Review

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Glutaredoxins and iron-sulfur protein biogenesis at the interface of redox biology and iron metabolism

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Abstract: The physiological roles of the intracellular iron and redox regulatory systems are intimately linked. Iron is an essential trace element for most organisms, yet elevated cellular iron levels are a potent generator and amplifier of reactive oxygen species and redox stress. Proteins binding iron or iron-sulfur (Fe/S) clusters, are particularly sensitive to oxidative damage and require protection from the cellular oxidative stress protection systems. In addition, key components of these systems, most prominently glutathione and monothiol glutaredoxins are involved in the biogenesis of cellular Fe/S proteins. In this review, we address the biochemical role of glutathione and glutaredoxins in cellular Fe/S protein assembly in eukaryotic cells. We also summarize the recent developments in the role of cytosolic glutaredoxins in iron metabolism, in particular the regulation of fungal iron homeostasis. Finally, we discuss recent

insights into the interplay of the cellular thiol redox balance and oxygen with that of Fe/S protein biogenesis in eukaryotes.

Keywords: CIA machinery; glutathione; ISC machinery; thioredoxin and glutaredoxin systems; transcriptional regulation.

Introduction

Iron is a key metal in biology. It functions as an essential co-factor in central cellular processes such as photosynthesis, respiration, metabolite conversion, DNA synthesis and repair, ribosome biogenesis, vertebrate oxygen transport, and the sensing of environmental cues. Iron ions are associated with proteins in mononuclear or dinuclear form, most commonly at the side chains of cysteine, histidine, aspartate, or glutamate residues. Occasionally, inorganic ligands such as molecular oxygen, CN^- , or CO are additionally involved in iron coordination. Moreover, iron is bound to proteins after being incorporated into heme co-factors or into iron-sulfur (Fe/S) clusters. The latter moieties are particularly sensitive to oxidation by oxygen and/or reactive oxygen species (ROS) and require protection from the cellular oxidative stress protection systems particularly under stress conditions (Figure 1). The delicate sensitivity of Fe/S clusters is prominently utilized by bacteria that employ transcription factors with sensitized Fe/S centers for orchestrating their adaptation to ambient oxygen and/or oxygen radical levels (Crack et al. 2014, 2018).

In all organisms, the maturation of Fe/S proteins essentially relies on complex proteinaceous biogenesis machineries. These systems, too, are particularly susceptible to changes in the redox status of the cell. Most non-plastidial eukaryotes utilize the mitochondrial iron-sulfur cluster assembly (ISC) system for the *de novo* synthesis of Fe/S clusters of cellular Fe/S apoproteins (Lill 2009; Lill and Freibert 2020). The formation of cytosolic and nuclear Fe/S proteins further

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depend on the more sensitive mitochondrial ISC system particularly rely on the cellular redox control and oxidative stress protection systems to sustain the maturation of cellular Fe/S proteins (Figure 1) (Gomez et al. 2014; Missirlis et al. 2003; Strain et al. 1998). Moreover, in non-green eukaryotes defects in the mitochondrial ISC system induce cellular iron overload, and a (post-)transcriptional deregulation of cellular iron homeostasis that is similar to that of an iron-deprived cell (Camaschella et al. 2007; Misslinger et al. 2018)). Excess intracellular iron usually accumulates within mitochondria causing oxidative damage which likely further aggravates existing defects in mitochondrial functions, again requiring responses by the cellular redox control and oxidative stress protection systems (Gomez et al. 2014; Hadzhieva et al. 2014; Herrero et al. 2008).

The members of the thioredoxin (Trx) and glutaredoxin (Grx) families make up an extensive array of proteins, which execute central roles in redox biology, including the antioxidant response to oxygen and ROS. Trxs and Grxs are found in nearly all known organisms, where they are located

in various cellular organelles (Figure 1A) (Jacquot and Zaffagnini 2019; Lu and Holmgren 2014). The yeast *Saccharomyces cerevisiae* encodes three thioredoxins, Trx1, Trx2, and Trx3, and four class I dithiol glutaredoxins, Grx1, Grx2, Grx8 and Mgp12 (Herrero et al. 2010). These proteins facilitate the reduction of other proteins by cysteine thiol-disulfide exchange. They harbor an active site with a dithiol CxxC motif that is essential for function. Trxs further act as electron donors to, e.g., peroxidases thereby linking cellular redox control with oxidative stress response (Figure 1). The related dithiol Grxs share many of the functions of Trxs, but are reduced by the tripeptide glutathione (GSH) which functions as a central reservoir for upholding the cellular thiol redox balance (Deponte 2017; Hanschmann et al. 2013; Jacquot and Zaffagnini 2019; Lillig and Berndt 2013; Lillig et al. 2008; Toledano and Huang 2017). GSH furthermore cooperates with, e.g., glutathione peroxidases (Gpx) and glutathione S-transferases and thus contributes significantly to the cellular response to ROS. Oxidized glutathione (GSSG) and Trxs are reduced by cognate NADPH-dependent reductases (Figure 1A). Simple eukaryotes such as *S. cerevisiae* tolerate the deletion of either their glutaredoxin reductases (Glr) or thioredoxin reductases (Trr), indicating that these proteins have partially overlapping functions. Yet, the simultaneous deletion of all genes encoding Trxs and Glrs, just like deletion of all Trx- and Grx-encoding genes is lethal for *S. cerevisiae* (Herrero et al. 2010; Herrero et al. 2008).

Several members of the cellular thiol redox systems play central roles in the biosynthesis of Fe/S proteins. Of these, GSH is an indispensable compound for the maturation of cytosolic and nuclear Fe/S proteins (Figures 1 and 2) (Kumar et al. 2011; Li and Cowan 2015; Lill et al. 2015; Schaedler et al. 2014, 2015; Sipos et al.; Srinivasan et al. 2014; Wachnowsky et al. 2018). Moreover, the mitochondrial glutaredoxin Grx5 and the cytosolic Grx3 serve as intermediate Fe/S cluster trafficking proteins in the mitochondrial ISC and the cytosolic CIA systems (Figures 1 and 2) (Berndt and Lillig 2017; Braymer and Lill 2017; Jia et al. 2020; Lill and Freibert 2020; Rouhier et al. 2010). These proteins belong to the class II Grxs with a conserved monothiol CGFS active-site motive and are, in contrast to most dithiol Grxs, not particularly active in catalyzing dithiol-disulfide redox reactions (Berndt and Lillig 2017; Lillig et al. 2008). Additionally, *S. cerevisiae* contains the two cis-Golgi-localized monothiol Grx6 and Grx7 with yet ill-defined cellular roles (Abdalla et al. 2016; Izquierdo et al. 2008; Mesecke et al. 2008) (Figure 1A).

As outlined above, the metabolism of iron and the process of Fe/S protein biogenesis are intimately entangled with the cellular redox control and oxidative stress

protection systems. In this review, we summarize the recent developments at the interface of these important cellular pathways in eukaryotes. We first focus on the role of GSH and Grxs as important components in cellular Fe/S protein maturation. Second, we review the recent developments of the roles of cytosolic Grxs in iron metabolism and the regulation of fungal iron homeostasis. Finally, we will address new insights into the interplay between iron and cellular redox control with a focus on how oxygen, ROS, and thiol redox systems impact Fe/S protein biogenesis and Fe/S proteins.

Role of glutathione and mitochondrial glutaredoxins in cellular Fe/S protein maturation

Overview of cellular Fe/S protein assembly pathways

The following is a brief description of eukaryotic Fe/S protein biogenesis. For in depth descriptions, we refer the reader to recent reviews (Ciofi-Baffoni et al. 2018; Dutkiewicz and Nowak 2018; Lill et al. 2015; Lill and Freibert 2020; Maio and Rouault 2015, 2020; Wachnowsky et al. 2018). One of the essential functions of the mitochondrion is the production of Fe/S clusters for the incorporation into Fe/S apoproteins by the mitochondrial ISC system. Maturation begins with the *de novo* synthesis of [2Fe-2S] clusters at the six-component protein complex of the core ISC system (Braymer and Lill 2017) (Figure 2). This complex comprises a cysteine-bound persulfide (CysS-SH)-generating cysteine desulfurase complex (Nfs1-Isd11-Acp1), a [2Fe-2S] scaffold protein (Isu1) for cluster assembly, reduced ferredoxin, and the Friedreich's Ataxia-causing protein frataxin (Yfh1) which acts as an allosteric effector (Boniecki et al. 2017; Fox et al. 2019). The three substrates, Fe²⁺, persulfide, and electrons are used to assemble a [2Fe-2S] cluster on the scaffold protein Isu1 (Boniecki et al. 2017; Gervason 2019 #2464; Weibert et al. 2014) (Figures 1 and 2). Holo-Isu1 binds its cluster with decent stability and hence is not an efficient Fe/S cluster donor without assistance. *In vivo*, Fe/S cluster release is assisted by the dedicated Hsp70 chaperone and Hsp40 co-chaperone system (Ssq1 and Jac1 in *S. cerevisiae*) which directly binds to Isu1 to transfer its bound [2Fe-2S] clusters to the monothiol Grx5. This ISC factor serves as a key Fe/S cluster trafficking protein (Uzarska et al. 2013).

Figure 2: Monothiol glutaredoxins as hubs for Fe/S cluster trafficking and Fe homeostasis.

Proposed trafficking pathways of [2Fe-2S]-cluster bound Grx5 (orange) in the mitochondrion (upper section). Grx5 serves as a donor of [2Fe-2S] clusters provided by the early ISC system for [2Fe-2S] and [4Fe-4S] apoproteins. Fe/S cluster transfer and insertion into some [2Fe-2S] target apoproteins can occur spontaneously, once their cysteine side chains are reduced by the cellular Grx/GSH or Trx systems (Weiler et al. 2020). Whether additional targeting factors (e.g., Bol1/3) binding to Grx5 are needed for other [2Fe-2S] target apoproteins remains open. Sequentially, the dislocation of the Grx5-bound [2Fe-2S] cluster, fusion of two [2Fe-2S] clusters to a [4Fe-4S] cluster, and insertion into apoproteins further requires the late ISC factors Isa1, Isa2, Iba57, and other late-acting targeting factors. Likewise in the cytosol (bottom section), multi-domain Grx3/Grx4 (orange) are responsible for a multitude of pathways. They are essential for the assembly of Fe/S clusters on the early CIA factor Dre2 (human CIAPIN1), likely by direct Fe/S cluster transfer. Together with Tah18 (human NDOR1), Dre2 provides electrons required for the built-up of [4Fe-4S] clusters on the CIA scaffold Cfd1-Nbp35 (human CFD1-NBP35) (Paul et al. 2015). Furthermore, Grx3/Grx4 are involved in the maturation of the diferric iron center of the small subunit of ribonucleotide reductase, Rnr2. The maturation reaction further involves the input of electrons from Tah18-Dre2. In *S. cerevisiae*, Grx3/Grx4 plays an important role in the formation of cytosolic iron- and heme-binding proteins and for the trafficking of iron to mitochondria and to the iron-storage vacuole (see Figure 1B). In fungi, heterodimeric Grx3/Grx4-Bol2 Fe/S cluster-binding complexes are sensors of the iron status of the cell. They physically interact with and thereby regulate iron-responsive transcription factors such as *S. cerevisiae* Aft1-Aft2 to control the expression of genes involved in the uptake and utilization of iron (Figure 3B).

The maturation of cytosolic Fe/S proteins by the CIA pathway involves 11 known proteins (Figure 2) (for a detailed description of the CIA system see (Ciofi-Baffoni et al. 2018; Lill 2020; Maio et al. 2020; Paul and Lill 2015)). The CIA factors Cfd1-Nbp35 are proposed to form the central scaffold complex of the CIA system on which the clusters for cytosolic [4Fe-4S] proteins are formed. Fe/S cluster formation on Cfd1-Nbp35 requires the compound X-S (see above) that is produced by the mitochondrial ISC system. Further, electrons are required that are provided by the cytosolic NADPH-Tah18-Dre2-dependent electron transfer chain. Tah18-Dre2 and Cfd1-Nbp35 of the early CIA system work in concert to generate [4Fe-4S] clusters, which are then passed to the CIA factor Nar1. Finally, the CIA targeting complex (CTC) formed by three late-acting CIA components, Cia1, Cia2, and Mms19, facilitates the transfer of [4Fe-4S] clusters into cytosolic and nuclear [4Fe-4S] proteins. The maturation of the ABC protein Rli1 involved in ribosome assembly and recycling further requires the deca-GX₃ proteins Yae1-Lto1 that recruit Rli1 to the CTC for [4Fe-4S] cluster insertion. The CIA system is also dependent on cytosolic Grx3/Grx4 that deliver [2Fe-2S] clusters to the CIA factor Dre2 (see below).

Role of Atm1 and GSH in the maturation of cytosolic and nuclear Fe/S proteins

The mitochondrial ISC and cytosolic CIA machineries are functionally connected via the mitochondrial ABC transporter Atm1 that exports the sulfur- and possibly iron-containing compound X-S that is produced by the mitochondrial ISC system (Kispal et al. 1999; Pandey et al. 2019). Atm1 has long been known to be involved in cellular iron homeostasis in yeast by performing a specific role in cytosolic Fe/S protein biogenesis (Kispal et al. 1997; Miao et al. 2009). Further support for a crucial role of Atm1 in exporting a substrate for the CIA pathway comes from studies of copper (Cu) stress in the yeast *Cryptococcus neoformans*. In this situation, Atm1 is overexpressed which may account for the disruption of [4Fe-4S] clusters on cytosolic proteins by Cu (Garcia-Santamarina et al. 2017). Homologues of Atm1 in other eukaryotes are well conserved in function (Bernard et al., 2009; Cavadini et al. 2007; Garcia-Santamarina et al. 2017; Kushnir et al. 2001; Pondarre et al. 2006; Wang et al. 2019; Zuo et al. 2017), including the human pathogenic protozoan parasite *Leishmania major* (Martinez-Garcia et al. 2016). The essentiality of Atm1 is also evident by its presence in the

highly reduced mitosomes of the microsporidian species *Encephalitozoon cuniculi* and *Trachipleistophora hominis*, where Fe/S protein biogenesis is proposed to be the sole essential biochemical function of these mitochondria-derived organelles (Braymer et al. 2020; Freibert et al. 2017).

Depletion of GSH induces a similar phenotype as the lowering of Atm1 levels, suggesting that GSH is playing its essential role in both iron regulation and cytosolic Fe/S protein biogenesis, either in the Atm1-assisted export process or as a cofactor of Grx3/Grx4 (Figures 1 and 2) (Kumar et al. 2011; Sipos et al. 2002). There is compelling evidence that the export of the X-S species is dependent on the core ISC system including monothiol Grx5, as well as GSH, which is likely a component of X-S (Figure 2, see review by (Lill et al. 2015)). *In vitro* transport assays have indicated the transport of various substrates by Atm1 including oxidized glutathione persulfide (GSSSG) or GSSG (Schäedler et al. 2014), and a tetra-GSH-bound [2Fe-2S] cluster ([2Fe-2S](GS)₄) (Li and Cowan 2015; Pearson et al. 2020; Qi et al. 2014). Structural and biochemical studies have further observed the binding of GSH and [2Fe-2S](GS)₄ to Atm1, respectively (Pearson et al. 2020; Srinivasan et al. 2014), yet *in vivo* experiments are still awaited to clarify the speciation of the transported species. To this regard, recent reports in yeast suggest that Atm1 is responsible for the export of two different types of sulfur-containing species, i.e. (Fe/S)_{int} for cytosolic Fe/S cluster biogenesis and S_{int} for tRNA thiouridinylation (Pandey et al. 2018; Pandey et al. 2019). While both pathways depend on the mitochondrial desulfurase Nfs1 and the scaffold Isu1, export of S_{int} is observed to be independent of the Hsp70 chaperone complex. These findings, along with the literature on Atm1 as a supplier of X-S for the CIA system support the hypothesis that both cytosolic Fe/S clusters and thiol modifications of tRNA are dependent on mitochondria-produced sulfur substrates (Figure 2) and not from a cytosolic desulfurase activity, as alternatively proposed for human cells (Maio et al. 2020). It is also of interest that the human homologue of Atm1, ABCB7, has been observed to be in complex with ferrochelatase, the last enzyme in heme synthesis (Maio et al. 2019; Takanani et al. 2003). However, a direct connection between Atm1 and heme trafficking remains undefined. The identity and specificity of the transported physiological Atm1 substrate(s) therefore remains to be determined. Their elucidation will have profound implications for iron homeostasis, Fe/S protein biogenesis, tRNA modification, and thiol redox balance.

Function of the mitochondrial [2Fe-2S] cluster-binding monothiol glutaredoxin 5

Most eukaryotes harbor the well-conserved mitochondrial monothiol Grx5 (Grx5 in *S. cerevisiae*; GLRX5 in human). This protein is a member of the class of single domain class II Grxs that are found in all kingdoms of life and contain an invariant CGFS active-site sequence motif (Berndt and Lillig 2017; Lillig et al. 2008). Its structure displays the typical Trx fold consisting of five α -helices and four β -sheets. Monothiol Grxs, including Grx5 bind a [2Fe-2S] co-factor that bridges two monomers. The cluster is coordinated symmetrically by the two active-site cysteine residues and the sulfhydryl group of two GSH molecules that are non-covalently bound to Grx5 in a specific GSH-binding pocket (Iwema et al. 2009; Johansson et al. 2011). The [2Fe-2S] cluster on monothiol Grxs is intrinsically labile and its rapid transfer to Fe/S apo-proteins *in vitro* is well documented (Banci et al. 2014; Bandyopadhyay et al. 2008; Berndt and Lillig 2017; Brancaccio et al. 2014; Iwema et al. 2009; Jia et al. 2020; Mapolelo et al. 2013; Moseler et al. 2015; Nasta et al. 2020; Rouhier et al. 2010). Consistent with a general role of monothiol Grxs in Fe/S cluster trafficking, *S. cerevisiae* Grx5 can be replaced by diverse monothiol Grxs with mobile Fe/S clusters from different species *in vivo*, including those with variations in the classical CGFS active site motif (Mapolelo et al. 2013; Molina et al. 2004; Moseler et al. 2015). Yet, most *in vitro* transfer assays involving monothiol Grxs as Fe/S cluster donors reported in the literature were carried out in the presence of the artificial thiol-specific reductants DTT (Banci et al. 2014; Brancaccio et al. 2014; Jia et al. 2020; Sen and Cowan 2017; Wachnowsky et al. 2016). DTT is a cytotoxic chemical that is known to support non-physiological Fe/S cluster-related reactions (Bridwell-Rabb et al. 2014; Gervason et al. 2019; Weber et al. 2014) raising the question in how far the conclusions drawn from these studies are relevant for the situation *in vivo*. *In vitro* studies in which artificial thiol-specific reductants have been avoided in favor of natural ones are so far rare and include the Fe/S cluster transfer from bacterial IscU to Grx5 by the Hsp70 chaperone system (Shakamuri et al. 2012), from cytosolic Grx4 to the transcription factor Aft2 (Poor et al. 2014), and the recent study on human GLRX5 (Weiler et al. 2020). In contrast to reactions involving DTT, *in vitro* Fe/S cluster transfer reactions that avoid this chemical in favor of a natural thiol-specific reducing system such as dithiol Grx/GSH show several features that are in line with *in vivo* observations indicating that such assay systems more faithfully reflect the physiological situation. First, the rate of Fe/S cluster

transfer from GLRX5 to apo-FDX1 is several orders of magnitude higher than those from other mitochondrial Fe/S cluster-binding ISC factors including ISCU2, ISCA1, ISCA2, and NUF1 (Weiler et al. 2020). This kinetic advantage of GLRX5 as a [2Fe-2S] cluster donor makes a similar role of the other ISC factors unlikely. This conclusion is in full agreement with *in vivo* findings, which confined the role of yeast and human ISCA1, ISCA2, and NUF1 proteins to the maturation of mitochondrial [4Fe-4S] proteins (Lill and Freibert 2020; Mühlenhoff 2011 #52; Sheftel et al. 2012). Second, Fe/S cluster transfer from human GLRX5 to human FDX1, a physiological client protein, is much faster than that to an apo-ferredoxin from a different species. This likely reflects an optimized interaction between physiological ISC donor and apoprotein acceptor pairs in a given organism (Weiler et al. 2020).

Grx5 deficiency in *S. cerevisiae*, zebrafish, or human diminishes the activities of all mitochondrial and extra-mitochondrial Fe/S proteins and impairs heme biosynthesis (Camaschella et al. 2007; Mühlenhoff et al. 2003; Rodriguez-Manzanique et al. 2002; Wingert et al. 2005; Ye et al. 2010). In yeast, Grx5 deficiency is further associated with a massive deregulation of the cellular iron uptake systems, which is a hallmark of all components of the core ISC assembly and export machineries. Moreover, yeast *grx5Δ* cells lose mitochondrial DNA, a phenotype they share with the late ISC factors Isa1, Isa2, and Iba57 that are essential for the formation of mitochondrial [4Fe-4S] proteins (Molina et al., 2004). The sum of these phenotypes indicates that Grx5 occupies a central position in the assembly line of the mitochondrial ISC system (Figures 1 and 2). Despite this central role and in contrast to several other core ISC components, Grx5 is not essential in *S. cerevisiae*, indicating that its function can be bypassed. Yet, taken the strong phenotype of *grx5Δ* cells, this bypass is inefficient. In metazoans where respiration is essential unlike in *S. cerevisiae*, *GLRX5* deletion is lethal, and mutations in *GLRX5* are associated with severe mitochondrial disorders in humans (Ast et al. 2019; Baker et al. 2014; Camaschella et al. 2007; Wingert et al. 2005; Ye et al. 2010).

In *S. cerevisiae*, Fe/S cluster formation on Grx5 *in vivo* depends on all components of the core ISC machinery, including the scaffold protein Isu1 and the Hsp70/Hsp40 chaperones Ssq1/Jac1 (Figure 2) (Uzarska et al. 2013). Moreover, depletion of yeast Grx5 results in the accumulation of Fe/S clusters on Isu1, a phenotype shared with cells depleted for the Hsp70 chaperone Ssq1 and its co-chaperone Jac1 (HSPA9 and HSC20 in human) (Mühlenhoff et al. 2003). This indicates that Grx5 cooperates with this dedicated chaperone system in order to facilitate Fe/S cluster transfer from Isu1 to recipient apoproteins

(Dutkiewicz and Nowak 2018; Kampinga et al. 2010; Uzarska et al. 2013; Vickery and Cupp-Vickery 2007). In *S. cerevisiae*, Isu1 binds to the specific peptide-binding domain of the Hsp70 chaperone Ssq1 in an ATP-dependent manner. Grx5 binding to a yet unidentified part of Ssq1 occurs independently of nucleotides. ATP hydrolysis by Ssq1, which is stimulated by both Isu1 and Jac1 but not Grx5, stabilizes the Ssq1-Grx5 interaction and weakens Fe/S cluster binding on Isu1. Simultaneous binding of holo-Isu1 and Grx5 on Ssq1 likely facilitates Fe/S cluster transfer from the scaffold protein to monothiol Grx5 (Uzarska et al. 2013). This Hsp70-mediated Fe/S cluster transfer is consistent with the observation that bacterial Hsp70 HscA and Hsp40 HscB strongly stimulate Fe/S cluster transfer from IscU to Grx5 *in vitro* (Bonomi et al. 2011; Shakamuri et al. 2012). An analogous *in vitro* transfer system has not been reported yet for the eukaryotic system.

Taken together, Grx5 occupies an important position in the ISC assembly line, connecting the early part of the ISC system that is dedicated to the *de novo* synthesis of [2Fe-2S] clusters on the scaffold protein Isu1 with the later parts of the ISC system that are involved in (1) the transfer of [2Fe-2S] clusters to recipient apoproteins, (2) the formation and export of X-S required for the maturation of cytosolic/nuclear Fe/S proteins, and (3) the conversion of [2Fe-2S] into [4Fe-4S] clusters and their insertion into target apoproteins (Berndt and Lillig 2017; Braymer and Lill 2017; Lill and Freibert 2020; Rouhier et al. 2010). Cluster transfer from Grx5 to model [2Fe-2S] apoproteins can occur spontaneously *in vitro*, yet whether additional targeting factors, i.e. Bol1/Bol3, are needed for certain client [2Fe-2S] proteins remains open (Figure 2). The maturation of [4Fe-4S] proteins essentially involves the late-acting ISC proteins Isa1-Isa2-Iba57 for the synthesis and Nfu1 for subsequent targeting of [4Fe-4S] clusters to client proteins (Brancaccio et al. 2014; Mühlenhoff et al. 2011) (Figure 1). This late reaction of the ISC system involves the transfer of [2Fe-2S] clusters from Grx5 to Isa1-Isa2-Iba57, where they are converted to [4Fe-4S] clusters by reductive fusion. Electrons are provided by NADPH via ferredoxin reductase Arh1 and the mitochondrial ferredoxin Yah1 (Banci et al. 2014; Brancaccio et al. 2014; Weiler et al. 2020).

Cellular roles of cytosolic multi-domain monothiol glutaredoxins

The cytosolic and nuclear multi-domain Grxs belong to a subgroup of class II Grxs and possess a tandem

organization with an N-terminal Trx domain followed by one or two C-terminal monothiol Grx domains (Alves et al. 2009; Berndt and Lillig 2017; Lillig et al. 2008; Rouhier et al. 2010). The Grx domains display the canonical architecture of single domain Grxs such as Grx5 and bind a bridging [2Fe-2S] cluster in a homodimeric complex using the same coordination sites as single domain Grxs (Haunhorst et al. 2010; Li et al. 2012; Sen and Cowan 2017). The Trx domain employs the typical Trx-fold, yet does not possess thiol-reductase activity because its WAXPC active site motif lacks the conserved nucleophilic cysteine of thioredoxins, and is not involved in Fe/S cluster binding (Chi et al. 2018). Multi-domain Grxs are ubiquitous in eukaryotes but are not present in bacteria. Cytosolic monothiol Grxs are essential for viability of many fungi including *Aspergillus fumigatus*, some strain backgrounds of *S. cerevisiae*, and fission yeast *Schizosaccharomyces pombe* under aerobic, but not semi-anaerobic conditions (Kim et al., 2011; Labbe et al. 2013; Misslinger et al. 2019; Mühlenhoff et al. 2010). In mammals, the cytosolic monothiol GLRX3 is essential for embryonic development, post-embryonic growth, and heart function (Cheng et al. 2011).

Role of cytosolic glutaredoxins in iron delivery

In all species analyzed, the cytosolic and nuclear multi-domain Grxs play a central role in cellular iron sensing. In addition, low levels of *S. cerevisiae* cytosolic Grx3/Grx4 result in multiple defects of cytosolic iron metabolism decreasing iron loading of several classes of iron-dependent proteins and the import of iron into mitochondria (Mühlenhoff et al. 2010). At the same time, cells display cytosolic iron overload and a massive increase of cellular GSH suggesting that yeast cytosolic Grxs are essential for intracellular trafficking of iron to make it bioavailable for the assembly of iron-containing proteins. In zebrafish embryos, cytosolic Grxs are required for the maturation of hemoglobin (Haunhorst et al. 2013). In human cell culture, too, depletion of cytosolic GLRX3 impairs the activity of cellular Fe/S proteins and causes a deregulated cellular iron uptake as a result of the reduced levels of Fe/S cluster binding to cytosolic iron-regulatory protein 1 (IRP1). Yet, in comparison to *S. cerevisiae*, these iron-related defects are mild (Frey et al. 2016; Haunhorst et al. 2013).

Cytosolic Grxs interact with several components of the cytosolic CIA system and play an important role in the maturation of cytosolic [4Fe-4S] proteins (Figure 2). In *S. cerevisiae* the paralogous Grx3/Grx4 physically interact with the early CIA factor Dre2 (human CIAPIN1) (Zhang

et al. 2014) and are required for Fe/S cluster assembly on Dre2 (Mühlenhoff et al. 2010; Zhang et al. 2014). Similar complex formation between cytosolic Grx3 and Dre2 has been detected in *Arabidopsis thaliana* and *A. fumigatus* (Inigo et al. 2016; Misslinger et al. 2019) as well as in human cells and in mice (Banci et al. 2015; Frey et al. 2016; Saito et al. 2011). This interaction essentially requires both the Fe/S cluster on Grx3 and the Trx domain (Banci et al. 2015). The complex of mammalian GLRX3 and the mammalian Dre2 homolog CIAPIN1 (anamorsin) also includes NDOR1 (yeast Tah18), the diflavin reductase that forms an electron transfer chain with Dre2/CIAPIN1 required for the generation of [4Fe-4S] clusters on the CIA factors CFD1 and NBP35 (Figure 2) (Frey et al. 2016; Netz et al. 2010; Paul and Lill 2015). Depletion of GLRX3 and the Grx-interacting protein BOLA2 reduces iron incorporation into CIAPIN1 in cultivated human cells *in vivo*, strongly suggesting that Fe/S clusters coordinated by GLRX3 are directly transferred to CIAPIN1 in cells (Frey et al. 2016). Transfer of [2Fe-2S] clusters from GLRX3 to CIAPIN1 via heterodimeric complex formation was also demonstrated *in vitro*. Yet, since this *in vitro* transfer was carried out in the presence of DTT, the physiological relevance of this finding requires further confirmation (Banci et al. 2015; Netz et al. 2016; Zhang et al. 2017). Cytosolic monothiol Grxs were reported to interact with several other CIA factors including those of the CIA targeting complex, Cia1 (CIAO1 in human), Mms19 (MMS19), and Cia2 (CIAO2B) (Frey et al. 2016; Inigo et al. 2016; Misslinger et al. 2019). The biochemical meaning of these interactions is as yet unclear.

The involvement of cytosolic Grxs in the maturation of iron-containing (non Fe/S) proteins is best studied for ribonucleotide reductase (RNR, Figure 2). RNR is an iron-, oxygen-, and thiol redox-dependent enzyme that is essential for DNA replication and repair, as it catalyzes the conversion of nucleoside 5'-diphosphates (NDPs) to the corresponding deoxynucleotides (Cotruvo and Stubbe 2011). Eukaryotic RNR is a multimeric protein complex composed of a large α subunit (yeast Rnr1, Rnr3) that binds substrates and allosteric effectors and a small β subunit (yeast Rnr2) that contains a diferric-tyrosyl radical ($\text{Fe}_2^{3+}\text{-Tyr}^\bullet$) site, which is used to initiate ribonucleotide reduction. *S. cerevisiae* RNR contains an unusual co-factor-less second β subunit (β' , Rnr4) that plays a critical role in facilitating iron insertion into the β subunit (Sommerhalter et al. 2004). *In vitro* assembly of the metal cofactor requires reducing equivalents, β' , Fe^{2+} , and O_2 . The iron source has been proposed to be the [2Fe-2S] cluster of Grx3/Grx4, whereas the electrons originate from the electron transfer chain NADPH-Tah18-Dre2 (Figure 2) (Li et al. 2017; Mühlenhoff et al. 2010; Zhang et al. 2014; Zhang et al. 2011). Since Grx3/Grx4 and Dre2 are Fe/S proteins,

cofactor biosynthesis on RNR indirectly depends on the mitochondrial ISC machinery. Once the radical at the di-iron site is formed, allosteric signals from the α subunit promote a proton-coupled electron transfer to the RNR active site, initiating the catalytic cycle (Cotruvo and Stubbe 2011). Upon reduction of NDP substrates, a disulfide bond must be reduced before the enzyme can turnover. In yeast, the thio-redoxin system is primarily responsible for activating the enzyme (Boronat et al. 2017; Camier et al. 2007). Hence, Fe/S protein biogenesis and thiol redox machineries work in concert to produce and turnover RNR.

Iron sensing involving fungal multi-domain monothiol glutaredoxins

Due to the low solubility of ferric iron in water, iron availability is scarce under aerobic conditions. Conversely, high intracellular iron levels are both a source and an amplifier of reactive oxygen species and thus are toxic. To acquire appropriate levels of intracellular iron and at the same time avoid iron-overloading, cells have developed sophisticated systems for assuring a balanced cellular iron homeostasis (Gupta and Outten 2020; Lane et al. 2015; Misslinger et al. 2020; Philpott and Jadhav 2019). At the cellular level, this balance is maintained through a strict coupling of cellular iron uptake at the plasma membrane to intracellular iron demands and a balanced distribution of iron between the cellular compartments involved in iron-utilization and storage. Fungi respond to low ambient iron levels by inducing cellular ionic iron and iron-siderophore uptake systems at the plasma membrane (Gupta and Outten 2020; Labbe et al. 2013; Misslinger et al. 2018; Mühlenhoff et al. 2015; Wang et al. 2019). This strategy to increase iron supply is paralleled by the mobilization of iron from the vacuole, the major iron-storage organelle in fungi, and the down-regulation of iron-consuming pathways in order to make iron available for the most essential tasks. The involvement of cytosolic monothiol Grxs in the post-translational adaptation to iron limitation in fungi is the best understood aspect of monothiol Grx function. In all fungi studied, depletion of cytosolic Grxs results in profound deregulation of the responsible transcription factors (Gupta and Outten 2020; Labbe et al. 2013; Misslinger et al. 2018; Mühlenhoff et al. 2015; Wang et al. 2019). The CGFS active-site cysteine residue which is essential for binding Fe/S clusters is absolutely required for the iron adaptation response, suggesting that the Fe/S cluster on cytosolic and nuclear monothiol Grxs functions as an iron sensor in fungi (Kim et al. 2011; Misslinger et al. 2019;

Mühlenhoff et al. 2010). The following sections outline this sensing function in various fungi.

Php4- and Fep1-dependent iron regulation in *S. pombe*

Most fungi orchestrate iron-responsive gene expression by the interplay of two conserved transcriptional repressors, a strategy that is widely employed in fungi (Figure 3A). This mode of regulation has been studied most intensively in the fission yeast *S. pombe* (Gupta and Outten 2020; Labbe et al. 2013). In this organism, the GATA-type transcription factor Fep1 represses genes involved in iron uptake under normal iron conditions (Brault et al. 2015; Jbel et al. 2009; Kim et al. 2016; Kim et al. 2011; Labbe et al. 2013). The repression of genes involved in iron-utilizing pathways is mediated by the transcriptional co-repressor Php4 that binds to the CCAAT-binding complex (Php2/3/5) under iron-limiting conditions. Both repressors are under mutual transcriptional control. Upon iron sufficiency, transcription of the *php4* gene is repressed by Fep1, while Php4 represses *fep1* under iron limitation (Brault et al. 2015; Labbe et al. 2013; Mercier et al. 2008). The mechanism of iron sensing by both transcription factors involves cytosolic Grx4, as *S. pombe* lacking Grx4 displays constitutively low levels of genes involved in cellular iron uptake (Jacques et al. 2014; Jbel et al. 2011; Kim et al. 2016; Kim et al. 2011). At the same time, genes of iron-consuming pathways remain constitutively low (Encinar del Dedo et al. 2015; Mercier and Labbe 2009; Vachon et al. 2012). In consequence, *S. pombe* cells lacking Grx4 or that harbor Grx4 variants in which the active site CGFS cysteine residue is replaced, are unable to properly respond to neither iron starvation nor iron sufficiency.

Php4, the negative regulatory subunit of the CCAAT-binding complex undergoes nuclear-cytoplasmic shuttling, being imported in the nucleus under low iron conditions and exported to the cytoplasm by the exportin Crm1 in response to iron sufficiency (Khan et al. 2014; Mercier and Labbe 2009; Vachon et al. 2012). In the nucleus, it interacts with monothiol Grx4. The role of Grx4 in iron sensing of Php4 is best explained by Fe/S cluster transfer from holo-Grx4 to Php4 which inactivates Php4's repressor function upon iron sufficiency and initiates its export from the cytosol (Figure 3A). Upon iron deprivation, Fe/S clusters on Grx4 and Php4 are lost resulting in the accumulation of Php4 in the nucleus and the repression of genes involved in iron-consuming pathways (Dlouhy et al. 2017; Encinar del Dedo et al. 2015; Jacques et al. 2014).

A: Monothiol glutaredoxins and transcriptional (co)repressors **B: Monothiol glutaredoxins and transcriptional activators**

Figure 3: Monothiol glutaredoxins as regulators of fungal iron homeostasis.

(A) Interplay of the two iron-responsive transcriptional repressors in fission yeast *S. pombe*. Right: Fep1 represses iron uptake genes under iron-replete conditions, while the co-repressor Php4 (left) binds to the CCAAT-binding complex (Php2/3/5) under iron-limiting conditions, in order to repress genes involved in iron utilization. In addition, Php4 blocks *fep1* gene expression during iron limitation, while Fep1 mutually represses the *php4* gene under iron sufficiency (B) Role of cytosolic and nuclear Grx3/Grx4 in the regulation of the iron-responsive transcriptional activators Aft1-Aft2 in budding yeast *S. cerevisiae*. Aft1-Aft2 bind to target promoters under iron-limiting conditions and activate the expression of iron uptake genes, including AFT1 and CTH2. Cth2 binds to mRNAs of genes involved in iron-consuming pathways inducing their degradation. Some iron-responsive transcription factors in (A) and (B) form physical complexes with cytosolic and nuclear Grx3 and Grx4 and likely are regulated on the post-translational level via the binding of iron or Fe/S clusters that are provided via Grx3 and Grx4 under iron-replete conditions.

Consequently, Php4 remains firmly bound to the CCAAT-binding complex in cells lacking Grx4. This model of regulation is supported by ample biochemical evidence. When co-expressed in *E. coli*, *S. pombe* Php4 and Grx4 form a stable complex that harbors a bridging [2Fe-2S] cluster that was verified by EPR spectroscopy and elemental analysis (Dlouhy et al. 2017). The spectroscopic characteristics are distinct from the [2Fe-2S] cluster on Grx4,

indicating that the [2Fe-2S] cluster is shared between Php4 and Grx4. The active-site cysteine on Grx4 and two conserved, catalytically important residues on Php4 are specifically required for Fe/S cluster binding and Php4-Grx4 complex formation. These residues are likely involved in iron-mediated inactivation of Php4 (Dlouhy et al. 2017).

In contrast to Php4, the GATA-type transcription factor Fep1 localizes constitutively to the nucleus. Its repressor function is regulated via physical complex formation with holo-Grx4 and the Grx-interacting protein BolA protein Bol2 (Figure 3A). Similar to Grx4, deletion of Bol2 results in the constitutive repression of iron uptake genes (Jacques et al. 2014; Jbel et al. 2011). Yet, Bol2 is not involved in regulating *S. pombe* Php4 (Dlouhy et al. 2017). Fep1 (and also Php4) interact with Grx4 via its N-terminal Trx- and C-terminal Grx domains (Jbel et al. 2011; Vachon et al. 2012). Binding to the Trx domain is constitutive, while association with the Grx-domain is weaker, iron-dependent, and requires the conserved Grx-active site cysteine residue (Encinar del Dedo et al. 2015; Kim et al. 2011; Mercier and Labbe 2009; Vachon et al. 2012). *In vitro*, the N-terminal domains of Fep1 from *S. pombe* and *Pichia pastoris* form [2Fe-2S]²⁺ cluster-binding homodimers (Cutone et al. 2016; Kim et al. 2016; Patti et al. 2018). Iron-responsive fungal GATA factors typically possess an N-terminal DNA-binding domain that harbors a Cys-X5-Cys-X8-Cys-X2-Cys motif located between two zinc fingers (Cutone et al. 2016; Patti et al. 2018). The four conserved cysteine residues serve as Fe/S cluster ligands *in vitro*, modulate Fep1's affinity for DNA and are essential for the repressor function of Fep1 under iron sufficiency *in vivo*. Yet, Fep1 lacking these four cysteine residues is still dimeric and binds a [2Fe-2S]²⁺ cluster ligated to two cysteine residues of the first zinc finger. This cluster is spectroscopically distinct from that of the wild-type protein and is also slowly formed on wild-type holo-Fep1 upon exposure to air. It was suggested that Fep1 might be both an iron and an oxygen sensor. *S. pombe* Fep1 was also isolated as a Fe-only protein (Encinar del Dedo et al. 2015), similar to the Fep1 homologs SRE from *Neurospora crassa* (Harrison and Marzluf 2002) and Sre1 from *Histoplasma capsulatum* (Chao et al. 2008). Iron binds to four conserved central cysteines that modulate the affinity of Fep1 for DNA, i.e. to the same site used for Fe/S cluster binding in the other studies. Ferric iron induces binding of *H. capsulatum* Sre1 to target promoters *in vitro*, indicating that iron alone is sufficient for its iron-responsive regulation. *In vitro*, *S. pombe* Fep1 transfers iron to apo-Grx4-Bol2, suggesting that the inactivation of Fep1 upon iron limitation possibly involves reverse metal transfer from Fep1 to Grx4-Fra2 (Encinar del Dedo et al. 2015). Taken together, Fe and/or Fe/S cluster

binding to Fep1 proteins explains how Grx4/Bol2 induces binding of Fep1 to target promoters under iron sufficiency (Figure 3A).

Iron regulation in fungal pathogens

The role of cytosolic Grxs as iron sensors has also been extensively studied in the fungal pathogens *A. fumigatus*, *C. neoformans*, and *Fusarium graminearum* (Gupta and Outten 2020; Misslinger et al. 2020; Misslinger et al. 2018; Trnka et al. 2020; Wang et al. 2019). The strategies for adaptation to iron starvation of these fungi are variations of those utilized by *S. pombe* and are important aspects of virulence (Figure 3A). *A. fumigatus* orchestrates iron-responsive gene expression by the repressors SreA (the ortholog of Fep1) and HapX (the ortholog of Php4). Similar to *S. pombe*, HapX represses transcription of *sreA* during iron limitation and, in turn, *hapX* transcription is repressed by SreA under iron sufficiency (Schrettl and Haas 2011). Yet, in contrast to *S. pombe*, *A. fumigatus* HapX is also essential for adaptation to toxic iron excess (Gsaller et al. 2014). The monothiol glutaredoxin GrxD from *A. fumigatus* simultaneously activates the HapX iron-starvation function and disables the repressor function of SreA at the post-translational level (Misslinger et al. 2019). Lack of GrxD results in intracellular iron deficiency as both SreA and HapX require physical interaction with GrxD to sense iron levels. Yet, in *Aspergilli* cytosolic Bol2 proteins are missing (Misslinger et al. 2019). GrxD binds to HapX irrespectively of the cellular iron status and, at least under iron sufficiency and iron excess, also to SreA. The N-terminal thioredoxin domain of GrxD is essential for interaction with SreA *in vivo*, yet dispensable for GrxD-HapX complex formation. Furthermore, the conserved active-site cysteine residue of GrxD is essential for the adaptation to iron starvation, but is not involved in complex formation with HapX. Remarkably, SreA represses the expression of *GrxD* under iron-replete conditions and by this regulates the levels of its own inhibitor (Misslinger et al. 2019). *A. fumigatus* GrxD and HapX form iron-binding hetero-tetramers upon co-expression in *E. coli* (Misslinger et al. 2019). The UV-Vis absorption spectrum of this complex indicates a bound [2Fe-2S] cluster, and recombinant HapX alone displays a similar spectrum. A more detailed biochemical analysis was not carried out yet. Thus, HapX is able to coordinate an Fe/S cluster without GrxD. *In vivo*, this Fe/S cluster is likely inserted via GrxD upon iron sufficiency. The cluster-ligating residues were not identified, yet HapX contains four cysteine-rich repeats that are reminiscent of the [2Fe-2S] cluster binding sites at the activator domain of

S. cerevisiae Yap5 (Misslinger et al. 2019; Rietzschel et al. 2015). Similar to *S. pombe* and *S. cerevisiae*, iron sensing in *A. fumigatus* requires an input of the mitochondrial, but not cytosolic Fe/S protein biogenesis machinery, which is consistent with the ISC-dependent and CIA-independent formation of Fe/S clusters on cytosolic Grxs, HapX and SreA (Misslinger et al. 2018; Mühlenhoff et al. 2010).

In *C. neoformans* the GATA-type regulator Cir1, the ortholog of *S. pombe*'s Fep1, is an important virulence factor that regulates iron uptake functions and the elaboration of virulence (Jung and Kronstad 2011). Cells lacking Grx4 display a deregulated iron homeostasis similar to cells lacking Cir1 (Attarian et al. 2018). Moreover, Grx4 deletion has negative effects on the formation of major virulence-related phenotypes and *grx4* mutants are unable to cause disease in mice. Regulation of Cir1 involves complex formation with Grx4 in the nucleus under the low-iron condition. Under iron-replete condition, complex formation is lost and Grx4 localizes to the cytoplasm while Cir1 remains in the nucleus as an active repressor.

Aft1/Aft2- and Yap5-dependent iron regulation in *S. Cerevisiae*

The regulation of cellular iron homeostasis has been intensively studied in budding yeast *S. cerevisiae* (Brault et al. 2015; Gupta and Outten 2020; Labbe et al. 2013). Its strategy of adaptation to iron limitation is unusual in that it employs transcriptional activators, Aft1 and Aft2, rather than repressors and is restricted to a small clade of yeasts (Gupta and Outten 2020) (Figure 3B). In contrast to other fungi, the CCAAT-binding complex (Hap) of *S. cerevisiae* is not iron-responsive and functions as global regulator of respiratory gene expression (Guarente 1995). In *S. cerevisiae*, the down-sizing of iron-consuming pathways is regulated by the RNA-binding proteins Cth1/Cth2 that bind to mRNAs of iron-consuming genes upon iron-limitation thereby inducing their degradation (Martinez-Pastor et al. 2013). Aft1 and its paralog Aft2, two Zn-finger DNA binding proteins of the WRKY-GCM1 family (Babu et al. 2006) bind to target promoters under iron-limiting conditions and activate the expression of the yeast iron regulon, a set of ~40 genes mostly involved in iron uptake and intracellular iron distribution (Courel et al. 2005). Aft1 also induces the transcription of its own gene and this auto-amplification circuit is essential for a robust response of *S. cerevisiae* to iron-limitation (U. Mühlenhoff, unpublished). Similar to other fungal iron responsive transcription factors, Aft1 and Aft2 require cytosolic Grx3/Grx4 function for iron sensing. Upon iron

sufficiency, they physically interact with Grx3/Grx4 and the Grx-interacting protein Bol2 (formerly termed Fra2) in the nucleus, which together promote dissociation of Aft1/Aft2 from target promoters and their subsequent nuclear export which is escorted by the specialized exportin Msn5 (Kumanovics et al. 2008; Ueta et al. 2012). Aft1 binds to Grx3/Grx4 at the C-terminal α -helical segment of the Grx domain that is opposite to the Grx active site, and this association is essential for Aft1 regulation (Hoffmann et al. 2011). This C-terminal region is also the binding interface of Grx3/Grx4 with Bol2 (Chi et al. 2018; Li and Outten 2019; Poor et al. 2014). The structure of the N-terminal domain of Aft2 bound to DNA has been solved (Poor et al. 2014). This functional domain contains two cysteine residues in a conserved Cys-Asp-Cys motif that are essential for deactivation of Aft1 and Aft2 under iron sufficiency. *In vitro*, Aft2 receives a [2Fe-2S] cluster from Grx3-Bol2 (Li and Outten 2019; Poor et al. 2014). The Fe/S cluster bridges two Aft2 monomers in a homodimer and is coordinated by the two conserved cysteine residues. This Fe/S cluster-induced dimerization reduces the affinity of Aft2 to target promoters resulting in the deactivation of Aft2. Moreover, physical complex formation of Aft2 with Bol2 likely plays an important role in the deactivation of Aft2 (Chi et al. 2018; Li and Outten 2019; Poor et al. 2014). By analogy, the regulatory mechanism of inactivation of Aft1 is most likely similar.

Physical Fe/S cluster binding using ^{55}Fe radiolabeling *in vivo* has directly been demonstrated for the high-iron sensing regulator Yap5 from *S. cerevisiae* (Rietzschel et al. 2015). It is important to note that for all other transcription factors discussed above, Fe/S cluster binding was so far documented only by *in vitro* analyses of truncated proteins expressed in *E. coli*. Interestingly, the [2Fe-2S] clusters on Yap5 are inserted in a Grx-independent fashion, as observed for HapX stated above. Their maturation also does not involve the CIA system, which strongly suggests that a subset of cytosolic/nuclear [2Fe-2S] proteins receive their cluster with the help of mitochondria, yet without further involvement of other known cytosolic Fe/S protein assembly factors including Grx3/Grx4 (Misslinger et al. 2019).

Taken together, the current literature uniformly suggests that fungal iron-responsive transcription factors are iron binding or, more likely, Fe/S cluster-containing proteins that are regulated on the post-translational level by cofactor occupancy (Figure 3). In many cases, the Fe/S cluster is provided by cytosolic monothiol Grxs, likely during physical complex formation between the partners, in some cases requiring the assistance by Bol2. The Fe/S cluster on Grxs is acquired from the mitochondrial core ISC system in a CIA-independent manner, explaining why

fungal iron uptake regulation is controlled by the status of the mitochondrial, but not the cytosolic Fe/S protein assembly machinery.

Redox-related functions of multi-domain monothiol glutaredoxins?

There is only limited evidence that cytosolic multi-domain monothiol Grxs execute roles independent of their Fe/S cluster-associated functions in iron metabolism. Yeast monothiol Grx3 possesses little thiol-reductase activity *in vitro*, amounting to $\sim 1\%$ of that of dithiol Grxs in the standard bis-(2-hydroxyethyl) disulfide (HEDS) assay (Chi et al. 2018). Therefore, this redox activity at best plays only a minor role in general thiol redox-related reactions. Grx3's thiol-reductase activity could still be important for a specific subset of proteins, yet, for structural reasons, this seems unlikely, as it was shown that a distinct loop structure adjacent to the active site forces the GSH thiol in monothiol Grxs into an position that is unfavorable for thiol redox activity (Liedgens et al. 2020; Trnka et al. 2020). Multi-domain monothiol Grxs possess glutathione S-transferase (GST) activity at a catalytic velocity similar to that of dithiol Grxs and 25% of that of canonical GSTs in standard assays (Chi et al. 2018). This indicates that these Grxs might be involved in the detoxification of xenobiotic compounds via conjugation to GSH. The GST activity is executed by the Grx-domain with assistance by the active-site cysteine of the Trx domain likely via a transient disulfide bond exchange with the Grx active-site cysteine. As a curiosity, yeast *grx4/ade2 Δ* cells do not form the typical red pigment of *ade2 Δ* cells, an end product of GSH-mediated detoxification in yeast (Jainarayanan et al. 2020). This phenotype is not found for any other member of the Trx superfamily pointing towards a specific glutathione S-transferase function of Grx4 (Jainarayanan et al. 2020). Further, *S. cerevisiae* Grx3/Grx4 S-glutathionylate the deacetylase Sir2 and thereby regulate its deacetylase activity (Vall-Llaura et al. 2016). Sir2 is essentially involved in transcriptional silencing at cryptic mating type loci, at telomeres, and some ribosomal rDNA loci, and is highly conserved from archaea to humans. Its mammalian counterpart also deacetylates substrates other than histones.

There are several studies that describe interactions of cytosolic multi-domain monothiol Grxs with kinases. *S. cerevisiae* Grx4 itself is phosphorylated by an atypical protein kinase Bud32, yet the role of the Grx4 phosphorylation remains unclear (Peggion et al. 2008). Further, *S. cerevisiae* Grx3/Grx4 form a physical complex with the stress-activated serine/threonine MAP kinase Slr2 that

plays a regulatory role in oxidative stress response (Pujol-Carrion and Torre-Ruiz 2017). Slt2 forms Fe/S cluster-bridged complexes with Grx3/Grx4 involving the Grx active-site cysteine, GSH, and specific cysteine residues on Slt2. The latter are also required for Slt2 dimerization, and mutations in these cysteine ligands display a severe impairment of Slt2 phosphorylation and Slt2 activation upon oxidative stress. Grx3/Grx4 are required for Slt2 phosphorylation under oxidative conditions and participate in the positive regulation of Slt2. In mammals, GLRX3 was initially described as protein kinase C θ -binding protein, PICOT, yet the relevance of this interaction remains unclear. PICOT plays a role in the development of stress-induced cardiac hypertrophy in mouse and rat models (Jeong et al. 2006; Kato et al. 2008). A characterization of iron-related functions of heart tissue from GLRX3-deficient mice was not carried out. Thus, whether this function is related to its role in iron metabolism or to other functions in cellular stress response is not yet resolved (Donelson et al. 2019; Pham et al. 2015).

The interplay between Fe/S clusters and cellular redox balance

The proper synthesis, trafficking, and functioning of redox-active Fe/S clusters is important to cellular redox balance at many levels. Even though the redox capabilities of Fe/S clusters make them so useful for biological reactions, these cofactors are prone to oxidative degradation by oxygen and ROS (Figure 1). Dissecting of how redox species affect Fe/S clusters and their biogenesis and vice versa has been important in understanding how Fe/S proteins interfere with cellular redox pathways. As a case in point, the first study on a yeast strain lacking monothiol Grx5 (*grx5 Δ*) attributed an observed increase in oxidative damage to the lack of thiol reducing capacity (Rodriguez-Manzanique et al. 1999). Subsequent studies clarified that under aerobic conditions oxygen and/or ROS react with the accumulated mitochondrial iron in *grx5 Δ* cells to create oxidative stress conditions (Mühlenhoff et al. 2003; Rodriguez-Manzanique et al. 2002). Hence, a deregulation of iron homeostasis and not a lack of reducing equivalents delivered to Grx5 by the Grx/GSH system cause the oxidative stress phenotype. This iron-overload phenotype is generally observed for cells with a defective mitochondrial core ISC system and for the disruption of Atm1 function, and is relevant to human mitochondrial disorders (Gomez et al. 2014; Miao et al. 2009; Mühlenhoff et al. 2015; Stehling et al. 2014). Sections 12 the next sections briefly discusses

examples of new work that broadens the existing knowledge of the known cellular redox pathways and redox species that influence Fe/S proteins in both apo- and holoforms as well as components of the Fe/S protein biogenesis machineries.

The impact of oxygen on Fe/S proteins and their utilization

As stated above, some Fe/S proteins are susceptible to oxidative damage by molecular oxygen and ROS derived from oxygen (Figure 1) (Imlay 2008). Highly susceptible Fe/S clusters can be found close to protein surfaces or involve [4Fe-4S] clusters with exposed non-protein-coordinated iron ions, e.g., in aconitase and isopropylmalate isomerase (Leu1) of the [4Fe-4S] dehydratase family (Figure 4) (Imlay 2006; Imlay et al. 2019). Therefore, the antioxidant proteins catalase and superoxide dismutase play important protective roles to maintain Fe/S proteins and/or their biogenesis (Figure 1) (Gomez et al. 2014; Missirlis et al. 2003; Strain et al. 1998). While the oxidative sensitivity of Fe/S clusters is detrimental in most Fe/S proteins to varying degrees, nature has also taken advantage of this feature. Many bacteria orchestrate their adaptation to ambient oxygen, iron, and/or oxygen radical levels by employing transcription factors with sensitive Fe/S centers for various regulatory tasks (Crack and Le Brun 2018; Mettert and Kiley 2015). In some α -proteobacteria, the global regulator RirA has been proposed to uniquely sense both oxygen and iron at the same [4Fe-4S] cluster (Pellicer Martinez et al. 2019). The release of free iron under low iron conditions from an uncoordinated site of the [4Fe-4S]²⁺ cluster is proposed to drive the iron-sensing mechanism, which may have important implications for many other [4Fe-4S] clusters that are not fully protein sidechain-coordinated (e.g., dehydratases, radical SAM enzymes). The resulting [3Fe-4S]⁰ species is then responsible for O₂ sensing. Other prominent examples of Fe/S-binding transcription factors are the oxygen-responsive transcriptional regulator FNR, the SoxR transcription activator that recognizes superoxide, and the Rrf2 type transcriptional regulator NsrR that regulates the response to NO stress (Kobayashi et al. 2014; Mettert and Kiley 2018; Volbeda et al. 2017). In eukaryotes, *S. cerevisiae*'s Aft1 and Yap5 contain reactive [2Fe-2S] clusters that elicit a functional response upon oxidative degradation or under iron limitation (Castells-Roca et al. 2011; Rietzschel et al. 2015).

Metazoans contain multiple levels of iron- and oxygen-sensing via Fe/S clusters bound to iron regulatory proteins (IRPs), in addition to the hydroxylation of hypoxia-

Figure 4: Influence of oxygen and cellular thiol redox levels on Fe/S protein biogenesis.

Examples of oxygen and thiol-responsive proteins in eukaryotic Fe/S protein biogenesis. Dark red: Components of cellular Fe/S protein biogenesis in yeast or human cells that have been demonstrated to be dispensable under anaerobic conditions. These proteins likely play an integral role by increasing Fe/S cluster synthesis and trafficking during oxidatively damaging conditions. Green stars: Fe/S proteins particularly sensitive to thiol redox imbalance (Braymer et al. 2019). Red stars: Target proteins with Fe/S clusters highly susceptible to oxidative damage (i.e., proteins of the dehydratase family) [**Ind1 pathway, for complex I (CI) maturation; absent in *S. cerevisiae*, but found in *Yarrowia* and humans (Bych et al. 2008; Jain et al. 2020; Sheftel et al. 2009)].

inducible factors (HIFs) by non-heme iron prolyl hydroxylases (Taabazuing et al. 2014; Wang et al. 2020). IRP1 and IRP2 are members of the aconitase family of proteins, where only the former is able to bind a [4Fe-4S] cluster (for detailed reviews see (Anderson et al. 2012; Kuhn 2015)). The CIA system is responsible for inserting the [4Fe-4S] cluster into IRP1 (Paul and Lill 2015). When cellular iron levels are low, IRP1 switches from an active cytosolic aconitase to a cluster-less protein that binds iron-responsive elements (IREs), i.e. stem-loop structures in the mRNAs of iron metabolism-related proteins, thereby dictating the low iron response (Anderson et al. 2012; Kuhn 2015). Oxygen tensions in mammalian tissues have been shown to regulate iron homeostasis via IRP1, and ROS have been observed to

deactivate IRP1 via oxidation of the [4Fe-4S] cluster (Mueller 2005). IRP2 regulation has recently been shown to be oxygen-responsive via a [2Fe-2S] cluster, which bridges IRP2 and the E3 ubiquitin ligase adaptor protein FBXL5 (Wang et al. 2020). Oxidation of the [2Fe-2S] cluster leads to ubiquitination and degradation of IRP2, and hence signals that the cell contains adequate iron levels under elevated oxygen levels.

The impact of oxygen on Fe/S protein biogenesis

Oxygen can also have a significant impact on the synthesis and trafficking of Fe/S clusters. This fact is well known in many (cyano-)bacteria and plastids, which utilize the more oxidative stress-resistant SUF system for Fe/S protein maturation as compared to the ISC system (Garcia et al. 2019). In eukaryotes that depend on the ISC and CIA systems, the question remains, which steps in the assembly line are most susceptible to redox perturbations induced by oxygen or redox stress? In *S. cerevisiae*, the ISC-factor Jac1 is not essential under anaerobic conditions, and cellular defects stemming from a lack of frataxin (Yfh1), Atm1, and Arh1 observed aerobically are recovered under anaerobic conditions (Ast et al. 2019; Bulteau et al. 2007; Snoek and Steensma 2006). Anoxic conditions rescue the [4Fe-4S] protein deficiency phenotype of cells lacking the late-acting Fe/S protein biogenesis component Nfu1, suggesting that Nfu1 is predominately important under normal oxygen levels (Melber et al. 2016). This either indicates that these five proteins are among the most oxygen-sensitive ISC components or that these factors can most easily be bypassed (Figure 4). In the CIA system, the lethal phenotype of Nar1 depletion is partially relieved under anaerobic conditions (Figure 4) (Fujii et al. 2009). Further, Lto1 and Yae1, two accessory CIA factors that facilitate [4Fe-4S] cluster insertion into Rli1 are not essential anymore under anaerobic conditions, indicating that they can be bypassed to some extent (Paul and Lill 2015; Snoek and Steensma 2006). Since Rli1 and/or its maturation is susceptible to oxidants (Alhebshi et al. 2012), this non-essentiality of Yae1 and Lto1 is explained by a higher stability of Rli1's Fe/S clusters or the CTC in the absence of oxygen.

In cultivated human cells, comparisons under ambient oxygen (21%) and hypoxic conditions (1%) show similar trends. FXN (frataxin homologue), HSCB (Jac1 homologue), NFU1, and NARFL (Nar1 homologue) have been classified as “selectively” essential under normoxic conditions (Ast et al. 2019; Jain et al. 2020). Additionally, GLRX5, its interaction partner BOLA3, and the

mitochondrial [4Fe-4S] targeting factor IND1 have been categorized as selectively essential, indicating their dispensability under anaerobic conditions (Figure 4). Taken together, the non-essentiality of several ISC and CIA factors under low oxygen and/or anaerobic conditions can be largely explained by a higher stability of Fe/S clusters in the absence of oxidants, the reduced demand for many Fe/S proteins (e.g., those involved in respiration), and the increased availability of iron, which collectively reduce the requirement for *de novo* Fe/S cluster synthesis. On the other hand, although the ISC system is severely impaired in the absence of frataxin, Jac1, or Grx5, it apparently retains a remnant functionality that is sufficient to produce enough Fe/S clusters to secure survival under these conditions (Figure 4). This reasoning parallels findings in bacteria, where the simplified NIF system (with only cysteine desulfurase NifS and the Fe/S scaffold NifU) can take the place of the ISC and SUF systems under anaerobic conditions (Tokumoto et al. 2004).

The protective role of thiol redox systems on Fe/S proteins and their biogenesis

In addition to the Fe/S cofactor, the cysteine thiols that ligate these clusters are susceptible to redox modifications. The binding of Fe/S clusters requires that the cysteine residues are in the reduced, thiol or thiolate state. Curiously, multiple studies have identified highly reactive cysteines in many Fe/S proteins, including cluster-binding residues (Backus et al. 2016; Bak et al. 2017; Brandes et al. 2011; Leichert et al. 2008; Weerapana et al. 2010). These reactive cysteines could be sites of relevant sulfhydryl modifications, e.g., persulfidation (-S-SH), glutathionylation (-S-SG), oxidation (-S-S-), nitrosylation (-S-NO), which remain to be further explored. With regards to Fe/S proteins, the U-type Fe/S cluster scaffolds Isu1 and bacterial NifU and SufB are Fe/S proteins that have been verified to bind functionally relevant persulfides at Fe/S cluster-ligating cysteines (Lill and Freibert 2020; Parent et al. 2015; Przybyla-Toscano et al. 2018). In yeast, this chemistry is orchestrated by the Nfs1-Isd11-Acp1-Isu1-Yah1-Yfh1 ISC complex for the *de novo* synthesis of [2Fe-2S] clusters and is dependent on cysteine desulfurase activity (Figure 4). Most other Fe/S proteins would likely be functionally hindered by thiol modifications on cysteine residues, as the Fe/S cluster-binding site would be inaccessible. However, direct evidence for sulfhydryl derivatization of apoproteins has been obtained in yeast cells where the ISC machinery is

impaired. Polysulfide adducts on a mitochondria-targeted Fe/S reporter protein distinctly increase upon depletion of early-acting ISC proteins, but not when late-acting ISC factors were diminished (Christ et al. 2016). Cytosolic Fe/S apoproteins do not show such modifications. Consistent with this observation, the reduction of target apoproteins by the dithiol-Grx/GSH or the Trx system is a prerequisite of Fe/S cluster insertion *in vitro* (Weiler et al. 2020).

If such per- and polysulfide modifications were physiologically relevant, they could function in protecting Fe/S cluster-binding sites in the apo state under stress conditions. Removal of these sulfur adducts may then be carried out by the Trx/Grx systems (Figure 1), which has been observed in human cells (Doka et al. 2016; Wedmann et al. 2016). Yeast cells lacking both the mitochondrial Trx and Grx systems by deleting the respective reductases (*trr2Δ* and *glr1Δ*, Figure 1) are viable (Trotter and Grant 2005) and the core ISC machinery remains functional as evidenced by normal iron homeostasis, a functional CIA machinery, and the normal function of various mitochondrial Fe/S proteins (Braymer et al. 2019; Song et al. 2006). A subset of mitochondrial Fe/S target proteins (e.g., succinate dehydrogenase and biotin synthase), however, indeed become inactivated indicating that certain Fe/S proteins are particularly sensitive to thiol redox perturbations (Figure 4). These observations agree with experiments in human cell lysates showing that succinate dehydrogenase has several reactive, cluster-coordinating cysteine residues (Bak et al. 2017). Surprisingly, the cytosol is more sensitive and the depletion of cellular Grx/Trx thiol oxidoreductases (*TRR1↓*, *trr2Δ*, and *glr1Δ*, Figure 1) prevents all cytosolic Fe/S cluster target proteins from being matured due to a defect starting at the CIA component Nar1 (Braymer et al. 2019). Therefore, Nar1 is not only implicated in oxygen sensitivity but is likely coupled to thiol redox balance (Figure 4). Taken together, the maintenance of Fe/S proteins and their biogenesis overlap with thiol redox balance. Further detailed studies are required to identify the exact Nar1 modifications and how they influence protein function. Similarly, studies in bacterial systems have suggested that thiol-reducing equivalents are required for Fe/S cluster biogenesis (Ding et al. 2005; Nomata et al. 2015; Zheng et al. 2019).

Lastly, thiol redox balance and Fe/S cluster biogenesis also appear to intersect in higher eukaryotes. In human cells, depletion of the mitochondrial dithiol glutaredoxin GLRX2 decreases activities of complex I and aconitase (Johansson et al. 2007; Lee et al. 2009; Lillig et al. 2005). GLRX2 is proposed to be a redox sensor in the cell via its [2Fe-2S] cluster, which is destabilized and disassembled upon oxidative stress or exposure to nitric

oxide, promoting either the dithiol-dependent oxidoreductase activity of GLRX2, or nitric oxide detoxification to combat oxidative stress (Berndt et al. 2007; Lepka et al. 2017; Lillig et al. 2005; Scalcon et al. 2019). Importantly, this cellular function is distinct from that of monothiol Grxs that are involved in Fe/S cluster trafficking. Various Trxs have also been characterized *in vitro* as Fe/S cluster-binding proteins. Whether they may act as additional cellular redox sensors awaits experimental verification *in vivo* (Bisio et al. 2016; Zannini et al. 2019; Zheng et al. 2019). In summary, a number of proteins in the glutaredoxin-thioredoxin family can bind Fe/S clusters and in this respect, the monothiol Grxs are the best functionally characterized members.

Outlook

In recent years many aspects of the intimate and multi-layered connection between cellular iron metabolism and components of the cellular thiol redox systems have been studied. The precise roles of Grxs and GSH in the maturation of Fe/S proteins and the regulation of cellular iron homeostasis have been characterized in detail. Yet, many important questions remain. In particular, the mechanistic details of the function of cytosolic glutaredoxins in various aspects of cellular iron metabolism remain to be elucidated and may be distinct in different organisms. Moreover, we expect that further connections between the cellular thiol redox systems and cellular Fe/S protein maturation will be elucidated in the future.

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