ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis

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Abstract | Reactive oxygen species (ROS) have been shown to be toxic but also function as signalling molecules. This biological paradox underlies mechanisms that are important for the integrity and fitness of living organisms and their ageing. The pathways that regulate ROS homeostasis are crucial for mitigating the toxicity of ROS and provide strong evidence about specificity in ROS signalling. By taking advantage of the chemistry of ROS, highly specific mechanisms have evolved that form the basis of oxidant scavenging and ROS signalling systems.

Iron–sulphur clusters
Iron–sulphur clusters are metal centres that consist of sulphide (S^{2-}) and iron. The most common structures are the diamond [2Fe–2S] and the cubane [4Fe–4S] clusters. Iron–sulphur clusters are usually coordinated by Cys or His residues at the iron atoms.

Reactive oxygen species (ROS) is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^•$). ROS are thought to mediate the toxicity of oxygen because of their greater chemical reactivity with regard to oxygen. They also operate as intracellular signalling molecules, a function that has been widely documented but is still controversial. This scepticism stems from the apparent paradox between the specificity that is required for signalling and the reactive nature of ROS that renders them indiscriminate and potentially lethal oxidants. Specificity in signalling is achieved through the non-covalent binding of a ligand to its cognate receptor by virtue of the complementarity of macromolecular shapes. By contrast, ROS operate in signalling through chemical reactions with specific atoms of target proteins that lead to covalent protein modifications[1]. Therefore, ROS molecular recognition occurs at the atomic and not at the macromolecular level, which necessarily expands the potential number of ROS-specific receptors because the atomic targets of ROS are the amino-acid building blocks of numerous proteins. So, how is specificity achieved in ROS signalling?

To provide answers to this question, we consider particular ROS signalling pathways that control ROS intracellular homeostasis. Studies of these pathways have provided (and are still providing) the fundamental molecular principles of ROS-based redox regulation that constitute solid demonstrations of high specificity in ROS signalling. These pathways generally make use of ROS sensors that 'measure' the intracellular concentration of ROS by a redox-based mechanism and proportionally set the expression of ROS-specific scavengers, thereby maintaining the concentration of ROS below a toxic threshold. These pathways regulate a physiological response that is fitted to a ROS signal, in which the ROS signal is the agonist and the sensor is a ROS-specific receptor.

In this Review, we discuss the redox mechanisms of such pathways in microbial models and their potential equivalents in mammals. We focus on pathway specificity and on the mechanistic features that generate specificity. Finally, we compare these mechanisms with other types of ROS signalling pathways.

ROS chemistry sets target specificity

Chemical reactivity distinguishes ROS from other signalling molecules (BOX 1; BOX 2). ROS have distinct biological properties, which include chemical reactivity, half-life and lipid solubility[2]. $HO^•$ has indiscriminate reactivity towards biological molecules, whereas O_2^- and H_2O_2 each have preferred biological targets. This preference is best exemplified by the striking early observation of the use of the redox-sensitive transcription factors SoxR and OxyR, which enable *Escherichia coli* to discriminate and regulate distinct responses towards ROS (see below). Such chemical discrimination is a hallmark of the high atomic reactivity of O_2^- with iron–sulphur clusters ([Fe–S] clusters), which constitute the SoxR redox centre, and of the reactivity of H_2O_2 with Cys residues, which constitute the OxyR redox centre.

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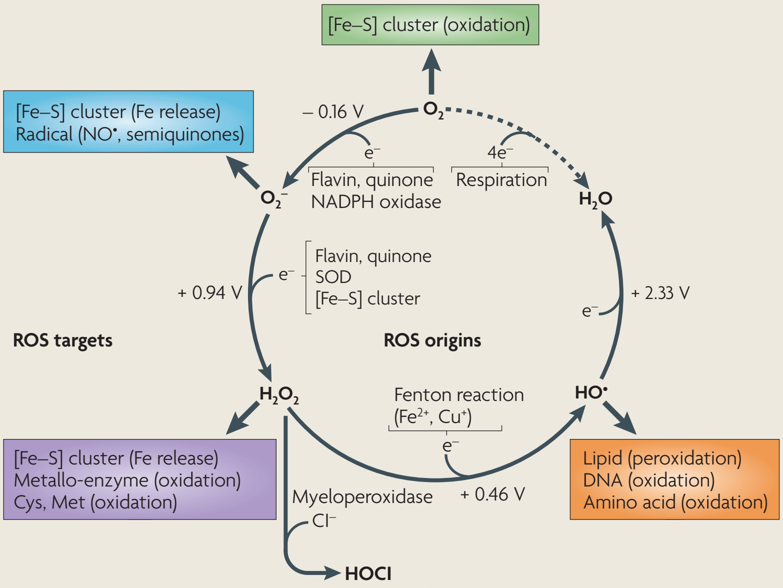
Box 1 | ROS sources and biochemical properties



Although molecular oxygen is a di-radical and rather unreactive, its univalent reduction leads to the formation of chemically more reactive species (known as reactive oxygen species; ROS). These are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$). Because of their intrinsic chemical properties, each ROS reacts with preferred biological targets (coloured boxes; see figure). As a rule, ROS reactivity dictates toxicity while decreasing signalling ability.

O_2^- is a by-product of respiration and is produced by NADPH oxidases. Due to high attraction, O_2^- oxidizes iron–sulphur ([Fe–S]) clusters at a rate that is almost diffusion-limited, and releases iron. O_2^- can react with thiols *in vitro*, but the slow reaction rates mean that this cannot occur *in vivo*[5]. In *Escherichia coli*, the steady-state concentration of O_2^- is very low (~10^{-11} M)[2], which reflects its instability; this is not only due to its reaction with the [Fe–S] cluster, but also to spontaneous and superoxide-dismutase-mediated O_2^- dismutation to H_2O_2. The instability of O_2^- and its inability to diffuse through membranes because of its negative charge make this ROS a poor signalling molecule.

H_2O_2 toxicity is essentially the consequence of its reduction to $HO^\bullet$ by metal-catalysed Fenton chemistry[3]. H_2O_2 is actually a poor oxidant and reacts mildly with [Fe–S] (rate constant of 10^2–10^3 $M^{-1}.s^{-1}$) and loosely bound metals (10^3–10^4 $M^{-1}.s^{-1}$), and very slowly with glutathione and free Cys (2–20 $M^{-1}.s^{-1}$) and with Met residues (10^{-2} $M^{-1}.s^{-1}$)[3,5]. By contrast, its reactivity towards Cys residues can significantly increase to 10–10^6 $M^{-1}.s^{-1}$ depending on the protein environment (BOX 2). As a corollary, H_2O_2 is relatively stable (cellular half-life ~1 ms, steady-state levels ~10^{-7} M). Diffusion of H_2O_2 might be modulated by changes in membrane permeability or by transport through aquaporins[114]. Its selective reactivity and diffusibility makes H_2O_2 fit for signalling.

The highly toxic $HO^\bullet$ has high indiscriminate reactivity, which limits its diffusion to sites of production (half-life 10^{-9} s)[2]. Despite this, $HO^\bullet$ seems to operate in H_2O_2 sensing.

Reactive nitrogen species
These are derived from the reaction of nitric oxide (NO) with oxygen or superoxide and include nitrogen trioxide (N_2O_3), peroxinitrite ($ONOO^-$) and nitrogen dioxide (NO_2).

The avidity of O_2^- for [Fe–S] clusters is a consequence of its high electrostatic attraction, which is not seen with H_2O_2 because it is uncharged. Indeed, [Fe–S] clusters are the main cellular target of O_2^--mediated toxicity[3] (BOX 1). These clusters are also sensitive to other molecules; they constitute the redox centre of regulators that respond to iron, oxygen and reactive nitrogen species (RNS). In contrast to SoxR, none of these regulators are specific O_2^- sensors. The molecular mechanisms that generate the specificity of [Fe–S] clusters towards a given signal are not understood.

The Cys residue is ideally suited for reacting with H_2O_2; it constitutes the catalytic centre of the thiol peroxidase class of H_2O_2 scavengers and the main regulatory target of H_2O_2. With the exception of the transcription factor peroxide operon regulator (PerR; a member of the Fur family), which uses an iron centre, all characterized H_2O_2-specific regulatory mechanisms involve the oxidation of unique Cys residues by H_2O_2. Two unique chemical features endow the Cys residue with its redox-regulatory properties: the exquisite H_2O_2 chemical reactivity it can reach (depending on its protein context), and its ability to cycle between different stable redox forms (BOX 2). Cys residues are not equal in their ability to undergo redox modifications[4–6], which provides the basis for selectivity and specificity[7–9]. For example, it is often overlooked that the low molecular weight thiol glutathione (GSH) cannot react with ROS *in vivo*[5]; it only participates in peroxide scavenging by assisting glutathione peroxidases (GPXs) or by forming *S*-glutathionylated adducts with protein-sulphenic acids (SOHs). Selective reactivity explains why the Cys residue is not a main target of H_2O_2 toxicity (BOX 1). Indeed, proteome-wide identification of oxidized protein thiols shows that H_2O_2 does not cause random protein thiol oxidation[10].

Cys can also serve in redox regulation by coordinating zinc, which provides a redox control of metal binding and metal control of Cys redox reactivity[11–13]. As a Lewis acid, zinc lowers the pK_a of its coordinating thiol and potentially modifies its reactivity. Reciprocally, Cys oxidation leads to zinc release, which results in a change of conformation that alters protein function.

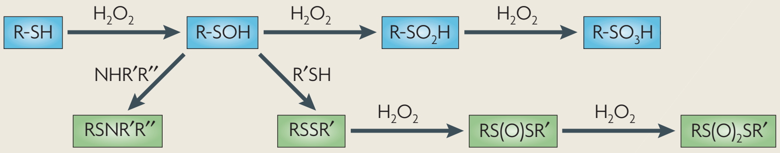
Met residues are another H_2O_2 target and can be oxidized at the sulphur atom to form methionine sulphoxide; however, the slow reaction rates are not compatible with an *in vivo* regulatory function (BOX 1). By contrast, selenocysteine, which is more reactive with H_2O_2 than Cys, may operate in H_2O_2-based redox regulation; for example, the thiol oxidase function of the selenothiol-based hydroperoxide glutathione peroxidase GPX4 (REFS 14,15).

Prokaryotic ROS homeostatic pathways

Prokaryotic pathways of ROS signalling constitute precise, fast and highly dynamic adjustment mechanisms of intracellular ROS homeostasis (FIG. 1). A key feature is feedback regulation: this originates from the nature of the pathway outputs, namely, ROS scavengers that extinguish ROS input signals. Tracking pathway outputs and physiological function is key to establishing the exact nature of the chemical(s) that are sensed, the knowledge of which is essential to understand the specificity of a redox mechanism. Accordingly, not all redox-regulatory mechanisms fit this definition of a ROS homeostatic pathway.

Sensing superoxide through [Fe–S] clusters. The SoxR transcription factor is an O_2^--specific sensor[16] that also has a minor role in the response to RNS[17] (FIG. 1a). Its specificity for O_2^- is attested by its target genes, the gene products of which are involved in O_2^- catabolism (for example, manganese-superoxide dismutase) and

Box 2 | Cys, the archetypal redox-regulatory amino acid



H_2O_2 Cys oxidation products

Sulphur-mediated nucleophilic attack of the peroxide O–O bond by the Cys thiol group (-SH) leads to H_2O release and formation of sulphenic acid (-SOH). The -SOH is highly reactive, its stability being influenced by the availability of a proximal -SH with which it condenses to form a disulphide bond, or by availability of a proximal nitrogen to form a sulphenamide[115,116] or by the presence of H_2O_2, which further oxidizes it to form sulphinic (-SO_2H) or sulphonic (-SO_3H) acids (see figure).

Cys residues are not equally reactive

The reactivity of Cys residues is dictated by their solvent-exposed localization and ionization state; thiolate anions ($-S^-$) are far more nucleophilic than their protonated counterpart[4]. When its pK_a is less than or equal to intracellular pH (6.8–7.2), a protein thiol will be deprotonated *in vivo* by >50%. Neighbouring positively charged residues stabilize $-S^-$ by electrostatic interaction, decreasing the pK_a of free Cys (8.3)[7]. However, there is an optimal value of pK_a for Cys reactivity. Indeed, as pK_a decreases, the stability of the $-S^-$ increases, decreasing negative charge availability and thus reactivity[4,6]. Reaction rates of H_2O_2 with thiols vary widely ($10–10^6\ M^{-1}.s^{-1}$)[4,5,117,118], which provides an extended reactivity range that is exploitable *in vivo* for selectivity.

Reversal of Cys oxidation

Only the forms of Cys that can be reversibly oxidized (-SOH, disulphide bond, sulphenamide, and occasionally -SO_2H) operate in redox signalling. Reduction of -SOH proceeds by condensation with a donor -SH to form a disulphide. Disulphides are reduced by either thioredoxin (TRX) or glutaredoxin (GRX), which themselves are reduced by NADPH-dependent TRX- and glutathione reductases. TRX and GRX catalyse fast and reversible thiol disulphide exchanges between their CXXC active-site residues and half-cysteines of disulphide substrates. Glutathione (GSH) can reduce the R-SOH that emanate from peroxide oxidation by formation of an *S*-glutathionylation adduct, which is then reduced by GRX. *S*-glutathionylation does not seem to derive from thiol-disulphide exchange between an R-SH and oxidized GSH. *S*-glutathionylation is important in protecting R-SOH from irreversible oxidation and might have protein-regulatory functions[119].

protection or repair of [Fe–S] clusters (for example, oxidation-resistant [2Fe–2S]-containing fumarase C and [Fe–S]-repair ferredoxin:NADPH oxidoreductase)[18]. Oxidation of the SoxR [2Fe–2S] cluster by O_2^- causes a change of SoxR conformation that alters the structure of the SoxR-bound DNA operator, resulting in gene activation.

The search for a SoxR reduction mechanism led to the discovery of the *rsxABCDGE* operon and *rseC* gene product, which together form a putative multicomponent NADPH-dependent reducing system that is associated with the membrane[19]. The stronger and more prolonged SoxR activation in strains that are impaired in $NADP^+$ reduction can be explained by defective SoxR reduction, which indicates that SoxR is not only linked to O_2^- levels but also to the ratio of NAD(P)H compared with $NAD(P)^+$ (REF. 18). Fridovich argued that SoxR may exclusively sense the NAD(P)H/$NAD(P)^+$ ratio, because paraquat and menadione — redox-cycling drugs that are used to activate SoxR — generate O_2^- while depleting cellular NAD(P)H[18]. Nevertheless, the fact that the SoxR output pathway specifies O_2^- catabolism indicates the O_2^- specificity of SoxR.

Sensing oxygen, iron and ROS through [Fe–S] clusters. Being chemically versatile, [Fe–S] clusters can sense other signals. The oxygen sensor FNR, which activates anaerobic gene transcription[20,21], requires a $[4Fe–4S]^{2+}$ cluster. This cluster is oxidized by oxygen, resulting in the formation of an unstable $[3Fe–4S]^+$ cluster and release of Fe^{2+} and O_2^-. H_2O_2 and O_2^- can both disassemble the cluster; however, oxygen is thought to be the specific FNR physiological signal because it reaches a much higher concentration than H_2O_2 and O_2^- during the aerobic switch[20].

The [Fe–S] cluster-containing transcription factor IscR senses both the [Fe–S] cluster biosynthesis status and H_2O_2, and exemplifies the coordinated regulation of iron and ROS metabolism, which is important for mitigating the synergistic toxicity of these compounds. When the [2Fe–2S] cluster is intact, IscR represses the transcription of genes involved in [Fe–S] cluster biosynthesis[22]. H_2O_2 derepresses IscR gene expression by disassembling the IscR [2Fe–2S] cluster and activates OxyR-dependent expression of an alternate [Fe–S] assembly pathway, which is more robust towards oxidative stress[23].

The coordination of iron and ROS responses is also carried out by the mammalian RNA-binding iron-response protein-1 (IRP1)[24]. IRP1 primarily senses iron through the assembly or disassembly of its [4Fe–4S] cluster (which is dependent on the cellular iron status) that regulates its RNA-binding activity. The RNA binding of IRP1 is also regulated by O_2^-, possibly by cluster disassembly, thereby decreasing iron availability and mitigating the potential toxicity of O_2^-. The biological importance of the IRP1 oxidative stress response is controversial.

Active Cys or iron centres for peroxide sensing. OxyR[25], PerR[26] and OhrR[27] are bona fide peroxide sensors, and each uses a distinct sensing mechanism (FIG. 1b). OxyR and PerR sense both H_2O_2 and organic peroxides, whereas OhrR only senses organic peroxides. PerR and OxyR are functional orthologues; each sensor is present in organisms that lack the other and they regulate many common target genes that specify peroxide scavenging and iron metabolism[28]. These genes include catalases, the peroxiredoxin AhpC and Fur — a transcriptional regulator of ferric uptake. OxyR also has a role in thiol redox control by regulating glutathione reductase, glutaredoxin (Grx) and thioredoxin (Trx). OhrR only regulates the organic peroxide-specific thiol-based peroxidase OhrA[27,29].

OxyR fulfils the definition of a peroxide receptor; it is activated by H_2O_2 at concentrations that just exceed cellular physiological levels (20 nM) and that are below the threshold toxic level (estimated at >1 µM)[30]. Based on these characteristics, it is used as a faithful monitor of intracellular H_2O_2 levels[31]. OxyR reacts with H_2O_2 through a unique Cys residue (Cys199) that presumably oxidizes to a SOH, which then condenses with Cys208 to form an intramolecular disulphide[25,32]. OxyR is deactivated by reduction by Grx1, which, together with AhpC and catalase, contributes to OxyR autoregulation. The reaction rate for SOH formation is rapid

Peroxidase
Peroxide-reducing enzymes that function by heterolytic cleavage of the O–O bond. Peroxidases fall into different classes depending on the nature of their catalytic site or according to the mechanisms that regenerate the active form.

pK_a
The equilibrium constant of proton (H^+) exchange reactions between acids and bases according to the Brönsted theory. It reflects the strength of an acid to donate its proton as pK_a decreases.

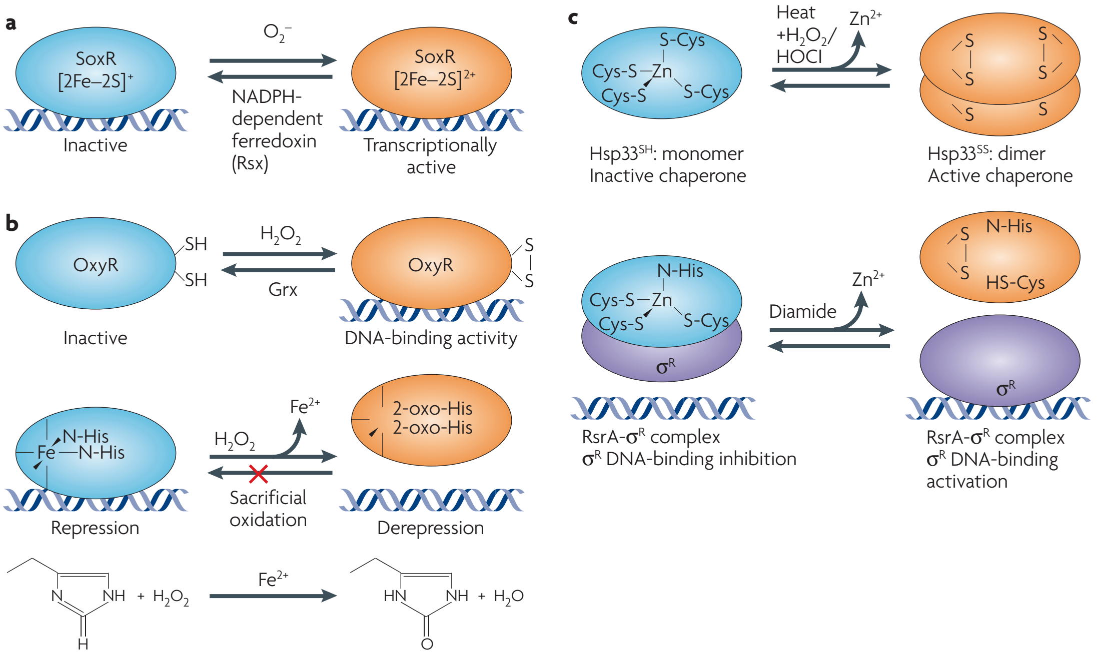


Figure 1 | **Microbial ROS receptors. a** | The SoxR transcription factor regulates the *Escherichia coli* response to the superoxide anion O_2^- (REF. 16). Oxidation of the SoxR [2Fe–2S] cluster by O_2^- causes a change in SoxR conformation, which alters the SoxR-bound operator DNA structure and results in gene activation. SoxR is reduced by NADPH-dependent ferredoxin (Rsx). **b** | The OxyR transcription factor regulates the H_2O_2 *E. coli* response. H_2O_2 oxidizes OxyR Cys199 to an R-SOH that reacts with Cys208 in an intramolecular disulphide bond[25]. The resulting allosteric change activates OxyR DNA binding. OxyR is reduced by glutaredoxin-1 (Grx). PerR is an OxyR functional orthologue that senses H_2O_2 through oxidation of two His residues to 2-oxo-His by $HO^•$, which is produced by iron-catalysed H_2O_2 reduction by the PerR iron centre. Oxidation releases iron and causes the loss of PerR DNA binding[26]. As no oxo-His reduction mechanism is currently known, PerR is viewed as a sacrificial regulator. **c** | Hsp33 is an *E. coli* redox-regulated chaperone that contains a Cys–zinc redox centre. Heat and H_2O_2 or hypochlorite (HOCl) lead to the formation of two disulphide bonds and zinc release, which together activate the Hsp33 chaperone[12]. RsrA is an anti-sigma factor with a Cys–zinc centre that negatively regulates sigma R (σ^R)[53]. Diamide-induced Cys oxidation and zinc release lead to the formation of two degenerate disulphide bonds, which causes the loss of σ^R binding by RsrA[50].

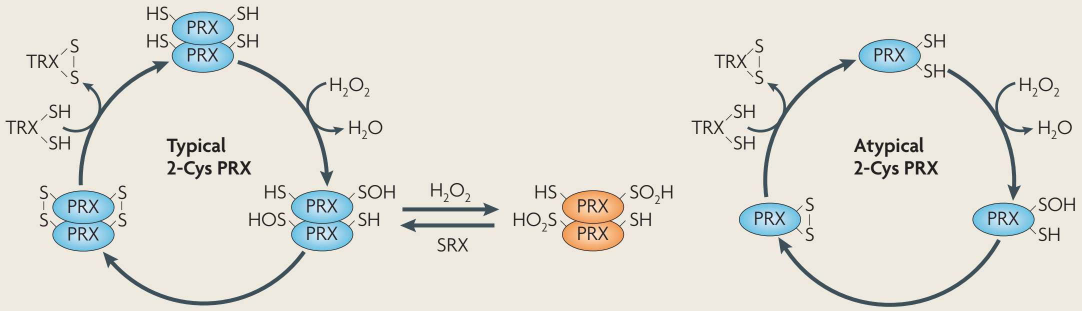
($\sim 1.1 \times 10^5$ $M^{-1}.s^{-1}$) and reflects the high H_2O_2 reactivity of Cys199 (REF. 33). Oxidation changes the conformation of dimeric OxyR, which triggers the activation of OxyR site-specific binding to DNA promoters[34]. X-ray crystallography and time-resolved structural changes that are associated with oxidation indicate a rapid extrusion of Cys199-SOH from its hydrophobic pocket, which brings Cys199 close to Cys208 — these residues are 17 Å away in reduced protein — followed by a slower second change that results from disulphide-bond formation[32,33]. These changes lead to alteration of the dimer geometry, which is thought to be the key to OxyR DNA-binding activation. Another study confirmed the H_2O_2 sensory role of Cys199 but questioned this model; it showed that an OxyR Cys208 substitution mutant did not have a strong phenotype, and identified SOH and *S*-glutathionylated forms of Cys199 instead of the Cys199–Cys208 disulphide in proteins that were reacted with H_2O_2 and oxidized GSH, respectively[35].

The thiol-specific oxidant diamide also activates OxyR, but the high concentrations that are required suggest an indirect effect either through inhibition of ROS scavengers, which increases H_2O_2 levels, and/or through GSH depletion, which decreases OxyR reduction. Similarly, constitutive activation of OxyR following inactivation of thiol-redox control pathways probably results from defective OxyR reduction rather than from OxyR redox equilibration with the oxidized cytoplasm of these cells[36]. Accordingly, OxyR might not sense disulphide stress *per se* as proposed[30,37] but, instead, might remain active under this condition owing to defective reduction. OxyR is also activated by nitrosothiols through *S*-nitrosylation of Cys199 (R-SNO)[38], but it has a minor role in the nitrosative stress response[17].

The transcriptional repressor OhrR senses peroxides using a unique reactive Cys residue that oxidizes to R-SOH[39]. In some prokaryotic species, the nascent OhrR-SOH forms a disulphide with a Cys of another subunit[40], which causes the conformation change that inhibits DNA binding. By contrast, OhrR has a unique Cys residue in *Bacillus subtilis*[41]. The OhrR-SOH form retains DNA-binding activity, but its reaction to form a mixed disulphide with either free Cys, coenzyme A or a novel 394-Da thiol, or to form a sulphenyl amide with a proximal nitrogen, leads to derepression. The structure of OhrR provides clues for its preference towards organic peroxides; a long strip of hydrophobic and aromatic residues surrounds its reactive Cys and could provide a docking site for the hydrophobic tail of organic peroxides[42].

S-nitrosylation
A modification to the –SNO form of the thiol moiety of a Cys residue, caused by reaction with peroxinitrite ($ONOO^-$) or nitrogen trioxide (N_2O_3).

Box 3 | Cys peroxiredoxins



2-Cys PRXs detect low levels of H_2O_2

2-Cys peroxiredoxins (PRXs) are a subclass of PRXs that provide unique examples of exquisite peroxide reactivity. Although they have poor catalytic constants (k_{cat} = 1–80 s^{-1}) compared to catalases (10^4–10^5 s^{-1}), they are good catalysts (k_{cat}/K_m ~10^4–10^7 $M^{-1}.s^{-1}$ for PRX versus 10^6–10^7 $M^{-1}.s^{-1}$ for catalase) because of their much higher affinity (reactivity) for substrates (PRX K_m = 1–10^3 µM versus catalase K_m = 10^3–10^5 µM) (see Supplementary information S1 (table)). Therefore, PRXs are efficient when H_2O_2 levels are low, whereas catalases are efficient when these levels are high[120].

The 2-Cys PRX cycle

Typical 2-Cys PRXs are homodimers that reduce H_2O_2 with a peroxidatic (catalytic) Cys that oxidizes to a -SOH. The -SOH then condenses with the resolving Cys of the other subunit to form a disulphide, reduced by thioredoxin (TRX). The resolving Cys is non-reactive due to shielding, but becomes solvent-exposed upon oxidation, which facilitates reduction. Homodimers form penta-homodimers that further increase the reactivity of the peroxidatic Cys by a conformational effect[121]. In atypical 2-Cys PRXs such as glutathione peroxidase (GPX)-like enzymes, the catalytic -SOH condenses with the resolving Cys on the same subunit to form an intramolecular disulphide (see figure).

Regulation of 2-Cys PRX by inactivation

PRXs exhibit a fascinating but puzzling redox twist; they undergo H_2O_2-mediated inactivation by overoxidation of their catalytic Cys to a sulphinic acid (R-SO_2H). This inactivation is unique to eukaryotic PRXs and is reversible by ATP-dependent reduction of the PRX Cys-SO_2H by sulphiredoxin (SRX) and sestrins[8,122–125], which suggests that this is an acquired gain-of-function that has been selected for regulatory purposes[4,8]. Inactivation is due to an additional C-terminal helix, which is absent in inactivation-insensitive PRXs, and slows down the rate of peroxidatic Cys-SOH condensation with the resolving Cys. This kinetic pause allows further oxidation of the R-SOH by H_2O_2 (REF. 8.) Overoxidation only occurs during enzymatic cycling and is proportional to the amount of substrate at non-saturating conditions. Excess substrate also increases the rate of overoxidation by increasing the likelihood of collision of the Cys-SOH with H_2O_2 (REF. 126).

The PerR repressor sensing mechanism is unprecedented. PerR uses a non-haem iron centre[28] and does not use a reactive Cys residue[26]. Either or both of the two His residues that contribute to iron coordination become oxidized to 2-oxo-His, presumably by HO$^\bullet$ that is produced *in situ* by iron-catalysed H_2O_2 reduction. Oxidation releases iron and causes the loss of PerR DNA binding. Full inactivation only occurs in iron-containing medium[43], which indicates that PerR not only senses H_2O_2 but also senses its toxic potential, which is a function of iron-catalysed Fenton chemistry (BOX 1). PerR transient inactivation[44] might indicate an unknown mechanism that reduces PerR 2-oxo-His or causes degradation of its oxidized form. PerR target genes are not all peroxide-inducible; some of them are differentially regulated by iron and manganese, which indicates a further degree of complexity in PerR control[28]. The PerR mechanism provides an absolute peroxide specificity because only these compounds can assist iron-catalysed His oxidation. As another manifestation of specificity, the PerR structural homologue Fur does not sense H_2O_2 but instead senses nitric oxide (NO) by dinitrosylation of the iron centre, which regulates a nitrosative stress response[17,45]. PerR can also respond to NO — not through the 2-oxo-His-based mechanism, but possibly through the mechanism described for Fur[46].

Other Cys-based redox-regulatory models exist in prokaryotes but have been omitted here because of space constraints. However, it is worth mentioning the *Staphylococcus aureus* MgrA virulence factor, which carries a unique Cys. The oxidation of MgrA by peroxide to an R-SOH leads to derepression of antibiotic resistance[47].

Cys–zinc redox switches. Cys coordination of zinc constitutes the redox centres of heat-shock protein-33 (Hsp33) (REFS 12,48) and the anti-sigma factor RsrA in prokaryotes (FIG. 1c) and probably of Kelch-like ECH-associated protein-1 (KEAP1) in mammals (KEAP1 is part of a putative ROS receptor complex in mammals; see below). These regulators differ from the aforementioned ROS receptors by their response to high levels of redox signals. Hsp33 is apparently exclusively activated by simultaneous heat and elevated H_2O_2 concentration or hypochlorite, but not by either stress alone[49]. RsrA is activated by diamide at elevated concentrations. The low reactivity is reflected by the reaction rates that are predicted from calculation of the half life ($t_{1/2}$) of zinc expulsion upon oxidation of Hsp33 by H_2O_2 at 43°C (REF. 48) and for oxidation of RsrA by diamide[50]; these rates are 10^5–10^6 below the reaction rates of OxyR[33] and PerR towards H_2O_2 (REF. 26).

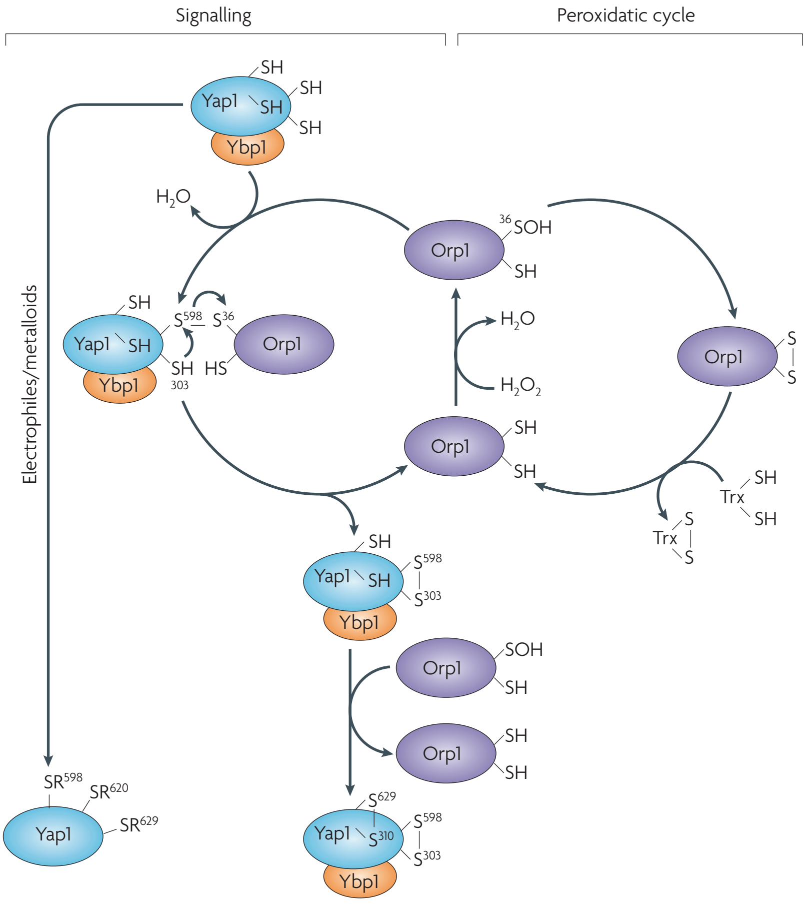


Figure 2 | **Model of the *Saccharomyces cerevisiae* Orp1–Yap1 redox relay.** Orp1 reacts with H_2O_2 at its catalytic Cys36, which oxidizes to a sulphenic acid (SOH). The Cys36-SOH then reacts with the Yap1 C-terminal Cys-rich domain (C-CRD) Cys598 to form an intermolecular disulphide bond that is transposed by thiol-disulphide exchange between Cys36 and N-terminal (N)-CRD Cys303 to form the first intramolecular disulphide bond (Cys303–Cys598). The second disulphide bond (Cys310–Cys629) presumably forms through a similar pathway on recruitment of another oxidized Orp1 molecule, only after the first disulphide bond has formed. Orp1 also functions as a peroxidase with a catalytic cycle that involves the condensation of Cys36-SOH with Orp1 Cys82 to form an intramolecular disulphide, which is then reduced by thioredoxin (Trx). Therefore, Cys36 sulphenic acid has two alternative reactive partners, Yap1 Cys598 or Orp1 Cys82. The Cys82 is preferred because of its higher efficient molarity in the vicinity of Cys36. A mechanism that brings oxidized Orp1 Cys36 close to Yap1 Cys598 and/or prevents its condensation with Orp1 Cys82 is thus needed, which might be provided by Ybp1. Note that a yeast mutant with substitution of Orp1 Cys36 to Ser cannot activate Yap1 in response to H_2O_2. Yap1 is also activated by several unrelated thiol-reactive chemicals and heavy metals through an Orp1-independent mechanism, which probably involves direct modification of Yap1 C-CRD Cys residues.

Hsp33 is a redox-regulated chaperone that substitutes for the ATP-dependent DnaK chaperone machinery, which loses activity under severe oxidative stress due to ATP depletion[49]. In Hsp33, tetrahedral zinc is coordinated by a C-terminal four-Cys zinc-binding motif, $CXCX_{(27-32)}CX_2C$, which stabilizes Hsp33 in an inactive conformation while keeping Cys residues in the thiolate form. The mechanism involves H_2O_2-promoted Cys oxidation with formation of two disulphide bonds between adjacent Cys residues and concomitant zinc release, resulting in unfolding of the C-terminal protein portion[51]. Unfolding unmasks the substrate-binding site and dimerization interface that promotes formation of the high-affinity dimer of the active chaperone[52]. Whether zinc release or Cys oxidation is the primary event that triggers Hsp33 is not established. Cys–zinc coordination carries the following theoretical paradox: Zn^{2+} is believed to keep Cys residues in the thiolate form poised to react with H_2O_2, but is also thought to decrease sulphur nucleophilicity by attracting sulphur negative charges. Furthermore, the high affinity of Zn^{2+} for Hsp33 Cys residues also prevents reaction of these residues, which might explain the need for the combination of heat and harsh oxidative stress for activation.

RsrA negatively regulates the sigma R (σ^R) transcription factor[53]. RsrA activation by diamide is consistent with the requirement of RsrA for diamide tolerance and for regulation of σ^R-dependent expression of the thioredoxin operon. RsrA is poorly activated by H_2O_2, in keeping with reaction rates that are one order of magnitude slower for this oxidant[50]. Zinc binds to a $CX_{25}HX_3CX_2C$ motif with very high affinity, which suggests a structural rather than a redox-regulated motif, and protects the Cys residue from reaction with mild oxidants[54]. Diamide-induced Cys oxidation and zinc release lead to the formation of a degenerate disulphide bond between Cys11 and either Cys41 or Cys44, causing loss of σ^R-binding by RsrA[50]. Elucidation of the natural signal mimicked by diamide should help us to understand the mechanism and function of this Cys–zinc redox switch.

Regulatory thiol peroxidases in yeast

Thiol peroxidases have unique H_2O_2 signalling functions and they contribute to the most important yeast H_2O_2-regulated pathways: namely, the *Saccharomyces cerevisiae* Yap1 and *Schizosaccharomyces pombe* Pap1 and Sty1 pathways. Thiol peroxidases consist of the peroxiredoxin (PRX)[9] and GPX-like enzyme families, and represent an important subgroup of non-haem peroxide-scavenging enzymes[15] (BOX 3). Thiol peroxidases function as both H_2O_2 receptors and redox transducers by uniquely combining high H_2O_2 reactivity and the ability to oxidize protein thiols other than their physiological reducing system[36], which is usually thioredoxin. Among these enzymes, 2-Cys PRXs can undergo reversible substrate-mediated inactivation, which supplies a second redox-switch that regulates both their H_2O_2 receptor and their scavenging functions. Yeast have other H_2O_2-signalling pathways, such as the Msn2–Msn4 transcription factors in budding yeast, but the corresponding receptors are not known.

The* S. cerevisiae *H_2O_2 sensor. In *S. cerevisiae*, the GPX-like enzyme Orp1 (oxidant receptor peroxidase-1; also known as Gpx3) and the basic leucine zipper (bZIP) transcription factor Yap1 regulate a typical H_2O_2 regulon, and constitute the yeast equivalent of OxyR[36]. The Orp1–Yap1 system operates a redox relay, which is initiated by a SOH that is formed at the Orp1 catalytic Cys (Cys36) upon its reaction with H_2O_2. This reaction leads to the consecutive formation of two Yap1 intramolecular

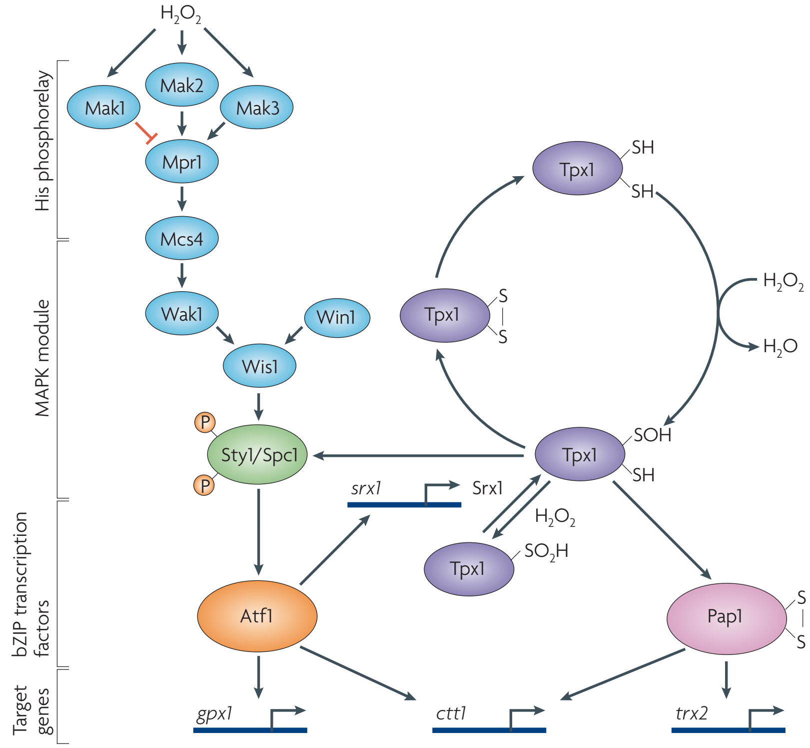


Figure 3 | **A PRX operates in the two parallel *Schizosaccharomyces pombe* H_2O_2 response pathways.** The peroxiredoxin Tpx1 and Yap1 homologue Pap1 constitute a pathway that operates as a redox relay and regulates H_2O_2 homeostasis in a similar way to Orp1–Yap1. The c-Jun N-terminal kinase (JNK)/p38 stress-activated protein kinase Sty1 (also known as Spc1) and the downstream bZIP transcription factor Atf1 constitute a second pathway, which is activated by multiple stresses. This pathway also responds to H_2O_2 and is important for surviving lethal doses of this oxidant. H_2O_2 signalling to Sty1 involves the Sty1 mitogen-activated protein kinase (MAPK) module and an upstream two-component-like His phosphorelay (similar to that in bacteria)[127,128] in which the Mak1 and Mak2 His kinases have been genetically identified as the proximal H_2O_2 sensors. Within this signalling network, Tpx1 functions downstream of the His phosphorelay, specifically enhancing H_2O_2-induced Wis1-dependent Sty1 phosphorylation by engaging its peroxidatic Cys in an H_2O_2-inducible disulphide linkage with Sty1 Cys35. Mak2 and Mak3 might actually not sense H_2O_2, but instead might respond to some as-yet-undefined metabolic cues because they contain PAS/PAC domains[129,130]. *ctt1*, cytosolic catalase-1; *gpx1*, glutathione peroxidase-1; Mpr1, stress-response regulatory protein; *srx1*, sulphiredoxin-1; *trx2*, thioredoxin-2; Win1, MAPK kinase kinase; Wis1, Ser/Thr kinase.

disulphides between N- and C-terminal Cys-rich domains (N- and C-CRD)[55] (FIG. 2). Ybp1, a protein of as-yet-undefined non-redox molecular function, is vital for the oxidation of Yap1 by Orp1 (REFS 56,57). Disulphide bond formation, by inhibiting Crm1 (also known as exportin-1)-dependent nuclear export of Yap1, promotes Yap1 nuclear accumulation and Yap1-dependent gene activation[58]. Disulphide bond formation produces a conformational change that conceals a C-CRD Crm1-cognate nuclear export signal (NES) within a hydrophobic core that is formed by the interaction of N- and C-CRD[59]. Reduction of disulphide bonds, probably by thioredoxin[60–62], exposes the NES and reactivates Yap1 nuclear export[58,63]. This oxidation-induced Yap1 allosteric change also seems to be important for transcriptional activation[56].

Two* S. pombe *H_2O_2-responsive pathways. *S. pombe* has two parallel H_2O_2-responsive pathways: one is dependent on the Yap1 homologue Pap1, and the other relies upon the stress-activated protein kinase Sty1 (FIG. 3) (for a review see REF. 64). Pap1 quickly responds to low H_2O_2 concentrations, but its activation is delayed as the concentration of H_2O_2 increases[65,66]. By contrast, Sty1 responds to high concentrations of H_2O_2. The reason for the Pap1 delayed response to high H_2O_2 levels lies in the use of the unique *S. pombe* PRX Tpx1 as the sensor device, instead of a GPX-like enzyme as in the Orp1–Yap1 pathway[67,68]. When the H_2O_2 concentration is <0.2 mM, Tpx1 is catalytically active; it senses H_2O_2 and oxidizes Pap1. When H_2O_2 concentration is >1 mM, Tpx1 becomes inactivated by Cys-sulphinic acid (-SO_2H) formation (FIG. 3), which interrupts redox signalling to Pap1. The relay is eventually reactivated as a consequence of Tpx1 SO_2H reduction by Sty1-induced Srx1 (BOX 3), and because of the antioxidant-dependent gradual decrease of the H_2O_2 concentration that prevents PRX re-inactivation. Both redox-active Tpx1 Cys residues are required for Pap1 oxidation through a mechanism that is not yet elucidated. Why is Pap1 transiently inactivated in the face of elevated H_2O_2 concentrations? Such inactivation, which evidently does not follow the logic of the antioxidant defence, could constitute a reversible Pap1 switch that allows a build-up of the intracellular H_2O_2 concentration to the levels required to switch on Sty1-dependent stress survival[68].

Tpx1 is also required for full Sty1 activation by H_2O_2 (REF. 69) (FIG. 3). By engaging its peroxidatic Cys in a H_2O_2-inducible disulphide linkage with Cys35 of Sty1, Tpx1 cancels out the inhibitory effect carried by this residue on Sty1 phosphorylation by the Ser/Thr protein kinase Wis1. It therefore enhances the response to the H_2O_2 signal that emanates upstream of Wis1. For reasons that are unclear, the effect of Tpx1 on Sty1 activation is not sensitive to Tpx1 inactivation by elevated H_2O_2 concentrations[67,69].

Yeast redox relays dissociate sensing from regulation. Yeast redox relays couple thiol peroxidases (that have high H_2O_2 reactivity) with regulators that carry stable disulphides[36]. The Yap1 oxidized form is stable. Stability is not only achieved by the two disulphides that link N- and C-CRDs but also by extensive hydrophobic interactions between these domains[59]. Using Yap1RD — a 'mini' Yap1 fusion that recapitulates the Yap1 redox-regulated domain — two redox potentials were established, with each potential attributed to one of the two Yap1 disulphide bonds[70]. The most stable of the two redox potentials (–330 mV) has a value below those of cytoplasmic thioredoxins (–275 mV for Trx1 and –265 mV for Trx2), which is a measure of high stability. It also indicates that Yap1 reduction is a function of the thioredoxin reduced/oxidized ratio, which decreases on exposure to H_2O_2. OxyR is less stable (–185 mV), maybe reflecting its intrinsic faster response compared to Yap1 (REF. 61).

The redox relay architecture of yeast sensors also restricts the signal that is transmitted, and thereby confers quasi-absolute specificity towards peroxides.

In *S. cerevisiae*, only the Orp1-SOH, which is exclusively generated by reaction with peroxides, can promote Yap1 oxidation. In fact, both Yap1 and Pap1 can respond to other thiol-reactive chemicals and heavy metals by alteration of their NES by direct, thiol-peroxidase-independent modification of C-CRD Cys residues[71,72].

Redox sensors in higher eukaryotes

ROS homeostasis in single-cell and multicellular organisms cannot be strictly compared because of differences in the external environments of the cells. Mammalian cells within their host habitat may not need the fast and highly dynamic ROS concentration-adjustment mechanisms that are crucial for the survival of single-cell organisms. Instead, mammalian cells respond to oxidative stress using either long-lasting protective responses that are usually part of global differentiation programmes or cell-death mechanisms.

Thiol-peroxidase-initiated H_2O_2 signalling. In multicellular organisms, thiol peroxidases affect several H_2O_2 signalling pathways; however, in contrast to yeast, none of these mechanisms regulate ROS homeostasis. Genetic data indicate that *Arabidopsis thaliana* ATGPX3, an enzyme similar to *S. cerevisiae* Orp1, functions as a transducer that relays a H_2O_2 signal in the abscisic acid (ABA) pathway of stomatal guard cells that controls drought stress tolerance[73]. In these cells, ATGPX3 is required to inhibit the *ABA INSENSITIVE-2* (*ABI2*)-encoded protein phosphatase type-2C, possibly by oxidation.

In mammals, although a wealth of data suggest the involvement of PRXs and GPX in signalling[74], these factors are not known to operate in the same way as in yeast; however, a redox-transducing function might be suggested for peroxiredoxin-1 (PRX1) on the basis of its requirement for the activation of the p38 mitogen-activated protein kinase (MAPK) by H_2O_2 and oxidized lipids[75]. Nevertheless, a thiol oxidase function has been clearly established for the selenothiol-based GPX4. GPX4 does not signal H_2O_2 but promotes polymerization of the sperm protective mitochondrial capsule by catalysing disulphide bond formation between structural keratin-like proteins and other proteins[15]. GPX4 also contributes to the condensation of chromatin in sperm by oxidation of protamine, which replaces histones during maturation[14]. GPX4-catalysed disulphide bond formation is presumably triggered by oxidation of GPX4 by peroxide, and requires a decrease in the concentration of the GPX4-physiological reducing system GSH, which occurs during sperm maturation.

Another mechanism by which thiol peroxidases may affect H_2O_2 signalling is by controlling the flux and concentration of the H_2O_2 that is produced upon growth-factor or cytokine-induced cellular signalling, especially within specific subcellular compartments[8,74]. For example, the most abundant mitochondrial peroxidase, PRX3, regulates apoptosis signalling by scavenging mitochondrial H_2O_2 (REF. 76). Another example of a process that also imparts specificity to the messenger function of H_2O_2 is the site-specific negative regulation of platelet-derived growth factor receptor (PDGFR) phosphorylation by peroxiredoxin-2 (PRX2), as established by studies of *Prx2*-knockout mice[77]. Upon PDGF stimulation, PRX2 associates with PDGFR, thereby possibly restricting the action radius of the H_2O_2 produced upon this stimulation, presumably by a neighbouring NADPH oxidase complex. This effect of PRX2 might ultimately lead to an alteration of the redox state of a putative PDGFR-cognate protein tyrosine phosphatase as the mechanism by which PRX2 specifically affects site-specific PDGFR phosphorylation.

The post-translational regulation of thiol peroxidases might add another layer of control to the signalling flux of H_2O_2 (REF. 9). In addition to being reversibly inactivated by their substrate (BOX 3), PRXs can be regulated by phosphorylation; PRX1 is reversibly inactivated during mitosis by phosphorylation by the CDC2-dependent kinase[78]. Similarly, GPX1 (REF. 79) and also catalase[80] are both activated by phosphorylation by c-ABL and Arg non-receptor tyrosine kinases.

ROS-inducible pathways in search of ROS receptors. Several signal circuitries regulate ROS-inducible oxidative stress-protective responses, but the corresponding upstream ROS receptors are still unknown. These pathways seem to differ from those of microbial organisms because they participate in large metabolic programmes, respond to many other physiological cues and provide either long-term oxidative stress protection or trigger apoptosis as a clearance mechanism for oxidatively damaged cells.

The main physiological function of the p53 tumour suppressor is to prevent the transmission of mutations to daughter cells either by restricting the proliferation of damaged cells and enhancing DNA repair or by inducing apoptosis. Another important component of the p53 tumour-suppressor function relies on a newly discovered antioxidant function. p53 prevents the accumulation of damage that could be caused by the continuous endogenous production of H_2O_2 by regulating GPX1 and sestrins, which are sulphiredoxin functional homologues[81]. Reminiscent of the distinct *S. pombe* responses to varying H_2O_2 concentration, low levels of H_2O_2 activate the p53 antioxidant response, whereas high levels trigger p53-dependent apoptosis through the induction of pro-oxidant activities.

The peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC1α), which stimulates mitochondrial biogenesis in response to increased energy demand, also regulates an H_2O_2-inducible antioxidant transcriptional programme that includes superoxide dismutases, catalase and GPX1 (REF. 82). PGC1α thus establishes an important link between the control of mitochondrial respiration that generates ROS and an anti-ROS programme.

Similarly, the oncogene c-Myc increases ROS tolerance by activating transcription of GSH biosynthesis genes in response to H_2O_2 (REF. 83). Transcription factors of the class O forkhead box (FOXO) family are activated by H_2O_2 and induce either cell death or a quiescent cell state that is characterized by improved tolerance to oxidative stress. This state is characterized by *SOD1* expression[84].

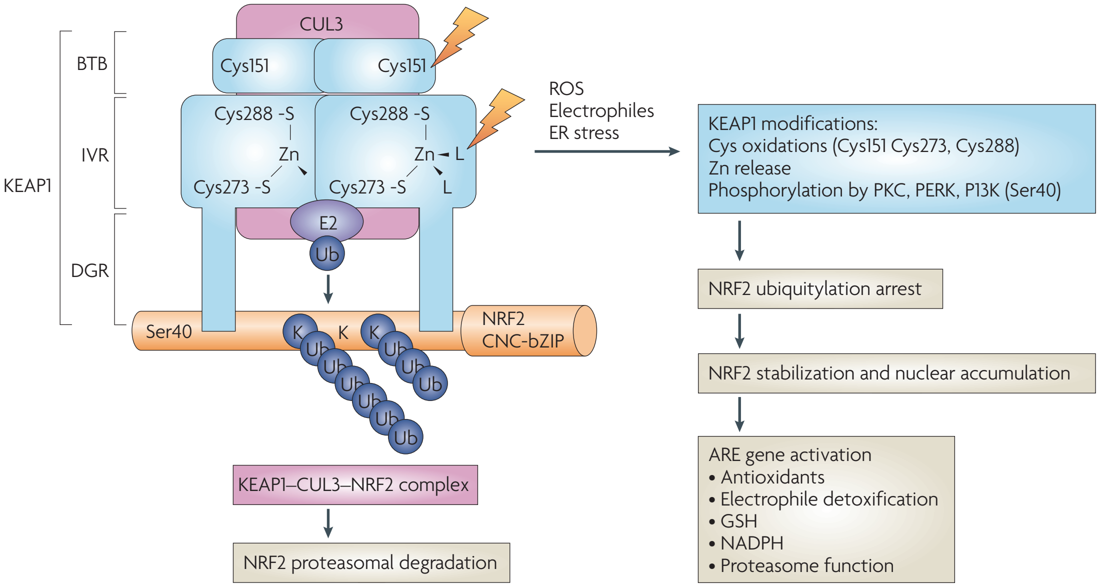


Figure 4 | **Schematic of the speculative two-site interaction model of KEAP1–NRF2.** The dimeric Kelch-like ECH-associated protein-1 (KEAP1) receptor contacts one NRF2 (nuclear factor (erythroid-derived-2)-like-2) molecule at two distinct N-terminal sites[103,131]. The KEAP1–cullin-3 (CUL3) complex constantly targets NRF2 for proteosomal degradation through ubiquitylation of NRF2 Lys residues that are located at its N terminus, between the two KEAP1 interaction sites. Electrophiles and signals from reactive oxygen species (ROS) modify KEAP1 Cys residues, leading to zinc release and a conformation that is non-permissible for ubiquitylation. An alternative speculative model proposes that two NRF2 molecules are each contacted by the Kelch domain of dimeric KEAP1 (REF. 104). Phosphorylation of Ser40 by phosphatidylinositol 3-kinase (PI3K), protein kinase RNA (PKR)-like endoplasmic reticulum (ER) kinase (PERK) or protein kinase C (PKC) might also lead to ubiquitylation arrest. KEAP1 dimerizes through the broad complex–tramtrack–bric-a-brac (BTB) domain and interacts with NRF2 through the Kelch domain. The KEAP1 intervening region (IVR) that carries reactive Cys residues is located between the BTB and Kelch domains. ARE, antioxidant response element; CNC-bZIP, cap 'n' collar-basic leucine zipper; DGR, double glycine repeat; GSH, glutathione.

This FOXO ROS-protective function is important for stem-cell survival[85]. Several mechanisms have been shown to transduce ROS signals to FOXO. The stress-activated Jun N-terminal kinase (JNK)[86] activates H_2O_2-induced FOXO protective functions, whereas the sterile-like kinase MST1 mediates H_2O_2-induced FOXO activation that leads to cell death[87]. FOXO activity is also regulated in response to H_2O_2 through acetylation by the cyclic-AMP responsive element binding (CREB)-binding protein (CBP)[88] and deacetylation by the sirtuin SIRT1[89].

The KEAP1–NRF2 pathway. The KEAP1–NRF2 complex constitutes the closest fit to a ROS receptor in mammals, and regulates environmental and xenobiotic stress-protective responses. NRF2 (nuclear factor (erythroid-derived-2)-like-2) is a CNC (cap 'n' collar)-bZIP transcription factor, whereas BTB (broad complex–tramtrack–bric-a-brac)-Kelch KEAP1 (REF. 90) functions as the receptor of the pathway and the NRF2-cognate adaptor of a cullin-3 (CUL3)–ring-box-1 (RBX1) ubiquitin-ligase complex that marks NRF2 for proteosomal degradation[91–94]. The functions of KEAP1–NRF2 in oxidative and environmental stress tolerance are attested by the nature of its inducers; these include H_2O_2, lipid oxidation products, nitric oxide, heavy metals and arsenicals and a multitude of natural and synthetic electrophilic compounds (for a detailed review see REFS 95,96). The function of this pathway in oxidative and environmental stress tolerance is also reflected by the NRF2 target genes, which comprise phase II xenobiotic enzymes and antioxidants. Moreover, *Nrf2*-null mice are characterized by susceptibility to several stresses, including acetaminophen, diesel exhaust fumes, butylated hydroxytoluene, hyperoxia, ultraviolet irradiation and carcinogens. Natural and synthetic NRF2 inducers are used in cancer chemoprevention based on the premise that higher NRF2 activity should help to detoxify carcinogens.

Chemical inducers, by modifying KEAP1, decrease ubiquitylation and proteosomal degradation of NRF2 and promote its nuclear accumulation[95,96]. KEAP1 sensing of NRF2 inducers involves reactive Cys residues[97–99], but a more complex mechanism that involves a Cys–zinc redox centre was suggested following the observation that KEAP1 binds to zinc with high affinity at stoichiometric amounts *in vitro*[95]. KEAP1 Cys273 and Cys288, which are among the few reactive Cys residues that have been identified in this protein, are indeed crucial for both zinc coordination and NRF2 degradation[96,98,99]. By contrast, KEAP1 Cys151 is not required for zinc binding, but this residue is reactive and important for derepression of NRF2 (REF. 99). Modification of these Cys residues and possibly others by alkylation, oxidation or disulphide bond formation[100] might displace zinc, thus switching KEAP1 to a conformation that is non-permissive for ubiquitylation. Zinc coordination by Cys residues in KEAP1 is reminiscent of Hsp33 and RsrA, suggesting that, in a similar way to

Alkylation
A chemical modification that involves the transfer of a carbon chain to any other atom.

these regulators, KEAP1 is not suited for detection of the low peroxide concentration that is characteristic of instant intracellular peroxide homeostasis.

How KEAP1 affects NRF2 ubiquitylation remains unclear. Chemical inducers might either disrupt the interaction of KEAP1 with NRF2 (REFS 94,96,101) or with CUL3 (REFS 94,102) or displace NRF2 within the KEAP1–CUL3 complex in a position that is not permissive for ubiquitylation[103]. A two-site interaction model of dimeric KEAP1 that contacts one NRF2 molecule at two distinct N-terminal sites of different affinities provides a basis for modularity in the position of NRF2 within the KEAP1–CUL3 complex[103] (FIG. 4), which is in keeping with the model that proposes displacement of NRF2 within the KEAP1–CUL3 complex. Alternatively, dimeric KEAP1 might contact two NRF2 molecules, with each KEAP1 monomer contacting one NRF2 molecule[104]. The KEAP1–NRF2 complex might also be controlled at the level of its subcellular localization; it undergoes nucleocytoplasmic shuttling by virtue of an NRF2 nuclear localization signal and a CRM1-cognate KEAP1 NES sequence[105,106]. The KEAP1 NES, which is close to Cys273 and Cys288, might also be altered by either zinc release and/or modification of Cys residues[106], leading to KEAP1–NRF2 nuclear accumulation in a manner that is similar to Yap1 and Pap1.

Conclusions

The ROS receptors that regulate ROS homeostasis define a distinct category of ROS signalling in which the ROS signal is the exclusive agonist that interacts with high specificity with its cognate receptor. Specificity is built up on the exceptionally high reactivity of agonists for their receptors, which is imprinted in the unique chemistry of the particular ROS and its atomic target within regulatory proteins. The ROS receptor function of thiol peroxidases provides a striking example of how nature has used the high H_2O_2 reactivity of these H_2O_2 scavengers to operate H_2O_2 homeostatic signalling. Surprisingly, these pathways, which are a hallmark of microbial organisms, have not yet been found in mammals.

A microarray analysis of the H_2O_2 response of human cells identified a large genomic response that includes genes involved in the cell cycle, cellular repair and death, many of which are targets of p53 (REF. 107). However, this study also showed the absence of fast and coordinated antioxidant transcriptional responses similar to those of microbes, leading to the hypothesis that mammalian ROS homeostasis is constitutive[107]. Mammalian ROS-specific responses, such as those regulated by FOXO, p53, c-Myc and PGC1α, seem to be part of global long-term differentiation programmes that integrate ROS protection together with multiple metabolic responses. In this view, the KEAP1–NRF2 pathway (which is devoted to xenobiotic and oxidant elimination) would be an exception to the rule. The lack of instant ROS homeostatic control in higher eukaryotes might be an inescapable consequence of ROS being used as diffusible signals that modulate multiple intracellular signalling pathways.

In multicellular organisms, ROS signals have been diverted from the primitive function of ROS homeostasis. These ROS signals originate from endogenous sources, such as the membrane NADPH oxidases or mitochondria, through poorly understood control mechanisms. They modulate a multitude of redox-sensitive signalling pathways, such as those downstream of growth-factor receptors[74,108]. Well-characterized targets of these endogenous ROS signals are the redox-sensitive catalytic Cys residues of Tyr and MAPK phosphatases, the oxidation of which reversibly abolishes enzymatic activity[109]. These ROS signals are generally not primary agonists but are superimposed co-signals that may allow the integration of cellular activities by recruiting, timing, and tuning growth-factor signalling pathways in accordance with the metabolic state of the cell. Specificity also exists in such situations, and is at least partly achieved by the highly compartmentalized production of H_2O_2 within the cell[110–112], and maybe also by the local modulation of H_2O_2 flux by peroxide scavengers[8,77]. However, the ubiquitous effects of these ROS signals, which potentially affect many pathways simultaneously, suggest a different kind of specificity that is less stringent than that of ROS receptors (as proposed by Nathan[1]). An instant ROS homeostatic control would mute ROS endogenous signals by elimination. In this view, inactivation of constitutively expressed PRX enzymes by either reversible overoxidation or phosphorylation might be an important mechanism in H_2O_2 signalling[77].

ROS homeostasis and ROS signalling are important in the ageing process and associated diseases, but their exact roles still remain puzzling. The study of ROS biology is made difficult because of the labile nature of ROS as well as their numerous cellular effects. The biochemistry of protein thiol oxidation is beginning to be mastered but rigorous methods of monitoring ROS cellular concentration are still vitally needed[113]. Mammalian ROS homeostatic pathways need to be more accurately described, especially regarding their genetic control and the biochemical mechanisms that operate ROS signal sensing. A genetic description of the cellular and organismal mechanisms of ROS tolerance is also needed. Further elucidation of the sources, cellular location, controlled production and regulatory targets of ROS as endogenous signalling molecules should provide the conceptual framework to understand the interplay between ROS signalling and ROS homeostasis.

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Competing interests statement

The authors declare no competing financial interests.

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