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Glutathione: subcellular distribution and membrane transport

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Abstract

Glutathione (γ -glutamylcysteinylglycine) is a small tripeptide found at millimolar concentrations in nearly all eukaryotes as well as many prokaryotic cells.

Glutathione synthesis is restricted to the cytosol in animals and fungi and to the cytosol and plastids in plants. Nonetheless, glutathione is found in virtually all subcellular compartments. This implies that transporters must exist, which facilitate glutathione transport into and out of the various subcellular compartments. Glutathione may also be exported and imported across the plasma membrane in many cells. However, in most cases, the molecular identity of these transporters remains unclear.

Whilst glutathione transport is essential for the supply and replenishment of subcellular glutathione pools, recent evidence supports a more active role for glutathione transport in the regulation of subcellular glutathione redox homeostasis. However, our knowledge of glutathione redox homeostasis at the level of specific subcellular compartments remains remarkably limited and the role of glutathione transport remains largely unclear.

In this review we discuss how new tools and techniques have begun to yield insights into subcellular glutathione distribution and glutathione redox homeostasis. In particular, we discuss the known and putative glutathione transporters and examine their contribution to the regulation of subcellular glutathione redox homeostasis.

Keywords: glutathione, transport, redox regulation, roGFP

Introduction

Glutathione (γ -glutamyl-cysteinyl-glycine) is found at a high concentration (1–30 mM) in essentially all eukaryotes as well as in many prokaryotic species (Deponte 2013, 2017; Schafer and Buettner 2001). Glutathione fulfils many different functions in the cell, including playing an important role in the detoxification of toxic electrophiles and heavy metals, in the reduction of certain reactive oxygen species, for example hydrogen peroxide, and is thought to have an essential role in the biogenesis of iron-sulfur clusters (Kumar et al. 2011).

Glutathione is synthesized in two enzyme-catalyzed, ATP-dependent steps. In the first step, γ -glutamylcysteine synthetase catalyzes the formation of a peptide bond between the amino group of cysteine and the side-chain γ -carboxylate group of glutamate, leading to the formation of γ -glutamylcysteine. In the second step, a peptide bond is formed between the cysteine carboxylate group of γ -glutamylcysteine and the amino group of a glycine residue, thereby forming one molecule of reduced glutathione (GSH). In most cell types these reactions take place exclusively in the cytosol. One exception to this rule is the kingdom Plantae. In plants, γ -glutamylcysteine synthetase is localized exclusively to the plastid, whereas glutathione synthetase is found in both the plastid and the cytosol (Maughan et al. 2010; Wachter et al. 2005). Nonetheless, despite the restricted subcellular localization of glutathione biosynthesis, it is believed that glutathione is present in the majority of, if not all, subcellular compartments. Glutathione is a negatively charged molecule at physiological pH and is unable to diffuse freely across lipid bilayers. Thus, taken together, these observations imply that each internal cell membrane contains transporters that are able to mediate import and/or export of glutathione.

In this review we focus on recent advances that have been made in understanding the subcellular distribution of glutathione. In particular, we discuss recent novel insights into the concentration and redox status of the glutathione pool in specific subcellular compartments. Furthermore, we review the known glutathione transporters and, where understood, examine how they contribute to the regulation of subcellular glutathione homeostasis.

Glutathione species present within living cells

Glutathione exists predominantly as a reduced tripeptide, which is referred to as GSH (**Figure 1**). Two GSH molecules can be covalently linked together via a disulfide bond between the cysteine side-chain sulfur atoms (**Figure 1**). This species is often referred to as oxidized glutathione, however, a more correct

nomenclature is glutathione disulfide (GSSG) as there are several possible oxidized glutathione species. Examples include *S*-nitrosoglutathione (Broniowska et al. 2013), glutathione persulfide (Libiad et al. 2014), glutathione trisulfide and glutathione polysulfides (**Figure 2**). However, the subcellular production, concentration, distribution and turnover of these species remains extremely unclear. These species will therefore not be discussed further in this review. Glutathione may also be linked via a disulfide bond to protein cysteine residues. In addition, glutathione can exist in the form of conjugates to numerous endogenously- and exogenously-derived toxic electrophiles and heavy metals (**Figure 3**).

Monitoring GSH and GSSG distribution within tissues, cells and subcellular compartments

Cell, tissue and organismal glutathione homeostasis has been extensively studied over the past 100 years. Typically, glutathione homeostasis inside living cells and tissues is monitored by means of biochemical analysis of cell or tissue extracts (Queval and Noctor 2007; Rahman et al. 2006). These methods enable an accurate determination of total cell or tissue GSH and GSSG content (with the caveat that there is always a risk of post-lysis GSSG formation) and have proven to be extremely useful in many instances. With knowledge of total cell or tissue [GSH] and [GSSG] it is possible to calculate both a GSH:GSSG ratio and an E_{GSH} for the cell or tissue of interest. However, one limitation of these measurements is that the temporal resolution is somewhat restricted, meaning that rapid changes in glutathione redox homeostasis might be missed. More importantly, the physiological relevance of whole cell/tissue GSH:GSSG ratios or E_{GSH} values has become increasingly questioned in recent years, for example, with the growing appreciation of the robust nature of subcellular GSSG compartmentalization (see the section on Cytosolic glutathione redox homeostasis) (Gutscher et al. 2008; Morgan et al. 2013; Schwarzlander et al. 2016).

Until recently the possibilities for gaining insight into glutathione homeostasis at the level of specific subcellular compartments were extremely limited. Classical biochemical analyses are of little use as the process of making whole cell/tissue lysates unavoidably mixes together the glutathione pools of the different subcellular compartments (Schwarzlander et al. 2016). As a result, our understanding of glutathione homeostasis at the level of specific subcellular compartments remains lacking in many regards. For example, there remains much uncertainty regarding the absolute and relative concentrations of GSH and GSSG in any subcellular compartment. We also do not understand the extent to which compartment-specific glutathione levels can change, for example under different environmental conditions or developmental stages. Furthermore, for most intracellular membranes we

do not know the identity of the proteins that mediate transport of glutathione across these membranes: The kinetics of glutathione transport across most intracellular membranes, inside living cells, are also poorly understood. In general, we do not know if, or to what extent, glutathione transport across intracellular membranes contributes to the regulation of subcellular compartment-specific glutathione redox homeostasis, for example the relevance of GSSG efflux from the cytosol. Finally, we do not understand the function(s) of glutathione in most subcellular compartments nor why cells harbor glutathione in such large quantities (Deponte 2017).

The reason for our historically poor understanding of subcellular glutathione homeostasis has, in large part, been due to a lack of appropriate investigative tools and techniques. Nonetheless, there is light on the horizon, new tools and approaches are constantly being developed and refined, and we now see a more comprehensive and quantitative picture of (sub)cellular glutathione homeostasis beginning to emerge. Below we describe two approaches that have driven the discovery of new insights over the past 10 years.

How can we monitor subcellular glutathione distribution?

There are at least three relevant parameters of glutathione homeostasis. These are: (i) The total concentration of free (i.e. non-protein-bound or conjugated) GSH and GSSG, referred to here as [GS-X]. (ii) The molar ratio between GSH and GSSG, i.e. GSH:GSSG. (iii) The glutathione redox potential (E_{GSH}). Knowledge of at least two of these parameters is required to calculate the third. For example, E_{GSH} cannot be calculated from the GSH:GSSG ratio alone as two GSH molecules come together to form one GSSG, leading to the inclusion of a square term in the Nernst equation. Thus, knowledge of [GS-X] and GSH:GSSG, or in other words, absolute GSH and GSSG concentrations, is required to determine E_{GSH} (Figure 4).

A major advance in our investigative toolbox came with the development of genetically encoded E_{GSH} sensors, for example Grx1-roGFP2 (Dooley et al. 2004; Gutscher et al. 2008; Hanson et al. 2004). These sensors permit real-time measurement of E_{GSH} in specific subcellular compartments, inside living cells and have proven instrumental in driving forward our understanding of subcellular glutathione dynamics. The mechanism, application and interpretation of these sensors has been extensively reviewed elsewhere and will not be covered again here (Meyer and Dick 2010; Schwarzlander et al. 2016).

Previously, E_{GSH} measurements have been combined with estimated [GS-X] values to allow for calculation of approximate subcellular compartment-specific [GSH] and [GSSG] values and therefore approximate GSH:GSSG ratios (**Figure 5**) (Morgan et al. 2013). Clearly, the accuracy of this approach depends upon the exactness of the assumption regarding compartment-specific [GS-X].

A second approach has recently been developed that enables a value for [GS-X] to be indirectly experimentally determined. This method employs a genetically-encoded sensor for E_{GSH} , as described above, in combination with a single cysteine variant of yeast Grx1, sCGrx1p, which equilibrates with the GSH:GSSG ratio (**Figure 6**) (Montero et al. 2013).

To date, combinations of sensors for GSH:GSSG and E_{GSH} have been employed in two separate studies to calculate subcellular compartment-specific [GS-X], one in the endoplasmic reticulum of HeLa cells and the second in the cytosol and endoplasmic reticulum of yeast (Montero et al. 2013; Ponsero et al. 2017).

In the following sections, we discuss the novel insights into subcellular-compartment-specific glutathione homeostasis that have been gained through the application of these sensors (**Figure 7**). Furthermore, we describe the known and putative intracellular and plasma membrane-localized glutathione transporters across all eukaryotes and examine how the activity of these transporters contributes to the regulation of glutathione homeostasis in the respective subcellular compartments.

Cytosolic glutathione redox homeostasis

The cytosol constitutes a large fraction of the total cell volume and is often assumed to contain the majority of the total cellular glutathione pool. In the past, this frequently led to the assumption that measurements of total cellular glutathione will be broadly representative of the total concentration and the redox state of the cytosolic glutathione pool. However, recent studies have clearly demonstrated that this is not the case. On the contrary, compartment-specific, genetically encoded E_{GSH} sensors have shown that the cytosolic glutathione pool is remarkably reduced, $E_{\text{GSH}} \leq -320$ mV (Gutscher et al. 2008; Morgan 2014; Morgan et al. 2013). Such highly reduced cytosolic glutathione pools have been observed across a diverse range of species including yeast, fruit flies, nematode worms, fish, plants, mice and human cells (Albrecht et al. 2011; Gutscher et al. 2008; Meyer et al. 2007; Morgan 2014; Morgan et al. 2013; Panieri et al. 2017). Furthermore, studies employing recently development genetically encoded sensors specific for the mycothiol and bacillithiol redox potential, E_{MSH} and E_{BSH} respectively, have revealed similar highly reduced pools of mycothiol and bacillithiol in the bacterial species studied

(Bhaskar et al. 2014; Loi et al. 2017). One possible exception to this rule may be mammalian cardiomyocytes, which seem to have a more oxidized cytosolic glutathione pool, in the range of -260 mV – -250 mV (Swain et al. 2016), although the reasons for this are unclear. In conclusion, it appears that the presence of an abundant and highly reduced cytosolic pool of a low molecular mass thiol species is a prevalent, if not completely ubiquitous, feature of life.

The [GS-X] of the cytosol is typically assumed to be ≤ 10 mM, based largely on inference from whole cell and tissue lysates. One recent study however suggests that cytosolic glutathione may be present at much higher concentrations than previously assumed, at least in yeast (Ponsero et al. 2017). A combination of an rxYFP E_{GSH} sensor and an sCGrx1 GSH:GSSG sensor was used to calculate cytosolic [GS-X], as described above. On this basis, a cytosolic [GS-X] of ~ 30 mM was reported. It was suggested that this value is compatible with values inferred from measurements of whole cell lysates if a correction is made for the large cell volume occupied by the yeast vacuole. Thus, it is plausible that the cytosolic glutathione concentration may be even higher than previously assumed. This is definitely an area that warrants further research.

If one takes cytosolic [GS-X] to be 30 mM, together with the reported $E_{\text{GSH}} = -320$ mV, the resultant cytosolic GSH:GSSG ratio would be $\sim 17,000:1$. This would imply a cytosolic [GSSG] of ~ 1.8 μM . A more conservative estimate of a 10 mM cytosolic [GS-X] implies a GSH:GSSG of $\sim 50,000:1$ and a [GSSG] of ~ 200 nM. In either case, cytosolic GSSG is present only at nanomolar to low micromolar concentrations.

Whilst a deeper understanding of cytosolic glutathione redox homeostasis is slowly coming into view, it remains much less clear how glutathione moves into and out of the cytosol. Clearly transport of GSH from the cytosol is important to supply and replenish the glutathione pools of other subcellular compartments. However, transport of GSH and GSSG into or out of the cytosol additionally has the potential to play an important role in the regulation of glutathione redox homeostasis in the cytosol and other subcellular compartments. In the following sections we discuss the emerging, although still limited, understanding of these processes and, where known, the transporters involved.

Vacuolar glutathione transport in yeast

Vacuolar import of GSSG

Acute and chronic stress conditions that promote the formation of GSSG, for example treatment with oxidants or deletion of the gene encoding glutathione reductase, lead to a large increase in GSSG levels measured in total cellular extracts (Muller 1996). However, measurements with genetically encoded glutathione sensors indicate that in these stress situations the cytosol remains robustly reduced (Morgan et al. 2013). This raises the question of the subcellular localization of GSSG.

In *Saccharomyces cerevisiae*, it was shown that the majority of cellular GSSG is found in the vacuole. The transporter responsible for shuttling GSSG from the cytosol into the vacuole was identified as the ABC-C transporter Ycf1 (**Figure 8**) (Morgan et al. 2013). The K_m of Ycf1 for GSSG is around 290 μM (Lazard et al. 2011), whilst the K_m for GSH was determined to be ~ 15 mM (Rebber et al. 1998), supporting a strong preference for transport of GSSG over GSH. Interestingly, in strains deleted for *GLR1* (the gene encoding glutathione reductase), which normally show high levels of GSSG in whole cell lysates, further deletion of *YCF1* was found to decrease whole cell lysate GSSG levels to less than those found in wild-type cell lysates. The reason for this was found to be that the cytosol contains efficient backup systems for the reduction of GSSG. Thus, when transport of GSSG to the vacuole is blocked, the GSSG that is trapped in the cytosol is efficiently reduced (Morgan et al. 2013). Changes in whole cell GSSG levels, at least in budding yeast, are therefore solely dependent upon changes in the level of vacuolar GSSG; whole cell GSSG levels cannot be interpreted to provide information regarding cytosolic GSSG levels. Ycf1-mediated transport of GSSG was also shown to be rapid enough to be able to contribute to the restoration of cytosolic glutathione redox homeostasis following an oxidative challenge. Therefore, this is a case whereby selective transport of a particularly species of glutathione, namely GSSG, appears to contribute to the maintenance of subcellular glutathione redox homeostasis under acute stress conditions.

Vacuolar export of GSSG?

Whilst the yeast vacuole has been shown to be capable of storing high concentrations of GSSG, the ultimate fate of this GSSG remains unclear. Time course experiments suggest that the GSSG stably remains in the vacuole for at least 60 mins after import and at least 45 mins after the cytosolic glutathione pool has recovered from an oxidative challenge (Morgan et al. 2013). However, the longer-term fate of this GSSG is less clear. Perhaps there are GSSG exporters in the yeast vacuolar membrane that return the GSSG to the cytosol for reduction when conditions are appropriate to do so or perhaps the GSSG is eventually degraded, for example by the vacuole-localized γ -glutamyltranspeptidase (Jaspers and Penninckx 1984; Mehdi et al. 2001).

Vacuolar import of glutathione conjugates

Transport of glutathione into the vacuole may also occur in the form of GSH conjugates, either with toxic electrophiles, which can be both endogenously or exogenously derived, or with heavy metals. Indeed, Ycf1 was first identified as an important factor in yeast resistance to cadmium stress (Li et al. 1997; Li et al. 1996). Ycf1 has additionally been shown to transport glutathione conjugates of Hg^{2+} (Gueldry et al. 2003), Pb^{2+} (Sousa et al. 2015), selenite (Lazard et al. 2011) and arsenite (Ghosh et al. 1999). Furthermore, Ycf1 was shown to transport the model glutathione conjugate, S-2,4-dinitrophenolglutathione (Li et al. 1996), as well as to be important for the accumulation of the red pigment in adenine biosynthetic yeast mutants (Sharma et al. 2003).

Ycf1 seems to be the most important transporter for the vacuolar import of all glutathione conjugates that have been studied, mirroring its importance in GSSG transport. Nonetheless, other ABC-C transporters in the yeast vacuolar membrane can also transport glutathione conjugates as well as GSH. Of these, Bpt1 is the best characterized and was shown to be able to transport GSH, glutathione cadmium conjugates, the model glutathione conjugate S-2,4-dinitrobenzeneglutathione, and to be involved in the accumulation of red pigmentation in adenine auxotrophs (Klein et al. 2002; Sharma et al. 2003; Sharma et al. 2002). Nonetheless, in each case the contribution of Bpt1 seems to be minor compared to Ycf1, although the K_m of Bpt1 for GSH at ~ 3 mM is lower than that for Ycf1 (Klein et al. 2002; Sharma et al. 2003; Sharma et al. 2002). Other ABC-C transporters in the yeast vacuolar membrane include Vmr1, Nft1 and Ybt1 (Paumi et al. 2009). Vmr1 has been shown to be capable of transporting glutathione conjugates (Wawrzycka et al. 2010). For Ybt1 and Nft1 it is unclear whether they can transport glutathione species or not.

Regulation of Ycf1 activity

The transcription of Ycf1 is induced by the general oxidative stress responsive transcription factor Yap1 (Wemmie et al. 1994). However, in addition to transcriptional regulation, several modes of post-translational regulation have been proposed to regulate Ycf1 activity. This includes activation of Ycf1 activity by phosphorylation of residues Ser 908 and Thr 911 (Eraso et al. 2004), negative regulation of Ycf1 activity by phosphorylation of residue Ser 251 (Paumi et al. 2008; Pickin et al. 2010), negative regulation by redox modification of Cys 436, possibly by S-glutathionylation (Wei et al. 2014), and

interaction with other proteins in the cell, including the Rho1 GTPase (Lee et al. 2011). These observations point to a well-regulated and fine-tuned control of Ycf1 activity. This may be particularly important, for example, to stop Ycf1 from completely depleting the cytosolic glutathione pool under conditions of high GSSG production or high rates of glutathione conjugate formation. Perhaps the cell needs to strike a careful balance between removal of GSSG/glutathione conjugates and avoiding over-depletion of the cytosolic glutathione pool. In any case, such a wide-range of post-translational modifications, indicates that Ycf1 activity is carefully coordinated with, and regulated by, a host of other cellular activities.

The observations discussed above are intriguing and warrant further investigation. Nonetheless, it should be considered that putative regulatory sites on Ycf1 were identified largely on the basis of their impact on yeast growth in the presence of Cd^{2+} or by assessing import of radiolabeled Ycf1 substrates into vacuoles isolated from strains expressing wild-type or mutant Ycf1 variants. The identification of Ycf1 activity as the principle determinant of cellular GSSG levels now provides an alternative method for assessing Ycf1 activity inside living cells. Potentially, insights gained into the post-translational regulation of Ycf1 activity may be transferable to disease-relevant Ycf1 homologs in humans, for example ABCC1/MRP1 and cystic fibrosis transmembrane conductance regulator (CFTR). Indeed there is some evidence to suggest that S-glutathionylation can regulate CFTR activity (Wang et al. 2005).

Other potential vacuolar glutathione transporters in yeast

The yeast proteins Gex1 and Gex2 were also recently reported as glutathione transporters that localize to both the vacuolar membrane and the plasma membrane (Dhaoui et al. 2011). At present however, the evidence in favor of a function for these proteins in glutathione transport is limited to changes in cell and growth media glutathione content upon deletion or overexpression of the protein. Further experiments would be required to directly demonstrate glutathione transport and to confirm a role for these proteins as *bona fide* glutathione transporters.

Vacuolar transport in plants

Vacuolar import of GSSG?

The plant vacuole has also been proposed as a storage site of GSSG (Noctor et al. 2013). Two Ycf1 homologs, the ABC-C transporters ABC-C1 and ABC-C2, in *Arabidopsis thaliana* have been suggested as candidate transporters for mediating vacuolar influx of GSSG (**Figure 8**) (Lu et al. 1998; Lu et al. 1997). ABC-C1 and ABC-C2 were heterologously expressed in yeast, from which vacuole-membrane enriched fractions were prepared and used for *in vitro* import studies. ABC-C1 and ABC-C2 were thus shown to be competent in the transport of GSSG as well as a range of glutathione conjugates including S-2,4-dinitrophenol-glutathione, cyanidin 3-glucoside-glutathione and metolachlor-glutathione (Lu et al. 1998; Lu et al. 1997). However, to date a direct demonstration of their relevance for vacuolar GSSG sequestration in *A. thaliana* remains lacking. Nonetheless, both proteins have been implicated in resistance to heavy metals including arsenic, cadmium and mercury suggesting that they transport glutathione conjugates *in vivo* (Park et al. 2012; Song et al. 2010).

Glutathione redox homeostasis in the endoplasmic reticulum

The redox potential of the ER glutathione pool

The redox state of the glutathione pool in the endoplasmic reticulum is believed to be considerably more oxidized than that of the cytosol (Appenzeller-Herzog 2011; Avezov et al. 2013; Bass et al. 2004; Birk et al. 2013; Delaunay-Moisan et al. 2017; Delic et al. 2010; Hudson et al. 2015; Hwang et al. 1992; Merksamer et al. 2008; Ponsero et al. 2017; van Lith et al. 2011). However, even with the development of genetically encoded sensors for monitoring E_{GSH} questions still remain regarding the exact redox state of the ER glutathione pool.

The first insights into the redox state of the glutathione pool in the endoplasmic reticulum came in 1992 with a seminal paper from the lab of Harvey Lodish (Hwang et al. 1992). They engineered a small peptide, N-acetyl-Asn-Tyr-Thr-Cys-NH₂, which can freely diffuse across cell membranes but becomes trapped in the endoplasmic reticulum upon N-glycosylation of the Asn-residue. This peptide engages in thiol-disulfide exchange with GSH/GSSG and thus, by monitoring the levels of reduced and oxidized peptide, the GSH:GSSG ratio could be estimated. This led to the textbook value of ER GSH:GSSG, i.e. between 1:1 and 3:1. However, the validity of this assumption rests on the exclusivity of the interaction with the glutathione redox couple; at the time of the study for example, Ero1 was still some years away from being identified. Furthermore, as was demonstrated in the paper, the peptide does not remain in the ER but is further trafficked along the secretory pathway. Thus, even assuming that the peptide redox

state is solely determined by the glutathione redox couple, the study only reports on the average glutathione redox state of the whole secretory pathway. As we have almost no understanding of the glutathione redox pool in the secretory pathway outside of the ER this complicates interpretation of these measurements.

Since the advent of genetically encoded sensors with midpoint potentials suitable for functioning in the ER, several studies have used these sensors to measure ER E_{GSH} , yielding values ranging from -208 mV – -236 mV (Birk et al. 2013; Delic et al. 2010; Hudson et al. 2015; Ponsero et al. 2017; van Lith et al. 2011), with a GSH:GSSG ratio perhaps $< 7:1$ (Montero et al. 2013).

However, all of these measurements should be treated with a degree of caution. The interaction of rxYFP/roGFP sensors and, when applicable, their fused glutaredoxin domains, with the myriad of different redox enzymes present within the ER is largely uncharacterized. Furthermore, there is some evidence to support the concept that roGFP sensors may interact with other ER redox couples. For example, overexpression of Pdi in yeast leads to increased roGFP oxidation (Delic et al. 2010), whilst human PDI has been shown to catalyze GSSG-mediated roGFP2 oxidation *in vitro* (Tsunoda et al. 2014).

Several studies have employed unfused rxYFP or roGFP1-iL, as ER E_{GSH} sensors. This is problematic as it has been shown that rxYFP or roGFP-based sensors require suitable glutaredoxin activity to mediate equilibration of the sensor thiol-disulfide redox couple with the 2GSH:GSSG redox couple (Gutscher et al. 2008; Meyer et al. 2007; Ostergaard et al. 2004). It is unclear where this glutaredoxin activity would come from in the ER. Some studies have reported similar E_{GSH} values when comparing unfused rxYFP/roGFP variants with their Grx1-fused counterparts (Birk et al. 2013; Ponsero et al. 2017), which might be seen to support the validity of rxYFP/roGFP-based E_{GSH} measurements in the ER. However, it also raises yet more questions: For example, it is not obvious why a probe for which there is no clear mechanism for equilibration with the glutathione pool (rxYFP/roGFP) should have a similar redox state compared to a probe that is assumed to equilibrate exclusively with the glutathione couple (Grx1-roGFP/Grx1-rxYFP). There are several possible explanations, including that both unfused and fused sensors predominantly equilibrate with an alternative redox couple in the ER, or perhaps that other ER enzymes can equilibrate roGFPs with the glutathione redox couple. These questions warrant and require further investigation. In summary, more work is necessary to ascertain whether rxYFP/roGFPs targeted to the ER faithfully report E_{GSH} or whether they are additionally influenced by other redox couples. This question also has important implications for the determination of ER [GS-X] as described below.

Is ER glutathione present at a higher concentration than cytosolic glutathione?

Two recent studies have attempted to measure [GS-X] in the ER of yeast cells and HeLa cells respectively (Montero et al. 2013; Ponsero et al. 2017) using a combination of E_{GSH} and GSH:GSSG measurements, as described above. These studies reported an ER [GS-X] >15 mM in HeLa cells and ~30 mM in yeast cells. Thus, the two studies seem broadly in line with each other and indicate that [GS-X] in the ER is considerably higher than may have been expected. However, they differ with respect to their conclusions regarding the difference between ER [GS-X] and cytosolic [GS-X]. In HeLa cells, the group of Christian Appenzeller-Herzog, estimated cytosolic [GS-X], on the basis of whole cell [GS-X], to be ~7 mM and thus conclude that there is a significant glutathione concentration gradient across the ER membrane (Montero et al. 2013). In contrast, the group of Michel Toledano, used a similar combination of E_{GSH} and GSH:GSSG probes to measure both ER and cytosolic [GS-X] in yeast, concluding that both pools harbor ~30 mM GS-X and thus are equally concentrated (Ponsero et al. 2017). The implications of each conclusion, in terms of the requirements for a glutathione transporter, are quite different. In the absence of a concentration gradient glutathione could be transported into the ER by means of a channel or by facilitated diffusion. However, in the case of a concentration gradient, glutathione would have to be actively transported into the ER. Recent research points to the former as being the correct model for GSH import into the ER, although it cannot currently be excluded that different mechanisms are employed in yeast and mammalian cells.

The identification of an ER glutathione importer

The group of Michel Toledano recently reported that GSH as well as GSSG, under conditions of high GSSG production, enters the ER via the Sec61 translocon, with transport also occurring through the Sec61 homolog, Ssh1 (Ponsero et al. 2017). These results therefore support a passive transport of GSH into the ER. Furthermore, there appears to be an elegant mechanism in place to regulate GSH influx (**Figure 9**). The ER Hsp70 (Kar2), regulates the flux of GSH through the Sec61 translocon in a redox-dependent manner. Kar2 contains a cysteine residue that can be oxidized, apparently to a sulfenic acid (Wang et al. 2014; Wang and Sevier 2016). In its oxidized form, Kar2 binds to, and 'plugs' the Sec61 translocon, thereby preventing further GSH influx, whilst in its reduced form Kar2 does not mediate Sec61 'plugging'. This is important as GSH can reduce the regulatory disulfide bond on the ER sulfhydryl oxidase Ero1, thereby activating Ero1 activity (Appenzeller-Herzog et al. 2008; Baker et al. 2008; Sevier et al. 2007), leading to increased disulfide bond and H₂O₂ production, as well as to the generation of oxidized Kar2.

Together, these factors permit a negative-feedback mechanism of ER redox homeostasis regulation, whereby the rate of Ero1-activating GSH influx is controlled by Ero1 activity (Ponsero et al. 2017). Furthermore, under conditions of unfolded protein stress in the ER, the UPR response triggers increased cytosolic GSH levels, leading to a net influx of GSH into the ER and a concomitant increase in Ero1 activity (Ponsero et al. 2017). It is unclear if this mechanism is conserved in mammalian cells. However, there are several studies presenting evidence that supports GSH transport through the ryanodine receptor channel 1 (RyR1), which is localized in the membrane of the sarcoplasmic reticulum of mammalian cells (Banhegyi et al. 2003; Csala et al. 2001, 2003). It is completely unclear how GSH transport through the ryanodine receptor channel might be coordinated with a potential transport through the Sec61 translocon.

Is there a requirement for GSSG export from the ER?

We still do not know how the glutathione redox potential is maintained in the ER, for example how would cells deal with either a reductive or an oxidative perturbation of the ER glutathione redox couple. One possibility is that GSSG can be exported from the ER for reduction in the cytosol. This export might be achieved either by a transporter that directly facilitates GSSG transport across the ER membrane, or alternatively, GSSG might conceivably pass along the secretory pathway via vesicular transport and be released into the cytosol via transport across the membrane of the vacuole or be secreted into the extracellular space.

Glutathione redox homeostasis in the Golgi apparatus

Almost nothing is known about glutathione redox homeostasis in the Golgi apparatus. Assuming that there is a Golgi glutathione pool there are several possible routes by which such a pool may be established and replenished. The first route would be the presence of a transporter in the Golgi membrane capable of mediating the passage of glutathione. The second would be via vesicular traffic from the endoplasmic reticulum, which in theory might supply the Golgi apparatus with glutathione. Presumably, such a pathway must occur to some degree, however, direct evidence for movement of glutathione in this way does not exist. The final possible pathway would be via retrograde vesicular trafficking from the vacuole to the Golgi.

Of note, we previously showed in yeast that the late Golgi-localized, chloride/proton antiporter, Gef1, plays a role in cytosolic glutathione homeostasis (Braun et al. 2010). Interestingly, this seemed to be specifically dependent upon the proton antiport function of Gef1 as mutants that function as chloride channels also displayed perturbed cytosolic glutathione homeostasis. Gef1 is known to be important for maturation of Fet3, a Ferro-O₂ oxidoreductase, which is important for high affinity iron uptake. However, the glutathione phenotype appears to be unrelated to disrupted iron homeostasis as the glutathione phenotype could not be recapitulated in a mutant strain lacking the Fet3 iron importer. These results therefore suggest that regulating the membrane potential / pH-gradient across the Golgi membrane, particularly under iron-limiting conditions, is important for cytosolic glutathione homeostasis. Possibly, this points to the existence of a proton-coupled glutathione transporter in the Golgi membrane. However, the identity of this transporter, if indeed it exists, is unclear.

Glutathione redox homeostasis in the peroxisome

Very little is also known about glutathione redox homeostasis in the peroxisome. However, we recently demonstrated that the glutathione redox potential in the peroxisome matrix is highly reduced, similar to the cytosol, with a redox potential in yeast of ~ -320 mV (Ayer et al. 2013; Elbaz-Alon et al. 2014). Similar results were observed in tobacco leaf epidermal cell peroxisomes (Schwarzlander et al. 2008).

We recently identified Opt2, as a peroxisome membrane-localized member of the oligopeptide transporter family, which affects cellular glutathione redox homeostasis. Interestingly, deletion of Opt2, particularly in combination with the peroxisomal catalase, Cta1, led to disrupted glutathione redox homeostasis in the cytosol, peroxisome and mitochondrial matrix. However, a mechanistic understanding of the link between Opt2 and cellular glutathione is unclear and it has not been demonstrated if Opt2 is a direct transporter of glutathione or not (Elbaz-Alon et al. 2014).

Mitochondrial glutathione redox homeostasis

Mitochondria are surrounded by two membranes, the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane. The mitochondrial matrix is enclosed by both the IMM and the OMM, whilst a second aqueous compartment, the intermembrane space (IMS), is sandwiched between the IMM and OMM. Consequently, the mitochondrion harbors two separate aqueous compartments, each of

which contain glutathione. For glutathione to be imported to the matrix it has to cross both the OMM and the IMM, whereas for glutathione to be transported to the IMS, only transport across the OMM is necessary (**Figure 10**).

Porins facilitate equilibration of the cytosolic and IMS glutathione pools

Regarding transport across the OMM the situation appears to be relatively straightforward. The OMM harbors porin proteins, which permit passage of all molecules $< \sim 5$ kDa (Kojer et al. 2012). This implies that both the total glutathione concentration and the glutathione redox potential in the IMS should be in equilibrium with that of the cytosol. Indeed, it was demonstrated that the E_{GSH} in the IMS is in equilibrium with the cytosol and depends upon cytosolic enzymes (Kojer et al. 2012).

One complication with regards to the above assumptions is a possible further subdivision of the IMS. The IMS can be thought of as two separate compartments, the peripheral IMS and the cristae space, which are separated from each other by cristae junctions. There is already some evidence for unequal distribution of proteins and protein complexes between the peripheral IMS and cristae space, as well as between the cristae-bounding IMM and the peripheral IMS-bounding IMM (van der Laan et al. 2016). It is possible that the physico-chemical environment, including the abundance of GSH and GSSG, is also different between the peripheral IMS and the cristae space. However, at present there is no clear evidence either for or against this idea.

What is the identity of the glutathione transporter in the inner mitochondrial membrane?

Clearly all glutathione that is destined for the matrix must first traverse the OMM, presumably via porin proteins. However, this still leaves the problem of the IMM, which is impermeable to most small molecules. Presumably this requires that the IMM contains dedicated GSH or GSSG transporters, or at least transporters that have a moonlighting function as glutathione transporters. Two IMM carrier proteins, the dicarboxylate carrier and the 2-oxoglutarate carrier were suggested as GSH transporters based on experiments in isolated human kidney mitochondria (Chen and Lash 1998; Chen et al. 2000). However, later experiments in which these carriers were overexpressed in *Lactococcus lactis* found no evidence for glutathione transport (Booty et al. 2015). Thus, the identity of the GSH importer of the IMM remains a matter of debate.

Mitochondrial export of glutathione

The yeast protein Atm1 is thought to be involved in export of Fe-S cluster assembly intermediates from the mitochondrial matrix to the cytosol (Schaedler et al. 2014). Atm1 activity is stimulated by GSSG and it seems to be capable of transporting GSSG as well as GSSSG across the IMM. The plant homolog, ATM3, was also shown to be capable of supporting GSSG transport (Schaedler et al. 2014). In addition, the crystal structure of Atm1 shows GSH bound to the transporter (Srinivasan et al. 2014). The question of whether transport of GSSG on Atm1/ATM3 has any physiological relevance remains unclear.

The matrix glutathione pool appears to be kinetically isolated from the cytosol

Experiments with rxYFP and roGFP-based sensors indicate that the matrix glutathione pool is highly reduced, $E_{\text{GSH}} < -300$ mV, similar to the cytosol (Calabrese et al. 2017; Hanson et al. 2004; Hu et al. 2008; Kojer et al. 2012; Morgan et al. 2011). However, irrespective of the identity of the glutathione transporters in the IMM, it appears that the transport of glutathione across the IMM is very slow. Thus, the matrix and cytosolic glutathione pools are effectively kinetically isolated from each other (Kojer et al. 2012). The evidence in support of this conclusion comes mainly from studies in yeast. In yeast, glutathione reductase is present in both the cytosol and the mitochondrial matrix. Both forms of Glr1 are encoded by the same gene. Differential start codons ensure the translation of forms with (matrix) and without (cytosolic) a matrix-targeting sequence (Outten and Culotta 2004). In strains expressing only a cytosolic form of Glr1, cytosolic glutathione redox homeostasis appears normal, whilst in the matrix the glutathione pool is more oxidized and unable to recover from an oxidative challenge (Hu et al. 2008; Kojer et al. 2012; Kojer et al. 2015). These observations indicate that GSSG cannot exit the mitochondrial matrix at a rate sufficient for observable reduction by cytosolic enzymes.

Glutathione transport into the nucleus

Several studies have suggested that nuclear glutathione levels can be selectively increased relative to the cytosol, particularly at specific phases of the cell cycle (Diaz-Vivancos et al. 2015; Diaz Vivancos et al. 2010; Markovic et al. 2007; Vivancos et al. 2010). However, it is unclear how this would be compatible

with a free passage of small molecules such as GSH and GSSG through the nuclear pore complexes (Bellomo et al. 1997).

One problem is that these studies have extensively relied on the fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA) as a glutathione dye. CMFDA is then used in combination with nuclear stains to track changes in 'nuclear glutathione'. There are several issues with this approach. First, reaction of CMFDA with GSH or indeed any other thiol does not render it fluorescent. CMFDA changes from a non-fluorescent to a fluorescent form only upon removal of its acetate groups by intracellular esterases (Poot et al. 1991). Thus, fluorescence reflects deacetylation of CMFDA, not reaction with thiols and certainly not a specific reaction with GSH. Secondly, the efficient reaction of CMFDA or the deacetylated fluorescent form with GSH requires glutathione S-transferase activity. Even if CMFDA is staining GSH in the nucleus it would be important to completely rule out that the observed changes in nuclear-localized CMFDA fluorescence are not due to changes in nuclear-localized glutathione S-transferase activity. Finally, although many researchers persist in using CMFDA as a glutathione dye, several studies have specifically shown that it is not suitable for this purpose (Sebastia et al. 2003; Tauskela et al. 2000).

In conclusion, in the absence of direct evidence for specific accumulation of glutathione in the nucleus we currently consider it unlikely that there are significant differences between cytosolic and nuclear free GSH and GSSG levels.

Glutathione homeostasis in plastids

Plastids consist of three separate aqueous compartments, the inter-envelope space, the stroma, and the thylakoid lumen. Similar to mitochondria, the plastid outer envelope contains porins that presumably render this membrane permeable to glutathione and impart the inter-envelope space with a similar glutathione concentration and redox state as the cytosolic glutathione pool, although this remains to be experimentally demonstrated.

The concentration of the stromal glutathione pool remains unclear although immunogold-labelling experiments have suggested a glutathione concentration that is broadly similar to that of the cytosol (Koffler et al. 2013; Muller et al. 2004; Zechmann and Muller 2010). Measurements with roGFP-based sensors indicate the presence of a highly reduced glutathione pool (~ -320 mV) (Maughan et al. 2010), which is consistent with the presence of a stroma-localized glutathione reductase.

With regard to the thylakoid lumen, the presence of a glutathione pool remains unclear. However, if glutathione is indeed present in the thylakoid lumen, glutathione transporters presumably must be present in the thylakoid membrane.

In plants, GSH1, the γ -glutamylcysteine synthetase, is localized exclusively to the plastids, whereas GSH2, glutathione synthetase, is localized to both the plastid and the cytosol. Thus, in order to support cytosolic GSH biosynthesis, γ -glutamylcysteine needs to be exported from the plastid. In *A. thaliana*, three homologs of the *Plasmodium falciparum* chloroquine-resistant transporter, PfCRT, were identified as mediating the export of γ -glutamylcysteine from the plastid into the cytosol (**Figure 11**). Plants harboring mutations in CLT1, CLT2 or CLT3 show significantly decreased cytosolic glutathione levels. Furthermore, reconstitution experiments in *Xenopus* oocytes demonstrated that CLT1, CLT2 and CLT3 are capable of transporting both γ -glutamylcysteine and GSH (Maughan et al. 2010).

Although both the plastid and the cytosol harbor GSH2, the plant cytosol appears to be the major site of GSH biosynthesis. In other words, the preferred mechanism for GSH synthesis appears to be synthesis of γ -glutamylcysteine in the plastid, followed by export to the cytosol, where the GSH2-mediated reaction preferentially occurs, rather than complete GSH synthesis in the plastid. Furthermore, it was shown that restricting glutathione biosynthesis exclusively to the cytosol is sufficient for normal plant development (Pasternak et al. 2008). These observations speak in favor of the existence of transporters mediating the transport of GSH from the cytosol into the plastid stroma. However, it is currently unclear if the CLT transporters are responsible for this function or whether other transporters fulfil this role.

Plasma membrane transport: cellular export and import of glutathione?

The first major insights into glutathione transport and organismal glutathione distribution were gained in studies spanning the early 1960s to the late 1990s. Evidence for cellular export and import of GSH, GSSG and glutathione conjugates came from a range of cell types, including, but not limited, to hepatocytes, renal proximal tubular cells, intestinal epithelial cells, erythrocytes, astrocytes and retinal epithelial cells (Ballatori and Dutczak 1994; Chen et al. 2000; Fan et al. 2017; Iantomasi et al. 1997; Lash 2005, 2009; Lee et al. 1997; Minich et al. 2006; Muller et al. 1996; Prchal et al. 1975; Reddy et al. 1973). These observations suggest that transporters capable of mediating the export and perhaps the import of glutathione species across the plasma membrane are present in most mammalian cell types.

Plasma membrane glutathione transport appears to have several purposes, including: (i) In the distribution of glutathione around the body, i.e. the so-called interorgan metabolism of glutathione. (ii) In the normal turnover of glutathione. (iii) In the cellular efflux of GSSG and glutathione conjugates. Each of these three functions has the potential to influence cellular and subcellular glutathione levels and thus influence subcellular glutathione redox homeostasis.

Interorgan metabolism of glutathione

The interorgan metabolism model of glutathione homeostasis was developed as a result of work from the 1970s to the 1990s, which pointed to considerable cell and tissue-specific differences in the synthesis and degradation of glutathione (Anderson et al. 1980; Bartoli and Sies 1978; Dass et al. 1992; Griffith and Meister 1979; McIntyre and Curthoys 1980; Ookhtens and Kaplowitz 1998). For example, liver hepatocytes are considered to be the major location of GSH synthesis. GSH can subsequently be secreted across both the sinusoidal membrane and the canalicular membrane into the blood plasma and the bile, possibly by MRP1 and MRP2 respectively (Ballatori et al. 2009). The liver is thereby thought to be the major determinant of blood plasma and bile GSH levels (Anderson and Meister 1980). In contrast to the liver, the kidneys are thought to be a sink for plasma GSH, with as much as 80% of GSH removed from blood plasma after a single pass through the kidneys (Lash 2005). Sodium dependent and sodium independent GSH transport processes were identified as mediating GSH influx across the basolateral membrane (Rankin and Curthoys 1982; Rankin et al. 1985; Scott and Curthoys 1987). OAT1, OAT3 and NaC3 were identified as putative GSH transporters in the basolateral membrane of renal proximal tubular cells (Chen et al. 2000; Lash 2005, 2009). However, this finding remains the subject of debate (Hagos et al. 2013). It is proposed that the interorgan transport of glutathione may serve as a way to effectively redistribute cysteine, for example for protein synthesis (McIntyre and Curthoys 1980). It has also been proposed that GSH can be transported across the intestinal epithelia, however, the identity of specific transporters remains elusive (Iantomasi et al. 1997 Vincenzini, 1989 #364).

Glutathione turnover: the γ -glutamyl (glutathione) cycle

The glutathione pool of most cell types is thought to be regularly turned over, with a half-life in the range of 2–6 hours. Until recently, it was thought that glutathione turnover was exclusively dependent upon the γ -glutamyl cycle, in which GSH or GSSG are exported from the cell, where they can be degraded and

the constituent amino acids re-imported into the cell (Orlowski and Meister 1970). In this model GSH and/or GSSG, must be exported from the cell in order to be degraded. This is because the enzyme γ -glutamyl transferase (GGT), is localized on the plasma membrane with its active site facing into the extracellular space. For a long time GGT was the only enzyme known to be capable of degrading GSH, which together with data implying a rapid turnover of the glutathione pool supported the need for extensive efflux of GSH across the plasma membrane. However, recently identified enzymes in yeast and mammalian cells clearly demonstrate that efficient glutathione degradation pathways are present in the cytosol. In yeast Dug1, Dug2 and Dug3 were shown to function in an alternative pathway for glutathione degradation (Ganguli et al. 2007). Dug2 and Dug3 form a heterodimeric complex, the assembly of which is required for the functional glutamine aminotransferase activity of Dug3 (Kaur et al. 2012). Dug1 subsequently acts as a Cys-Gly dipeptidase (Kaur et al. 2009). These enzymes and thus glutathione degradation are thought to play an important role in regulating cellular glutathione homeostasis (Baudouin-Cornu et al. 2012). In mammalian cells, ChaC1 and its homolog ChaC2, were identified as a γ -glutamylcyclotransferases, mediating the cleavage of GSH into 5-oxoproline and Cys-Gly (Crawford et al. 2015; Kaur et al. 2017; Kumar et al. 2012). These observations together with research challenging other aspects of the originally proposed γ -glutamyl cycle have led to the proposal that it be renamed the glutathione cycle (Bachhawat and Kaur 2017; Bachhawat and Yadav 2018; Inoue 2016).

In summary, it now remains to be determined how glutathione degradation is distributed between the external GTT and the cytosolic degradation pathways. Perhaps the relative importance of each degradation pathway differs in different cell types and speculatively represents yet another mechanism by which glutathione homeostasis can be controlled.

Redox regulation and detoxification: export of GSSG and glutathione conjugates

The third function of plasma membrane glutathione transporters is in the efflux of GSSG and glutathione conjugates, apparently for regulation of cytosolic glutathione redox homeostasis and detoxification purposes. GSSG efflux has been observed from numerous cell types, especially under conditions of oxidative stress, including rat and mouse astrocytes, cardiomyocytes and human endothelial cells (Hirrlinger et al. 2001; Krause et al. 2007; Minich et al. 2006; Mueller et al. 2005; Suzuki and Sugiyama 1998; Widder et al. 2007). MRP1 (ABCC1) seems to be particularly important in this regard. Furthermore, MRPs (ABCCs) are capable of transporting a wide-range of glutathione conjugates, including conjugates to therapeutic and antineoplastic drugs, pesticides, herbicides and metals (Cole 2014; Cole and Deeley

2006; Johnson and Chen 2017; Leslie 2012). The investigation of MRP1 and related proteins is of particular interest due to their role in anticancer drug resistance (Lu et al. 2015). In addition to these transporters many other confirmed and candidate plasma membrane-localized glutathione transporters have now been identified and characterized. We briefly discuss the different known plasma membrane transporters below.

Plasma membrane transporters in mammalian cells

Plasma membrane glutathione exporters in mammalian cells

The ABCC family of transporters.

Humans express 13 members of the ABCC/MRP family, which includes ABCC1–6/MRP1-6 and ABCC 8–13/MRP8-13, as well as the cystic fibrosis transmembrane conductance regulator (CFTR), which is also known as ABCC7 (Vasiliou et al. 2009). These proteins are all thought to reside in the plasma membrane and, for the most part, use energy from ATP hydrolysis to power transport of substrates. These proteins have been a focus of intense research efforts over the past years due to their role in multi-drug resistance and the causal role of CFTR mis-function in the pathology of cystic fibrosis.

ABCC1/MRP1 is the closest human homolog of Ycf1 and appears to share a conserved function. Similar to Ycf1, ABCC1 was shown to transport GSSG with a K_m of $\sim 93 \mu M$, whilst its affinity for GSH is much lower (Leier et al. 1996). Together, with the demonstration of GSSG efflux to the yeast vacuole by the ABCC transporter, Ycf1, and the likelihood that a similar mechanism is employed in plants, this observation implies that efflux of GSSG is a mechanism for regulating cytosolic glutathione redox homeostasis that is conserved across the eukaryotes (**Figure 8**).

ABCC1, as well as ABCC2 are thought to be important for the release of GSH from hepatocytes into the sinusoidal space and bile canaliculus respectively (Ballatori et al. 2009), as described above. There is also good evidence for the transport of GSH, GSSG and GSH conjugates on ABCC2, ABCC3, ABCC4 and ABCC5, GSH conjugates may also be transported on ABCC6, ABCC7 and ABCC8 (Ballatori et al. 2005; Borst et al. 2000; Linsdell and Hanrahan 1998; Rius et al. 2006; Wijnholds et al. 2000).

CFTR

Despite its homology to other members of the ABCC family, CFTR functions as an ATP-gated ion channel, mediating the transport of Cl⁻ ions down their concentration gradient. Almost 300 cystic fibrosis-causing mutations have been identified in CFTR, with the most common being a deletion of residue F508.

CFTR has also been shown to be permeable to both GSH and GSSG (Gao et al. 1999; Jungas et al. 2002; Kogan et al. 2003; Linsdell and Hanrahan 1998). It has even been speculated in the past that abnormal glutathione transport may contribute to the development of cystic fibrosis pathology, although no further evidence has since been presented that would support this conjecture (Childers et al. 2007; Hudson 2001, 2004). It remains unclear as to the physiological role of GSH and GSSG transport by CFTR, as well as the consequences if this transport is disrupted in patients carrying CFTR mutations.

ABCG2

The human ABC transporter ABCG2 belongs to the ABCG subfamily of ABC transporters. Recently, ABCG2 was expressed and characterized in yeast cells lacking Opt1. It was found that cells expressing ABCG2 had ~2.5 times higher GSH levels in the growth media as compared to wild-type cells. Furthermore it was shown that treatment of the yeast cells with the ABCG2 substrate methotrexate abolished GSH efflux, supporting a competitive inhibition. Based on these data ABCG2 was concluded to function as a GSH exporter (Brebuhl et al. 2010). However, later studies performed in HEK293 and MCF7 cells, as well as monitoring ³H-GSH transport in insect Sf9-derived inside-out membrane vesicles, concluding that ABCG2 is not a GSH transporter, although ABCG2 overexpression may alter intracellular GSH levels (Gauthier et al. 2013).

LABCG2 from *Leishmania major* was found to be involved in virulence, oxidative stress resistance (Manzano et al. 2017) and resistance to antimony (Perea et al. 2016). In the latter case, the mechanism of resistance was proposed to be ABCG2-mediated transport of glutathione-antimony conjugates (Perea et al. 2016). Recently LABCG2 was reconstituted in insect Sf9-derived inside-out vesicles. These vesicles were found to be able to transport ³H-GSH thereby supporting the idea that transporters from this subfamily of ABC transporters can facilitate GSH transport (Perea et al. 2018). Finally, evidence was produced for a role of the yeast ABCG2 protein, Gxa1, in GSH export across the plasma membrane, in the context of biotechnological GSH production (Kiriya et al. 2012). These observations were based on increased glutathione levels in the growth media although direct evidence for GSH transport was not produced. Interestingly, of the 10 ABCG transporters present in yeast, Gxa1 is the only example of a

'half-transporter', i.e. a half-length transporter that needs to form homo- or hetero-oligomers to become functional (Paumi et al. 2009). ABCG2 from *Leishmania* and humans are also half-transporters. Full length ABCG transporters have only been identified in plants, fungi, oomycetes, slime molds and brown algae (Kang et al. 2011). Perhaps this is indicative of some aspect of the structure of ABCG half-transporters that renders them especially suitable for evolving the ability to transport GSH. Clearly, the ABCG subfamily of transporters warrant further investigation to better understand their role in glutathione transport.

RLIP76

RLIP76 (RalA-binding protein 1) is a non-ABC, ATP-dependent, plasma membrane-localized transporter, which has been shown to be capable of transporting glutathione conjugates. Indeed, in erythrocytes RLIP76 was reported to be the most important ATP-dependent glutathione conjugate transporter (Awasthi et al. 2000; Sharma et al. 2001; Singhal et al. 2009). RLIP76 is also thought to be an important mediator of drug and radiation resistance in many cancer cells types (Awasthi et al. 2005; Singhal et al. 2008; Singhal et al. 2005) and RLIP76 inhibition is currently being investigated as a therapy for treatment of neuroblastoma and kidney cancer (Singhal et al. 2017; Singhal et al. 2010; Singhal et al. 2015).

Plasma membrane glutathione exporters in yeast

Yor1

The ABCC transporter Yor1 was recently suggested to be important for cadmium detoxification in *Saccharomyces cerevisiae* (Nagy et al. 2006). Indirect evidence indicates that Yor1 transports bis-glutathionato cadmium conjugates. Overexpression of Yor1 rescued the cadmium sensitivity of a *Δycf1* yeast strain supporting an overlapping function of these two transporters and implying that yeast secrete glutathione-cadmium conjugates in addition to sequestering them in the vacuole. More work is required to understand the physiological importance of Yor1.

Plasma membrane glutathione importers in mammalian cells

OAT1, OAT3 and NaC3

OAT1 and OAT3 are organic anion transporters, whilst NaC3 is a sodium-dicarboxylate carrier. All three proteins are localized to the renal basolateral membrane and were proposed to have a role in GSH import into renal proximal tubular cells (Lash 2009). However, later studies concluded that, at least for OAT1 and OAT3, they have no role in GSH uptake but rather mediate transport of glutamate derivatives (Hagos et al. 2013). Thus, there remain no undisputed mammalian GSH importers identified.

Plasma membrane glutathione importers in yeast

Yeast cells contain a member of the oligopeptide transporter family, Opt1/Hgt1, in their plasma membrane (Lubkowitz 2011). Opt1 was shown to be a high-affinity glutathione importer capable of mediating the transport of both GSH and GSSG (Bourbouloux et al. 2000). Indeed, yeast cells deleted for the gene encoding Gsh1, the γ -glutamylcysteine synthetase that catalyzes the first step in GSH biosynthesis, are viable providing that GSH is available in the growth media (Grant et al. 1996; Wu and Moye-Rowley 1994). However, simultaneous deletion of both *GSH1* and *OPT1* is lethal in any condition implying that Opt1 is the only, or by far the most important, GSH importer in yeast (Bourbouloux et al. 2000).

Interestingly however, it appears that the levels of functional Opt1 in the plasma membrane are limiting for GSH import. For example, it was shown that even in the presence of 100 μ M GSH Δ *gsh1* cells only contain about 30% of the amount of glutathione as found in wild-type cells (Kumar et al. 2011). However, upon overexpression of Opt1/Hgt1 cells incubated with GSH accumulate cellular GSH up to 7-8 times as high as found in a wild-type cell (Kumar et al. 2011). Indeed this eventually leads to cell death. This would argue in favor of Opt1 activity being regulated largely or exclusively by expression level, with the expression level normally being very low. This allows sufficient GSH import to maintain viability of Δ *gsh1* cells but not sufficient to restore GSH levels to that found in wild-type cells. More work is required to fully understand the functional relevance of Opt1 for cellular glutathione homeostasis in wild-type yeast cells.

Homologs of Opt1 have been identified in many other species of yeast and in several cases have been functionally characterized. Hgt1-like transporters with a $K_m < 200 \mu$ M for either GSH or GSSG come from species including *Schizosaccharomyces pombe* (Dworeck et al. 2009; Thakur et al. 2008), *Kluyveromyces lactis*, *Cryptococcus neoformans*, *Schizosaccharomyces japonicus* and *Pichia guilliermondii* (Thakur and Bachhawat 2010). Interestingly, in *Candida albicans*, the closest homolog of Opt1 was found not to

transport GSH or GSSG. Instead, a more distant homolog, Opt7, was found to facilitate GSH uptake, with a K_m for GSH of 204 μM (Desai et al. 2011).

Plants also contain multiple transporters of the oligopeptide transporter family (Lubkowitz 2011). In *A. thaliana* AtOPT4 and AtOPT6 have both been confirmed as glutathione transporters (Cagnac et al. 2004; Koh et al. 2002; Pike et al. 2009; Wongkaew et al. 2018; Zhang et al. 2016). Furthermore, the oligopeptide transporters, OsGT1 and BjGT1, from rice (*Oryza sativa*) and mustard (*Brassica juncea*) respectively were shown to mediate glutathione transport (Cagnac et al. 2004; Zhang et al. 2004). Together, these results indicate that members of the oligopeptide transporter family function throughout the yeast and plants to mediate the transport of glutathione.

Conclusion

A large body of knowledge and insights have been gained since the first description of glutathione some 120 years ago. For example, we have extensive knowledge of many of the *in vivo* roles of glutathione and the biochemistry underpinning these functions.

However, although it is clear that glutathione transport plays important roles in a wide-range of physiological and pathological contexts, for example in the distribution of glutathione around multicellular organisms, in multidrug resistance, drug resistance in parasites and, at the level of single cells, in the supply of glutathione to various subcellular compartments, we still do not know the identity of the majority of the glutathione transporters that presumably exist. Their identification and characterization is therefore clearly an important focus for future research. It will be particularly exciting to apply new genetically encoded tools to help in the search for these transporters and subsequently to develop our understanding of how the function and activity of these glutathione transporters impacts upon subcellular glutathione homeostasis.

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Figure legends

Figure 1. The structure of GSH and GSSG.

The molecular structure of GSH and GSSG indicating the constituent amino acids.

Figure 2. The structures of different forms of oxidized glutathione.

An illustration showing the structure of glutathione persulfide (GSSH), S-nitrosoglutathione (GSNO) and glutathione trisulfide (GSSSG).

Figure 3. The structures of glutathione S-conjugates.

An illustration depicting the structure of the glutathione-cadmium conjugate (bisglutathionato cadmium complex) and the conjugate between glutathione and dinitrophenyl (S-2,4.dinitrophenyl-glutathione).

Figure 4. The relationship between [GS-X], GSH:GSSG and E_{GSH}

A 3D surface plot showing how [GS-X] and the GSH:GSSG ratio together influence E_{GSH} .

Figure 5. Determination of E_{GSH} and [GSSG] with genetically encoded Grx1-roGFP2 sensors.

The diagram illustrates the mechanism of the Grx1-roGFP2 sensor. Grx1 catalyzes the thermodynamic equilibration between the roGFP2 dithiol/disulfide redox couple and the 2GSH/GSSG redox couple. The redox state of the roGFP2 moiety can be determined by fluorescence emission as described elsewhere (Meyer and Dick 2010). Subsequently it is possible to determine E_{GSH} using the Nernst equation (Equation 1), where R is the universal gas constant ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), F is the Faraday constant ($96,485 \text{ C} \cdot \text{mol}^{-1}$) and T is absolute temperature in Kelvin. Determination of E_{GSH} allows calculation of [GSH] and [GSSG] according to the Nernst equation (Equation 2), if an estimation of total glutathione concentration ([GS-X]), i.e. ([GSH] + 2 x [GSSG]) is made. Equation 2 can be re-written in terms of [GS-X] and [GSSG] (Equation 3) and subsequently solved for GSSG according to Equation 4 (Morgan et al. 2013). Calculation of [GSSG] thus enables determination of [GSH] and thereby calculation of the GSH:GSSG ratio.

Figure 6. Experimental determination of compartment-specific total glutathione concentration.

An approach has recently been developed that enables a value for [GS-X] to be indirectly experimentally determined. This method employs both a genetically-encoded sensor for E_{GSH} and a single cysteine variant of yeast Grx1, sCGrx1p, which equilibrates with the GSH:GSSG ratio according to Equation 5 (Montero et al. 2013). The K_{ox} for the reaction in Equation 5 can be determined according to Equation 6. K_{ox} can then be used to determine the GSH:GSSG ratio according to Equation 7. The measured value for $\frac{[\text{GSH}]}{[\text{GSSG}]}$ can be combined with a measured value for $\frac{[\text{GSH}]^2}{[\text{GSSG}]}$, determined on the basis of E_{GSH} by rearrangement of Equation 1 in Figure 5 (Equation 8), to calculate [GS-X] (Equation 9).

Figure 7. The subcellular distribution of GSH and GSSG

A cartoon of an idealized yeast cell, showing, where known, [GS-X] and E_{GSH} in specific subcellular compartments. There is a clear distinction between the highly reduced plasmatic glutathione pools, i.e. the cytosol, mitochondrial intermembrane space, mitochondrial matrix, peroxisome and in plants the plastid stroma, and the more oxidized glutathione pools found in secretory pathway compartments, for example the endoplasmic reticulum and the vacuole.

Figure 8. GSSG export as an evolutionarily conserved mechanism for regulating cytosolic glutathione redox homeostasis?

Cartoons showing an idealized yeast, mammalian and plant cell. GSSG is transported from the cytosol to the vacuole by the ABCC transporter, Ycf1, in yeast. In mammalian cells, ABCC1/MRP1 mediates efflux of GSSG from the cytosol to the extracellular space in a number of cell types. The plant ABC transporters ABCC1 and ABCC2 are localized in the tonoplast and have been shown to transport GSSG when heterologously expressed in yeast cells. It is likely they fulfill a similar function in plant cells. These observations suggest that active efflux of GSSG is a highly conserved mechanism for regulating cytosolic glutathione redox homeostasis under conditions of high oxidative stress.

Figure 9. Sec61 mediates the regulated import of GSH and GSSG into the ER.

The protein translocon, Sec61, was shown to mediate the import of GSH and GSSG from the cytosol into the ER. GSH leads to the reduction of regulatory disulfide bonds on the ER sulfhydryl oxidase, Ero1 and a subsequent increase in the production of H_2O_2 . Increased H_2O_2 leads to oxidation of a cysteine residue on the ER Hsp70, Bip, which causes it to plug the Sec61 channel, thereby preventing further import of GSH.

Figure 10. Glutathione transporters in mitochondria

Porins are thought to mediate the passive diffusion of GSH and GSSG across the OMM. This would imply that the both $[GS-X]$ and E_{GSH} in the IMS are in equilibrium with the cytosolic glutathione pool. However, it is possible that there is further compartmentalization between the cristae space and the peripheral IMS; it is unclear whether small molecules freely diffuse through the cristae junction or not. The transport of GSH into the matrix is less clear. The dicarboxylate carrier protein (DCC) and 2-oxoglutarate carrier (OGC) were proposed as low affinity glutathione transporters, although this notion has recently been questioned. Atm1 in yeast and its plant homolog, ATM3, are important for cellular Fe-S cluster biogenesis and have been shown to mediate the efflux of GSSG and GSSSG.

Figure 11. Glutathione transporters in plastids

In plant cells, glutathione biosynthesis is split between the cytosol and the plastids. Gsh1, the γ -glutamylcysteine synthetase is exclusively localized to plastids whilst Gsh2, the glutathione synthetase is present in both the cytosol and plastids. Nonetheless, the majority of Gsh2 activity appears to be present in the cytosol. CLT1, CLT2 and CLT3 were identified as transporters mediating the efflux of γ -glutamylcysteine, as well as GSH, from the plastid to the cytosol. However, it was also shown that although glutathione is apparently essentially required in plastids, plant cells are viable if glutathione synthesis is exclusively localized to the cytosol. These observations imply the existence of transporters mediating the influx of GSH from the cytosol to the plastid. It is not clear if the CLT protein can fulfil this role or if other transporters are responsible.

Table 1. Known and putative glutathione transporters.

Organism	Transporter	Substrate(s)	Affinity	Localization
Mammals				
Active transporters: ABC-C transporters				
<i>Mammals</i>	MRP1 (ABCC1)	GSH GSSG GSH-conjugates	$K_m = 10 \text{ mM}$ $K_m = 93 \text{ }\mu\text{M}$	Plasma membrane
<i>Mammals</i>	MRP2 (ABCC2)	GSH GSSG GSH-conjugates		Plasma membrane
<i>Mammals</i>	MRP3 (ABCC3)	GSH-conjugates		Plasma membrane
<i>Mammals</i>	MRP4 (ABCC4)	GSH GSH-conjugates		Plasma membrane
<i>Mammals</i>	MRP5 (ABCC5)	GSH GSH-conjugates		Plasma membrane
<i>Mammals</i>	CFTR (ABCC7)	GSH GSSG		Plasma membrane
Active transporters: ABC-G transporters				
<i>Mammals</i>	ABCG2	GSH		Plasma membrane
Active transporters: Non-ABC ATP-dependent multidrug exporters				
<i>Mammals</i>	RLIP76 (RalBP1)	GS-dinitrophenol	$K_m = 60 \text{ }\mu\text{M}$	Plasma membrane
Secondary active transporters: Major facilitator superfamily (Na^+-independent)				
<i>Mammals</i>	OAT1 (SLC22A6) ?	GSH		Plasma membrane
<i>Mammals</i>	OAT3 (SLC22A8) ?	GSH		Plasma membrane
Secondary active transporters: Major facilitator superfamily (Na^+-dependent)				
<i>Mammals</i>	NaC3 (SLC13A3) ?	GSH		
Secondary active transporters: Mitochondrial carrier protein				
<i>Mammals</i>	DCC (SLC25A10) ?	Dicarboxylate GSH		Mitochondria
<i>Mammals</i>	OGC (SLC25A11) ?	2-oxoglutarate GSH		Mitochondria
Channels and pores				
<i>Mammals</i>	RYR1	Calcium GSH		Sarcoplasmic reticulum
Yeast				
Active transporters: ABC-C transporters				

<i>S. cerevisiae</i>	Ycf1	GSH GSSG GS-Cd ²⁺ Selenodiglutathione GS-dinitrophenol	<i>Km</i> = 15 mM <i>Km</i> = 290 μM <i>Km</i> = 39 μM <i>Km</i> = 110 μM <i>Km</i> = 14 μM	Vacuole
<i>S. cerevisiae</i>	Bpt1	GSH GSH-conjugates	<i>Km</i> = 3 mM	Vacuole
<i>S. cerevisiae</i>	Vmr1	GSH-conjugates		Vacuole
<i>S. cerevisiae</i>	Yor1	GS-Cd ²⁺		Plasma membrane
Active transporters: ABC-B transporters				
<i>S. cerevisiae</i>	Atm1	GSSG GS-S-SG	<i>Km</i> = 109 μM	Mitochondrial inner membrane
Active transporters: ABC-G transporters				
<i>S. cerevisiae</i>	Gxa1 (ABCG2) ?	GSH		Plasma membrane
Secondary active transporters: Oligopeptide transporters				
<i>S. cerevisiae</i>	Hgt1 (Opt1)	GSH	GSH <i>Km</i> = 54 μM GSSG <i>Km</i> = 91 μM	Plasma membrane
<i>S. pombe</i>	Pgt1p	GSH GSH conjugates	GSH <i>Km</i> = 62 μM	Plasma membrane
<i>K. lactis</i>	Kl-Hgt1p	GSH	GSH <i>Km</i> = 64 μM	Plasma membrane
<i>S. japonicus</i>	Sj-Hgt1p	GSH	GSH <i>Km</i> = 204 μM	Plasma membrane
<i>P. guilliermondii</i>	Pg-Hgt1p	GSH		Plasma membrane
<i>C. neoformans</i>	Cn-Hgt1p	GSH	GSH <i>Km</i> = 83 μM	Plasma membrane
<i>C. albicans</i>	Opt7p	GSH	GSH <i>Km</i> = 206 μM	Plasma membrane
Secondary active transporters: Proton-coupled antiporters				
<i>S. cerevisiae</i>	Gex1p	GSH		Vacuole/ plasma membrane
<i>S. cerevisiae</i>	Gex2p	GSH		Vacuole/ plasma membrane
Passive transporters: Channels and pores				
<i>S. cerevisiae</i>	Sec61	GSH GSSG		Endoplasmic reticulum
<i>S. cerevisiae</i>	Ssh1	GSH GSSG		Endoplasmic reticulum

<i>S. cerevisiae</i>	Por1	GSH GSSG		Mitochondrial outer membrane
<i>S. cerevisiae</i>	Por2	GSH GSSG		Mitochondrial outer membrane
Plants				
Active transporters: ABC-C transporters				
<i>A. thaliana</i>	At-Mrp1	GS-dinitrophenol GS- metolachlor GSSG	$K_m = 49 \mu\text{M}$ $K_m = 64 \mu\text{M}$ $K_m = 219 \mu\text{M}$	Vacuole
<i>A. thaliana</i>	At-Mrp2	GS-dinitrophenol GS-metolachlor GSSG	$K_m = 66 \mu\text{M}$ $K_m = 75 \mu\text{M}$ $K_m = 73 \mu\text{M}$	Vacuole
Active transporters: ABC-B transporters				
<i>A. thaliana</i>	At-ATM3	GSSG GS-S-SG		Mitochondrial inner membrane
Secondary active transporters: Oligopeptide transporters				
<i>A. thaliana</i>	At-OPT6	GSH GSSG	$K_m = 566 \mu\text{M}$	Plasma membrane
<i>A. thaliana</i>	At-OPT4	GSH	$K_m = 1.4 \text{ mM}$	plasma membrane
<i>Brassica juncea</i>	Bj-GT1	GSH		Plasma membrane
<i>Oryza sativa</i>	Os-GT1	GSH GSSG GSH-conjugates		Plasma membrane
Secondary active transporters: Drug/metabolite transporter superfamily				
<i>A. thaliana</i>	At-CLT1	GSH		Plastids- Inner envelope
<i>A. thaliana</i>	At-CLT2	GSH		Plastids- Inner envelope
<i>A. thaliana</i>	At-CLT3	GSH		Plastids- Inner envelope
Parasites				
Active transporters: ABC transporters				
<i>Leishmania</i>	LABCG2	GSH GSSG		Plasma membrane

Transporters marked with a question mark are those for which there are conflicting reports regarding their ability to transport glutathione.

Figure 1.

Figure 2.

GSSH

GSNO

GSSSG

Figure 3.

Figure 4.

Figure 5.

Equation 1.

$$E_{\text{GSH}} = E_{\text{roGFP2}} = E_{\text{roGFP2}}^{\circ'} - \frac{RT}{2F} * \ln \frac{[\text{roGFP2}_{\text{red}}]}{[\text{roGFP2}_{\text{ox}}]}$$

Equation 2.

$$E_{\text{GSH}} = E_{\text{GSH}}^{\circ'} - \frac{RT}{nF} * \ln \frac{[\text{GSH}]^2}{[\text{GSSG}]}$$

Equation 3.

$$E_{\text{GSH}} = E_{\text{GSH}}^{\circ'} - \frac{RT}{nF} \times \ln \left(\frac{[\text{GSX} - 2 \times \text{GSSG}]^2}{[\text{GSSG}]} \right)$$

Equation 4.

$$[\text{GSSG}] = \frac{1}{8} \left(e^{\frac{2 \times (E_{\text{GSH}}^{\circ'} - E_{\text{GSH}}) \times F}{RT}} \right) + 4 \times \text{GSX} - \sqrt{\left(-e^{\frac{2 \times (E_{\text{GSH}}^{\circ'} - E_{\text{GSH}}) \times F}{RT}} - 4 \text{GSX} \right)^2 - 16 \times \text{GSX}^2}$$

Figure 6.

Equation 6.
$$K_{ox} = \frac{[\text{sCGrx1pSH}][\text{GSH}]}{[\text{sCGrx1pSSG}][\text{GSSG}]}$$

Equation 7.
$$\frac{[\text{GSH}]}{[\text{GSSG}]} = K_{ox} \frac{[\text{sCGrx1pSH}]}{[\text{sCGrx1pSSG}]}$$

Equation 8.
$$e^{\frac{2 \times (E'_{\text{GSH}} - E_{\text{GSH}}) \times F}{RT}} = \frac{[\text{GSH}]^2}{[\text{GSSG}]}$$

Equation 9.
$$[\text{GSX}] \text{ (M)} = \frac{\left(\frac{[\text{GSH}]^2}{[\text{GSSG}]}\right)}{\left(\frac{[\text{GSH}]}{[\text{GSSG}]}\right)} + \left(2 \times \frac{\left(\frac{[\text{GSH}]^2}{[\text{GSSG}]}\right)}{\left(\frac{[\text{GSH}]}{[\text{GSSG}]}\right)^2}\right)$$

Figure 7.

Yeast Cell

Figure 8.

Figure 9.

Figure 10.

Figure 11.