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Minireview

Microbial 2-Cys Peroxiredoxins: Insights into Their Complex Physiological Roles

Michel B. Toledano*, and Bo Huang

The peroxiredoxins (Prxs) constitute a very large and highly conserved family of thiol-based peroxidases that has been discovered only very recently. We consider here these enzymes through the angle of their discovery, and of some features of their molecular and physiological functions, focusing on complex phenotypes of the gene mutations of the 2-Cys Prxs subtype in yeast. As scavengers of the low levels of H_2O_2 and as H_2O_2 receptors and transducers, 2-Cys Prxs have been highly instrumental to understand the biological impact of H_2O_2, and in particular its signaling function. 2-Cys Prxs can also become potent chaperone holdases, and unveiling the in vivo relevance of this function, which is still not established, should further increase our knowledge of the biological impact and toxicity of H_2O_2. The diverse molecular functions of 2-Cys Prx explain the often-hard task of relating them to peroxiredoxin genes phenotypes, which underscores the pleiotropic physiological role of these enzymes and complex biologic impact of H_2O_2.

INTRODUCTION

Reactive oxygen species (ROS), which include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$), are produced by the incomplete reduction of oxygen. It is in the fifties that ROS were first proposed to mediate the toxicity of elevated oxygen tension, i.e. poor growth, inflammation, genome instability, loss of cell fitness and cell death, because of their chemical reactivity that is much greater than that of oxygen (Imlay, 2013). The discovery of superoxide dismutase (SOD) by McCord and Fridovich, the enzyme that scavenges $O_2^{\bullet -}$ (McCord and Fridovich, 1969), in addition to the long known catalases and peroxidases, the enzymes that degrade H_2O_2, strongly supported this hypothesis, as indicating that H_2O_2 and $O_2^{\bullet -}$ are not only present in living organism, but must be also harmful because enzymes exist to destroy them. However, definite proofs that ROS are the perpetrators of oxygen toxicity came in the eighties with the advent of genetic studies in microorganisms, which showed that disabling ROS scavengers indeed exacerbates the phenotypes of hyperbaric oxygen, thus linking oxygen toxicity to the ROS scavenged by a particular enzyme (Carlioz and Touati, 1986). The peroxiredoxins constitute a very large and highly conserved family of thiol-based peroxidases that has been discovered only very recently, in the nineties. They are *bona fide* antioxidants that are attracting tremendous attention due to their extra functions in ROS signaling and protein quality control, and to their association with cancer, aging, inflammation and vascular diseases.

We will consider here these enzymes through the angle of their discovery, and of some features of their molecular and physiological functions, focusing on complex phenotypes of the gene mutations of the 2-Cys Prxs subtype in yeast. Echoing the importance of SOD in establishing ROS as culprit of oxygen toxicity, 2-Cys Prxs have been highly instrumental as a tool to understand the biological impact of H_2O_2, and in particular its signaling function, and to begin untangling H_2O_2 toxicity from its regulatory functions. As we will see, the difficulty that has prevailed in incriminating ROS as the vectors of oxygen toxicity reproduces in the often-hard task of relating peroxiredoxin genes phenotypes to the diverse molecular functions of the encoded enzymes, which underscores the pleiotropic role of these enzymes and the complex biologic impact of H_2O_2.

DISCOVERY OF PEROXIREDOXINS

In 1989, an alkyl hydroperoxide reductase activity was purified from *S. typhimurium* in Ames's laboratory, based on its ability to reduce the organic peroxide cumene hydroperoxide with NADH or NADPH as cofactor, but in a manner independent of selenium, glutathione and heme (Jacobson et al., 1989). Two polypeptides made up this activity, a 52-kDa flavoprotein and a 22-kDa protein, named AhpF and AhpC, respectively. AhpC was proposed to mediate peroxide reduction by a thiol-disulfide based mechanism, and AhpF to couple NAD(P)H oxidation to peroxide reduction by electron transfer between an AhpF redox-active dithiol and the AhpC disulfide.

Concomitantly in Stadtman's lab, Rhee and colleagues purified from yeast extracts a 25-kDa polypeptide that protected glutamine synthase from oxidative inactivation in the presence of a mixed function oxidation system (MFO)(O_2, iron, and a reducing agent, dithiothreithol or β-mercaptoethanol) (Kim et al.,

CEA, DSV, IBITECS, SBIGEM, Laboratoire Stress Oxydant et Cancer (LSOC), CEA-Saclay, 91191 Gif-sur-Yvette, France
*Correspondence: michel.toledano@cea.fr

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1988), therefore naming it protector protein. MFO generates the highly reactive radical $HO^{\bullet}$, but also reactive sulfur species ($RS^{\bullet}$, $RSSR^{\bullet}$, $RSOO^{\bullet}$) when sulfur is present in the mixture. As the protector protein only afforded protection in the presence of a thiol as reductant, but not ascorbate, its role against a sulfur radical was suspected and the protein was named thiol-specific antioxidant (TSA). Ambiguity regarding the actual enzymatic function of TSA remained however, as the observed strong induction of the TSA-encoding gene (*TSA1*) by hyperoxia, and the very poor growth of the corresponding null yeast strain to this same condition rather suggested an antioxidant function (Kim et al., 1989). A turn in the story came in the beginning of the nineties, with the advent of gene databases searches, which revealed a high homology between *TSA1* and *ahpC*, the genes of the human and Rat TSA at that time identified as retaining protector activity, and with orthologous sequences from diverse organisms from all phyla, thus leading Rhee and colleagues to propose that AhpC and Tsa1 constitute a new family of thiol-based peroxidases (Chae et al., 1994b). The presence of a conserved N-terminal cysteine (Cys) residue in all sequences, and of a C-terminal Cys residue present in most but not all sequences, separated the AhpC/Tsa1 family into 2-Cys and 1-Cys enzymes (Chae et al., 1994b). The Rhee group then renamed Tsa1, thioredoxin peroxidase (Tpx) by analogy with glutathione peroxidase (Gpx), to acknowledge its true enzymatic function, and on the basis of the purification of thioredoxin and thioredoxin reductase from yeast extracts as the hydrogen donor system of the enzyme peroxidase activity (Chae et al., 1994a), and then as peroxiredoxin (Prx) when it was found that enzymes of the 1-Cys group rely on glutathione as hydrogen donor instead of thioredoxin (Chen et al., 2000). The peroxiredoxin enzyme family is since known to scavenge not only H_2O_2, but also organic peroxides and is the first enzyme capable of scavenging peroxynitrite (Trujillo et al., 2007).

THE ALTERNATIVE CATALYTIC CYCLES OF 2-CYS PRXS

The Prx1 subgroup of peroxiredoxins, also known as typical 2-Cys Prxs, is the most widespread of the six Prx subfamilies, from archaea, bacteria to eukaryotes (Nelson et al., 2011; Soito et al., 2011). Eukaryotic 2-Cys Prxs share with their prokaryotic counterpart fast catalytic rates (k_{cat}/K_m 10^7-10^8 M^{-1} s^{-1} ~ 10^7 M^{-1} s^{-1}) (Parsonage et al., 2008; Peskin et al., 2007), and an extraordinary high reactivity towards peroxides, but are distinguished from them by undergoing inactivation by hyperoxidation at elevated H_2O_2 levels (Woo et al., 2003; Wood et al., 2003; Yang et al., 2002). This peculiar attribute individualizes two alternative catalytic cycles, the peroxidatic one and the sulfinic acid one, displayed in Fig. 1.

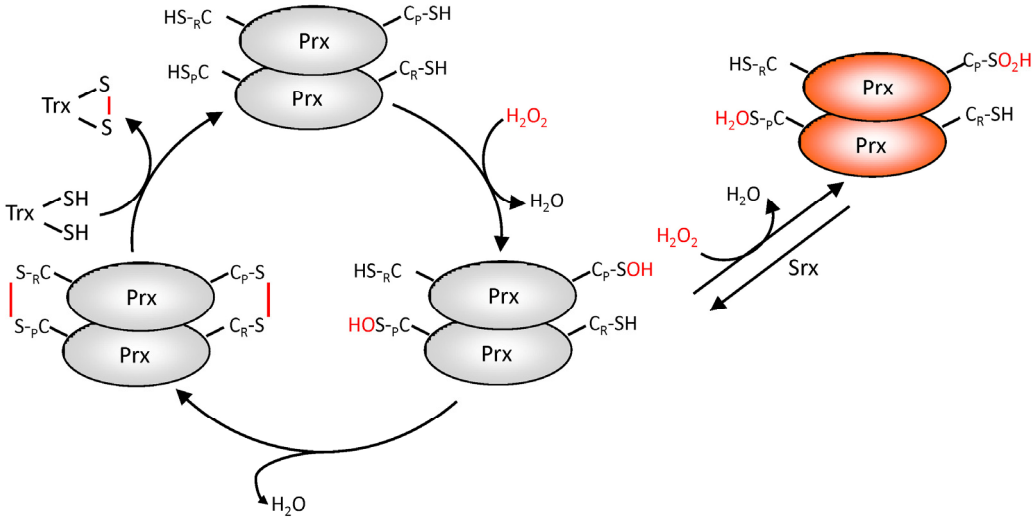

Fig. 1. The two catalytic cycles of 2-Cys Prxs (Noichri et al., 2015). 2-Cys Prx are obligate head-to-tail B-type homodimers, each with two catalytic Cys residues. In the peroxidatic cycle, the N-terminal Cys, named C_P for peroxidatic Cys, reduces H_2O_2 by direct reaction with release of one H_2O molecule, and is in turn oxidized to a sulfenic acid (C_P-SOH) (Wood et al., 2003). The Cys-sulfenic acid moiety then condenses with the C-terminal catalytic Cys residue of the other subunit, or resolving Cys (C_R) into an intermolecular disulfide, with release of the second H_2O molecule. Disulfide formation causes an important structural remodeling both at the C_P-active site pocket and C_R-containing C-terminal domain, which switches the enzyme structure form a fully folded (FF) to a locally unfolded (LU) conformation (Hall et al., 2011; Wood et al., 2003). Karplus and coworkers have elegantly shown that the enzyme FF conformation both stabilizes the deprotonated reactive form of C_P and provides a steric and electrostatic environment that activates H_2O_2, hence establishing the observed C_P extraordinary high reactivity for H_2O_2 (Hall et al., 2010; Karplus, 2015). The catalytic intermolecular disulfide is subsequently reduced by thioredoxin, which completes the catalytic cycle, returning the enzyme to the FF conformation. In the sulfinic acid cycle however, the C_P-SOH further reacts with H_2O_2 instead of condensing with C_R, thus becoming oxidized to the corresponding sulfinic acid (-SO_2H), which exit the enzyme from the peroxidatic cycle. Sulfinylated Prx undergoes a slow ATP-dependent reduction by the enzyme sulfiredoxin (Srx), which returns the enzyme into the peroxidatic cycle (Biteau et al., 2003; Woo et al., 2003). Sensitivity of eukaryotic enzymes to hyperoxidation is linked to the presence of two sequence fingerprints absent in other family enzymes, an insertion in the loop between α4 and β5 carrying the conserved GGLG motif, and an additional helix (α7) occurring as a C-terminal extension and containing the conserved YF motif (Wood et al., 2003). Such a structural configuration is thought to slow down the FF to LU transition rate, thereby favoring hyperoxidation.

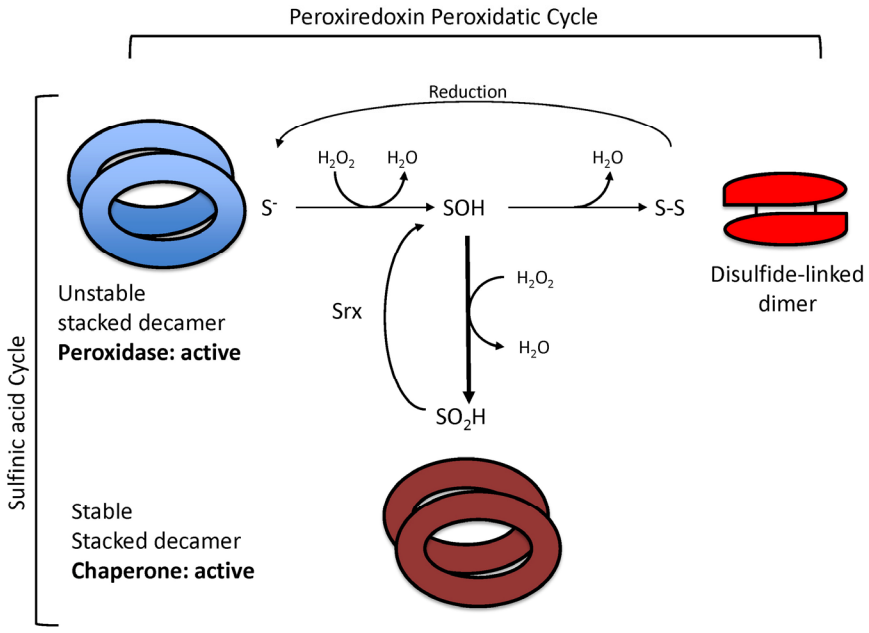

Fig. 2. 2-Cys Prxs enzymatic cycling involves dramatic changes in quaternary structure (Noichri et al., 2015). See the text.

Enzymatic cycling involves dramatic changes in quaternary structure (Fig. 2). Reduced 2-Cys Prxs are typically in the form of decamers arranged in a ring-like toroid structure. During peroxidatic cycling, decamers dissociate into dimers upon disulfide formation, and are regained upon disulfide reduction (Barranco-Medina et al., 2008; 2009; Hall et al., 2011; Parsonage et al., 2005; Sarma et al., 2005; Wood et al., 2002). In contrast, enzyme hyperoxidation stabilize the decameric structure and triggers the stacking of decamers, up to filaments (Cao et al., 2005; Gourlay et al., 2003; Lim et al., 2008; Noichri et al., 2015; Phalen et al., 2006; Saccoccia et al., 2012).

MICROBIAL PEROXIREDOXINS ARE PEROXIDE SCAVENGERS THAT EXCEL TOWARDS LOW ENDOGENOUS H_2O_2

Eukaryotic 2-Cys Prxs combine extremely high H_2O_2 reaction rates, reflected by very low K_m values (Nelson et al., 2008), and the enzyme oxidative inactivation at elevated H_2O_2 levels (Cordray et al., 2007; Wood et al., 2003; Yang et al., 2002). Together with usually very high expression levels, these catalytic features should restrict Prxs scavenging activity towards the low metabolically produced H_2O_2 levels (Fourquet et al., 2008; Imlay, 2013).

In *Escherichia coli,* AhpC indeed came out from microbial genetics and physiological studies as the primary scavenger of the low endogenous H_2O_2 produced during normal metabolism (Seaver and Imlay, 2001). In *E. coli* mutants, an aerobic growth defect is only observed upon simultaneously inactivating all three major peroxide scavenging enzymes, AhpC and the two catalases (KatG and KatE), suggesting overlapping functions between AhpC and catalases (Park et al., 2005; Seaver and Imlay, 2001). However, measure of cellular H_2O_2 catabolic efficiencies established that while AhpC is very efficient at scavenging H_2O_2 at low, but not at high concentration, the reversely is true for the catalases mutant, which fit the distinct catalytic attributes of these enzymes (Imlay, 2013). In addition, OxyR was constitutively active in the ahpC but not the catalases mutant, further indicating the unique role of AhpC in scavenging the low endogenous levels of H_2O_2 (Seaver and Imlay, 2001). Although AhpC is now known as a highly efficient H_2O_2 scavenger (Parsonage et al., 2008), AhpC was initially named AhpC, based on its ability to reduce organic peroxides *in vitro* (Jacobson et al., 1989) and on the dissociated peroxide phenotype of the ahpC null mutant, which displayed hypersensitivity only towards organic peroxides, but not towards H_2O_2 (Storz et al., 1989). However, this phenotype is a consequence of the constitutive upregulation of the OxyR-target gene KatG, which is active only towards H_2O_2, but not organic peroxides (Seaver and Imlay, 2001).

S. cerevisiae has five Prxs, two cytosolic 2-Cys Prxs, Tsa1 and Tsa2, two atypical 2-Cys Prx, Ahp1 that is cytosolic (Lee et al., 1999b) and nTpx that is nuclear, and a mitochondrial 1-Cys mTpx (Fourquet et al., 2008; Park et al., 2000). *S cerevisiae* also carries three other thiol peroxidases, Gpx1, Gpx2 and Gpx3 (also known as Orp1 and Hyr1), which are thiol-based enzymes and not selenoenzymes and are dependent upon thioredoxin and not glutathione (Avery and Avery, 2001; Delaunay et al., 2002; Inoue et al., 1999; Tanaka et al., 2005). What are the distinctive and overall physiological role of the eight thiol-peroxidases (Tpxs) in peroxide catabolism? Tsa2, a structural and functional duplicate of Tsa1, is not abundant but stress-inducible (Godon et al., 1998; Lee et al., 1999a), and its protective function is controversial (Munhoz and Netto, 2004; Wong et al., 2004). mTPx has a specific protective role towards peroxides produced during respiration, consistent with its localization (Pedrajas et al., 2000). nTpx does not have any known function. Of the Gpxs, although they have all peroxidase activity *in vitro* (Avery et al., 2004), only the inactivation of Orp1/Gpx3 leads to defective tolerance to H_2O_2 and t-BOOH (Delaunay et al., 2002), which is a consequence of defective activation of the peroxide-stress-responsive Yap1 transcriptional regulator (see below). The remaining Tpxs, Tsa1 and Ahp1 are in fact the primary peroxide scavengers, as indicated by the peroxide-induced growth inhibition phenotypes of their corresponding null mutants; however, whereas the *TSA1* null strain is more sensitive towards H_2O_2 than organic peroxide, the reverse is observed with *AHP1* (Chae et al., 1994a; Jeong et al., 1999;

Lee et al., 1999b). The *TSA1* null strain (Δ*tsa1*) also displays a high genome instability not shared with the other Tpx mutants (Huang et al., 2003), which indicates that, as AhpC in *E. coli*, this enzyme has a unique role in scavenging the low levels of endogenous H_2O_2. Such a primary role of Tsa1 in genome protection is further emphasized by the synthetic lethal phenotypes observed when combining Δ*tsa1* with null mutations of the genes encoding recombinational or post-replication repair (Huang and Kolodner, 2005). Prx null mutants with simultaneous deletion of *TSA1*, *TSA2*, *AHP1*, *nTPX* and *mTPX* are still viable (Wong et al., 2004), as is a mutant lacking all eight *S. cerevisiae* thiol-based peroxidases named Δ8 (Fomenko et al., 2011). However, reduced fitness and genome instability phenotypes exacerbates in proportion with the number of isotype inactivated, indicating that the other thiol-based peroxidase can substitute Tsa1 in protecting the genome against endogenous H_2O_2 toxicity (Fomenko et al., 2011; Kaya et al., 2014; Wong et al., 2004). As a probable consequence of its genomic instability, Δ*tsa1* displays a significantly shorter replicative life span, and this phenotype is also worsened by successive inactivation of the other Tpxs (Fomenko et al., 2011). Further examination of the Δ8 mutant has recently shown however that it remains viable only by virtue of constitutively overexpressing of both the inner mitochondrial membrane cytochrome C peroxidase (Ccp1) and Uth1, a gene of unknown function in mitochondrial biogenesis (Kaya et al., 2015). Overexpression of Ccp1 and Uth1 in this strain is the result of the duplication of chromosome XI that carries these two loci, thus providing an example of adaptive aneuploidy in H_2O_2 tolerance. Ccp1, which scavenges H_2O_2 using cytochrome *c* as electron donor, requires a functional respiratory chain, which explains the paradoxical anaerobic lethality of Δ8 (Kaya et al., 2015). Uth1 overexpression is probably also required for rescue of Δ8 as a mean of increasing mitochondrial mass, and therefore Ccp1 activity.

FUNGI PEROXIREDOXINS ARE SENSORS AND TRANSDUCERS OF THE H_2O_2 SIGNAL

The 2-Cys Prx H_2O_2 extraordinary reactivity also endow them the function of sensor and transducer of the H_2O_2 signal. This reactivity is such that, with a very few exceptions, no Cys residue other than C_P might react with H_2O_2 at the concentration at which signaling by this molecule occurs. Exceptions to this rule are enzymes such as the glycolytic enzyme glyceradehyde-3-phosphate dehydrogenase, the catalytic core of which contains a Cys residue highly sensitive to H_2O_2 that contributes to the metabolic adaption to H_2O_2 stress (Peralta et al., 2015).

The role of the *S. cerevisiae* thiol peroxidase Orp1 (Gpx3) in the activation of the bZip transcription factor Yap1 constitutes the first description of the H_2O_2 sensing and signaling function of these enzymes (Toledano et al., 2004). Yap1, which regulates an H_2O_2-inducible transcriptional adaptive response to this oxidant, is activated by oxidation when intracellular H_2O_2 levels rise (Delaunay et al., 2000). Orp1 and not Yap1 however is the H_2O_2 sensor of the pathway, hence its name as oxidant receptor peroxidase, which initiates a redox relay that leads to the oxidation of Yap1 into its active form (Delaunay et al., 2002). In this relay, Orp1 C_P forms a sulfenic acid by reaction with H_2O_2, which then condenses with one of Yap1 Cys residues into an intermolecular disulfide, then transposed into a Yap1 intramolecular disulfide by a thiol-disulfide exchange reaction between Orp1 C_P and a second Yap1 Cys residue (Delaunay et al., 2002; Ma et al., 2007). This redox relay also requires the Yap1-binding protein Ybp1 (Veal et al., 2003), which somehow helps the redox interaction between Orp1 and Yap1. In yeast strains lacking Ybp1, Tsa1 and not Orp1 operates as oxidant receptor in Yap1 activation by H_2O_2 (Ross et al., 2000; Tachibana et al., 2009; Veal et al., 2003). In *S. pombe*, it is the 2-Cys Prx Tpx1 that initiates the H_2O_2 redox relay leading to the activation of the *S. pombe* Yap1 homologue, Pap1 (Bozonet et al., 2005; Vivancos et al., 2005). It is not clear however, whether Tpx1 directly oxidizes Pap1, or instead oxidizes its reductase thioredoxin, which in turn oxidizes Pap1 (Brown et al., 2013; Calvo et al., 2013). The Pap1 response is unique in being gradually delayed upon increasing H_2O_2 levels, as a result of Tpx1 sulfinylation, which transiently shuts off signaling to Pap1 until Tpx1 sulfinic-acid reduction by the MAP kinase Sty1-induced Srx1 (Bozonet et al., 2005; Vivancos et al., 2005). Pap1 inactivation might be required for a build-up of H_2O_2 at levels sufficient to switch on Sty1, which regulates a response to H_2O_2 that is different from that of Pap1 (Vivancos et al., 2005). In *S. pombe*, Tpx1 also contributes to the H_2O_2-induced activation of the MAP kinase Sty1 by engaging C_P into a disulfide linkage with a Sty1 Cys residue, thereby canceling out an inhibitory effect carried by this residue on Sty1 phosphorylation by the upstream MAPKK Wis1 (Veal et al., 2004).

If indeed H_2O_2 requires Orp1, Tsa1 or Tpx1 for regulating adaptive oxidative stress responses, would other cellular H_2O_2 responses make similar use of specific receptors? This question was addressed by monitoring the genome-wide response to H_2O_2 in the Δ8 strain that lacks all eight thiol-based peroxidases (see above) (Fomenko et al., 2011). The massive genomic response to H_2O_2 —about 1000 genes induced and 500 others repressed, was totally abated in Δ8, irrespective of the concentration of the oxidant and duration of exposure. Hence, at least in *S. cerevisiae*, H_2O_2 never directly reaches its regulatory targets but is instead funneled to them by ways of thiol peroxidase relays. These data further establish the role of thiol peroxidases as receptors/sensors in H_2O_2 signaling, which is now also emerging in higher eukaryotes (Sobotta et al., 2015).

MICROBIAL PRXS AS CHAPERONES

Lee and coworkers showed that hyperoxidation of the *S. cerevisiae* Tsa1 by H_2O_2 triggers the polymerization of the enzyme into high molecular weight (HMW) forms, which have lost peroxidase activity, but have acquired the ability to prevent aggregation of heat-denatured model substrates (Jang et al., 2004), a function that fulfills the definition of a chaperone holdase. This switch from a peroxidase to a chaperone is reversed by reduction of Tsa1 by sulfiredoxin (Srx1) (Jang et al., 2004; Moon et al., 2013). A similar chaperone holdase activity was then described for the human cytosolic 2-Cys Prxs, Prdx1 (Jang et al., 2006; Pan et al., 2014; Park et al., 2011) and Prdx2 (Moon et al., 2005), plant chloroplastic 2-Cys Prxs (Konig et al., 2013), *S. mansoni* 2-Cys Prx SmPrx1 (Angelucci et al., 2013; Saccoccia et al., 2012), *L. infantum* mitochondrial 2-Cys Prx mTXNPx (Teixeira et al., 2015), *H. pilori* 2-Cys Prx AhpC (Chuang et al., 2006), cyanobacterial *Anabaena* PCC7120 2-Cys Prx alr4641 (Banerjee et al., 2015) and *P. aeruginosa* 2-Cys Prx PaPrx (An et al., 2010). In all these cases but two, available data fit the model of Jang whereby H_2O_2-induced C_P hyperoxidation triggers 2-Cys Prx assembly into HMW structures with chaperone activity (Jang et al., 2004; Moon et al., 2013; Noichri et al., 2015). For the A. *thaliana* chloroplastic 2-Cys Prx, the chaperone function is triggered by H_2O_2 and requires C_P sulfinylation, but the chaperone active form of the enzyme is a decamer and not a higher order oligomer (Konig et al., 2013). In contrast to most Prxs, for the *Leishmania infantum* mitochondrial 2-Cys

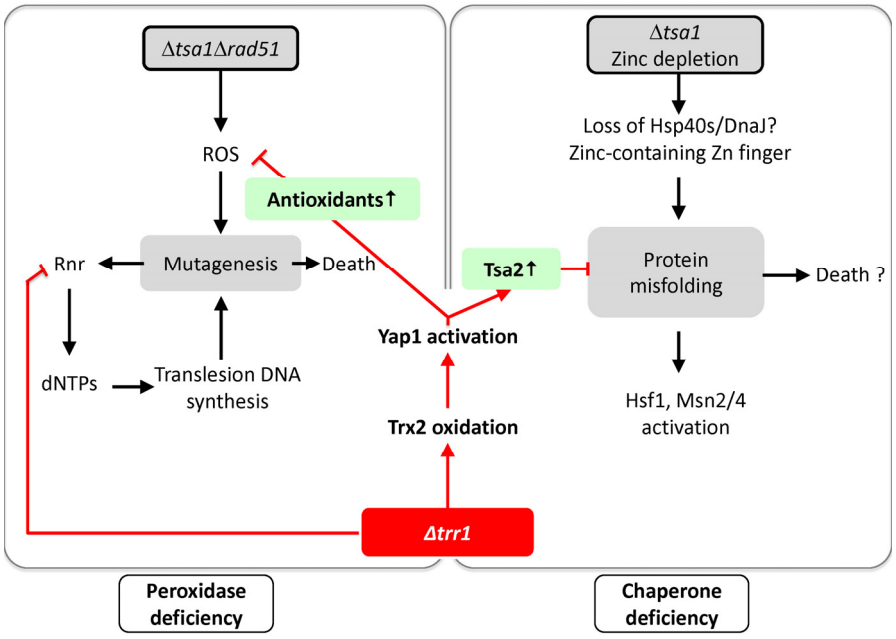

Fig. 3. Loss-of function mutations of cytosolic thioredoxin reductase-encoding *TRR1* suppress defects in H_2O_2 scavenging and protein quality control linked to the loss of TSA1 (MacDiarmid et al., 2013; Ragu et al., 2014). Suppression is indicated in red arrows (see text).

Prx mTXNPx, activation of the chaperone function is triggered on the reduced decamer by elevated temperature and not H_2O_2-induced enzyme hyperoxidation, which leads to major secondary and tertiary structural changes that increase surface hydrophobicity (Castro et al., 2011; Teixeira et al., 2015). As shown by electron microscopy, mTXNPx single decameric rings use direct contacts to position substrates in their ring center. The *L. infantum* case might be akin to the observation of Jang and coworkers that heat treatment can also trigger Tsa1 chaperone activity in a manner independent of C_P, which suggested that this stress and H_2O_2 operate the functional switch of 2-Cys Prx through distinct mechanisms (Jang et al., 2004).

The *L. infantum* mTXNPx is not only unique by the biochemical features of its chaperone function, but also by providing the first example so far of a physiological role of a Prx as a chaperone. *L. infantum* mTXNPx is indeed essential for host virulence, and for thermotolerance of the insect form of the parasite (Castro et al., 2011). These phenotypes are dependent, not on the enzyme C_P, thus ruling out the loss of its peroxidase function as causal, but on the enzyme powerful general chaperone function, specifically protecting proteins against temperature-induced aggregation, and maintaining them in a folding-competent conformation (Teixeira et al., 2015). The insect form of the mTXNPx null mutant displayed a substantially higher amount of temperature-induced protein aggregation than its wild type counterpart, thus providing a biochemical signature of the chaperoning role of mTXNPx.

THE COMPLEX PHENOTYPES OF MICROBIAL 2-CYS PRXS

As carrying the functions of H_2O_2 scavengers, H_2O_2 signaling devices and chaperones, microbial 2-Cys Prxs necessarily have diverse physiological functions, and not surprisingly their mutation can cause complex phenotypes, as illustrated below.

In the first two studies, loss-of function mutations of cytosolic thioredoxin reductase-encoding *TRR1* were selected as suppressors of totally different defects of Δ*tsa1* (MacDiarmid et al., 2013; Ragu et al., 2014) (Fig. 3). *TSA1* is one of several genes induced in zinc-depleted yeast cells and is essential for growth under this condition, a requirement initially interpreted as symptomatic of the presence of oxidative stress caused by zinc depletion (Wu et al., 2007). A selection of transposon-insertion mutations that bypass the requirement of *TSA1* upon zinc depletion identified a mutation of the *TSA2* promoter that increased its expression by several folds (MacDiarmid et al., 2013). Spontaneous suppressor mutants were also selected, one of which was identified as a loss-of function mutation of *TRR1*. As the latter condition constitutively activate Yap1 by impairing its reduction, upregulation of the Yap1-target gene *TSA2* was invoked as the mechanism of *TRR1* suppression. However, as TRR1 is essential for Prx peroxidatic cycling, the idea that Tsa1 is needed to sustain zinc depletion-induced oxidative stress felt. Authors could show instead that Tsa1 is required as a chaperone, based on the following data. They used a Tsa1 resolving Cys residue mutation (C_R), which significantly weakens the enzyme scavenging activity, that fully restored the defective growth of Δ*tsa1* in a zinc-depleted medium. The Δ*tsa1* zinc phenotype was also rescued by overexpression of the chaperones Hsp26 and Hsp42. Lastly, the presence in zinc-depleted Δ*tsa1* of a potent activation of Hsf1 which is usually seen upon cytosolic accumulation of misfolded proteins, and of GFP-Hsp104 foci, which form at sites of protein aggregation, concurred to convince that Tsa1 prevents the accumulation of misfolded proteins upon zinc depletion. How zinc depletion causes protein misfolding and aggregation might be impairing the metallation of the 10% of cellular zinc-containing proteins, thereby preventing their folding, as suggested by authors.

In the study of Ragu et al. (2014), a *TRR1* mutation was selected as a suppressor of a Tsa1-dependent peroxide stress phenotype, in contrast to the previous observation (MacDiarmid et al., 2013) (Fig. 3). As discussed above, Δ*tsa1* is synthetic lethal with *RAD51*, with other DNA strand break repair genes and *RAD6* (Huang and Kolodner, 2005; Smith et al., 2004). This synthetic lethality is a consequence of H_2O_2 genome toxicity, as rescued by anaerobiosis (Ragu et al., 2007), and is due to the combined loss of the major endogenous H_2O_2 scavenger

and a major DNA repair enzyme. Upregulation of Yap1 was also invoked as the *trr1* suppression mechanism, as improving peroxide scavenging through increased expression of its target genes that comprise most yeast reductases and scavenging activities. In this case however, *TSA2* was dispensable for suppression, in keeping with the notion that anyhow it would be inactive in the *trr1* mutant (Ragu et al., 2014). A second suppression mechanism was also suggested however. Through the activation of checkpoint pathways, H_2O_2-induced DNA damage stimulates the production of dNTPs needed for DNA repair (Chabes et al., 2003), which also paradoxically increases mutation rates by more efficient translesion DNA synthesis (Sabouri et al., 2008). A vicious circle between the accumulation of DNA lesions and the chronic activation of checkpoint pathways was invoked to explain the abnormally high mutagenesis rate of Δ*tsa1* (Tang et al., 2009). As dNTP synthesis involves the thioredoxin-dependent redox cycling of ribonucleotide reductase, inactivation of Trr1 also rescued the Δ*tsa1*Δ*rad51* lethal phenotype by decreasing this synthesis (Ragu et al., 2014).

A role of Tsa1 in preventing protein damage was recently shown, but this effect was shown to involve its peroxidase and not chaperone function (Weids and Grant, 2014). In this study, Δ*tsa1* was shown to be highly sensitive to the proline analogue azetidine-2-carboxylic acid (AZC), which causes nascent-protein misfolding and aggregation. Hypersensitivity was seen under aerobiosis, but not anaerobiosis, and was totally rescued by the loss of mitochondrial DNA, which led authors to conclude that the protein aggregates induced by AZC cause the toxic mitochondrial production of H_2O_2, and Tsa1 prevent this toxicity by scavenging H_2O_2.

Deletion of *TSA1* was also shown to worsen the growth defect and the accumulation of carbonylated proteins resulting from inactivation of the vacuolar iron transporter Ccc1, which lead to cytosolic accumulation of iron (Lin et al., 2011). Exacerbation of the iron toxicity of Δcc1 by the deletion of *TSA1* was seen both under aerobiosis and anaerobiosis, which led these authors unable to decide between the peroxidase or chaperone function of Tsa1 as important in this setting.

The last study considered, which describes a novel function of *TSA1* and *SRX1* in yeast replicative aging, also opens the question of which is the function of Tsa1 that underlies a given *TSA1* phenotype (Molin et al., 2011). In this study, the ability of caloric restriction to increase yeast replicative lifespan was shown to be lost in strains lacking either *SRX1* or *TSA1*, therefore establishing these two genes products as effectors or facilitators of the effects of CR on replicative aging. Furthermore, increasing the expression of *SRX1* in the absence of CR also increased life span by about 20%, but in the presence and not the absence of *TSA1*, thus indicating that Srx1 and Tsa1 have a linked function in aging. The nutrient sensitive cAMP-PKA pathway was established as the link between CR and Tsa1 and Srx1, as this pathway, which is downregulated by CR, was shown to repress Tsa1 and Srx1 expression by imposing a translation block involving the activation of the eIF2 kinase Gcn2 (Molin et al., 2011).

FAILURE OF GENETICS IN LINKING 2-CYS PRX MOLECULAR FUNCTIONS AND PHENOTYPES

When trying to link phenotypes and molecular functions, it is easy to ascribe the genomic instability to defective Prx scavenging, and alterations of H_2O_2 genomic responses and tolerance to the loss of Prx-dependent H_2O_2 signaling. However, as illustrated by the studies considered above, it is not as easy to ascribe a given phenotype to the alteration of Prx chaperone function, and in other cases to elucidate the process responsible for a given phenotype. The demonstrated role of the *L. infantum* mTXNPx in protein quality control (PQC) and its importance for parasite virulence is compelling, and perhaps the unique example so far of a chaperoning function for a peroxiredoxin (Texeira et al., 2015). The requirement of Tsa1 as a chaperone for yeast growth under zinc depletion also provide some good evidence of this function (MacDiarmid et al., 2013), still how to explain this requirement in view of the very numerous and efficient chaperone systems that already exist in the cytosol of an eukaryotic cell? One possible answer could be, as suggested by authors, that the other PQC systems become disabled as a consequence of loss of the zinc-finger-containing DnaJ enzymes, which activate Hsp70 ATPase activity and contribute with the latter to the recognition of folding substrates. Accordingly, 2-Cys Prxs would backup PQC systems, only becoming important under the physiological conditions that mimic zinc depletion. Conversely, the *CCP1* rescue of the now known unviable phenotype of the yeast Δ8 strain, which lacks all yeast thiol peroxidases (Fomenko et al., 2015), indicates that these enzymes essential nature is linked to their scavenging functions. Accordingly, the Δ8 strain-oxygen auxotrophy is the consequence of the respiratory chain requirement for Ccp1 activity. However, if these enzymes are indeed essential as H_2O_2 scavengers, why then is Δ8 not viable under anaerobiosis, a condition that should lower if not abate H_2O_2-dependent phenotypes? In the absence of H_2O_2, the 2-Cys Prx redox relay function cannot be invoked, but what about the chaperone function, and why then under anaerobiosis? By linking CR restriction-induced replicative life span extension to the role of Tsa1 and Srx1 in H_2O_2 resistance, the study of Molin apparently indicated that Tsa1/Srx1 affect lifespan as peroxide scavengers (Molin et al., 2011). However, this linkage was only correlative, and Tsa1 and Srx1 could also influence lifespan as a chaperone. One could even ask whether 2-Cys Prx contributes to H_2O_2 stress resistance as chaperones. Similarly in the study of Lin et al. (2011), authors could not conclude as to which of the function of Tsa1 is preventing iron toxicity. The difficulty in linking molecular functions and phenotypes resides in the intertwined nature 2-Cys Prx peroxidase and chaperone functions, and hence in the lack of mutations that could unambiguously separate them. Except for the *L. infantum* mTXNPx, the 2-Cys Prx C_P is essential both for H_2O_2 scavenging and for chaperoning. Weids and Grant (2014) and Lin et al. (2011) used the C_P mutation-induced loss of Tsa1 peroxidase activity to establish the importance of this function in preventing AZC and iron toxicity, respectively, which is fair in view of the lack of robust data regarding the absolute requirement of C_P for chaperoning. Mutation of the 2-Cys resolving Cys C_R has been used by Molin et al. (2011) and MacDiarmid et al. (2013), to disable scavenging without hampering chaperoning, based on the known effect of this mutation to weaken scavenging, but whether it also alters chaperoning is not known, due to very limited knowledge of the molecular details of this function.

CONCLUSION

Studies of the bacterial and yeast 2-Cys Prxs have clearly established a primary role of these enzymes in scavenging the low levels of H_2O_2 produced during normal growth, and their inefficiency in the face of H_2O_2 onslaughts, which fit their *in vitro* peroxidase function and its catalytic features, in particular their extraordinary H_2O_2 reactivity. They have also revealed the role of 2-Cys Prx, and more generally of thiol peroxidases, as re-

ceptors and transducers of the H_2O_2 signal, a function conserved in mammals (Sabotta MC et al., 2014), which is also based on these enzymes high H_2O_2 reactivity. Lastly they indicate that 2-Cys Prxs can become potent chaperone holdases, but apart a few exceptions, knowledge of this function is mainly based on *in vitro* data, and so far not a hint of an *in vivo* molecular function, of potential substrates and physiological scope is yet available. Furthermore, as we just saw above, establishing which of the 2-Cys Prx molecular function(s) underlie their physiological action in aging and PQC remains a difficult task, leading sometimes to ambiguous answers. Such ambiguity is reminiscent of the one that prevailed in the old days in establishing the role of ROS as the culprit of oxygen toxicity and antioxidants as ROS scavengers and in the discovery of Tsa1 by Rhee and colleagues (Chae et al., 1988). This ambiguity can lead to the provocative question of whether Tsa1 was identified based on its chaperone activity, rather than on its scavenging function, by protecting glutamine synthase from oxidative damage by $HO^{\bullet}$. Rhee ruled out this possibility, based on the Tsa1 protective effect seen only in the presence of a thiol donor in the MFO system (DTT, thioredoxin), but not in the presence of ascorbate, which was taken as a proof of the requirement of Tsa1 peroxidatic cycling. However peroxidatic cycling is part of the chaperone activation process, which requires, at least in some cases C_P hyperoxidation.

It will thus be important in the future to establish biochemical signatures of the 2-Cys Prx chaperone function, and growth conditions that require this function. It will be also important to address the 2-Cys Prx structure-chaperone function relationship, which will help design mutations that non ambiguously separate this function from the enzyme peroxidase function.

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