity has been measured in yeast and is associated with the vacuole; however, the open reading frame for the enzyme has yet to be identified but might be the recently identified Dug1 pathway (82).

The degradation of glutathione in the yeast vacuole is paralleled by the degradation of glutathione by extracellular peptidases in mammalian cells. In both human cells and yeast, glutathione alone or in a complex, for example with xenobiotics, is expelled from the cytosol by ABC transporters [Ycf1 in yeast (74, 75, 89, 109) and MRP1 and MRP5 in mammals (8, 64, 84)]. In mammalian cells, γ-glutamyltranspeptidase is localized to the outer face of the plasma membrane (48). This degradation pathway not only

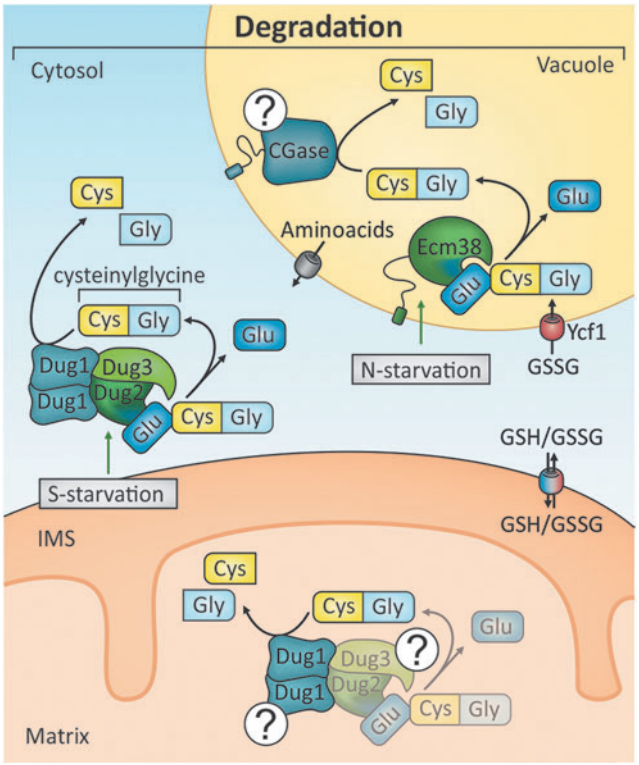

FIG. 5. Glutathione degradation. Similar to glutathione synthesis, degradation is a two-step process, which in yeast takes place in the vacuole and the cytosol. Speculatively, a pathway for glutathione degradation may also be present within the mitochondrial matrix. In mammalian cells, glutathione degradation involves extracellular peptidases. CGase, hypothetical protein with L-cysteinylglycine dipeptidase function; Ecm38, vacuolar γ-glutamyltranspeptidase; Dug1, Cys-Gly metallo-di-peptidase; Dug2, component of glutamine amidotransferase working in a complex with Dug3.

facilitates rapid removal of xenobiotics from the cytoplasm but also serves to remove excess amounts of GSSG during acute oxidative stress and to mediate transport of cysteine and glutathione precursors between cells and organs (58, 130).

Yeast harbors an alternative glutathione degradation system involving a complex of three proteins, Dug1, Dug2, and Dug3 (35, 61). In addition to GSH degradation, the Dug system can also degrade other tri- and tetrapeptides *in vitro* and might, thus, be less specific and exhibit lower activity compared with the γ-glutamyltranspeptidase/L-cysteinylglycine dipeptidase system. It might also account for the previously measured L-cysteinylglycine dipeptidase activity. Dug1 is a metallo-dipeptidase with Cys-Gly dipeptidase activity and is believed to be present in both the cytosol (35) and mitochondria (53, 110, 111). Dug1 is able to form homodimers and can operate in a Dug2-Dug3-independent manner as a dipeptidase with high specificity for cysteinylglycine. The human homologue CNDP2 can complement the loss of the dipeptidase activity in a Δ*dug1* yeast strain (62). Dug2 and Dug3 act as heterodimers and are required to cleave glutathione into glutamate and cysteinylglycine. Dug3 exhibits glutamine amidotransferase (GATase) activity, whereas Dug2 is responsible for the interaction with GSH and exhibits no peptidase activity. Both genes are constitutively repressed and only become induced under sulfur starvation in an Met4-dependent manner, thus pointing to a highly regulated crosstalk between glutathione degradation and cysteine availability (61). Dug3 and Dug2 are believed to mainly localize to the cytosol; however, prediction tools such as Predotar, MitoProt, and PSORT place Dug3 in the mitochondrial matrix. High-throughput mass spectrometric protein complex identification experiments also suggest that Dug2 interacts with matrix proteins (83, 108). Thus, in principle, the matrix might harbor a complete glutathione degradation system. If so, this would further emphasize the importance of a glutathione import pathway into the matrix.

Mitochondrial import and export of glutathione

Due to the exclusive localization of glutathione synthesis to the cytosol, it is crucial that glutathione is imported into the IMS and the matrix (Fig. 6). Glutathione is negatively charged and unable to pass freely across a lipid bilayer. Clearly, both the OMM and IMM must harbor transporters or channels that facilitate the entry of GSH. In addition, there may be a requirement for GSH and/or GSSG to exit from the matrix and the IMS to the cytosol, which would also need to be mediated by transporters.

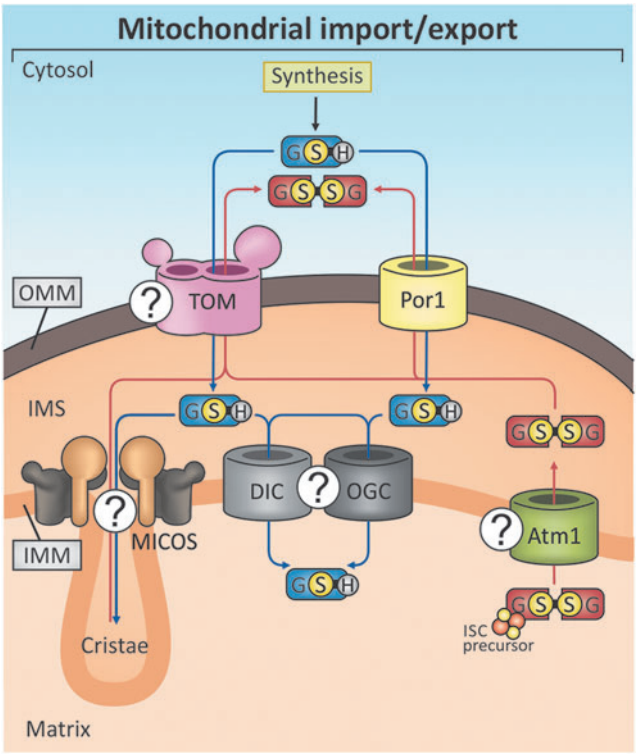

FIG. 6. Glutathione import into mitochondria. Glutathione traverses the outer membrane (OMM) of mitochondria *via* porin/voltage-dependent anion channels and likely *via* the TOM40 protein translocase. Glutathione transport across the OMM appears to be rapid, allowing for equilibration of the cytosolic and IMS glutathione pools. No evolutionarily conserved IMM glutathione transporter has been identified. IMM, inner mitochondrial membrane; TOM, translocase of the outer membrane; Por1, mitochondrial porin; MICOS, mitochondrial inner membrane complex involved in maintenance of crista junctions; DIC, dicarboxylate carrier; OGC, oxoglutarate carrier; Atm1, mitochondrial inner membrane ABC transporter involved in the export of precursors of iron–sulfur clusters.

The mechanism of glutathione transport across the OMM appears to be relatively straightforward. The OMM is rich in porins (in mammalian cells, voltage-dependent anion channels), large transport proteins, which form aqueous channels through the lipid bilayer. Porins allow molecules smaller than ~5 kDa to diffuse between the IMS and the cytosol, including small proteins and glutathione, although it remains to be strictly proved whether this is the case for both GSH and GSSG (25). In the absence of porins, the translocase of the outer membrane (TOM) can also facilitate small-molecule transport across the OMM (72, 124). However, it remains unclear as to what extent this channel contributes to small-molecule transport in the presence of porins. The free passage of small molecules across the OMM, including glutathione, would indicate that the small-molecule environment of the IMS is equivalent to that of the cytosol, as discussed later in detail. One possible caveat to this conclusion would be that it is still unclear as to what extent the OMM slows free diffusion, which might be important, for example, during rapidly changing stress conditions. Another important consideration is that the IMM serves to divide the IMS into two further subcompartments, the cristae space and the peripheral IMS, which are separated from each other by cristae junctions. These junctions not only have a structural role but might also serve in regulating protein distribution in the IMM and perhaps control small-molecule diffusion between the cristae space and the peripheral IMS (12, 45, 47, 49, 131, 136). The accumulation of the proton-pumping and ROS-generating respiratory chain complexes in the cristae membrane likely results in differences in the small-molecule composition (pH, ROS, glutathione) between the peripheral IMS and cristae, but this remains to be extensively explored. Nonetheless, porins are likely the common initial entry point for glutathione destined for both subcompartments of the IMS and for the matrix.

The IMM and OMM differ strongly in terms of protein and lipid composition (13, 23, 127). In contrast to the OMM, the IMM is impermeable to most of the solutes that can pass the OMM, including glutathione. Instead, dedicated transporters facilitate the transport of specific substrates into the matrix. Compared with the situation for the OMM, the identity of the proteins that facilitate transport of GSH or GSSG across the IMM is much less clear, although there are some suggestions. Two anion carriers, the dicarboxylate carrier (DIC) and the oxoglutarate carrier (OGC), were identified as transporters of GSH based on experiments in isolated human kidney mitochondria and mitoplasts. GSH import was found to be hampered in the presence of dicarboxylates and inhibitors of the transporters (21, 22). In contrast, in *Lactococcus lactis*, DIC and OGC do not transport GSH (16), suggesting the existence of alternative glutathione transporters in this organism. In yeast, it remains unclear as to whether the DIC and OGC homologs transport glutathione. Thus, a conclusive identification of an evolutionarily conserved mitochondrial glutathione transporter is still missing. An alternative possibility is that glutathione import to the matrix relies on multiple low-affinity transporters that typically transport alternative substrates but might ''moonlight'' in replenishing the matrix with glutathione. This might also be in line with the lack of any influence of the charge of GSH on IMM transport, which is pH dependent due to the pK_a of the cysteine moiety of 9 (16).

Glutathione can also be exported from the matrix. This takes place *via* the IMM transporter Atm1, which is involved in the export of mitochondrially synthesized iron–sulfur cluster assembly intermediates to the cytosol. Atm1 is stimulated by GSSG but not by GSH, and it appears to transport GSSG across the IMM in yeast (116). Further, the crystal structure of Atm1 includes GSH bound to the transporter, supporting its role in GSH or GSSG transport (120). The plant homologue Atm3 fulfills the same function (116).

The matrix and IMS glutathione pools are highly reduced

A recent major advance in terms of our ability to investigate cellular glutathione homeostasis came with the development of genetically encoded fluorescent E_{GSH} sensors, which now permit real-time E_{GSH} measurements in defined subcellular compartments in living cells (5, 6, 30, 90, 117). These probes are based on a genetic fusion between a redox-sensitive GFP (roGFP) and a glutaredoxin that mediates the thermodynamic equilibration of the roGFP thiol/disulfide with the GSH/GSSG redox couple. These probes have already yielded significant new understanding of mitochondrial glutathione homeostasis.

Both the matrix and the IMS glutathione pools have been investigated with roGFP-based sensors. Interestingly, the matrix and IMS glutathione pools were found to be extremely reduced with E_{GSH} values ≤ -300 mV in both compartments in yeast and mammalian cells (33, 44, 46, 68, 90) (Fig. 3). These observations imply a GSH:GSSG ratio >10,000:1, with GSSG present only in trace amounts. Further, both compartments can rapidly restore E_{GSH} after an oxidative challenge with diamide or hydrogen peroxide (H_2O_2) (68), suggesting that GSSG is robustly removed or reduced (50, 68).

The matrix and the IMS independently regulate E_{GSH}

Perhaps surprisingly, given their very similar glutathione pools, recent experiments in yeast have clearly revealed that the matrix and the IMS employ independent mechanisms to maintain glutathione redox homeostasis and suggest that there is very limited communication between the IMS and matrix glutathione pools (68) (Fig. 7). The major evidence in support of this conclusion comes from experiments on yeast strains with differential targeting of glutathione reductase (Glr1) (39, 68). Glr1 reduces GSSG to GSH by using an FAD cofactor and NADPH. Glr1 localizes to both the cytosol and the mitochondrial matrix, but it has not been detected in the IMS. The cytosolic and the matrix forms of Glr1 are encoded by the same gene and translated from two different start codons in the mRNA (99); this translational control is generally believed to be conserved in mammalian systems. Interestingly, in a strain lacking the *GLR1* gene, E_{GSH} in the matrix cannot recover after an oxidative challenge, whereas IMS E_{GSH} can (50, 68, 69). In this respect, the IMS mirrors the situation in the cytosol where E_{GSH} can be restored in the absence of Glr1 due to the presence of other mechanisms for GSSG removal, including Trx2-mediated GSSG reduction and ABC transporter-mediated export of GSSG (88, 89, 125). In yeast, Ycf1 mediates GSSG transport to the vacuole (89); whereas in mammalian cells, GSSG is exported *via* plasma membrane-localized transporters (9, 84, 139). On generation of a yeast strain that exclusively produces the cytosolic form of Glr1, it was observed that both cytosolic and IMS E_{GSH} is restored to its correct, highly reduced, state, whereas matrix E_{GSH} remains oxidized. In addition, in a yeast strain

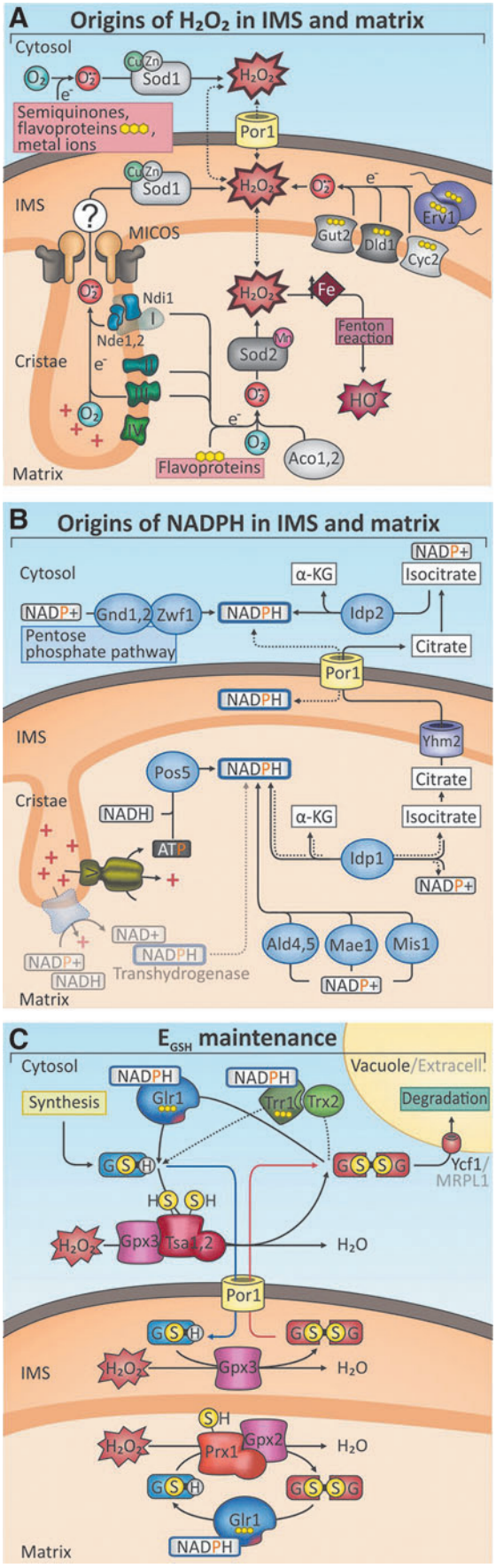

FIG. 7. Glutathione pool maintenance and dynamics in the IMS and matrix. The glutathione pools of the cytosol, IMS, and matrix are influenced by multiple factors, including levels of other cellular redox species, NADPH availability, and redox enzymes. **(A)** $O_2^{\bullet-}$ and H_2O_2 arise from the incomplete reduction of molecular oxygen by electrons originating from the electron transport chain, flavoproteins, semiquinones, or metal ions. The copper–zinc superoxide dismutase (Sod1, cytosol, and IMS) and the manganese superoxide dismutase (Sod2, matrix) convert $O_2^{\bullet-}$ to H_2O_2. H_2O_2 can diffuse across membranes or (more rapidly) *via* channels. **(B)** NADPH is the source of cellular-reducing equivalents. In the cytosol, it is synthesized by dehydrogenases of the pentose phosphate pathway (Gnd1, Gnd2, and Zwf1). In the matrix, the process involves dehydrogenases (Ald4, Mae1, Mis1, and Idp1) and kinases (Pos5). The latter is absent in mammals. Mammalian cells rely on the membrane potential-dependent NAD(P) transhydrogenase (NNT). Idp1 and Idp2 are responsible for the NADPH shuttling from the matrix to the cytosol by using the citrate transporter Yhm2 and the porin Por1 to cross the IMS. **(C)** The IMS glutathione pool is believed to be in equilibrium with the cytosolic glutathione pool due to the free passage of GSH and GSSG across the OMM. Effectively, the IMS depends on cytosolic enzymes using reductive equivalents provided by cytosolic NADPH to reduce GSSG. In contrast, the matrix harbors its own pools of glutathione-metabolizing enzymes, and it maintains independent pathways for the production of NADPH. The matrix glutathione pool must be replenished by GSH synthesized in the cytosol, but the rate of glutathione transport across the IMM appears to be so slow that the matrix glutathione pool is kinetically isolated from the IMS and cytosolic glutathione pools. Sod1, cytosolic/IMS copper–zinc superoxide dismutase; Por1, mitochondrial porin of the outer membrane; Erv1, flavin-linked sulfhydryl oxidase of the mitochondrial IMS (in humans ALR); Gut2, mitochondrial glycerol-3-phosphate dehydrogenase; Dld1, mitochondrial D-lactate dehydrogenase; Cyc2, mitochondrial peripheral inner membrane oxidoreductase involved in ligation of heme to apocytochromes *c* and *c1*; Nde1,2 and Ndi1, IMS and matrix NADH dehydrogenases; Sod2, mitochondrial manganese superoxide dismutase; Aco1,2, mitochondrial aconitases; Gnd1,2, 6-phosphogluconate dehydrogenases; Zwf1, cytosolic glucose-6-phosphate dehydrogenase; Idp1,2, cytosolic and mitochondrial isocitrate dehydrogenase; Yhm2, citrate and oxoglutarate carrier; Pos5, mitochondrial NADH kinase; Ald4,5, mitochondrial aldehyde dehydrogenases; Mae1, mitochondrial malic enzyme; Mis1, mitochondrial C1-tetrahydrofolate synthase (in humans MTHFD); Glr1, cytosolic and mitochondrial glutathione reductase; Trr1, cytoplasmic thioredoxin reductase; Trx2, cytoplasmic thioredoxin; Ycf1, vacuolar glutathione S-conjugate ABC-C transporter; Gpx3, thiol peroxidase involved in the response to high H_2O_2 levels; Tsa1,2, cytosolic thioredoxin peroxidases; Prx1, mitochondrial peroxiredoxin with thioredoxin peroxidase activity; Gpx2, phospholipid hydroperoxide glutathione peroxidase. The enzymes represented in semi-transparency are enzymes only present in mammals, for example, NAD(P) transhydrogenase NNT.

deleted for *ZWF1*, the gene encoding glucose 6-phosphate dehydrogenase, the major source of cytosolic NADPH, the recovery of cytosolic and IMS E_{GSH} is significantly impaired after an oxidative challenge but recovery of matrix E_{GSH} is unaffected (100). The matrix independently produces NADPH from a variety of sources, including malic enzyme, the NADH kinase Pos5, and aldehyde dehydrogenases, for example, Ald4 (59, 85, 98) and in higher eukaryotes by the proton-dependent NAD(P) transhydrogenase (56, 93) and methylene tetrahydrofolate dehydrogenase (MTHFD2, in yeast Mis1), a central enzyme in one-carbon metabolism (31, 94) (Fig. 7).

The experiments described earlier indicate that the IMS glutathione pool is effectively regulated by cytosolic enzymes and that the cytosolic and IMS glutathione pools must be in close communication with the rapid transport of glutathione across the OMM (Fig. 7). The finding that the deletion of *POR1* (the gene encoding the major yeast porin isoform) in yeast results in a more oxidized E_{GSH} in the IMS compared with the cytosol supports this notion. In contrast, although the IMM presumably must harbor transporters that facilitate the import of GSH in addition to a transporter, Atm1, that can facilitate the export of GSSG, it appears that these processes are too slow to mediate any detectable equilibration of the matrix with the IMS/cytosolic glutathione pool, at least on a timescale of seconds to minutes. Thus, although glutathione transport across the IMM is clearly sufficient to resupply the matrix with glutathione that is lost by export or degradation, it appears to be too slow to impact the dynamic regulation of matrix E_{GSH}.

Functions of Glutathione in the Mitochondrial Matrix

In addition to the independent mechanisms for regulation of the IMS and matrix glutathione pools, it is now apparent that the two compartments maintain very different populations of glutathione-utilizing redox enzymes and glutathione seems to fulfill different functions in each compartment.

The matrix harbors several enzymes that react with or utilize glutathione (Figs. 2 and 8). Some of these enzymes are dually localized and, thus, also found in the cytosol, including Glr1 and the dithiol glutaredoxin Grx2 (99, 105, 107). However, in addition, the matrix also contains two glutathione transferases, Gtt1 and Gtt2, and a selection of enzymes that are found exclusively in the matrix, including the monothiol glutaredoxin Grx5, a single cys-peroxiredoxin, Prx1, a thioredoxin, Trx3, and a thioredoxin reductase Trr2 (86, 101, 103, 105, 114, 118, 134). Each of these enzymes may interact with glutathione and/or be important for catalyzing reactions between glutathione and protein thiols.

Glutathione regulates Trx3 redox state

Glutathione was recently implicated in the maintenance of Trx3 redox state in yeast (128). Intriguingly, in a Δ*trr2* strain deleted for the yeast mitochondrial thioredoxin reductase, Trx3 remains in a fully reduced state. On the contrary, deletion of the cytosolic thioredoxin reductase leads to oxidation of both cytosolic thioredoxins. However, when *TRR2* was deleted in combination with glutathione reductase, Trx3 was found to accumulate in an oxidized form (128). Thus, counterintuitively, it seems that in the matrix the glutathione system has a more prominent role in maintaining Trx3 in a reduced state compared with the situation in the cytosol (43, 128).

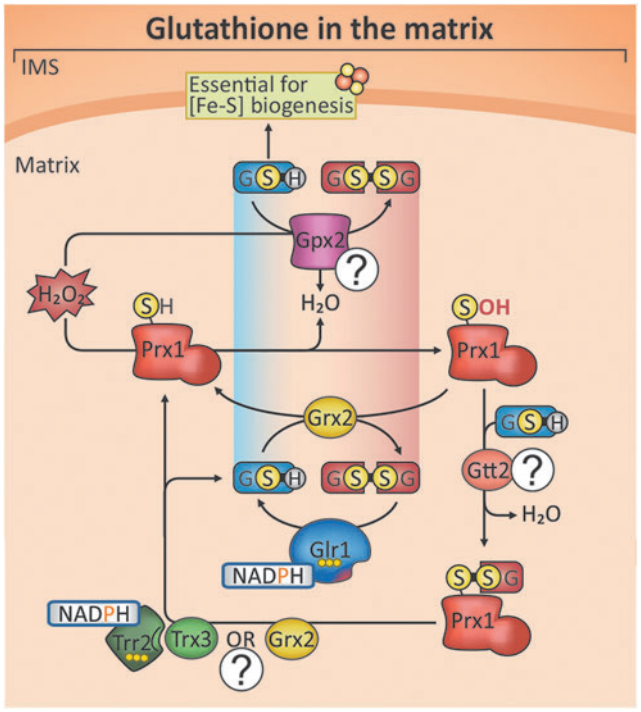

FIG. 8. Roles of glutathione in the matrix. Glutathione fulfills a number of roles in the matrix, including an essential role in the biogenesis of iron–sulfur clusters. Glutathione is also important for the reduction of H_2O_2, serving as a reductant for the matrix peroxiredoxin Prx1. However, the exact mechanism remains unclear and several models have been proposed (see text for details). Gpx2, phospholipid hydroperoxide glutathione peroxidase; Prx1, mitochondrial peroxiredoxin with thioredoxin peroxidase activity; Glr1, cytosolic and mitochondrial glutathione reductase; Grx2, cytoplasmic and mitochondrial glutaredoxin; Gtt2, mitochondrial glutathione S-transferase; Trr2, mitochondrial thioredoxin reductase; Trx3, mitochondrial thioredoxin.

Glutathione may be important for H_2O_2 reduction in the matrix

The yeast matrix harbors Prx1, a 1-cys peroxiredoxin that is believed to be the major pathway for the reduction of matrix H_2O_2.. Peroxiredoxins usually rely on the thioredoxin system to provide electrons for reduction. However, the situation appears to be more complex in the matrix and several models for Prx1 reduction have been proposed. First, it was suggested that Prx1 is reduced by a combination of Trr2 and GSH (42). It was proposed that the sulfenic acid on the Prx1 peroxidatic cysteine initially reacted with GSH, yielding a glutathionylated Prx1. Trr2, subsequently, attacked the glutathionlyated Prx1, releasing GSH and yielding a mixed disulfide between Prx1 and Trr2, which was, subsequently, reduced by two GSH molecules, leading to the production of GSSG. Thus, in this scheme, the reduction of one H_2O_2 molecule yielded one GSSG, thereby linking the glutathione pool to H_2O_2 production.

A second scheme was proposed, which, as with scheme 1, relies on an initial glutathionylation of the peroxidatic cysteine of Prx1. However, it was suggested that the glutathionylation was subsequently reduced by the dithiol Grx2, ultimately yielding a GSSG molecule as in scheme 1. Such Grx-mediated reduction of a peroxiredoxin was not

unprecedented (19, 104). Indeed, *Haemophilus influenzae* harbors a Prx-Grx fusion protein, which can reduce H_2O_2 completely independently of a Trx system. The Prx-Grx fusion protein forms a tetramer in which the active site interfaces of Grx and Prx from different monomers alternatively interact with each other, thereby facilitating the electron transfer from the Grx to the Prx domain (66). Another similar possibility is that the monothiol Grx5 could react with the oxidized Prx1 and form an intermolecular disulfide bond that is then resolved by GSH (27).

A third alternative model proposes that Trx3 reduces the glutathionylated Prx1 (102). This implies that GSH was not oxidized directly during the reaction (102). However, the subsequent reduction of Trx3 may be glutathione dependent and, therefore, even this mechanism may indirectly lead to glutathione oxidation, although the relative contribution of glutathione and Trr2 for Trx3 reduction remains to be fully determined. Interestingly, however, when Prx1 is absent, even in a Δ*glr1*Δ*trr2* strain, Trx3 is no longer oxidized, even in the presence of H_2O_2, pointing to Prx1 as the key translator of H_2O_2 to matrix protein targets and matrix E_{GSH} (43).

A fourth model for Prx1 reduction has been presented, based on experiments with purified bovine 1-cys peroxiredoxin. Here, the Prx first required an activation step *via* heterodimerization with a glutathione transferase, followed by glutathionylation of its oxidized catalytic cysteine. The successive separation into monomers yielded a glutathionylated Prx, which became catalytically active only after spontaneous reaction with GSH, generating a GSSG molecule (80). This process, thus, also resulted in GSSG generation.

It is clear that more experiments are required to fully dissect the complex interplay between mitochondrial glutathione, H_2O_2, and the thioredoxin system. Perhaps the roGFP2 fusions with yeast Grx1, Grx2, and Prx1 (89, 91) can help to untangle the complexity in intact living cells. It will certainly be exciting to investigate this in future.

S-glutathionlyation of proteins as a regulatory mechanism

Protein S-glutathionylation involves the covalent linkage of a GSH molecule to a protein cysteine residue *via* a disulfide bond. Protein S-glutathionylation has been proposed to be an important mechanism for regulating protein function and activity in mitochondria. Although many questions remain, there are several plausible routes by which protein S-glutathionylation may be achieved in mitochondria, which are described later.

The first of these involves Grx-catalyzed glutathionylation. Grxs are traditionally considered to mediate protein deglutathionylation reactions. However, it is clear that the Grx-catalyzed reaction is fully reversible and, thus, Grxs may actually catalyze S-glutathionylation of some proteins in response to an increase in E_{GSH}. Indeed, the E_{GSH}-sensing Grx1-roGFP2 probes require a fully reversible catalytic activity of Grxs for their function. In response to an increase in E_{GSH}, that is, an oxidation of the glutathione pool, Grx1 is glutathionylated. Grx1, subsequently, passes this glutathionylation onto a cysteine residue in the roGFP2 protein. A second cysteine in roGFP2 then resolves the glutathionylation, leading to the formation of an intramolecular disulfide bond in roGFP2. This reaction is fully reversible; hence, when the glutathione pool becomes more reduced, GSH attacks the roGFP2 disulfide and Grx1 catalyzes the deglutathionylation of the roGFP2. Effectively, Grx1 catalyzes thermodynamic equilibration between the glutathione redox couple and roGFP2. Although roGFP2 is clearly not a physiological substrate, it, nonetheless, nicely illustrates the feasibility of Grx-mediated S-glutathionylation. Both yeast Grx1 and Grx2 were shown to be capable of mediating the fully reversible equilibration of roGFP2 with the glutathione redox couple (89). The dithiol glutaredoxin Grx2 is present in the cytosol, matrix, and the IMS in yeast. Similar to Glr1, it is encoded by one gene that gives rise to one mRNA. This mRNA contains two alternative start codons, which lead to the production of either a short cytosolic form of Grx2 or a long form that additionally harbors a mitochondrial targeting sequence (105–107). On import into the matrix, this sequence is cleaved, leaving behind a mature form of Grx2 that contains four additional amino acid residues (STPK) compared with its cytosolic counterpart (134). This tetrapeptide extension results in a markedly higher activity of mitochondrial Grx2 in the catalysis of dihydrolipoamide-driven GSSG reduction (106). A portion of the long mitochondrial Grx2 also localizes as an unprocessed form in the OMM (106). The IMS harbors the cytosolic form of Grx2 as well as small amounts of its homolog Grx1, which is also a cytosolic protein (69, 133). The mechanism for import of Grx1 and Grx2 into the IMS is unknown. Given the multiple localization patterns of Grx2 and Grx1 and their feasibility to promote protein S-glutathionylation, it will be particularly interesting in the future to determine their importance for endogenous mitochondrial proteins.

A second mechanism by which proteins could become S-glutathionylated is *via* the reaction of a cysteinyl thiol group with a GSH thiyl radical. Grxs may also be important in this context as human Grx2 has been shown to stabilize GSH thiyls (121).

The third possible mechanism that has the potential to mediate protein S-glutathionylation in mitochondria is *via* Gst activity. Gsts are generally known for their ability to conjugate glutathione to xenobiotics to improve water solubility and facilitate elimination. Moreover, they can be involved in ROS handling due to their Gpx activity and their ability to conjugate GSH to lipid peroxides (3, 4, 54, 126). Yeast contains six Gsts: Gto1–3, and Gtt1–3 (24, 36, 115). Of these, only Gtt2 has been proposed to be dually localized to the cytosol and matrix, and it has been suggested that the enzyme exhibits some functional overlap with Grx1 and Grx2 (38, 110, 118). Gsts catalyze glutathionylations, which involve the binding of GSH to the amino acids of the active sites that then promote thiolate formation in the GSH by lowering its pK_a. After deprotonation, the glutathionylate is transferred to the target thiol, forming a reversible disulfide bond (27). Interestingly, Gtt2 lacks classical catalytic residues, including a cysteine residue (77). Absence of the cysteine might, therefore, allow Gtt2 to perform glutathionylations without itself becoming a target of any of them in a highly dynamic redox environment such as the matrix.

Glutathionylation may also proceed in a non-enzyme catalyzed manner. For example, protein thiols that have become oxidized to a sulfenic acid or to a thiyl radical may react directly with GSH, leading to the formation of a disulfide bond between the protein thiol and GSH. Irrespective of the mechanism of formation, it still remains unclear as to how protein S-glutathionylation exists as a stable modification in a highly

reduced glutathione pool such as that typically found in the mitochondrial matrix, in which Grxs would normally be expected to efficiently catalyze deglutathionylation reactions.

Of the processes proposed to be regulated by S-glutathionylation in mitochondria, perhaps the most studied is the regulation of metabolism-related proteins, including proteins in complex I, III, and V, as well as enzymes of the TCA cycle, including aconitase, 2-oxoglutarate dehydrogenase, and succinyl coenzyme A synthetase (2, 20, 37, 60). Other notable proposed S-glutathionylation targets include the IMM uncoupling proteins UCP2 and UCP3 (78, 79). However, in our opinion, the physiological relevance of these modifications remains to be fully demonstrated. The majority of work looking at protein S-glutathionylation has been performed on isolated mitochondria or mitochondrial membranes (32). In these systems, the evidence for protein S-glutathionylation is convincing; however, it is important to be aware that significant glutathione oxidation likely takes place very rapidly on opening cells. Further, during isolation, mitochondria will unavoidably be exposed to oxygen levels that are many times higher than would be expected inside an intact cell. These results raise the question of whether the S-glutathionylation of mitochondrial proteins that are observed in these samples is indicative of the physiological cellular situation.

Glutathione serves an essential role in iron–sulfur cluster biogenesis in the matrix

Besides its role in redox reactions, GSH plays an important role in iron homeostasis and iron–sulfur cluster assembly, and its essential role in yeast viability appears to be directly linked to this function (71). The mitochondrial matrix contains a single monothiol Grx, Grx5, which plays a role in iron metabolism by directly interacting with iron–sulfur cluster-binding proteins (10, 11, 65, 137, 138). Therefore, a GSH-containing Grx5 complex binds iron–sulfur clusters and helps to transfer them toward apoproteins. Absence of Grx5 leads to iron accumulation in the cell and inactivation of enzymes requiring iron–sulfur clusters for their activity (114). Since Grx5 is involved in the biogenesis of all mitochondrial iron–sulfur cluster proteins, it belongs to the core assembly machinery (10, 65, 114). Moreover, it is hypothesized that export of a sulfur component from mitochondria that is required for cytosolic iron–sulfur cluster biogenesis relies on glutathione. Further findings support the role of glutathione in iron homeostasis: Both GSH depletion in a *Δgsh1* strain and GSH over-accumulation in *HGT1*-overexpressing cells led to an iron starvation-like response. The phenotype in Δ*gsh1* cells can be partially rescued by the addition of ferric iron (71). Deletion of the mitochondrial NADH kinase (Pos5) that supplies NADPH to efficiently maintain high levels of reduced glutathione leads to hyperaccumulation of iron in mitochondria, with phenotypes similar to the yeast Δ*isa2* mutant, known to be defective in iron–sulfur cluster biogenesis and to be auxotrophic for arginine (98). Moreover, Δ*gsh1* yeast strains with additionally impaired Trx activity (Δ*trr1 vs.* Δ*gsh1*Δ*trr1*) required higher external glutathione levels for survival in contrast to strains with a functional Trx system (wt *vs.* Δ*gsh1*) (71). An interesting effect of GSH over-accumulation was that it impaired the activity of the cytosolic iron–sulfur cluster-containing protein Leu1 but not the mitochondrial Aco1, indicating that the matrix is affected differently under these conditions (71).

Glutathione in the Mitochondrial IMS

In contrast to the matrix, the IMS does not appear to contain any of its own glutathione-related enzymes. Instead, the IMS harbors small populations of cytosolic redox enzymes, including the dithiol glutaredoxins Grx1 and Grx2, a member of the glutathione peroxidase family Gpx3, a thioredoxin, Trx1, and a thioredoxin reductase, Trr1 (133) (Fig. 9). At present, it remains unclear as to how these enzymes are imported into the IMS. Further, it is not known whether these proteins are equally distributed between the peripheral IMS and the cristae space or whether they are preferentially localized to either one of the two IMS subcompartments.

Enzymes control the translation of E_{GSH} to IMS proteins

The extremely reduced E_{GSH} values determined for the cytosol/IMS and matrix arguably have only limited meaning in a physiological context, since the rate constants for the spontaneous reaction of GSH and GSSG with proteins are

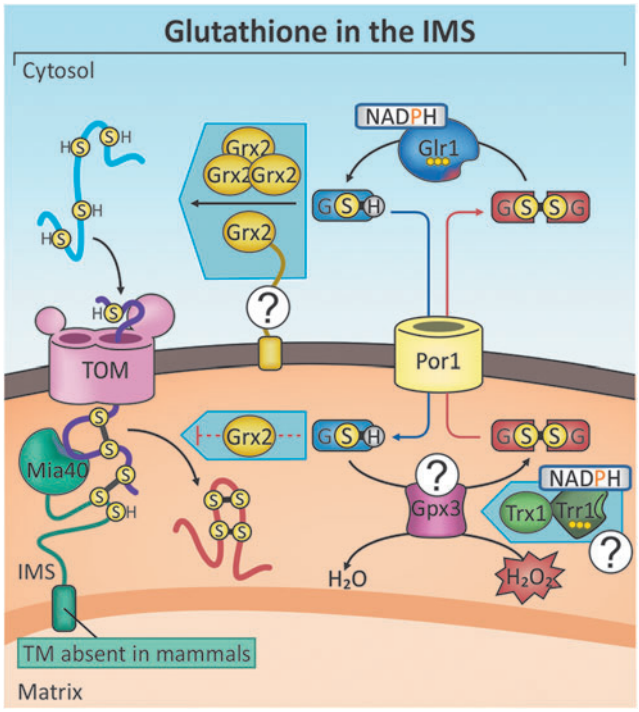

FIG. 9. Roles of glutathione in the IMS. The role of glutathione in the IMS has not been extensively investigated. However, glutathione was shown to regulate the redox state of Mia40 in a Grx2-mediated reaction. Consequently, glutathione influences oxidative folding of Mia40-substrate proteins. It remains unclear as to whether Grx2 also directly reduces substrates in the IMS as it does in the cytosol where it is present in large amounts. The IMS also harbors small pools of several other cytosolic redox enzymes (*e.g.*, Trx, Trr, Gpx) that may also interact with glutathione in the IMS, but this remains to be investigated. Grx2, cytoplasmic and mitochondrial glutaredoxin; Glr1, cytosolic and mitochondrial glutathione reductase; Por1, mitochondrial porin of the outer membrane; Mia40, import and assembly protein of the mitochondrial intermembrane space; Gpx3, thiol peroxidase involved in the response to high hydrogen peroxide levels; Trr1, cytoplasmic thioredoxin reductase; Trx1, cytoplasmic thioredoxin.

extremely slow compared with enzyme-catalyzed ones (34, 132). Thus, if changes in E_{GSH} are at all able to affect protein thiol oxidation and, consequently, protein function, it is likely that the abundance of enzymes such as Grxs, which are capable of catalyzing the reaction between glutathione and proteins, will be extremely important in determining the impact of glutathione redox changes.

Although the IMS contains a full set of GSH-utilizing enzymes (68, 133), their effective concentrations and activities in the compartment remain largely unclear. One exception is Grx (69): Grx activity can be measured and compared between different compartments by comparing measurements obtained with Grx1-roGFP2, roGFP2, and redox-sensitive yellow fluorescent protein (rxYFP) sensors. The latter two ''unfused'' sensors rely on endogenous Grx activity to facilitate the equilibration of their thiol/disulfide pair with the local glutathione pool. In this respect, they can be employed to investigate how putative, endogenous, thiol-containing proteins may be affected by changes in E_{GSH} and Grx availability. In the cytosol and the matrix, no difference can be detected in the oxidation state of fused or unfused sensors, implying that endogenous Grxs are sufficiently abundant to mediate the equilibration of roGFP2/rxYFP with the glutathione pool (26, 44, 90, 96, 97). Conversely, in the IMS, an unfused sensor was found to be more oxidized and to recover much slower from an oxidative insult compared with a fused sensor, that is, Grx1-roGFP2 (50, 69). These results are strongly suggestive of limiting Grx activity in the IMS compared with the cytosol and the matrix (69). It also reinforces the notion that Grxs are essential to mediate the equilibration of roGFP/rxYFP with the glutathione redox couple. In the absence of Grxs, this equilibration appears to be too slow to have any relevance on physiologically meaningful timescales. Importantly, it seems that Grx-mediated equilibration of protein thiols with the glutathione redox couple is not only restricted to non-physiological proteins such as roGFPs but also relevant to endogenous mitochondrial proteins as described later.

Oxidative protein folding in the IMS is influenced by E_{GSH} and glutaredoxin levels

The IMS houses many proteins that contain intramolecular disulfide bonds, which are introduced after import into the IMS. To facilitate disulfide bond formation, the IMS possesses a complete disulfide-generating system consisting of the oxidoreductase Mia40 and the sulfhydryl oxidase Erv1. It is responsible for the import and folding of a large set of substrates, including several respiratory chain subunits. Conceptually, this pathway closely resembles the analogous systems in the ER and the bacterial periplasm (1, 18, 28, 29, 63, 113). However, in contrast to the ER and the bacterial periplasm, which contain relatively oxidized glutathione pools or very little to no glutathione, protein disulfide bond formation in the IMS must take place in a highly reduced glutathione pool. *In vitro* studies showed that physiological concentrations of GSH, in the range of 5–10 m*M*, actually accelerated the oxidation of Mia40 substrates such as Cox19 and Tim10. This effect was ascribed to the reduction of long-lived reaction intermediates that impede the function of Mia40. At higher GSH concentrations, this effect was reverted and that rate of oxidative folding was decreased. However, it is important to note that these experiments were performed in the absence of any Grx activity (14).

Recent research looking at the effect of glutathione depletion on intact cells also observed an impact on the Mia40 redox state (69). Further, modulation of Grx levels led to impaired oxidative protein folding kinetics. On overexpression of IMS-targeted Grxs, oxidative folding and protein import in the IMS became impaired and the redox state of Mia40 became more reduced. Presumably, at higher concentrations, Grxs mediate the equilibration of Mia40 with the glutathione pool, thereby impeding disulfide bond formation in substrate proteins. Interestingly, deletion of Grxs also resulted in decreased rates of oxidative protein folding, perhaps because Grx/GSH is involved in isomerization of incorrectly formed disulfide bonds. Thus, it seems that there is an optimal ''Goldilocks'' level of endogenous Grx in the IMS. Any more Grx would lead to equilibration of the protein folding machinery with the highly reduced IMS glutathione pool. Any less Grx would, speculatively, prevent essential disulfide bond isomerization or reduction of disulfide bonds after acute oxidative stress (Fig. 9).

At present, it remains unclear as to whether any other pathways are affected by the limiting amounts of Grx in the IMS. It will be interesting to explore this in future. Likewise, it will now be interesting to elucidate whether the concentration and activity of other IMS-localized, GSH-utilizing enzymes are adapted to the special environment of the IMS. In addition, given the further subcompartmentalization of the IMS, it might be important as to which of these subcompartments enzyme activity is localized.

Open Questions

There are many open questions remaining surrounding the regulation and role of glutathione in mitochondria. Here, we focus on a small selection of questions that we believe will be particularly exciting to address in the near future.

Interplay of H_2O_2, thioredoxin, and glutathione in the matrix

The complex interaction between Prx1, Trx3, Trr2, Grx2, and GSH in the matrix demonstrates that, in contrast to other subcellular compartments, there is extensive crosstalk between these systems. This implies that a change in H_2O_2, for example, might readily impact E_{GSH} and/or the redox state of Trx3. Changes in E_{GSH} may also directly impact the Trx3 redox state. This could be particularly interesting as it was recently shown that the oxidation of Trx3 is an important trigger of apoptosis in yeast cells (43). It is, thus, tempting to speculate that Trx3 serves as a hub to integrate signals from H_2O_2, glutathione, and thioredoxin reductase as well as NADPH levels to initiate cell death under conditions of extreme oxidative stress, for example due to severe malfunction of the respiratory chain. It will be particularly interesting to try to better untangle the complex relationships between these matrix redox systems and dissect their impact on mitochondrial and cellular function. Newly available sensors for H_2O_2 such as roGFP2-Tsa2ΔC_R, as well as roGFP2-Prx1 fusions that may allow the direct monitoring of changes in Prx1 oxidation (91), together with E_{GSH} sensors, might aid us in making previously unachievable new insights in this area.

Glutathione turnover in the matrix

Although we now have an understanding of the redox poise of the mitochondrial matrix glutathione pool, we have little idea of the concentration of glutathione in the matrix and whether it is similar to the concentration of cytosolic glutathione or not. Also completely unclear is the rate of turnover of the matrix glutathione pool. This will be dependent on many factors of which we have little or no knowledge, including the rate of glutathione import and export and the question of whether the matrix harbors glutathione-degrading enzymes.

Experiments with E_{GSH} sensors suggest that the rate of glutathione transport into the matrix is very slow and that the glutathione pool of the matrix is effectively isolated from the cytosolic and IMS glutathione pools (68). However, it is important to consider what the E_{GSH} sensors are actually measuring, or more specifically, how E_{GSH} will be affected by changes in GSH and GSSG concentration. At the extremely reduced redox poise of the matrix glutathione pool, large changes in GSH concentration would only give rise to small changes in E_{GSH} and, thus, be difficult to reliably detect with E_{GSH} sensors. Thus, it could be that GSH is readily imported into the matrix. Resolution of this question will require more extensive investigation, employing alternative techniques, to yield a deeper understanding. It is also unclear as to whether GSH can be exported from the matrix; again, this would be difficult to discern with E_{GSH} sensors. In contrast, it seems clear that the IMM is largely impermeable to GSSG (17, 68), as small changes in GSSG levels would lead to large changes in E_{GSH} in a glutathione pool with the redox poise found in the matrix and, thus, be easily measurable with genetically encoded sensors. The identification and characterization of the IMM glutathione transporter/s will certainly help to clarify the uncertainties surrounding GSH transport across the IMM. A final factor that could profoundly affect matrix glutathione homeostasis is the possible existence of a matrix glutathione degradation pathway. It will be extremely interesting to investigate whether the putative matrix localization of Dug enzymes in yeast reflects the actual situation and if so, to address the implication of this for matrix glutathione homeostasis.

Acknowledgments

Research in the authors' laboratories is funded by the German research council (DFG) to J.R. (RI2150/1-2, RI2150/2-1 [SPP1710], SFB1218 TP B02) and by the Forschungsinitiative Rheinland-Pfalz BioComp and the University of Kaiserslautern Nachwuchsring and the DFG priority program SPP1710 (MO 2774/2-1) to B.M.

References

1. Allen S, Balabanidou V, Sideris DP, Lisowsky T, and Tokatlidis K. Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome c. *J Mol Biol* 353: 937–944, 2005.
2. Applegate MA, Humphries KM, and Szweda LI. Reversible inhibition of alpha-ketoglutarate dehydrogenase by hydrogen peroxide: glutathionylation and protection of lipoic acid. *Biochemistry* 47: 473–478, 2008.
3. Avery AM and Avery SV. *Saccharomyces cerevisiae* expresses three phospholipid hydroperoxide glutathione peroxidases. *J Biol Chem* 276: 33730–33735, 2001.
4. Avery AM, Willetts SA, and Avery SV. Genetic dissection of the phospholipid hydroperoxidase activity of yeast gpx3 reveals its functional importance. *J Biol Chem* 279: 46652–46658, 2004.
5. Ayer A, Fellermeier S, Fife C, Li SS, Smits G, Meyer AJ, Dawes IW, and Perrone GG. A genome-wide screen in yeast identifies specific oxidative stress genes required for the maintenance of sub-cellular redox homeostasis. *PLoS One* 7: e44278, 2012.
6. Ayer A, Sanwald J, Pillay BA, Meyer AJ, Perrone GG, and Dawes IW. Distinct redox regulation in sub-cellular compartments in response to various stress conditions in *Saccharomyces cerevisiae*. *PLoS One* 8: e65240, 2013.
7. Ayer A, Tan SX, Grant CM, Meyer AJ, Dawes IW, and Perrone GG. The critical role of glutathione in maintenance of the mitochondrial genome. *Free Radic Biol Med* 49: 1956–1968, 2010.
8. Ballatori N, Hammond CL, Cunningham JB, Krance SM, and Marchan R. Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins. *Toxicol Appl Pharmacol* 204: 238–255, 2005.
9. Ballatori N, Krance SM, Marchan R, and Hammond CL. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. *Mol Aspects Med* 30: 13–28, 2009.
10. Banci L, Brancaccio D, Ciofi-Baffoni S, Del Conte R, Gadepalli R, Mikolajczyk M, Neri S, Piccioli M, and Winkelmann J. [2Fe-2S] cluster transfer in iron-sulfur protein biogenesis. *Proc Natl Acad Sci U S A* 111: 6203–6208, 2014.
11. Bandyopadhyay S, Gama F, Molina-Navarro MM, Gualberto JM, Claxton R, Naik SG, Huynh BH, Herrero E, Jacquot JP, Johnson MK, and Rouhier N. Chloroplast monothiol glutaredoxins as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters. *EMBO J* 27: 1122–1133, 2008.
12. Barbot M, Jans DC, Schulz C, Denkert N, Kroppen B, Hoppert M, Jakobs S, and Meinecke M. Mic10 oligomerizes to bend mitochondrial inner membranes at cristae junctions. *Cell Metab* 21: 756–763, 2015.
13. Becker T, Gebert M, Pfanner N, and van der Laan M. Biogenesis of mitochondrial membrane proteins. *Curr Opin Cell Biol* 21: 484–493, 2009.
14. Bien M, Longen S, Wagener N, Chwalla I, Herrmann JM, and Riemer J. Mitochondrial disulfide bond formation is driven by intersubunit electron transfer in Erv1 and proofread by glutathione. *Mol Cell* 37: 516–528, 2010.
15. Birk J, Meyer M, Aller I, Hansen HG, Odermatt A, Dick TP, Meyer AJ, and Appenzeller-Herzog C. Endoplasmic reticulum: reduced and oxidized glutathione revisited. *J Cell Sci* 126: 1604–1617, 2013.
16. Booty LM, King MS, Thangaratnarajah C, Majd H, James AM, Kunji ER, and Murphy MP. The mitochondrial dicarboxylate and 2-oxoglutarate carriers do not transport glutathione. *FEBS Lett* 589: 621–628, 2015.
17. Carbonera D and Azzone GF. Permeability of inner mitochondrial membrane and oxidative stress. *Biochim Biophys Acta* 943: 245–255, 1988.
18. Chacinska A, Koehler CM, Milenkovic D, Lithgow T, and Pfanner N. Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138: 628–644, 2009.

19. Chae HZ, Chung SJ, and Rhee SG. Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269: 27670–27678, 1994.
20. Chen YR, Chen CL, Pfeiffer DR, and Zweier JL. Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation. *J Biol Chem* 282: 32640–32654, 2007.
21. Chen Z and Lash LH. Evidence for mitochondrial uptake of glutathione by dicarboxylate and 2-oxoglutarate carriers. *J Pharmacol Exp Ther* 285: 608–618, 1998.
22. Chen Z, Putt DA, and Lash LH. Enrichment and functional reconstitution of glutathione transport activity from rabbit kidney mitochondria: further evidence for the role of the dicarboxylate and 2-oxoglutarate carriers in mitochondrial glutathione transport. *Arch Biochem Biophys* 373: 193–202, 2000.
23. Cogliati S, Enriquez JA, and Scorrano L. Mitochondrial cristae: where beauty meets functionality. *Trends Biochem Sci* 41: 261–273, 2016.
24. Collinson EJ and Grant CM. Role of yeast glutaredoxins as glutathione S-transferases. *J Biol Chem* 278: 22492–22497, 2003.
25. Cummings BS, Angeles R, McCauley RB, and Lash LH. Role of voltage-dependent anion channels in glutathione transport into yeast mitochondria. *Biochem Biophys Res Commun* 276: 940–944, 2000.
26. Dardalhon M, Kumar C, Iraqui I, Vernis L, Kienda G, Banach-Latapy A, He T, Chanet R, Faye G, Outten CE, and Huang ME. Redox-sensitive YFP sensors monitor dynamic nuclear and cytosolic glutathione redox changes. *Free Radic Biol Med* 52: 2254–2265, 2012.
27. Deponte M. Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochim Biophys Acta* 1830: 3217–3266, 2013.
28. Deponte M and Hell K. Disulphide bond formation in the intermembrane space of mitochondria. *J Biochem* 146: 599–608, 2009.
29. Endo T and Yamano K. Multiple pathways for mitochondrial protein traffic. *Biol Chem* 390: 723–730, 2009.
30. Ezerina D, Morgan B, and Dick TP. Imaging dynamic redox processes with genetically encoded probes. *J Mol Cell Cardiol* 73: 43–49, 2014.
31. Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, and Rabinowitz JD. Quantitative flux analysis reveals folate-dependent NADPH production. *Nature* 510: 298–302, 2014.
32. Filipovska A and Murphy MP. Measurement of protein glutathionylation. *Curr Protoc Toxicol* Chapter 6: Unit6 11, 2006.
33. Fischer M, Horn S, Belkacemi A, Kojer K, Petrungaro C, Habich M, Ali M, Kuttner V, Bien M, Kauff F, Dengjel J, Herrmann JM, and Riemer J. Protein import and oxidative folding in the mitochondrial intermembrane space of intact mammalian cells. *Mol Biol Cell* 24: 2160–2170, 2013.
34. Flohe L. The fairytale of the GSSG/GSH redox potential. *Biochim Biophys Acta* 1830: 3139–3142, 2013.
35. Ganguli D, Kumar C, and Bachhawat AK. The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in *Saccharomyces cerevisiae*. *Genetics* 175: 1137–1151, 2007.
36. Garcera A, Barreto L, Piedrafita L, Tamarit J, and Herrero E. *Saccharomyces cerevisiae* cells have three omega class glutathione S-transferases acting as 1-Cys thiol transferases. *Biochem J* 398: 187–196, 2006.
37. Garcia J, Han D, Sancheti H, Yap LP, Kaplowitz N, and Cadenas E. Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates. *J Biol Chem* 285: 39646–39654, 2010.
38. Gaudet P, Livstone MS, Lewis SE, and Thomas PD. Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. *Brief Bioinform* 12: 449–462, 2011.
39. Gostimskaya I and Grant CM. Yeast mitochondrial glutathione is an essential antioxidant with mitochondrial thioredoxin providing a back-up system. *Free Radic Biol Med* 94: 55–65, 2016.
40. Grant CM, Collinson LP, Roe JH, and Dawes IW. Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. *Mol Microbiol* 21: 171–179, 1996.
41. Grant CM, MacIver FH, and Dawes IW. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol Biol Cell* 8: 1699–1707, 1997.
42. Greetham D and Grant CM. Antioxidant activity of the yeast mitochondrial one-Cys peroxiredoxin is dependent on thioredoxin reductase and glutathione in vivo. *Mol Cell Biol* 29: 3229–3240, 2009.
43. Greetham D, Kritsiligkou P, Watkins RH, Carter Z, Parkin J, and Grant CM. Oxidation of the yeast mitochondrial thioredoxin promotes cell death. *Antioxid Redox Signal* 18: 376–385, 2013.
44. Gutscher M, Pauleau AL, Marty L, Brach T, Wabnitz GH, Samstag Y, Meyer AJ, and Dick TP. Real-time imaging of the intracellular glutathione redox potential. *Nat Methods* 5: 553–559, 2008.
45. Hackenbrock CR. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. *J Cell Biol* 30: 269–297, 1966.
46. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, and Remington SJ. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem* 279: 13044–13053, 2004.
47. Harner M, Korner C, Walther D, Mokranjac D, Kaesmacher J, Welsch U, Griffith J, Mann M, Reggiori F, and Neupert W. The mitochondrial contact site complex, a determinant of mitochondrial architecture. *EMBO J* 30: 4356–4370, 2011.
48. Heisterkamp N, Groffen J, Warburton D, and Sneddon TP. The human gamma-glutamyltransferase gene family. *Hum Genet* 123: 321–332, 2008.
49. Horvath SE, Rampelt H, Oeljeklaus S, Warscheid B, van der Laan M, and Pfanner N. Role of membrane contact sites in protein import into mitochondria. *Protein Sci* 24: 277–297, 2015.
50. Hu J, Dong L, and Outten CE. The redox environment in the mitochondrial intermembrane space is maintained separately from the cytosol and matrix. *J Biol Chem* 283: 29126–29134, 2008.
51. Huang CS, Anderson ME, and Meister A. Amino acid sequence and function of the light subunit of rat kidney gamma-glutamylcysteine synthetase. *J Biol Chem* 268: 20578–20583, 1993.

52. Huang CS, Chang LS, Anderson ME, and Meister A. Catalytic and regulatory properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. *J Biol Chem* 268: 19675–19680, 1993.
53. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, and O'Shea EK. Global analysis of protein localization in budding yeast. *Nature* 425: 686–691, 2003.
54. Inoue Y, Matsuda T, Sugiyama K, Izawa S, and Kimura A. Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. *J Biol Chem* 274: 27002–27009, 1999.
55. Inoue Y, Sugiyama K, Izawa S, and Kimura A. Molecular identification of glutathione synthetase (GSH2) gene from *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1395: 315–320, 1998.
56. Jackson JB. Proton translocation by transhydrogenase. *FEBS Lett* 545: 18–24, 2003.
57. Jaspers CJ and Penninckx MJ. Glutathione metabolism in yeast *Saccharomyces cerevisiae*. Evidence that gamma-glutamyltranspeptidase is a vacuolar enzyme. *Biochimie* 66: 71–74, 1984.
58. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, and Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* 56: 988–994, 1996.
59. Jo SH, Son MK, Koh HJ, Lee SM, Song IH, Kim YO, Lee YS, Jeong KS, Kim WB, Park JW, Song BJ, and Huh TL. Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial NADP+-dependent isocitrate dehydrogenase. *J Biol Chem* 276: 16168–16176, 2001.
60. Kang PT, Chen CL, and Chen YR. Increased mitochondrial prooxidant activity mediates up-regulation of Complex I S-glutathionylation via protein thiyl radical in the murine heart of eNOS(-/-). *Free Radic Biol Med* 79: 56–68, 2015.
61. Kaur H, Ganguli D, and Bachhawat AK. Glutathione degradation by the alternative pathway (DUG pathway) in *Saccharomyces cerevisiae* is initiated by (Dug2p-Dug3p)2 complex, a novel glutamine amidotransferase (GATase) enzyme acting on glutathione. *J Biol Chem* 287: 8920–8931, 2012.
62. Kaur H, Kumar C, Junot C, Toledano MB, and Bachhawat AK. Dug1p Is a Cys-Gly peptidase of the gamma-glutamyl cycle of *Saccharomyces cerevisiae* and represents a novel family of Cys-Gly peptidases. *J Biol Chem* 284: 14493–14502, 2009.
63. Kawano S, Yamano K, Naoe M, Momose T, Terao K, Nishikawa S, Watanabe N, and Endo T. Structural basis of yeast Tim40/Mia40 as an oxidative translocator in the mitochondrial intermembrane space. *Proc Natl Acad Sci U S A* 106: 14403–14407, 2009.
64. Keppler D, Leier I, Jedlitschky G, and Konig J. ATP-dependent transport of glutathione S-conjugates by the multidrug resistance protein MRP1 and its apical isoform MRP2. *Chem Biol Interact* 111–112: 153–161, 1998.
65. Kim KD, Chung WH, Kim HJ, Lee KC, and Roe JH. Monothiol glutaredoxin Grx5 interacts with Fe-S scaffold proteins Isa1 and Isa2 and supports Fe-S assembly and DNA integrity in mitochondria of fission yeast. *Biochem Biophys Res Commun* 392: 467–472, 2010.
66. Kim SJ, Woo JR, Hwang YS, Jeong DG, Shin DH, Kim K, and Ryu SE. The tetrameric structure of *Haemophilus influenza* hybrid Prx5 reveals interactions between electron donor and acceptor proteins. *J Biol Chem* 278: 10790–10798, 2003.
67. Kistler M, Maier K, and Eckardt-Schupp F. Genetic and biochemical analysis of glutathione-deficient mutants of *Saccharomyces cerevisiae*. *Mutagenesis* 5: 39–44, 1990.
68. Kojer K, Bien M, Gangel H, Morgan B, Dick TP, and Riemer J. Glutathione redox potential in the mitochondrial intermembrane space is linked to the cytosol and impacts the Mia40 redox state. *EMBO J* 31: 3169–3182, 2012.
69. Kojer K, Peleh V, Calabrese G, Herrmann JM, and Riemer J. Kinetic control by limiting glutaredoxin amounts enables thiol oxidation in the reducing mitochondrial intermembrane space. *Mol Biol Cell* 26: 195–204, 2015.
70. Kumar A, Tikoo S, Maity S, Sengupta S, Sengupta S, Kaur A, and Bachhawat AK. Mammalian proapoptotic factor ChaC1 and its homologues function as gamma-glutamyl cyclotransferases acting specifically on glutathione. *EMBO Rep* 13: 1095–1101, 2012.
71. Kumar C, Igbaria A, D'Autreaux B, Planson AG, Junot C, Godat E, Bachhawat AK, Delaunay-Moisan A, and Toledano MB. Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control. *EMBO J* 30: 2044–2056, 2011.
72. Lee AC, Xu X, Blachly-Dyson E, Forte M, and Colombini M. The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. *J Membr Biol* 161: 173–181, 1998.
73. Lee JC, Straffon MJ, Jang TY, Higgins VJ, Grant CM, and Dawes IW. The essential and ancillary role of glutathione in *Saccharomyces cerevisiae* analysed using a grande gsh1 disruptant strain. *FEMS Yeast Res* 1: 57–65, 2001.
74. Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, and Rea PA. A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc Natl Acad Sci U S A* 94: 42–47, 1997.
75. Li ZS, Szczypka M, Lu YP, Thiele DJ, and Rea PA. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. *J Biol Chem* 271: 6509–6517, 1996.
76. Lu SC. Glutathione synthesis. *Biochim Biophys Acta* 1830: 3143–3153, 2013.
77. Ma XX, Jiang YL, He YX, Bao R, Chen Y, and Zhou CZ. Structures of yeast glutathione-S-transferase Gtt2 reveal a new catalytic type of GST family. *EMBO Rep* 10: 1320–1326, 2009.
78. Mailloux RJ and Harper ME. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radic Biol Med* 51: 1106–1115, 2011.
79. Mailloux RJ, Seifert EL, Bouillaud F, Aguer C, Collins S, and Harper ME. Glutathionylation acts as a control switch for uncoupling proteins UCP2 and UCP3. *J Biol Chem* 286: 21865–21875, 2011.
80. Manevich Y, Feinstein SI, and Fisher AB. Activation of the antioxidant enzyme 1-CYS peroxiredoxin requires glutathionylation mediated by heterodimerization with

pi GST. *Proc Natl Acad Sci U S A* 101: 3780–3785, 2004.
81. Mehdi K, Thierie J, and Penninckx MJ. gamma-Glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae* and its role in the vacuolar transport and metabolism of glutathione. *Biochem J* 359: 631–637, 2001.
82. Meister A and Anderson ME. Glutathione. *Annu Rev Biochem* 52: 711–760, 1983.
83. Mering C, Krause R, Snel B, Cornell M, Oliver SG, Fields S, and Bork P. Comparative assessment of large-scale data sets of protein-protein interactions. *Nature* 417: 399–403, 2002.
84. Minich T, Riemer J, Schulz JB, Wielinga P, Wijnholds J, and Dringen R. The multidrug resistance protein 1 (Mrp1), but not Mrp5, mediates export of glutathione and glutathione disulfide from brain astrocytes. *J Neurochem* 97: 373–384, 2006.
85. Miyagi H, Kawai S, and Murata K. Two sources of mitochondrial NADPH in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 284: 7553–7560, 2009.
86. Molina MM, Belli G, de la Torre MA, Rodriguez-Manzaneque MT, and Herrero E. Nuclear monothiol glutaredoxins of *Saccharomyces cerevisiae* can function as mitochondrial glutaredoxins. *J Biol Chem* 279: 51923–51930, 2004.
87. Montero D, Tachibana C, Rahr Winther J, and Appenzeller-Herzog C. Intracellular glutathione pools are heterogeneously concentrated. *Redox Biol* 1: 508–513, 2013.
88. Morgan B. Reassessing cellular glutathione homoeostasis: novel insights revealed by genetically encoded redox probes. *Biochem Soc Trans* 42: 979–984, 2014.
89. Morgan B, Ezerina D, Amoako TN, Riemer J, Seedorf M, and Dick TP. Multiple glutathione disulfide removal pathways mediate cytosolic redox homeostasis. *Nat Chem Biol* 9: 119–125, 2013.
90. Morgan B, Sobotta MC, and Dick TP. Measuring E(GSH) and H_2O_2 with roGFP2-based redox probes. *Free Radic Biol Med* 51: 1943–1951, 2011.
91. Morgan B, Van Laer K, Owusu TN, Ezerina D, Pastor-Flores D, Amponsah PS, Tursch A, and Dick TP. Real-time monitoring of basal HO levels with peroxiredoxin-based probes. *Nat Chem Biol* 12: 437–443, 2016.
92. Ng CH, Tan SX, Perrone GG, Thorpe GW, Higgins VJ, and Dawes IW. Adaptation to hydrogen peroxide in *Saccharomyces cerevisiae*: the role of NADPH-generating systems and the SKN7 transcription factor. *Free Radic Biol Med* 44: 1131–1145, 2008.
93. Nickel AG, von Hardenberg A, Hohl M, Loffler JR, Kohlhaas M, Becker J, Reil JC, Kazakov A, Bonnekoh J, Stadelmaier M, Puhl SL, Wagner M, Bogeski I, Cortassa S, Kappl R, Pasieka B, Lafontaine M, Lancaster CR, Blacker TS, Hall AR, Duchen MR, Kastner L, Lipp P, Zeller T, Muller C, Knopp A, Laufs U, Bohm M, Hoth M, and Maack C. Reversal of mitochondrial transhydrogenase causes oxidative stress in heart failure. *Cell Metab* 22: 472–484, 2015.
94. Nilsson R, Jain M, Madhusudhan N, Sheppard NG, Strittmatter L, Kampf C, Huang J, Asplund A, and Mootha VK. Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer. *Nat Commun* 5: 3128, 2014.
95. Ohtake Y and Yabuuchi S. Molecular cloning of the gamma-glutamylcysteine synthetase gene of *Saccharomyces cerevisiae*. *Yeast* 7: 953–961, 1991.
96. Ostergaard H, Henriksen A, Hansen FG, and Winther JR. Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein. *EMBO J* 20: 5853–5862, 2001.
97. Ostergaard H, Tachibana C, and Winther JR. Monitoring disulfide bond formation in the eukaryotic cytosol. *J Cell Biol* 166: 337–345, 2004.
98. Outten CE and Culotta VC. A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. *EMBO J* 22: 2015–2024, 2003.
99. Outten CE and Culotta VC. Alternative start sites in the *Saccharomyces cerevisiae* GLR1 gene are responsible for mitochondrial and cytosolic isoforms of glutathione reductase. *J Biol Chem* 279: 7785–7791, 2004.
100. Outten CE, Falk RL, and Culotta VC. Cellular factors required for protection from hyperoxia toxicity in *Saccharomyces cerevisiae*. *Biochem J* 388: 93–101, 2005.
101. Park SG, Cha MK, Jeong W, and Kim IH. Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. *J Biol Chem* 275: 5723–5732, 2000.
102. Pedrajas JR, McDonagh B, Hernandez-Torres F, Miranda-Vizuete A, Gonzalez-Ojeda R, Martinez-Galisteo E, Padilla CA, and Barcena JA. Glutathione is the resolving thiol for thioredoxin peroxidase activity of 1-Cys peroxiredoxin without being consumed during the catalytic cycle. *Antioxid Redox Signal* 24: 115–128, 2016.
103. Pedrajas JR, Miranda-Vizuete A, Javanmardy N, Gustafsson JA, and Spyrou G. Mitochondria of *Saccharomyces cerevisiae* contain one-conserved cysteine type peroxiredoxin with thioredoxin peroxidase activity. *J Biol Chem* 275: 16296–16301, 2000.
104. Pedrajas JR, Padilla CA, McDonagh B, and Barcena JA. Glutaredoxin participates in the reduction of peroxides by the mitochondrial 1-CYS peroxiredoxin in *Saccharomyces cerevisiae*. *Antioxid Redox Signal* 13: 249–258, 2010.
105. Pedrajas JR, Porras P, Martinez-Galisteo E, Padilla CA, Miranda-Vizuete A, and Barcena JA. Two isoforms of *Saccharomyces cerevisiae* glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments. *Biochem J* 364: 617–623, 2002.
106. Porras P, McDonagh B, Pedrajas JR, Barcena JA, and Padilla CA. Structure and function of yeast glutaredoxin 2 depend on postranslational processing and are related to subcellular distribution. *Biochim Biophys Acta* 1804: 839–845, 2010.
107. Porras P, Padilla CA, Krayl M, Voos W, and Barcena JA. One single in-frame AUG codon is responsible for a diversity of subcellular localizations of glutaredoxin 2 in *Saccharomyces cerevisiae*. *J Biol Chem* 281: 16551–16562, 2006.
108. Prokisch H, Scharfe C, Camp DG, 2nd, Xiao W, David L, Andreoli C, Monroe ME, Moore RJ, Gritsenko MA, Kozany C, Hixson KK, Mottaz HM, Zischka H, Ueffing M, Herman ZS, Davis RW, Meitinger T, Oefner PJ, Smith RD, and Steinmetz LM. Integrative analysis of the mitochondrial proteome in yeast. *PLoS Biol* 2: e160, 2004.
109. Rebbeor JF, Connolly GC, Dumont ME, and Ballatori N. ATP-dependent transport of reduced glutathione on YCF1, the yeast orthologue of mammalian multidrug resistance associated proteins. *J Biol Chem* 273: 33449–33454, 1998.

110. Reinders J, Zahedi RP, Pfanner N, Meisinger C, and Sickmann A. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res* 5: 1543–1554, 2006.
111. Renvoise M, Bonhomme L, Davanture M, Valot B, Zivy M, and Lemaire C. Quantitative variations of the mitochondrial proteome and phosphoproteome during fermentative and respiratory growth in *Saccharomyces cerevisiae*. *J Proteomics* 106: 140–150, 2014.
112. Richman PG and Meister A. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* 250: 1422–1426, 1975.
113. Riemer J, Bulleid N, and Herrmann JM. Disulfide formation in the ER and mitochondria: two solutions to a common process. *Science* 324: 1284–1287, 2009.
114. Rodriguez-Manzaneque MT, Tamarit J, Belli G, Ros J, and Herrero E. Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell* 13: 1109–1121, 2002.
115. Samanta MP and Liang S. Predicting protein functions from redundancies in large-scale protein interaction networks. *Proc Natl Acad Sci U S A* 100: 12579–12583, 2003.
116. Schaedler TA, Thornton JD, Kruse I, Schwarzlander M, Meyer AJ, van Veen HW, and Balk J. A conserved mitochondrial ATP-binding cassette transporter exports glutathione polysulfide for cytosolic metal cofactor assembly. *J Biol Chem* 289: 23264–23274, 2014.
117. Schwarzlander M, Dick TP, Meye AJ, and Morgan B. Dissecting redox biology using fluorescent protein sensors. *Antioxid Redox Signal* 24: 680–712, 2016.
118. Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schonfisch B, Perschil I, Chacinska A, Guiard B, Rehling P, Pfanner N, and Meisinger C. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci U S A* 100: 13207–13212, 2003.
119. Springael JY and Penninckx MJ. Nitrogen-source regulation of yeast gamma-glutamyl transpeptidase synthesis involves the regulatory network including the GATA zinc-finger factors Gln3, Nil1/Gat1 and Gzf3. *Biochem J* 371: 589–595, 2003.
120. Srinivasan V, Pierik AJ, and Lill R. Crystal structures of nucleotide-free and glutathione-bound mitochondrial ABC transporter Atm1. *Science* 343: 1137–1140, 2014.
121. Starke DW, Chock PB, and Mieyal JJ. Glutathione-thiyl radical scavenging and transferase properties of human glutaredoxin (thioltransferase). Potential role in redox signal transduction. *J Biol Chem* 278: 14607–14613, 2003.
122. Stephen DW, Rivers SL, and Jamieson DJ. The role of the YAP1 and YAP2 genes in the regulation of the adaptive oxidative stress responses of *Saccharomyces cerevisiae*. *Mol Microbiol* 16: 415–423, 1995.
123. Sugiyama K, Izawa S, and Inoue Y. The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. *J Biol Chem* 275: 15535–15540, 2000.
124. Szczechowicz A, Hryniewiecka L, and Kmita H. The influence of depletion of voltage dependent anion selective channel on protein import into the yeast *Saccharomyces cerevisiae* mitochondria. *Acta Biochim Pol* 48: 719–728, 2001.
125. Tan SX, Greetham D, Raeth S, Grant CM, Dawes IW, and Perrone GG. The thioredoxin-thioredoxin reductase system can function in vivo as an alternative system to reduce oxidized glutathione in *Saccharomyces cerevisiae*. *J Biol Chem* 285: 6118–6126, 2010.
126. Tanaka T, Izawa S, and Inoue Y. GPX2, encoding a phospholipid hydroperoxide glutathione peroxidase homologue, codes for an atypical 2-Cys peroxiredoxin in *Saccharomyces cerevisiae*. *J Biol Chem* 280: 42078–42087, 2005.
127. Tatsuta T, Scharwey M, and Langer T. Mitochondrial lipid trafficking. *Trends Cell Biol* 24: 44–52, 2014.
128. Trotter EW and Grant CM. Overlapping roles of the cytoplasmic and mitochondrial redox regulatory systems in the yeast *Saccharomyces cerevisiae*. *Eukaryot Cell* 4: 392–400, 2005.
129. Ubiyvovk VM, Blazhenko OV, Gigot D, Penninckx M, and Sibirny AA. Role of gamma-glutamyltranspeptidase in detoxification of xenobiotics in the yeasts *Hansenula polymorpha* and *Saccharomyces cerevisiae*. *Cell Biol Int* 30: 665–671, 2006.
130. Ubiyvovk VM, Maszewski J, Bartosz G, and Sibirny AA. Vacuolar accumulation and extracellular extrusion of electrophilic compounds by wild-type and glutathione-deficient mutants of the methylotrophic yeast *Hansenula polymorpha*. *Cell Biol Int* 27: 785–789, 2003.
131. van der Laan M, Horvath SE, and Pfanner N. Mitochondrial contact site and cristae organizing system. *Curr Opin Cell Biol* 41: 33–42, 2016.
132. Van Laer K, Hamilton CJ, and Messens J. Low-molecular-weight thiols in thiol-disulfide exchange. *Antioxid Redox Signal* 18: 1642–1653, 2013.
133. Vogtle FN, Burkhart JM, Rao S, Gerbeth C, Hinrichs J, Martinou JC, Chacinska A, Sickmann A, Zahedi RP, and Meisinger C. Intermembrane space proteome of yeast mitochondria. *Mol Cell Proteomics* 11: 1840–1852, 2012.
134. Vogtle FN, Wortelkamp S, Zahedi RP, Becker D, Leidhold C, Gevaert K, Kellermann J, Voos W, Sickmann A, Pfanner N, and Meisinger C. Global analysis of the mitochondrial N-proteome identifies a processing peptidase critical for protein stability. *Cell* 139: 428–439, 2009.
135. Wheeler GL, Quinn KA, Perrone G, Dawes IW, and Grant CM. Glutathione regulates the expression of gamma-glutamylcysteine synthetase via the Met4 transcription factor. *Mol Microbiol* 46: 545–556, 2002.
136. Wilkens V, Kohl W, and Busch K. Restricted diffusion of OXPHOS complexes in dynamic mitochondria delays their exchange between cristae and engenders a transitory mosaic distribution. *J Cell Sci* 126: 103–116, 2013.
137. Wingert RA, Galloway JL, Barut B, Foott H, Fraenkel P, Axe JL, Weber GJ, Dooley K, Davidson AJ, Schmid B, Paw BH, Shaw GC, Kingsley P, Palis J, Schubert H, Chen O, Kaplan J, Zon LI, and Tubingen Screen C. Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature* 436: 1035–1039, 2005.
138. Yang M, Cobine PA, Molik S, Naranuntarat A, Lill R, Winge DR, and Culotta VC. The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2. *EMBO J* 25: 1775–1783, 2006.
139. Zechmann B, Liou LC, Koffler BE, Horvat L, Tomasic A, Fulgosi H, and Zhang Z. Subcellular distribution of glutathione and its dynamic changes under oxidative stress in

the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res* 11: 631–642, 2011.

140. Zhu Y, Sun J, Zhu Y, Wang L, and Qi B. Endogenic oxidative stress response contributes to glutathione over-accumulation in mutant *Saccharomyces cerevisiae* Y518. *Appl Microbiol Biotechnol* 99: 7069–7078, 2015.

Address correspondence to:
Jun. Prof. Dr. Bruce Morgan
Department of Cellular Biochemistry
University of Kaiserslautern
Kaiserslautern 67663
Germany

E-mail: morgan@bio.uni-kl.de

Prof. Dr. Jan Riemer
Institute of Biochemistry
University of Cologne
Cologne 50674
Germany

E-mail: jan.riemer@uni-koeln.de

Date of first submission to ARS Central, May 12, 2017; date of acceptance, May 22, 2017.

Abbreviations Used

DIC = dicarboxylate carrier
ER = endoplasmic reticulum
Glr = glutathione reductase
Gpx = glutathione peroxidases
Grx = glutaredoxins
GSH = reduced glutathione
GSSG = oxidized glutathione
Gst = glutathione-S-transferases
H_2O_2 = hydrogen peroxide
IMM = inner mitochondrial membrane
IMS = intermembrane space
OGC = oxoglutarate carrier
OMM = outer mitochondrial membrane
roGFP = redox-sensitive GFP
ROS = reactive oxygen species
rxYFP = redox-sensitive yellow fluorescent protein
TOM = translocase of the outer membrane