EXPERT
RECOMMENDATION

Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology

Helmut Sies[1,2]✉, *Vsevolod V. Belousov*[3,4], *Navdeep S. Chandel*[5], *Michael J. Davies*[6], *Dean P. Jones*[7], *Giovanni E. Mann*[8], *Michael P. Murphy*[9], *Masayuki Yamamoto*[10] *and Christine Winterbourn*[11]

Abstract | 'Reactive oxygen species' (ROS) is a generic term that defines a wide variety of oxidant molecules with vastly different properties and biological functions that range from signalling to causing cell damage. Consequently, the description of oxidants needs to be chemically precise to translate research on their biological effects into therapeutic benefit in redox medicine. This Expert Recommendation article pinpoints key issues associated with identifying the physiological roles of oxidants, focusing on H_2O_2 and $O_2^{\cdot-}$. The generic term ROS should not be used to describe specific molecular agents. We also advocate for greater precision in measurement of H_2O_2, $O_2^{\cdot-}$ and other oxidants, along with more specific identification of their signalling targets. Future work should also consider inter-organellar communication and the interactions of redox-sensitive signalling targets within organs and whole organisms, including the contribution of environmental exposures. To achieve these goals, development of tools that enable site-specific and real-time detection and quantification of individual oxidants in cells and model organisms are needed. We also stress that physiological O_2 levels should be maintained in cell culture to better mimic in vivo redox reactions associated with specific cell types. Use of precise definitions and analytical tools will help harmonize research among the many scientific disciplines working on the common goal of understanding redox biology.

Electrophile
A molecule with an electron-deficient centre that reacts with electron-rich (nucleophile) species.

Redox tone
The oxidation–reduction steady states of redox-active elements in cells and tissues.

Radical
An atom or molecule with one or more unpaired electrons.

✉*e-mail:* sies@hhu.de

https://doi.org/10.1038/s41580-022-00456-z

'Reactive oxygen species' (ROS) is a generic term for a large family of oxidants derived from molecular oxygen. They are part of a family of reactive species, including reactive nitrogen, sulfur, carbon, selenium, electrophile and halogen (RHS) species that can undergo redox (reduction–oxidation) reactions and form oxidative modifications on biological macromolecules, thereby contributing to redox signalling and biological function. However, it is well established that supraphysiological concentrations of ROS react non-specifically with proteins, lipids, nucleic acids and carbohydrates, and also generate other reactive species with potentially toxic consequences[1]. Powerful cellular stress response systems maintain homeostasis and protect against this damage by sensing deviations from the steady-state set point of oxidant levels in different cell compartments and then initiating appropriate countermeasures[2,3]. Elucidation of mechanisms underlying physiological (beneficial) oxidative stress, referred to as 'eustress', and supraphysiological (damaging) oxidative stress, referred to as 'distress', is ongoing, as is research to understand how oxidative stress response systems control the cellular redox tone. In oxidative eustress, oxidants are present at low levels and react with specific targets for physiological redox signalling, whereas in oxidative distress an increased oxidant concentration drives aberrant or disrupted redox signalling, when oxidants react with unspecific targets.

This Expert Recommendation addresses key open questions associated with defining the impact of oxidants on physiology and their contribution to disease. We provide directions for research in this field and recommendations for use of validated analytical methods and biomarkers (key recommendations are summarized in BOX 1). As the existing literature is vast, we mainly focus on hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_2^{\cdot-}$) as major redox signalling agents, as well as on secondary oxidants and electrophiles such as 4-hydroxynonenal (HNE) generated by lipid peroxidation, and peroxynitrite ($ONOO^-$) formed from $O_2^{\cdot-}$ and nitric oxide ($^{\cdot}NO$). Comprehensive coverage is not intended; instead, we give our opinion and guidance on key issues to provide a framework for

Redox medicine
The use of concepts and strategies of redox biology for applications in diagnosis and therapy.

Redox relay mechanisms
Mechanisms by which interacting molecules (often proteins) undergo reversible and consecutive redox reactions, transferring a redox signal from one site to another.

Peroxiredoxins
A family of six ubiquitous, highly reactive, thiol-containing enzymes that control hydrogen peroxide (H_2O_2) levels and mediate signal transduction.

Transition metal ion
A metal ion from the central block of the periodic table.

Fe–S cluster
Geometrical clusters of iron and sulfur. These occur in multiple forms including [2Fe–2S], [4Fe–3S], [3Fe–4S] and [4Fe–4S] and are often involved in electron transfer chains.

Uncoupling protein 1
(UCP1). A protein of the inner mitochondrial membrane that dissipates the proton electrochemical potential gradient across the membrane, thereby uncoupling oxidation from ATP production, leading to thermogenesis.

future redox medicine. For the background on oxidant signalling we refer readers to the textbook by Halliwell and Gutteridge[4] and recent key reviews[5–10].

Basics of redox signalling

It is important to consider that 'ROS' is a convenient but chemically ambivalent catch-all term in studying redox biology and medicine (BOX 2). We thus recommend, whenever possible, to specify the oxidant species under investigation, and when this is not available to use the term 'oxidant' to refer to these agents[11,12]. We here focus on two specific oxidants: H_2O_2 and superoxide ($O_2^{\cdot-}$), which have extensive documented functions.

In physiology, H_2O_2 is an essential redox signalling agent, not a dangerous by-product. It occurs in normally metabolizing cells at a controlled steady-state level[13] and is formed continuously, together with $O_2^{\cdot-}$, by mitochondrial NADH-dependent systems and by extra-mitochondrial NADPH-dependent systems and numerous oxidases (FIG. 1). Physiological intracellular pools are typically in the range of low nanomolar for H_2O_2 and low picomolar for $O_2^{\cdot-}$ (REF.[14]). Signalling by H_2O_2 in the nanomolar range behaves similarly to the signals generated by Ca^{2+}. $O_2^{\cdot-}$, at three orders of magnitude lower concentration than H_2O_2, leads to more localized spatial responses. As with other second messenger systems, signalling by oxidants can be amplified by triggering kinase cascades or can be transmitted over longer distances by conversion to more stable species, such as lipid peroxides or HNE. Signalling outside cells is expected to differ from intracellular signalling due to the higher oxidant concentrations present outside cells (extracellular fluids or plasma typically have 1–5 μM H_2O_2 (REF.[15])).

Signalling by H_2O_2 occurs mainly through reversible oxidation of specific protein Cys thiolate residues (RS^-) to sulfenic acid (RSOH) species and subsequent redox relay mechanisms, often involving peroxiredoxins[16,17]. These signalling events are coupled to metabolism, phosphorylation cascades, transcriptional regulation, cytoskeletal rearrangements, cell replication and other critical cell functions[6,10,18]. Other Cys modifications including Cys-SNO (*S*-nitrosothiol formation), Cys-SSH (persulfidation) and Cys-SSG (glutathionylation), and a recently described reversible Lys-Cys redox switch[19], are also active in redox signalling, but are not discussed here in detail. A redox species interactome has been described to cover this multilevel, multispecies redox regulatory system[20,21].

Signalling by $O_2^{\cdot-}$ is less well understood, but most probably proceeds by affecting the redox state of transition metal ion complexes in proteins[22]. Examples include $O_2^{\cdot-}$-mediated disruption of the Fe–S cluster in the citric acid cycle enzyme aconitase, which impacts mitochondrial activity[23]; $O_2^{\cdot-}$-mediated activation of uncoupling protein 1 (UCP1), thereby protecting against excessive membrane potential[24]; and $O_2^{\cdot-}$-induced inactivation of the Fe–S form of mitochondrial glutaredoxin 2 (GRX2) (REF.[25]). $O_2^{\cdot-}$ signalling pathways can be modulated by superoxide dismutases[26].

Control of redox signalling via feedback mechanisms occurs at multiple levels that are not discussed here in detail, but one example is the sirtuins, which are NAD^+-dependent enzymes that deacetylate histones and other proteins, modulating key oxidative stress genes and inducing oxidant production as well as elimination[27]. As a consequence, the effects of oxidants on metabolic fluxes and levels of metabolites are bidirectional.

Cellular redox signalling

Specificity of redox signalling depends upon spatially regulated generation and distribution of oxidants across subcellular compartments within the cell[28,29]. Considerable knowledge on redox signal generation and transduction at the plasma membrane, nucleus, mitochondria, peroxisomes and endoplasmic reticulum has accumulated[1]. However, further research is recommended on critical questions about the mechanisms and orchestration of redox regulation across the cell[30], the molecular identification of targets of redox signalling and the larger scope of cellular and intercellular redox networks. Furthermore, deeper understanding of the functioning and mechanisms of adaptive responses to oxidative challenge is an important subject for future research.

Subcellular compartments in redox regulation. The extracellular face of the plasma membrane is a major cellular site of $O_2^{\cdot-}$ and H_2O_2 generation[31] via NADPH oxidases (NOXs)[32] under physiological conditions[33]. A lateral concentration profile of oxidants along the membrane surface has been reported[34], providing a potential basis for spatial selectivity of redox signalling, such as within caveolae at the plasma membrane or at contact sites between organelles. However, the variety of cell types and physiological context, the existence of polarity across membrane leaflets and the limited rigorous quantification of site-specific H_2O_2 generation mean that each metabolic condition needs to be systematically verified.

The mitochondrial electron transport chain (ETC) produces $O_2^{\cdot-}$, which is then converted to H_2O_2 and other oxidants[35–37]. Complex I produces $O_2^{\cdot-}$ on the matrix side, whereas complex III generates $O_2^{\cdot-}$ towards the matrix

Author addresses

[1]Institute for Biochemistry and Molecular Biology I, Faculty of Medicine, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany.
[2]Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany.
[3]Federal Center of Brain Research and Neurotechnologies, Federal Medical Biological Agency, Moscow, Russia.
[4]Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Pirogov Russian National Research Medical University, Moscow, Russia.
[5]Division of Pulmonary & Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA.
[6]Department of Biomedical Sciences, Panum Institute, University of Copenhagen, Copenhagen, Denmark.
[7]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Emory University, Atlanta, GA, USA.
[8]King's British Heart Foundation Centre of Research Excellence, School of Cardiovascular Medicine and Sciences, King's College London, London, UK.
[9]MRC Mitochondrial Biology Unit, University of Cambridge, Cambridge, UK.
[10]Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan.
[11]Department of Pathology and Biomedical Science, University of Otago, Christchurch, New Zealand.

Box 1 | **Key recommendations for studies in redox biology**

General

- Focus research on specific oxidants, for example hydrogen peroxide (H_2O_2) or superoxide anion radical ($O_2^{\cdot-}$), and only use the generic term 'reactive oxygen species' (ROS) when referring collectively to multiple species. Use the term 'oxidant' when the identity of the species is not defined.
- Identify specific Cys residues in proteins targeted by H_2O_2 signalling, and the role of redox relay pathways; develop an atlas of redox network responses.
- Consider production of H_2O_2 and $O_2^{\cdot-}$ as physiological, not as a 'by-product' or 'leakage'.
- Be aware of the intricate relations to other reactive species (reactive nitrogen species, reactive sulfur species and so on) and their interactions within the 'redox interactome'.
- Define thresholds ('tipping points') between redox eustress and distress in molecular terms.

Technical

- Use a physiological O_2 atmosphere in cell culture experiments, tailored to the specific cell type; choose a consistent time of day for sampling to minimize circadian (diurnal) fluctuations.
- Use in vivo model systems (zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, mammals).
- Use non-invasive genetically encoded probes to identify and quantify oxidant formation.
- Restrict the use of redox probes, and if using these tools be aware of their non-specificity and other pitfalls.
- Use 'omic' technologies and redox biomarkers to examine clusters of genes, proteins and metabolites that respond together to oxidant generation, rather than measure a single 'omic' level.

Cell physiology

- Analyse the dynamics of the cellular 'redox landscape', including the subcellular oxidant profile and associated changes in the redox proteome.
- Study mitochondria in intact cells, not as isolated organelles. Consider redox interactions between organelles (for example, mitochondria, peroxisomes, endoplasmic reticulum, nucleus).
- Explore patterns of oxidative post-translational modifications.

Organism level

- Interpret pharmacological and genetic intervention at the redox network/systems biology level.
- Consider exogenous factors ('exposome'): nutrition, exercise, lifestyle, environment.
- Develop practical interventions to improve health through modulation of oxidant level or oxidant signalling mechanisms.

and intermembrane space. Succinate accumulation (for example, during ischaemia) can also lead to $O_2^{\cdot-}$ generation from complex I by reverse electron transfer[38]. Molecules that selectively suppress $O_2^{\cdot-}$ production[36] may act as therapeutics to ameliorate mitochondrial ETC-dependent oxidant-linked diseases. It is a widely held view that oxidant production by the mitochondrial respiratory chain represents unavoidable leakage of the ETC, but this mitochondrial production of oxidants may actually be a controlled physiological signalling process. Hence, use of the term 'leakage' — which suggests an unfavourable reaction — is discouraged (BOX 1). Critical questions remain on whether site-specific generation of $O_2^{\cdot-}$, H_2O_2 and other oxidants in mitochondria have specific signalling functions. Mitochondrial matrix H_2O_2 is reduced by peroxidases, including glutathione (GSH) peroxidases and peroxiredoxins 3 and 5 (REF.[39]). Multiple post-translational modifications of peroxiredoxins may also add to the specificity of signalling[40].

Isolated mitochondria can release H_2O_2 from the matrix to the extra-mitochondrial space, but the rate at which this occurs physiologically in intact cells remains unclear. This highlights the problem of identifying the mechanisms and rates of mitochondrial H_2O_2 release required to exert biological effects. Acute (minutes) bursts of mitochondrial H_2O_2 production such as during reperfusion injury do not appear to be sufficient to reach the cytosol[41]. However, longer-term (hours) generation, such as during immune activation of macrophages, can enhance cytosolic oxidant levels, perhaps reflecting an eventual override of matrix antioxidant defence systems[42]. To address this problem, mitochondria should preferentially be studied within intact cells, not as isolated organelles.

Peroxisomes contain multiple H_2O_2-producing and scavenging enzymes and are active in H_2O_2 metabolism[43]. In addition to intraorganellar peroxisomal reactions, there is considerable interaction with extra-peroxisomal sites[44,45]. Peroxisomal catalase can modulate oxidative stress at the cellular level[46,47], possibly via suppression of catalase import into the peroxisomes[48], allowing for higher extra-peroxisomal catalase activity. As an organelle characterized by specialized functions in H_2O_2 metabolism, there is a critical need to improve understanding of the integration of peroxisomal and extra-peroxisomal oxidant signalling.

The lumen of the endoplasmic reticulum is a major source of H_2O_2 production during disulfide bond formation in protein folding, and the endoplasmic reticulum also contains cytochrome P450 (Cyp) family enzymes functioning in steroid synthesis and oxidative detoxification of xenobiotics. These systems can generate considerable fluxes of $O_2^{\cdot-}$ and H_2O_2, and impact protein translation, pro-apoptotic signalling and other pathways for sensing and responding to H_2O_2 (REF.[49]). The diversity of endoplasmic reticulum structures, the functional continuity of the endoplasmic reticulum with the Golgi and lysosomal compartments, and the spatial proximity to mitochondria, nuclei and other subcellular structures emphasize a need to analyse the redox integration of the endoplasmic reticulum within different cell types. Aquaporin 11 is of particular interest as a regulator of endoplasmic reticulum redox homeostasis and signalling, because it serves as a peroxiporin for H_2O_2 transport through the endoplasmic reticulum membrane[50].

In situations where mitochondria are densely packed, as occur in cardiomyocytes, H_2O_2 diffusion is thought to synchronize responses across the cell[51]. Furthermore, the distribution of mitochondria within the cell via the mitochondrial adapter protein Miro1 is thought to regulate oxidant distribution[52]. In addition, redox nanodomains exist at the endoplasmic reticulum–mitochondrial interface, which are functionally linked to signalling between these organelles[53]. Mitochondria, the endoplasmic reticulum and peroxisomes form a 'redox triangle' where redox signalling is coordinated[54]. Altogether, this complexity of cellular oxidant networks highlights a need for more refined studies on the subcellular regulation of oxidants, their localized signalling, distribution across the cell and redox crosstalk at specialized membrane contact sites between different organelles.

Superoxide dismutases
Enzymes which convert two molecules of superoxide ($O_2^{\cdot-}$) to one molecule of oxygen and one molecule of hydrogen peroxide (H_2O_2), a process called dismutation.

Redox networks
Networks of interacting molecules that undergo redox reactions.

NADPH oxidases
(NOXs). Membrane-bound enzyme complexes, assembled from multiple protein components, that use NADPH to reduce O_2 to the superoxide anion radical ($O_2^{\cdot-}$) and/or hydrogen peroxide (H_2O_2).

Caveolae
Invaginations of the plasma membrane that bud off internally, which are thought to form from lipid rafts.

Ischaemia
A condition in which blood flow (and hence the supply of O_2 and other materials) to a tissue is impaired.

Reverse electron transfer
Flow of electrons through an electron transport chain (ETC) in the reverse direction to that which normally occurs, for example in ischaemia.

It should also be noted that oxidants are present and modulated extracellularly. We thus emphasize the need to integrate the cell biology of redox signalling across organs. Plasma membrane-located NOXs, together with extracellular superoxide dismutase (SOD3), generate a pool of extracellular H_2O_2, the concentration of which is two to three orders of magnitude higher than that present intracellularly, as mentioned above. H_2O_2-dependent oxidant signalling in blood plasma emerges as a redox-integrating characteristic between different organs. The release of redox-active enzymes such as protein disulfide isomerase (PDI)[55], thioredoxin (TRX) and peroxiredoxin into plasma[56] increases during infection and inflammation. Catalase is associated with the plasma membrane in cancer cells[57,58] and, thus, becomes an interesting extracellular therapeutic target[2].

Dissecting redox signalling targets. A full understanding of the role of specific oxidants in cell biology requires determination of their targets across the entire cell, which can be achieved by combined 'omic' approaches and description of the redox interactome, including information on interactions between different oxidants and their downstream targets[20,21]. A major issue is how to identify key redox-sensitive Cys residues in proteins. They can be identified by redox proteomics (for example, the Oximouse data set)[59] or, potentially, predicted using machine-learning methods[60]. Replacement of key Cys with redox-insensitive residues, leading to signal

Box 2 | **Terminology of reactive oxygen species and their characteristics**

'Reactive oxygen species' (ROS) is a general term that provides no information on the species being reported, thereby limiting the given biological information; whenever possible, specific designation — for example, superoxide anion radical ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2) — is preferred. 'Oxidant' should be used when unspecified species are referred to. This family is divided into radicals (sometimes unnecessarily called 'free' radicals) and non-radical species.

Radical (one-electron) ROS

Superoxide anion radical ($O_2^{\cdot-}$)
Commonly called superoxide, $O_2^{\cdot-}$ exists at very low concentrations. Most assay methods are selective but not specific (see BOX 5). Generated by one-electron transfer to O_2 in electron transport chains (ETCs) (mitochondria, endoplasmic reticulum, plasma membrane) and by enzymes. A weak oxidant (electron or hydrogen atom removal) and a reductant (electron donation). Reacts with Fe–S clusters, releasing iron, and with some transition metal ions (reduction of Fe^{3+} to Fe^{2+}). A major fate is dismutation with another $O_2^{\cdot-}$, giving O_2 and H_2O_2. This occurs spontaneously but is enzymatically accelerated by superoxide dismutases. The protonated form (perhydroxyl radical, $HO_2^{\cdot}$) occurs at significant concentrations in acidic environments (pK_a 4.9) and can, unlike $O_2^{\cdot-}$, diffuse through membranes and abstract a hydrogen atom from conjugated methylene groups of polyunsaturated fatty acids (PUFAs) to give carbon-centred radicals ($R^{\cdot}$). $O_2^{\cdot-}$ reacts rapidly with other radicals, for example nitric oxide ($^{\cdot}NO$), to give peroxynitrite ($ONOO^-$).

Hydroxyl radical ($HO^{\cdot}$)
$HO^{\cdot}$ is the most reactive biological oxidant which reacts non-specifically at diffusion-controlled rates ($k \sim 10^9\ M^{-1}\ s^{-1}$) with biomolecules. Products of its reaction with proteins, DNA, RNA and lipids serve as biomarkers of oxidative damage (BOX 5). It cannot be readily scavenged. Little evidence for a direct signalling function, but generation by ionizing radiation and one-electron reduction of H_2O_2 (for example, by Fe^{2+} or Cu^+) may affect other signalling processes.

Peroxyl radical ($ROO^{\cdot}$)
Formed during lipid and other peroxidation chain reactions, $ROO^{\cdot}$ can also be derived from enzyme-generated lipid hydroperoxides. May serve as a signalling agent, as it can diffuse over considerable distances.

Alkoxyl radical ($RO^{\cdot}$)
Highly reactive species formed by one-electron reduction of organic hydroperoxides (ROOH) by metal ions that can amplify radical chain reactions. Limited evidence for a direct role in signalling, but can rearrange to longer-lived radicals.

Non-radical (two-electron) ROS

Hydrogen peroxide (H_2O_2)
A pleiotropic oxidant signalling agent. When validated with appropriate methods, it should be explicitly named. A two-electron oxidant, but poorly reactive, except with specific protein Cys residues ($k \leq 10^7\ M^{-1}\ s^{-1}$), allowing selective and specific signalling. There is a need for detailed understanding of its downstream signalling mechanisms and the role of redox relays in these processes. Such relays can occur via direct thiol oxidation or via haeme enzymes (peroxidases), Fe–S clusters, redox-active metal ions and bicarbonate, which forms a more reactive oxidant, peroxymonocarbonate (HCO_4^-).

Singlet molecular oxygen ($^1O_2$) and triplet carbonyls ($RR'C{=}O^*$)
Electronically excited state species formed by energy transfer from ultraviolet or visible light with a sensitizer as well as by chemical and enzymatic reactions. Important mediators of damage to surface organs (for example, skin and eye). Can be quenched by dietary carotenoids. Limited information on signalling functions.

Ozone (O_3)
A moderately reactive atmospheric oxidant and air pollutant. Reacts with double bonds (PUFAs, cholesterol) to form peroxides, which can act systemically. Elucidation of species formed in the lungs and surface-exposed tissues, and involvement in downstream signalling, may be important for designing protective agents against this oxidant.

Organic hydroperoxides (ROOH)
Formed enzymatically by lipoxygenases and cyclooxygenases with specific PUFAs, ROOH is important in enzyme-mediated and non-enzymatic cell signalling. Also formed via $ROO^{\cdot}$ during lipid and protein peroxidation. Can function directly in signalling through the nuclear factor erythroid 2 (NRF2)–KEAP1 system and oxidation of protein Cys.

Hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN)
Reactive species generated by myeloperoxidase and related enzymes from H_2O_2 and halide/pseudohalide ions (Cl^-, Br^-, SCN^-), important in phagolysosomal pathogen killing. Also released extracellularly, and consequently these species and their products are potential secondary signalling agents.

Reactive carbonyls ($RR'C{=}O$) and α,β-unsaturated carbonyls ($-C{=}C{-}C{=}O$)
Reactive electrophilic metabolites generated by endogenous metabolism (glyoxal, methylglyoxal), oxidation of phenols and catechols to quinones, metabolism of xenobiotics (chemicals, drugs, pollutants) and lipid peroxidation (4-hydroxynonenal (HNE)). They form adducts with Cys residues at significant rates, and less rapidly with other nucleophiles (Lys, His and Arg), and function in signalling through NRF2–KEAP1 and, potentially, other redox-sensitive transcription systems.

Peroxynitrite ($ONOO^-$)
$ONOO^-$ is the anion of peroxynitrous acid (ONOOH; pKa 6.8) formed physiologically from reaction of $NO^{\cdot}$ and $O_2^{\cdot-}$. A powerful two-electron oxidant and nitrating agent studied extensively in pathobiology, warranting examination for signalling activities. Reacts rapidly with CO_2 to form $CO_3^{\cdot-}$ and $NO_2^{\cdot}$, which are responsible for much of its impact on physiology.

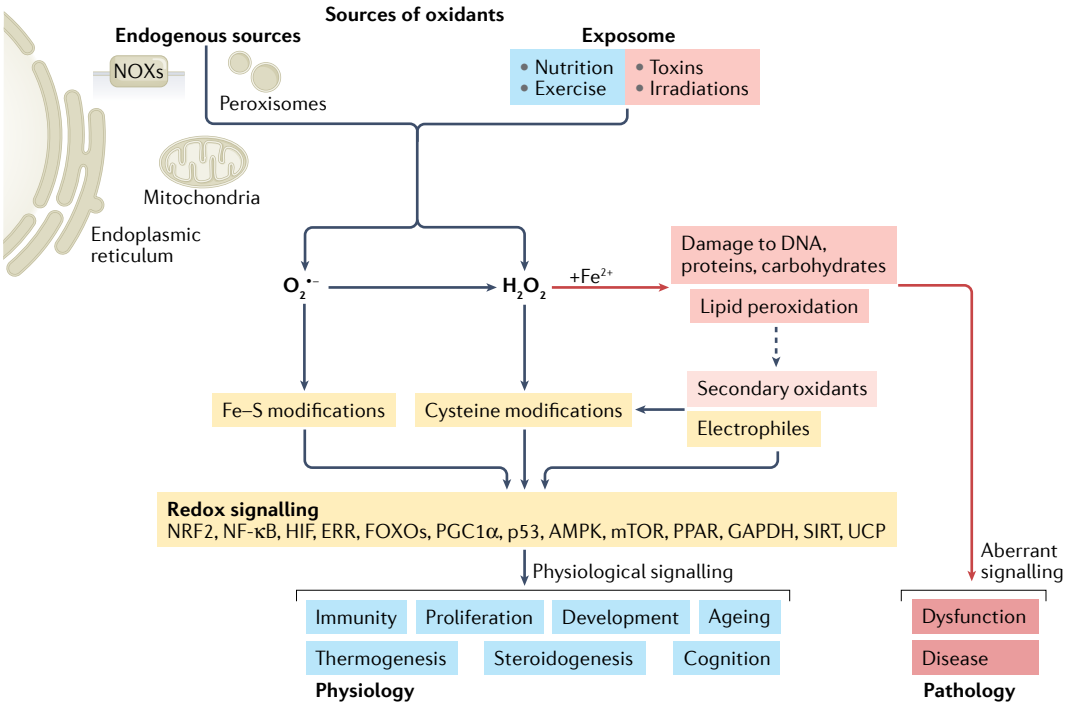


Fig. 1 | **Generation of superoxide and hydrogen peroxide and their relation to redox signalling.** Superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are generated by multiple enzymatic and non-enzymatic processes including NAPDH oxidases (NOXs), mitochondria, endoplasmic reticulum, peroxisomes and external stimuli (exposome). Reaction with iron–sulfur cluster proteins (Fe–S), or ionized form of cysteine residues (thiolate) on multiple redox-sensitive proteins, results in modifications that induce redox signalling and downstream biological effects (yellow boxes), with multiple physiological processes controlled by redox signals (blue boxes). Alternative reactions of oxidants induce damage to biomolecules that can generate additional electrophiles and secondary species implicated in aberrant signalling and pathology (red boxes). AMPK, 5′-adenosine monophosphate-activated protein kinase; ERR, oestrogen-related receptor; FOXO, forkhead box O transcription factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; NF-κB, nuclear factor-κ light chain-enhancer of activated B cells; NRF2, nuclear factor erythroid 2-related factor 2; p53, cellular tumour antigen p53; PGC1α, peroxisome proliferator-activated receptor-γ co-activator 1α; SIRTs, sirtuin protein family; UCP1, uncoupling protein 1.

Glutathione
(GSH). A key cysteine-containing tripeptide (γ-glutamyl-cysteine-glycine) that acts as a reducing cofactor and direct antioxidant.

Catalase
A haeme-containing protein that enzymatically dismutates hydrogen peroxide (H_2O_2) to O_2 and H_2O (catalatic reaction) or reduces H_2O_2 to H_2O by oxidizing a hydrogen donor AH_2 to A (peroxidatic reaction).

Xenobiotics
Chemical substances not naturally present in an organism.

Peroxiporin
An aquaporin that transports hydrogen peroxide (H_2O_2) across membranes.

Membrane contact sites
A location where membranes come into close proximity, facilitating transfer of molecules and signals.

Protein disulfide isomerase
(PDI). An enzyme typically, but not exclusively, found in the endoplasmic reticulum of eukaryotes that catalyses the formation and breakage of disulfide bonds between cysteine residues.

disruption, can validate their relevance in specific contexts. The considerable circadian (diurnal) variation of oxidant levels needs to be considered in experimental settings as well[61]. Overall, specificity of the redox signal is given by spatio-temporal control of the oxidant and by the enormous span of reactivity of target thiols (million-fold differences between different protein thiols[62]), and there is a need to develop more refined research tools in cell biology enabling specific capture of redox targets in a context-dependent manner.

A second key issue is how oxidants find these Cys residues on target proteins, as they are orders of magnitude less reactive than the oxidant-consuming protective proteins of antioxidant systems. This implies that oxidation may involve redox relays[16,63], whereby oxidation signals are passed from one protein to another via Cys residues on each protein, eventually leading to oxidation of the intended target[64]. Alternatively, a signal that increases oxidant production could cause localized inactivation of the oxidant-consuming proteins, thus facilitating H_2O_2-mediated oxidation of target proteins. For example, peroxiredoxins may be oxidized to an inactive state, preventing H_2O_2 degradation and, thereby, increasing the pool of H_2O_2 available for signalling. This is known as the floodgate model[65]. Delineation of these possible pathways, each of which may occur in different situations, is a key issue.

Oxidant signals impact on a plethora of cellular processes and enable adaptation to the environment. They are transmitted to enzyme cascades and other targets which induce changes in gene expression patterns[66]. Substantial progress has been made in integrating redox sensors within a redox network[67] and the next step should be the development of atlases of network responses.

Major redox hubs in mammalian cells include nuclear factor erythroid 2 (NRF2) (REF.[68]), nuclear factor-κ light chain-enhancer of activated B cells (NF-κB)[69], hypoxia-inducible factor (HIF)[70], oestrogen-related receptor (ERR)[71], forkhead box O transcription factor (FOXO)[72,73], peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) (REF.[74]), p53 (REF.[75]), 5′-adenosine monophosphate-activated protein kinase (AMPK)[76], glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[77] and UCP[24] (FIG. 1). A notable feature of the first three of these is the regulation of their turnover and activation by a Cys redox signal on an associated protein, not the transcription factor itself (for example, KEAP1 for NRF2). This ultimately leads to nuclear accumulation of the active transcription factor. The other hubs

Thioredoxin
(TRX). A small ubiquitous redox-active protein that plays a role in redox signalling via the maintenance of cysteine residues in their thiol form.

Redox interactome
A collective term for all of the layers of omics space with redox interactions. This includes both reversible and irreversible redox reactions. The proteome has the largest number of reversible oxidizable elements.

Redox hubs
Central nodes in a redox network through which redox changes can impact multiple downstream components.

Selenocysteine
A selenium analogue of cysteine, where selenium replaces sulfur.

Fenton reaction
The reaction of Fe^{2+} with H_2O_2 that generates the hydroxyl radical (HO·).

Hormesis
A biologic process in which low-dose exposure to a stressor activates mechanisms that protect against future toxic exposures.

typically contain intrinsic redox-sensitive Cys residues that react with the oxidant directly. Methods to visualize fundamental mechanisms involved in oxidation within the redox hubs are needed to provide understanding of real-time responses to the presence of oxidants and how these oxidant-sensitive systems function together within redox networks.

Contribution of the antioxidant network. Antioxidant defences predominantly consist of powerful antioxidant enzymes and, to a lesser degree, low molecular-mass compounds (LMMCs), including vitamins, micronutrients and cofactors. These molecules together shape the redox landscape, having both beneficial roles (scavenging excess oxidants and preventing oxidative distress) and pathological consequences (disruption of physiological oxidant signalling)[30].

The role of LMMCs in redox regulation is commonly overstated. Although they are essential cofactors for many antioxidant enzymes, LMMCs do not generally undergo catalytic reactions on their own. Exceptions are vitamins C and E, which act as one-electron radical scavengers. The inactivation of non-radical (two-electron) oxidants is largely enzyme-catalysed[62], predominantly mediated by selenocysteine as well as Cys-containing or haeme-containing proteins. Thus, we emphasize that the major biologically active 'antioxidants' are these enzymes, and not LMMCs. The micronutrient selenium in the form of selenocysteine is essential for the activity of several critical antioxidant systems[78]. Other antioxidant enzymes rely on the controlled redox activity of transition metal ions such as iron within haeme groups in catalase or copper and manganese ions in superoxide dismutases.

Importantly, when redox-active transition metal ions, in particular iron and copper, are free — that is, not chelated to metal ion-binding proteins — they generate the highly reactive HO· radical via the Fenton reaction. The toxicity of high levels of extraneous transition metal ions is well documented in environmental and occupational health, consistent with the importance of transition metal ions as an oxidant source. However, the activities of low-concentration 'labile pools' of redox-active ions within the physiological redox architecture need to be further described. Substantial geographical variations in metal ion exposure from soil, water, dust and diet warrant additional investigation of their associated effects on oxidant signalling mechanisms. NRF2 and HIF1α provide pivotal control of systemic iron homeostasis[79], and the role of these systems in regulating iron, copper, manganese and other essential redox-active metals needs to be understood in relation to redox signalling.

Differentiating between oxidative eustress versus distress. Maintenance of redox balance via multiple adaptive response mechanisms is essential for metabolic control. The concept of 'oxidative stress' describes the imbalance of oxidants over antioxidants and repair processes, leading to a disruption of redox signalling and control and/or molecular damage[80]. A pivotal consideration is the distinction between eustress (good stress) and distress (bad stress)[81]. Oxidative eustress describes physiological deviations from the steady-state redox set point[80,82], which has been called the 'golden mean of healthy living'[83]. At higher levels of oxidative challenge[84], there is a transition to oxidative distress, associated with biomolecular damage. As a corollary, a deficiency of oxidants manifests as 'reductive stress'[85], and there are cellular mechanisms to detect and counteract reductive stress[86]. Defining the thresholds between eustress and distress in molecular terms is an important task for further research[87].

Understanding adaptive responses to oxidative stress. Adaptation to changing conditions is accomplished by oxidative stress response systems (see above), which can be divided into reactive (feedback, counter-regulation) and predictive (feedforward, anticipatory) modes. The preconditioning response to cues is described as hormesis[88,89] or, more specifically, 'mitohormesis'[90], as the key driving mechanism of adaptation involves changes in mitochondrial function, including transient increase in mitochondrial production of oxidants in response to mild stress. This has been shown to confer resistance to repeated stress and promote the lifespan and healthspan in multiple organisms. Eventually, as a result of the hormetic response, a new gene expression pattern is established in the cell, including increased expression of antioxidant enzymes and other stress response pathways. This adaptation to altered conditions has been termed 'allostasis'[91] or 'adaptive homeostasis'[92]. Even with eustress, there is constant monitoring and fine-tuning to maintain redox homeostasis[93]. The variety of human endogenous and exogenous exposures (exposome, discussed below) warrants extension of these response concepts. Key issues in analysis of redox signalling in hormesis relate to identifying the spatiotemporal thresholds of oxidant levels for induction of a protective response and the mechanism of on/off switching of relevant mediators of the responses, such as the NRF2 system[88,94].

Dissecting oxidant physiology

Considerable evidence supports the role of oxidants as signalling molecules, regulating processes including thermogenesis[95], immunity[96,97], fibrosis[98,99], cognition[100], responses to exercise[101], ischaemia–reperfusion injury[102,103], development[104,105], steroidogenesis[106], cancer[3,107,108], ageing[109–111] and oxygen sensing[112] (FIG. 1). Below, we delineate two main research areas that require further study to dissect the impact of oxidants on physiology and pathophysiology of entire organisms.

Determining oxidant function. Many of the initial targets in redox signalling are phosphatases and kinases, which are regulated by oxidants and then propagate the signal leading to a biological response. The problem of studying redox modification of enzymes is that mutation of catalytic Cys residues renders these proteins inactive, making it challenging to determine whether they are regulated by oxidation. An example is the glycolytic enzyme GAPDH, in which oxidation of the catalytic Cys (C152 in humans) to RSOH attenuates activity in glycolysis[77].

The conserved C156 is not required for catalytic function, and mutation to Ser or Ala does not affect normal activity; this mutation does, however, attenuate the sensitivity of C152 to H_2O_2-induced oxidation. Another consideration for determining oxidant function is potential spatio-temporal alterations in protein structure, which may expose cryptic Cys residues, thereby allowing redox regulation, as reported for epidermal growth factor (EGF) signalling[113]. Functional in vivo testing of oxidant signalling will require development of tissue and cell-specific animal models for individual target proteins with mutations in specific Cys residues that prevent H_2O_2-induced protein oxidation.

Consideration of lifelong exposures that impact oxidant biology. The exposome is defined as the cumulative lifelong exposure to external factors which complement the genome in generating phenotypic diversity[114,115]. Oxidant signalling responses to diet, microbiome, pharmaceuticals, dietary supplements, cosmetics and environmental pollutants illustrate that redox systems respond to various environmental cues.

Oxidant exposure comes from an array of environmental agents, including ozone (O_3) and other oxidant gases, ionizing radiation, ultraviolet light, sound waves and heat[116]. Of note, physiological external stimuli are sensed and processed by the transient receptor potential channels, whose functional state is subject to modification by H_2O_2 and electrophiles[117]. Supraphysiological oxidant production can occur directly from the environmental agent by non-enzymatic chemical reactions, or from hyperactivation of endogenous pathways of oxidant production as well as by inactivation of antioxidant pathways. The spectrum of exposome effects on redox balance is complex and illustrated by airborne particulate exposure[118]. These widespread air pollutants contain ultrafine atmospheric particulates ($PM_{2.5}$) in addition to chemical oxidants such as O_3, and sulfur and nitrogen oxides (SO_x, NO_x). Immune system functioning to eliminate foreign matter can be chronically activated to generate oxidants by non-degradable particulates[119]. Oxidant generation can also be enhanced by metal ions bound on particulates. Other oxidant sources are engineered nanoparticles[120], noise[121] and electromagnetic fields[122], indicating potential for widespread exposures from industrial products, cosmetics, microplastics and different forms of radiation exposure. External oxidants first come into contact with the skin and the eyes, the lungs and the gastrointestinal tract, potentially causing harm in these organs. Indeed, there is evidence that exposome components can induce extrinsic skin ageing[123].

A target of environmental stressors is the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that integrates immune responses[124–126]. This receptor controls redox homeostasis and shapes the tumour microenvironment[127]. AHR induces cytochrome p450s that, besides serving to metabolize xenobiotics, contribute to oxidant production and can cause developmental toxicity in response to exposure to $PM_{2.5}$ particles as shown in zebrafish embryos[128]. We recommend further exploration of the role of AHR in environmental redox biology.

Nutritional components can be beneficial and often are essential, but they may also be toxic and detrimental, depending on the context and amount. The gut is a major interface between nutritive input and the organism, and host–microbiome relationships include redox interactions[129]. For example, oxidants from host mitochondria influence gut microbiome diversity[130], and gut-resident *Lactobacilli* activate hepatic NRF2 to protect against oxidative liver injury[131]. Dietary polyphenols generate H_2O_2, activate NRF2 and affect redox signalling[132], and polyphenol consumption has been linked to attenuation of oxidative stress and disease[133,134]. Certain dietary factors such as polyphenols or carotenoids can counteract exposomal skin damage by chemical or physical quenching reactions[135,136]. Among lifestyle factors, physical activity has a potential to modulate levels of oxidants. For example, adaptive processes to exercise in skeletal muscle[137], including epigenetic responses[138], are mediated via H_2O_2. This indicates that redox regulation is central to exercise physiology[101,139].

In the future, it will be important to determine the exact contributions of these different exposures and behaviours to redox balance and to link them mechanistically to (patho)physiological outcomes. These efforts will require the integration of molecular studies with systems biology, and network medicine will enable clinicians to stratify the health outcomes associated with the different exposures within populations[140–142]. These approaches will be assisted by the development of wearable devices to monitor air pollution and health habits, such as physical activity, along with in-depth biomonitoring tools for micronutrients from food and supplements. Together, these developments may impact the diverse pro-oxidant and antioxidant roles in human health and enable individuals to manage lifestyle factors and exposures to limit the damaging effects of the exposome on redox balance and to promote the behaviours that support physiological redox signalling[143,144].

Applications to redox medicine

With better understanding of the biology of specific oxidants, a horizon is opening for 'precision redox medicine'[145], including the use of nanomaterials with oxidant-modulating properties[146]. There are several conceptually distinct approaches to redox medicine, and mechanism-based research is needed for each (FIG. 2). Drug development is usually targeted to specific proteins to inhibit or activate specific processes. As recently reviewed[1], redox medicine is advancing with strategies that address selective disease-relevant mechanisms while avoiding disruption of important signalling processes. Increased knowledge of cancer-causing mutations which result from oxidative DNA damage and associated redox mechanisms is a priority for redox medicine research.

Unlike earlier efforts to alter the *global* balance of oxidants and antioxidants, current pharmacological approaches are focused on *selective* modulation of enzymatic oxidant sources, such as inhibitors of NOXs[147] or myeloperoxidase[148], taking advantage of the knowledge of

Transient receptor potential channels
A group of ion channels mostly localized on the plasma membrane of animal cells, mediating various sensations such as pain or temperature.

Myeloperoxidase
A leukocyte (mainly neutrophil and monocyte)-derived haeme enzyme that catalyses the conversion of hydrogen peroxide (H_2O_2) to multiple reactive oxidant species including hypochlorous acid (HOCl). A major component of the innate immune response against invading pathogens, but also strongly implicated in tissue damage at sites of inflammation.

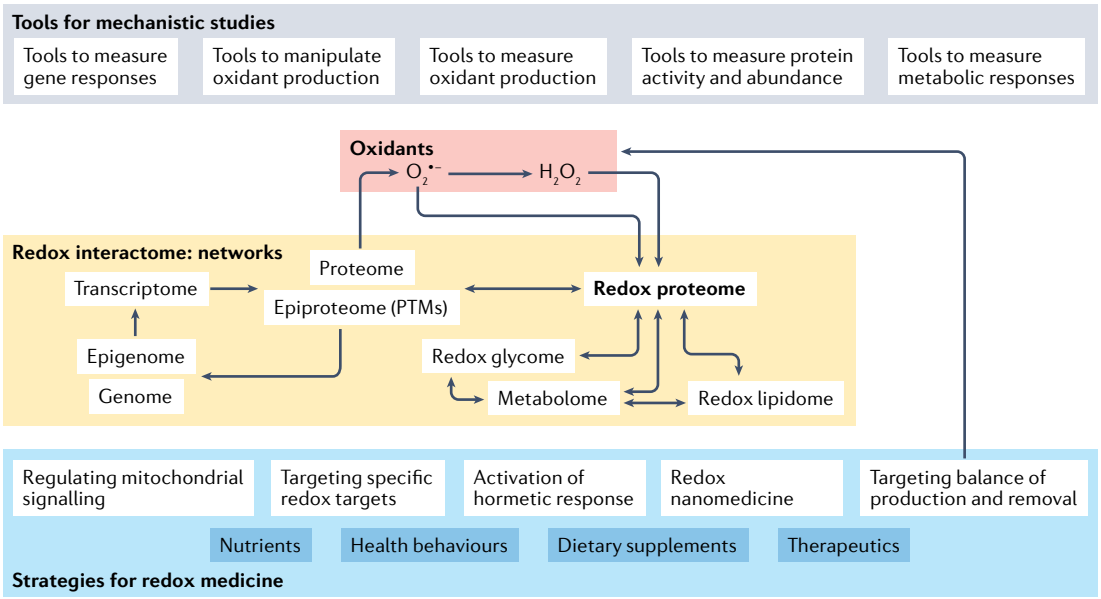


Fig. 2 | **Redox interactome of hydrogen peroxide and superoxide.** The redox interactome is a functional network linking hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) with all 'omics' domains. In this depiction, redox interactome is a collective term for redox-sensitive components of these 'omics' domains formed by reversible oxidation reactions, principally in the proteome and metabolome. Detailed knowledge is available for specific cysteine residues and other oxidizable components within the proteome, termed the 'redox proteome'. Similarly, there is a 'redox lipidome' and a 'redox glycome'. The proteome includes many types of post-translational modifications (PTMs), termed the epiproteome, which interact with the redox proteome and also are involved in control of the genome, epigenome and transcriptome. As indicated in FIG. 1, all macromolecular systems are also subject to direct oxidative damage, and this can accumulate and contribute to overall homeostatic balance of the redox interactome of cells and tissues. These omics domains are often measured as scalar entities with little or no spatial reference, but new advanced tools for mechanistic studies are becoming available to manipulate and measure H_2O_2 and $O_2^{\bullet-}$ and should be used to enable specificity and quantification in analyses and reporting. Enhanced capabilities for spatial and temporal resolution create new opportunities for use of these tools to manipulate and measure changes in omics domains, including epigenome and gene expression, proteomics measures of protein activities, oxidation and abundance, and metabolomics analyses of metabolic responses, support precision in research on oxidants and oxidant signalling. Strategies for redox medicine should build upon understanding of the redox interactome and the new tools used for mechanistic studies. Prior research focused extensively upon balance of production and removal of oxidants. Although this balance remains important, new tools for mechanistic studies and omics capabilities to measure the redox interactome enable more targeted manipulations of nutrients, micronutrients, dietary supplements, health behaviours and therapeutics to impact redox mechanisms underlying physiologic regulation and disease. Ongoing advances in pharmacologic approaches and redox nanomedicine will enable targeted delivery to cellular and subcellular sites. Application of this spectrum of approaches should be incorporated into new diagnostic methods for health and disease evaluations, with an ultimate goal to deliver practical devices for personal monitoring of redox health.

mechanisms specific for a given conditon[149]. The central NRF2 redox sensor system is a prime therapeutic target for cancer[3,150,151] and chronic disease[152]. Several agents that activate NRF2 are available, either as approved drugs or being tested in phase III trials[68], but it is not yet established whether the beneficial effects observed are mediated by the increased NRF2 activity. As with oxidant generation, selective and cell/tissue-specific targeting of oxidant-modulating drugs is needed for effective applications[2,3]. The ability of oxidants to alter the epigenetic landscape[153,154] offers further perspectives for designing effective drugs to modulate redox signalling. Furthermore, it is often the case that multiple causes converge to result in a disease phenotype, thus providing functional hubs to serve as potential therapeutic targets[149]. The emerging field of redox regulation of immunometabolism, with points of control of proliferation, survival and function of T cells, B cells and macrophages via the NRF2 pathway[96], underscores the utility of targeting central regulatory hubs as an approach in redox medicine.

The fine line between beneficial (preventive) or detrimental effects of oxidants and antioxidants is a key challenge for redox medicine. For example, H_2O_2 has both stimulating and inhibiting effects on insulin action (the so-called peroxide dilemma)[155]. Similarly, application of low molecular-mass antioxidants has been shown to have both beneficial and detrimental effects on the progression of several diseases, including cancer[2]. Identifying and exploring the ways to modulate the 'tipping points' between oxidative eustress and distress will be essential steps towards therapeutic applications in redox medicine. In principle, the accumulating knowledge of oxidant signalling via central transcriptional regulatory systems will lead to strategies for enhancing antioxidant defences, redirecting inflammatory signalling, hypoxia adaptation, mitochondrial biogenesis and other key mechanisms to alleviate disease.

Epiproteome
The set of post-translational modifications of the proteome supported by evolved mechanisms for control of protein activities. These are distinct from post-translational modification by reactive environmental chemicals. Many of these modifications, such as phosphorylation, are reversible and are integrated with redox systems in the redox interactome.

Oxystat
A device to maintain a constant O_2 concentration despite fluctuations in the O_2 consumption rate. These generally operate by having an O_2 sensor and feedback system to regulate the rate of introduction of O_2.

Boronates
A family of compounds derived from boric acid of general structure [R–B(OH)$_2$], where R is an alkyl or aryl group. The hydroxyl groups can be derivatized to esters [R–B(OR')$_2$] to form redox-active probes.

Each of these transcriptional systems has multiple positive and negative regulators as well as important targets (such as kinase signalling systems, as well as GAPDH, mitochondrial UCP1 and kinase adaptor proteins), thereby providing different possibilities to enable disease prevention or management.

Technical considerations

There are many experimental challenges associated with defining the biological role of oxidants. This section provides our recommendations on technical approaches that can be used for these experiments. It gives guidance on cell culture conditions, methods for manipulating production and identification of specific oxidant species, and on assessment of their impact on cells and organisms, their effects in cells and organisms, and how to manipulate them.

Recapitulating physiological context in cell culture. Control of environmental factors including ambient O_2 (REF.[156]) and CO_2 (REF.[157]), pH and the composition of media is critical to ensure physiologically relevant cellular function[158]. Culture of primary cells and immortalized cell lines in vitro is routinely conducted under atmospheric O_2 levels, which means that the cells are exposed to hyperoxia, as their physiological O_2 environment in vivo is substantially lower. Such a pro-oxidant environment significantly affects their redox phenotype, upregulates antioxidant defence genes and reduces the replicative lifespan. We thus recommend recapitulating physiological O_2 levels in cell culture to enhance the translation of in vitro findings into in vivo scenarios[156] (BOX 3).

Analytical methods for detecting specific oxidant species. A major challenge is to identify which specific oxidants are produced in living systems and establish when they are produced, in which location and in what quantity, which requires real-time monitoring with live cells. To date, most methodologies have relied on redox-active fluorescent and luminescent probes such as dihydrodichlorofluorescein ($DCFH_2$). These are still commonly used and form the basis of most commercial kits. However, they have significant limitations and give results that are frequently misinterpreted (see TABLE 1 and critical reviews[159–162]). At best, they can provide a preliminary assessment, and we do not recommend using them for analysis of specific oxidants. Small-molecule caged fluorophores are less prone to artefacts (TABLE 1). Below we provide our recommendations for detecting the main oxidant species.

In systems where expression is possible, genetically encoded fluorescent protein probes are recommended for intracellular H_2O_2 detection[163] (BOX 4). These probes provide spatio-temporal detection and can be expressed in single cells, organoids, organs and whole organisms (for example, zebrafish[164], nematodes[165], flies[166,167], frogs[168], mammals[169,170]) (BOX 4). HyPer-based[41,171] and roGFP-based sensors[172,173] are available, with HyPer7 (REF.[41]) and roGFP2-Tsa2ΔC$_R$ (REF.[173]) being the most sensitive[174]. Enzyme-based (chemogenetic) generation of H_2O_2 is a powerful approach to study the role of H_2O_2 using D-amino acid oxidase (DAO) or glucose oxidase (GOX) [175] (BOX 4). Expression of DAO in the cell type of interest and further targeting it to a defined subcellular compartment that is the primary source of oxidant generation is recommended. Chronic exposure to D-amino acids provides a means to mimic pathological oxidative stress, for example by causing cardiac dysfunction through selectively enhancing mitochondrial H_2O_2 production in the heart[176]. Chemogenetic H_2O_2 generators can be co-expressed with genetically encoded probes to visualize subcellular H_2O_2 production and diffusion[41,177,178].

Small-molecule probes can also be used to detect intracellular H_2O_2, provided appropriate caution and caveats are understood (TABLE 1). Probes that react with H_2O_2 to release a caged fluorophore are strongly recommended over redox-active probes, which do not react directly with H_2O_2 (REFS[179–181]). Focus has been on boronates, and a wide range has been developed that enable detection of localized intracellular or transcellular activity of H_2O_2 (REF.[182]). Nevertheless, boronates display low sensitivity to H_2O_2 levels relevant for signalling, which can be a limitation[161,183], and H_2O_2 generation must be distinguished from peroxynitrous acid (ONOOH) and HOCl, which react much more rapidly with these probes[184]. Redox-active probes such as Amplex Red in combination with horseradish peroxidase to trap H_2O_2 reliably measure extracellular or released H_2O_2 (REF.[161]).

Unequivocal evidence for H_2O_2 in intact mammalian tissues and sensitive, quantitative detection of intracellular H_2O_2 can be obtained by optical spectroscopy.

Box 3 | Recommendations for achieving physiological normoxia in cell culture

For standard cell culture, as well as co-culture models and 3D organoids[156,229], we recommend protocols that provide long-term adaptation to in vivo O_2 levels. Standard air incubator O_2 conditions (18% at 75% humidity) result in non-physiological pericellular O_2 for most cells. For lung and aorta, normoxia is ~13% O_2 but for others, including microvascular cells, neurons and stem cells, O_2 needs to be 2–7% (REFS[156,230,231]). To achieve normoxic phenotypes, adaptation to a defined O_2 level for 5 days or more is recommended. Responses to hypoxic O_2 levels are mediated via hypoxia-inducible factor 1α (HIF1α), the stability of which is modulated by oxidant levels[70]. Adaptation is critical for establishing a 'normoxic' phenotype in the absence of HIF1α-mediated signalling[232–234]. This is preferable to acutely lowering ambient O_2 from 18% to <1% O_2.

If cultured cells are not maintained under physiological normoxia, the pro-oxidant environment significantly affects their phenotype. Notably, in endothelial cells, stabilization of HIF1α and HIF1α-mediated hypoxic responses are suppressed[156]. Compared with cells adapted to 5% O_2, endothelial cells at 18% show an approximately twofold increase in nuclear factor erythroid 2 (NRF2)-dependent antioxidant gene expression[234], their replicative lifespan is reduced and embryonic fibroblasts have a decreased ability to upregulate antioxidant defences in response to hydrogen peroxide (H_2O_2) (REF.[235]). Culture of stem cells under physiological O_2 levels (3%) improves cell survival and the potential for tissue repair[156].

To achieve physiological pericellular (in vivo mimetic) O_2 levels in cell culture, workstations are available[156] in which O_2, CO_2, N_2 and humidity can be regulated. Miniaturized O_2-controlled portable platforms enable short-term maintenance of cells under defined O_2 levels for imaging. The permeability of plasticware, volume of medium and rates of O_2 equilibration should be considered. Cellular consumption, together with limited diffusion into solution, generates an O_2 gradient between ambient levels and those experienced by cell monolayers[156,236]. Thus, when cultured under the same O_2 concentration, cell types consuming less O_2 experience a greater intracellular O_2 level compared with cells that consume O_2 more rapidly, such as contracting cardiomyocytes. Feedback-controlled Oxystat systems can be used to maintain O_2 levels[237]. In the absence of an O_2-controlled workstation or system, flasks can be gassed with defined O_2–CO_2–N_2 mixtures and maintained in a Tri-Gas incubator at a preset O_2 value.

Table 1 | **Advantages and disadvantages of redox-active and caged fluorescent or luminescent probes for detection and quantification of oxidants**

Probe type	Redox-active probes	Caged probes
Examples	Dihydrodichlorofluorescein ($DCFH_2$), dihydrorhodamine, hydroethidine (and MitoSOX), luminol, lucigenin and related compounds	Boronates (H_2O_2, $ONOO^-$), sulfonyl fluorescein derivatives ($O_2^{\cdot-}$), thioethers (hypohalous acids)
Mechanism	Oxidized by multistep radical mechanism to fluorescent (luminescent) product	One-step reaction of oxidant with blocking group releases caged fluorescent product by non-oxidative process
Advantages	Sensitive Easy to use Widely available as commercial kits Can provide a read-out of cellular redox environment Used appropriately with a peroxidase, can give sensitive, quantitative detection of extracellular H_2O_2 (Amplex Red)	Less prone to interference by media components and cell metabolites Improved selectivity Reaction with H_2O_2 is direct Standardization possible
Disadvantages and cautions	React non-specifically with a wide range of oxidants Require a transition metal catalyst to react with H_2O_2 Intermediates can react in a multiplicity of ways, including with O_2, $O_2^{\cdot-}$ and antioxidants Variations in interactor concentrations amplify or suppress the signal, independent of the amount of initial oxidant produced Probe can self-generate oxidants through light exposure	Limited commercial availability or in kit form Selective rather than specific Relatively few extensively characterized and specificity fully tested For H_2O_2, slow reactivity can limit sensitivity to physiological levels Boronate assays must be independently validated to distinguish H_2O_2 from ONOOH, HOCl or other species
Recommendations	Be aware of multiple potential pitfalls Avoid if possible or use as an indicator of cellular redox environment and preliminary assay for further analysis Do not rely on these assays alone Do not assign to a specific oxidant unless independently validated Regardless of whether used as a kit, or specified by the supplier, always include appropriate controls and interpret results cautiously	Preferred as are less prone to interference and show improved specificity, but assignment to a specific oxidant requires validation

H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $O_2^{\cdot-}$, superoxide anion radical; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid.

Cytochrome *c*
A small (~12 kDa) haeme protein usually found loosely associated with the outer face of the inner mitochondrial membrane where it functions to transfer electrons between complex III and complex IV of the electron transport chain (ETC) via cycling between the Fe^{3+} and Fe^{2+} states. Its release into the cytosol is commonly used as a marker of mitochondrial damage and apoptosis.

Tetrazolium salts
Salts (including MTT, XTT, MTS and WSTs) with a tetra-nitrogen heterocycle that are reduced by the superoxide anion radical ($O_2^{\cdot-}$) and also act as substrates for active cellular dehydrogenases and reductases. In the presence of NADH/NADPH, they are reduced to formazans which have strong, distinct optical absorption spectra. Widely used as a means of distinguishing metabolically active from inactive (dead) cells.

Lucigenin
An organic compound (10,10′-dimethyl-9,9′-bisacridinium nitrate) used, often inappropriately, as a chemiluminescent probe for the detection of superoxide anion radical ($O_2^{\cdot-}$) in cells and tissue.

Luciferin
A small-molecule substrate for the enzyme luciferase that reacts with oxygen to release energy as light.

A specific optical read-out for H_2O_2 is the charge transfer band of catalase Compound I (~660 nm) in the difference spectrum with the resting enzyme[185]. Using this method, H_2O_2 was first detected as a physiological metabolite in normal aerobic cell metabolism in perfused liver[13]. Steady-state titration with a hydrogen donor for Compound I, for example methanol, permits determination of the rate of H_2O_2 production per gram of tissue[186].

Another approach to monitor H_2O_2 is to take advantage of its high reactivity with peroxiredoxins[16,40]. The distinct locations and binding partners of the five human 2-Cys forms of peroxiredoxins constitute a basis for selective H_2O_2 signalling[17]. The redox state of peroxiredoxins is monitored by separating the oxidized dimer from the reduced monomer[187], and real-time monitoring is possible using genetically encoded fluorescent analogues of these enzymes[188].

No genetically encoded probes for $O_2^{\cdot-}$ are currently available. The best validated fluorescent probe is hydroethidine, or its mitochondrially targeted analogue MitoSOX. Importantly, an increase in fluorescence of these probes alone is not sufficient evidence for $O_2^{\cdot-}$ generation, because hydroethidine is oxidized non-specifically to give fluorescent ethidium[189]. Specificity requires validation by chromatography or mass-spectrometric post analysis of the $O_2^{\cdot-}$-generated product, 2-hydroxyethidium[190], and interpretation of 2-hydroxyethidium formation is subject to caveats[189]. Improved, although not yet widely used, fluorescent probes based on NeoD are more selective and also more physiological, as they do not intercalate with DNA, unlike ethidine compounds[191]. Other sensors for detection and imaging of $O_2^{\cdot-}$ (REFS[37,192]), including redox probes that show a preference for $O_2^{\cdot-}$ over H_2O_2 and other oxidants[193], have promise but require more testing. Assays using cytochrome *c* or tetrazolium salts[194] provide reliable quantification of $O_2^{\cdot-}$ released by inflammatory cells but lack sensitivity with cells that produce less $O_2^{\cdot-}$. Although greater sensitivity is obtained from the chemiluminescent reaction of $O_2^{\cdot-}$ with lucigenin, the assay itself generates $O_2^{\cdot-}$, so is self-defeating in reporting physiological $O_2^{\cdot-}$ generation[159]. Luciferin analogues such as coelenterazine produce less self-generated $O_2^{\cdot-}$, but the

L-NAME
The L isomer of N^G-nitro-arginine methyl ester, which is used as an inhibitor of nitric oxide synthase enzymes.

read-out of $O_2^{\cdot-}$ levels is still confounded by $O_2^{\cdot-}$ reacting with other radicals in a chain reaction[159]. The most definitive evidence for $O_2^{\cdot-}$ uses electron paramagnetic (spin) resonance spectroscopy with spin trapping[195]. Signals are characteristic, and this method is recommended for unequivocal identification of $O_2^{\cdot-}$. However, limited sensitivity plus the metabolism of spin adducts within cells restricts it applicability. Immuno-spin trapping, which combines the specificity of spin trapping and the high sensitivity of immunological techniques[196,197], is useful for detecting stable adducts generated from radical reactions.

Reactive nitrogen species and RHS contribute to oxidative eustress and distress, and need to be distinguished from other oxidants. $ONOO^-$ can be monitored in cells in real time using boronate probes[161,184,198], but needs to be distinguished from H_2O_2 using appropriate inhibitors or scavengers, such as L-NAME to inhibit nitric oxide synthase. Hypochlorous acid (HOCl) is a major oxidant produced by inflammatory cells, and numerous HOCl-sensitive probes have been described[199]; they also react with most other RHS. A genetically encoded RHS probe, Hypocrates, was developed, which likely will become a useful tool in systems where expression of transgenes is possible[200].

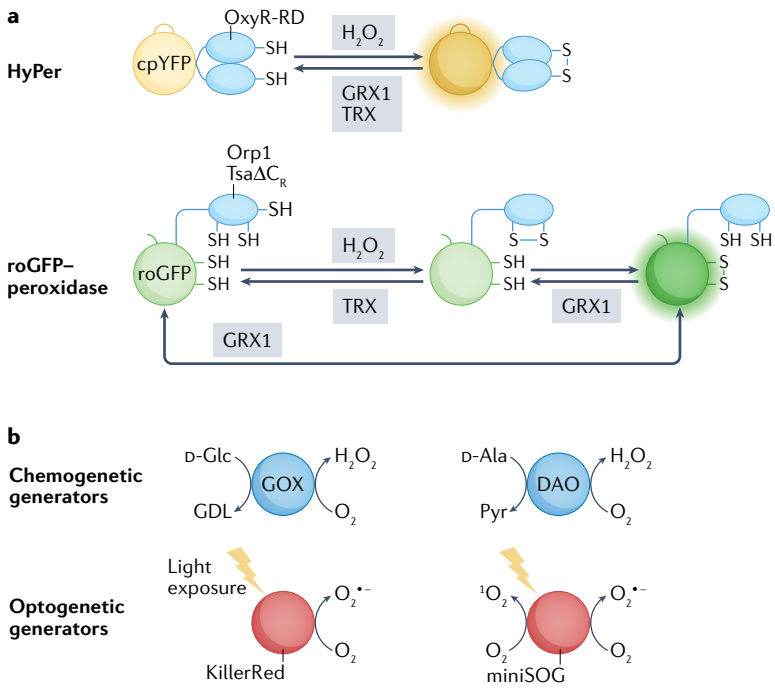
To sum up, newer-generation genetically encoded sensors and small-molecule probes are now available for detection of oxidants in cellular and organismal studies, and we recommend that they are used rather than the common redox probes and kit assays. There are multiple promising approaches under development that use novel technologies. For $O_2^{\cdot-}$, these include a nanoprobe based on chemiluminescence resonance energy transfer[201], and a positron emission tomography radiotracer[202]. For studies of $O_2^{\cdot-}$ and/or singlet molecular oxygen ($^1O_2$) on the time scales of seconds, genetically encoded (optogenetic)

Box 4 | **Genetically encoded tools for specific oxidant detection and production in cells and organisms**

Genetically encoded fluorescent probes for H_2O_2

These include the HyPer and roGFP-based sensors (see the figure, part **a**). Both consist of a modified fluorescent protein linked to a hydrogen peroxide (H_2O_2) sensing domain that translates oxidation by H_2O_2 into ratiometric changes in the fluorescence spectrum. HyPer7 (REF.[41]) and roGFP2-Tsa2ΔC$_R$ (REF.[173]) are recent versions that detect H_2O_2 in the low-nanomolar range. The chemical basis of probe function is oxidation of Cys residues in the fluorescent protein to a disulfide, which is reversed by cellular thiol reducing systems. Therefore, the oxidation state of the probe reflects both the presence of H_2O_2 and reducing system activity. Advantages over synthetic dyes include subcellular localization to the organelle of interest, expression in transgenic animals under cell and tissue-specific promoters, and greater selectivity and less phototoxicity compared with synthetic dyes. Successful applications in model organisms have been performed, for example, in zebrafish[164], *Caenorhabditis elegans*[165], *Drosophila melanogaster*[166], *Xenopus laevis*[168] and mammals[169].

In HyPer probes, oxidation of the OxyR regulatory domain (OxyR-RD) directly induces conformational rearrangement of the integrated circularly permuted fluorescent protein (cpFP). roGFP-based probes operate by a multistep mechanism in which H_2O_2 oxidizes the peroxidase domain and then a redox relay from the peroxidase leads to oxidation of roGFP. Oxidized roGFP can be reduced by the glutathione (GSH)-dependent reducing system. The peroxidase Orp1 can be also reduced by thioredoxin (TRX). roGFP alone or fused with non-peroxidase thiol exchange domains (for example, glutaredoxin 1 (GRX1)-roGFP2) should not be used to detect oxidants as they, rather, sense the redox state of the cellular thiol pool. Even when roGFP is linked to an H_2O_2 sensor, this sensitivity remains. HyPer7 and roGFP2-based H_2O_2 probes give pH-stable signals, but HyPer1–3 and HyPerRed are pH-sensitive and should be used only in combination with an appropriate pH control (SypHer)[238]. HyPer7 and roGFP2-Tsa2ΔC$_R$ are preferred for detecting basal intracellular H_2O_2 levels. However, under conditions of high H_2O_2 these can be completely oxidized, and less sensitive probes (for example, HyPer1–3, HyPerRed, roGFP2-Orp1) should be used.



Chemogenetic tools for generation of oxidants

Chemogenetic approaches to modulating H_2O_2 generation use glucose oxidase (GOX) or D-amino acid oxidase (DAO) (see the figure, part **b**, top). GOX oxidizes glucose, releasing H_2O_2 (REF.[239]), and when added to cell culture medium containing glucose, allows controlled steady-state extracellular H_2O_2 production. The presence of glucose in the cell culture medium does not allow substrate control, but H_2O_2 production can be regulated by enzyme concentration. By contrast, DAO can be expressed intracellularly and regulated by the D-alanine concentration[175,240]. The system can be calibrated using HyPer probes. Heterologous yeast DAO is stereoselective and inactive in the absence of D-amino acids that are minimally present in mammalian cells. Addition of D-amino acids, which are taken up by non-stereoselective amino acid transporters, results in the generation of nanomolar to low-micromolar H_2O_2.

Optogenetic tools for generation of oxidants

Optogenetic probes use fluorescent proteins with 'open' structures that enable their photo-excited chromophores to interact with O_2, generating superoxide anion radical ($O_2^{\cdot-}$) or singlet molecular oxygen ($^1O_2$) on illumination with light of appropriate wavelength (see the figure, part **b**, bottom). These genetically encoded photosensitizers include GFP-like proteins (for example, KillerRed, KillerOrange or SuperNova[241–243]) or flavin mononucleotide-binding domain proteins (miniSOG)[244]. A limitation of these tools is the low tissue penetration of visible light, mainly restricting their use to cell culture experiments.

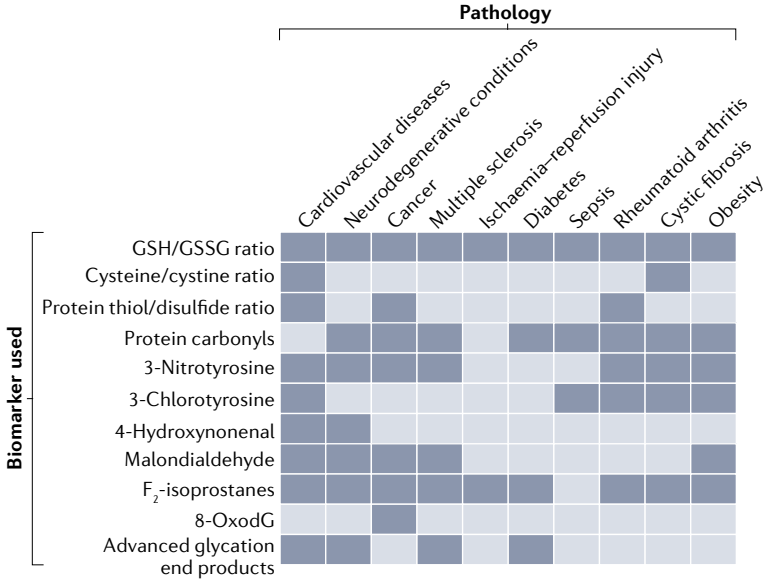
photosensitizers are recommended[203,204] (BOX 4). Examples of their use include inactivation of certain cellular proteins and induction of photoinduced cell death[203,205]. For H_2O_2, surface-enhanced Raman spectroscopy shows promise[206], with nanosensors anchored to the outer surface of the cell, or mitochondrially localized, allowing site-specific detection[34,207]. Electron paramagnetic resonance in combination with low-field magnetic resonance imaging to delineate tissue architecture has been used to obtain information on in vivo redox status[208]. Single-photon counting of low-level (ultra-weak) chemiluminescence generated by the transition of electrons from an electronically excited state to the ground state in carbonyl groups of cellular metabolites has been used for dynamic monitoring of oxidative stress metabolism in intact cells and organs[209,210]. We recommend that redox biology researchers monitor upcoming technological developments and adapt their experimental design to use state-of-the-art techniques as they become available.

Oxidant biomarkers and multi-omics approaches. To probe the roles of oxidants in biology, development of reliable biomarkers of redox reactions is essential. Ideal techniques provide detection, identification, location and ready quantification, enabling changes in oxidant levels and the associated molecular change to be inferred[211]. The most commonly used technique for detecting biomarkers of redox reactions is mass spectrometry-based proteomics. Here, oxidation products can be determined directly or after chemical derivatization. The latter increases sensitivity but often prohibits accurate quantification as derivatization is usually not 100% efficient. Enrichment techniques, for example specific binding columns and immunoprecipitation[212,213], allow mass spectrometry methods to be used on complex systems including ex vivo human samples. Mass spectrometry analysis can be carried out at various levels: the level of individual components after processing to release these; the peptide level for proteins; or the intact molecule level (for example, phospholipids, cholesterol esters, proteins, lipoproteins). Each has advantages and disadvantages (BOX 5). Mass spectrometry-based analysis at a single-cell level is now within reach[214].

The synthesis of data from different 'omic' methods (for example, proteomics, transcriptomics, metabolomics, epigenomics) is an important tool in biomarker discovery. For example, protein glycosylation pathways enormously amplify the proteome[215], and there are fascinating reciprocal interrelationships between glycan biosynthesis and the redox state[216]. The emerging field of 'glyco-redox'[217] may uncover relations between the glycome, glycoproteome and redox proteomes. A key advantage of 'omic' methods is that they simultaneously provide information on multiple oxidant targets[218]. The rapid development in 'omic' techniques is transforming current understanding of the molecular details of oxidant signalling (BOX 5). Measurements of mitochondrial H_2O_2 production with mitoPy1 (a targeted boronate probe), MitoSOX oxidation, aconitase oxidation, cellular thiol oxidation, transcriptomics, metabolomics and redox proteomics in a cell system exposed to increasing doses of manganese recently showed that signalling responses are integrated across the 'omic' layers; that is, they induce effects at the RNA, protein and metabolite levels[219]. Readily accessible databases, such as the Oximouse data set for redox proteomics[59], Metabolomics Workbench for metabolomics and Gene Expression Omnibus for transcriptomics, allow mining of pre-existing data to enhance statistical power of analysis of redox targets and avoid costly experimental duplication. From the perspective of redox medicine, measuring a combination or patterns of various biomarkers across the 'omics' spectrum is generally recommended for clinical studies evaluating oxidative stress[220–223], and can be used as a diagnostic for diseases associated with excessive oxidant generation (for example, cardiovascular disease, neurodegenerative disease, diabetes, obesity)[224].

Genetic and pharmacological manipulation of oxidant production. In order to determine the function of a specific oxidant and assess its impact on physiology, it is helpful to be able to modulate its subcellular production and monitor the effects of this manipulation. This can be achieved using genetic or pharmacological approaches. These can be applied to specific oxidants and their production in a specific subcompartment, although off-target effects limit both approaches[149]. A genetic approach to diminish mitochondrial $O_2^{\cdot-}$ is the expression of the alternative oxidase (AOX) from *Ciona intestinalis*[225], which takes electrons from ubiquinol and transfers them directly to O_2 to form water. This limits electron flow from ubiquinol to complex I or III of the ETC, thereby also lowering the $O_2^{\cdot-}$ generation associated with these complexes[226]. A direct way to assess the impact of H_2O_2 is to genetically express catalase targeted to different subcellular compartments. Mice have been generated that overexpress catalase in either the nucleus (nCAT) or mitochondria (mCAT), as well as wild-type peroxisomal catalase (pCAT) to study ageing and age-related diseases[110]. Strikingly, mCAT mice, unlike pCAT or nCAT mice, displayed a significant increase in maximal and median lifespan[110]. mCAT overexpression diminished insulin resistance, atherosclerosis, cardiac failure, pulmonary hypertension, muscle atrophy and various types of cancers[109]. Conditional overexpression of mCAT in adult astrocytes demonstrated that mitochondrial H_2O_2 is necessary to control astrocyte metabolism and supports normal cognitive function[100]. Future work involving conditional organelle overexpression of catalase in different cells will help elucidate the requirement for H_2O_2 in physiology and its contributions to pathology. Oxidant modulation can also be achieved by conditional knockout of oxidant-generating enzymes. Such a strategy is particularly encouraged for the dissection of the role of the different NOXs in physiology. This needs to take into account the regional and cellular localization of NOXs as examined, for example, for brain diseases[227]. Conditional knockouts of NOX4 in either cancer or stromal cells indicated that NOX4 production of H_2O_2 specifically within cancer cells, and not stroma, prevents the initiation of carcinogen-induced cancer[228]. An exciting development in the field has been the translation of the NOX1/NOX4 inhibitor setanaxib (previously known as GKT137831) into clinical trials as a drug to treat fibrotic diseases[147].

Box 5 | **Biomarkers of oxidant signalling in biology and disease**



Many biomarkers of oxidant-induced modifications of proteins, lipids, nucleic acids and carbohydrates have been studied and, although useful, often do not measure species directly involved in redox-dependent signalling but, rather, indicate oxidative damage as surrogate markers. For clinical assessments, patterns rather than single biomarkers are recommended (see the figure; modified from REF.[224]).

Oxidation products of proteins and peptides include oxidized Cys in proteins and glutathione (GSH) (with the Oximouse data set[59] recommended as a key resource), which provides information on redox balance; protein carbonyls (formed directly or from hydroperoxides), which provide a general marker of oxidative stress and correlate with some diseases; products formed by particular reactive species (3-nitrotyrosine, 6-nitrotryptophan from NO_2/peroxynitrous acid (ONOOH); 3-chlorotyrosine from hypochlorous acid (HOCl)/myeloperoxidase), which correlate with some diseases; and protein glycation biomarkers, including advanced glycation end products (AGE)[245], which provide information on specific protein modifications and the oxidants involved.

Detection of protein oxidation products can be achieved via mass spectrometry[246,247]. Technical advances that improve mass spectrometry sensitivity now allow increasingly smaller samples to be examined, for example from laser dissection of tissues[248], and proteomic analysis of single cells obtained by fluorescence-activated cell sorting[249]. Methods that target modifications at specific residues, such as redox western and biotin-conjugated iodoacetamide (BIAM) blots, are also available to measure oxidation of protein thiols. For instance, 2-Cys peroxiredoxin oxidation by hydrogen peroxide (H_2O_2) causes a monomer–dimer conversion detectable by non-reducing SDS-PAGE[187] and has been used along with redox western analysis of thioredoxins (TRXs) to measure compartmental H_2O_2 production. Derivatization of Cys sulfenic acid (RSOH) species using dimedone-type probes[250] and of carbonyls using hydrazines requires long incubation times, although reagents that allow enrichment of the product species[251] and those with higher rate constants are being developed to allow greater temporal resolution[250].

Biomarkers for oxidized polyunsaturated fatty acids (PUFAs) and phospholipids include F_2-isoprostanes[252], 4-hydroxynonenal (HNE) and related aldehydes[253]. F_2-isoprostane levels correlate with disease. Cholesterol oxidation products 7-ketocholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol, measured by mass spectrometry, can indicate oxidant production and correlate with disease.

Oxidative products of DNA and RNA have been extensively studied for oxidative DNA and RNA damage[254,255]. Oxidatively generated base modifications in DNA have been implicated in redox signalling[256]. The main nucleic acid oxidation products are 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG, 8-OH-deoxyguanosine; from DNA) and 7,8-dihydro-8-oxo-guanosine (8-oxoG; from RNA). These are detectable in plasma, urine, cells and tissues. Elevated 8-oxodG and 8-oxoG correlate with ageing and multiple diseases. Their detection is performed via antibody-based assays (such as ELISA) or liquid chromatography combined with ultraviolet, electrochemical or mass spectrometry detection. In addition to individual nucleobase-derived products, other forms of oxidative DNA damage (strand breaks, DNA adducts, excision repair sites and cross-links) can be detected by the Comet assay (single-cell gel electrophoresis)[257].

Chlorinated products are formed by HOCl, and together with the enzyme myeloperoxidase (that generates HOCl), are markers of inflammation and correlate with disease. Nitrated products (8-nitro-2′-deoxyguanosine, 8-nitro-guanosine) are generated by peroxynitrite ($ONOO^-$) and myeloperoxidase and can be detected by mass spectrometry.

The glutathione disulfide (GSSG) to GSH ratio (cells or tissues) and the cystine to cysteine ratio (plasma) are oxidative stress markers and are perturbed in many diseases. Plasma cystine and GSH concentrations are independent predictors of death in patients with coronary artery disease[258]. The serum free thiol status associates with disease outcome[259].

A valuable pharmacological tool has been provided by S1QEL and S3QEL small molecules[36]. These compounds inhibit the electron transfer to oxygen in complex I and complex III, respectively, without affecting forward electron transfer to complex IV through the ETC. Their use could alleviate various pathologies associated with $O_2^{\cdot-}$. These molecules have advantages over genetic strategies, including specificity for particular sites in the respiratory complexes and relative ease in experimental usage[36]. However, a caveat is that it is not known how they prevent $O_2^{\cdot-}$ production without interfering with the normal function of the ETC.

Concluding remarks

New roles for H_2O_2 and $O_2^{\cdot-}$, the major physiologically occurring oxidants, in redox signalling are emerging throughout cell biology and biomedical research. These oxidants impact master regulators of gene transcription and cell homeostasis, with pleiotropic functions in physiology and pathology. It is clear that future studies to develop redox-based therapeutics require detailed understanding and quantification of the species involved in signalling, as well as their sites and rates of production within cells. This requires more precise definitions of oxidants and improved analytical tools, including the development of real-time imaging methods which detect localized oxidant generation and their signal transduction. These advances should lead to rational modulation of processes such as antioxidant defences, mitochondrial biogenesis, cell quality control, or hypoxia adaptation to improve health or counteract disease. We hope that the recommendations provided here (BOX 1) will form a foundation to harmonize research among the many scientific disciplines working towards the common goal of understanding redox biology and its translation to redox medicine.

Published online: 21 February 2022

1. Sies, H. & Jones, D. P. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nat. Rev. Mol. Cell Biol.* **21**, 363–383 (2020).
2. Forman, H. J. & Zhang, H. Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. *Nat. Rev. Drug Discov.* **20**, 689–709 (2021).
3. Hayes, J. D., Dinkova-Kostova, A. T. & Tew, K. D. Oxidative stress in cancer. *Cancer Cell* **38**, 167–197 (2020).
4. Halliwell, B., Gutteridge, J. M. C. *Free Radicals in Biology and Medicine* 5th edn (Oxford Univ. Press, 2015).
This work is an essential textbook for everyone studying redox biology.
5. Schieber, M. & Chandel, N. S. ROS function in redox signaling and oxidative stress. *Curr. Biol.* **24**, R453–R462 (2014).
6. Holmstrom, K. M. & Finkel, T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat. Rev. Mol. Cell Biol.* **15**, 411–421 (2014).
7. Jones, D. P. & Sies, H. The redox dode. *Antioxid. Redox. Signal.* **23**, 734–746 (2015).
This work is a comprehensive review of the underlying principles of redox organization.
8. Egea, J. et al. European contribution to the study of ROS: a summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS). *Redox. Biol.* **13**, 94–162 (2017).
9. Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q. & Griendling, K. K. Reactive oxygen species in metabolic and inflammatory signaling. *Circ. Res.* **122**, 877–902 (2018).
10. Parvez, S., Long, M. J. C., Poganik, J. R. & Aye, Y. Redox signaling by reactive electrophiles and oxidants. *Chem. Rev.* **118**, 8798–8888 (2018).
This work is a comprehensive review of the role of electrophiles in redox biology.
11. Murphy, M. P. et al. Unraveling the biological roles of reactive oxygen species. *Cell Metab.* **13**, 361–366 (2011).
12. Brandes, R. P., Rezende, F. & Schröder, K. Redox regulation beyond ROS: why ROS should not be measured as often. *Circ. Res.* **123**, 326–328 (2018).
13. Sies, H. & Chance, B. The steady state level of catalase Compound I in isolated hemoglobin-free perfused rat liver. *FEBS Lett.* **11**, 172–176 (1970).
This paper establishes H_2O_2 as a physiologically occurring metabolite in eukaryotic cells.
14. Chance, B., Sies, H. & Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59**, 527–605 (1979).
15. Forman, H. J., Bernardo, A. & Davies, K. J. A. What is the concentration of hydrogen peroxide in blood and plasma? *Arch. Biochem. Biophys.* **603**, 48–53 (2016).
16. Stöcker, S., Van, L. K., Mijuskovic, A. & Dick, T. P. The conundrum of hydrogen peroxide signaling and the emerging role of peroxiredoxins as redox relay hubs. *Antioxid. Redox Signal.* **28**, 558–573 (2018).
This paper describes how redox signals are transmitted by protein redox relays.
17. van Dam, L. et al. The human 2-Cys peroxiredoxins form widespread, cysteine-dependent- and isoform-specific protein–protein interactions. *Antioxid. (Basel)* https://doi.org/10.3390/antiox10040627 (2021).
18. Forman, H. J., Ursini, F. & Maiorino, M. An overview of mechanisms of redox signaling. *J. Mol. Cell Cardiol.* **73**, 2–9 (2014).
19. Wensien, M. et al. A lysine-cysteine redox switch with an NOS bridge regulates enzyme function. *Nature* **593**, 460–464 (2021).
20. Cortese-Krott, M. M. et al. The reactive species interactome: evolutionary emergence, biological significance, and opportunities for redox metabolomics and personalized medicine. *Antioxid. Redox Signal.* **27**, 684–712 (2017).
This paper describes how reactive species react with themselves and their targets.
21. Malard, E., Valable, S., Bernaudin, M., Pêrès, E. & Chatre, L. The reactive species interactome in the brain. *Antioxid. Redox Signal.* **35**, 1176–1206 (2021).
22. Buettner, G. R. Superoxide dismutase in redox biology: the roles of superoxide and hydrogen peroxide. *Anticancer. Agents Med. Chem.* **11**, 341–346 (2011).
23. Lushchak, O. V., Piroddi, M., Galli, F. & Lushchak, V. I. Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species. *Redox Rep.* **19**, 8–15 (2014).
24. Echtay, K. S. et al. Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**, 96–99 (2002).
25. Lillig, C. H. et al. Characterization of human glutaredoxin 2 as iron–sulfur protein: a possible role as redox sensor. *Proc. Natl Acad. Sci. USA* **102**, 8168–8173 (2005).
26. Wang, Y., Branicky, R., Noë, A. & Hekimi, S. Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. *J. Cell Biol.* **217**, 1915–1928 (2018).
27. Singh, C. K. et al. The role of sirtuins in antioxidant and redox signaling. *Antioxid. Redox Signal.* **28**, 643–661 (2018).
28. Sun, Y. et al. ROS systems are a new integrated network for sensing homeostasis and alarming stresses in organelle metabolic processes. *Redox Biol.* **37**, 101696 (2020).
29. Herb, M., Gluschko, A. & Schramm, M. Reactive oxygen species: not omnipresent but important in many locations. *Front. Cell Dev. Biol.* **9**, 716406 (2021).
30. Santolini, J., Wootton, S. A., Jackson, A. A. & Feelisch, M. The redox architecture of physiological function. *Curr. Opin. Physiol.* **9**, 34–47 (2019).
31. Nordzieke, D. E. & Medraño-Fernandez, I. The plasma membrane: a platform for intra- and intercellular redox signaling. *Antioxidants* **7**, 7110168 (2018).
32. Schroeder, K. NADPH oxidases: current aspects and tools. *Redox Biol.* **34**, 101512 (2020).
33. Zhang, Y. & Wong, H. S. Are mitochondria the main contributor of reactive oxygen species in cells? *J. Exp. Biol.* **224**, 221606 (2021).
34. Hosogi, S. et al. Plasma membrane anchored nanosensor for quantifying endogenous production of H_2O_2 in living cells. *Biosens. Bioelectron.* **179**, 113077 (2021).
35. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1–13 (2009).
This paper describes how mitochondria produce ROS.
36. Brand, M. D. Riding the tiger — physiological and pathological effects of superoxide and hydrogen peroxide generated in the mitochondrial matrix. *Crit. Rev. Biochem. Mol. Biol.* **55**, 592–661 (2020).
37. Mailloux, R. J. An update on methods and approaches for interrogating mitochondrial reactive oxygen species production. *Redox Biol.* **45**, 102044 (2021).
38. Chouchani, E. T. et al. A unifying mechanism for mitochondrial superoxide production during ischemia–reperfusion injury. *Cell Metab.* **23**, 254–263 (2016).
39. Cox, A. G., Winterbourn, C. C. & Hampton, M. B. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. *Biochem. J.* **425**, 313–325 (2009).
40. Bolduc, J. et al. Peroxiredoxins wear many hats: factors that fashion their peroxide sensing personalities. *Redox Biol.* **42**, 101959 (2021).
41. Pak, V. V. et al. Ultrasensitive genetically encoded indicator for hydrogen peroxide identifies roles for the oxidant in cell migration and mitochondrial function. *Cell Metab.* **31**, 642–653 (2020).
This paper describes the most recent version of HyPer probes and reveals new details of mitochondrial H_2O_2 transport.
42. Malinouski, M., Zhou, Y., Belousov, V. V., Hatfield, D. L. & Gladyshev, V. N. Hydrogen peroxide probes directed to different cellular compartments. *PLoS ONE* **6**, e14564 (2011).
43. Walker, C. L., Pomatto, L. C. D., Tripathi, D. N. & Davies, K. J. A. Redox regulation of homeostasis and proteostasis in peroxisomes. *Physiol. Rev.* **98**, 89–115 (2018).
This work is a comprehensive review of peroxisomal redox regulation.
44. Fransen, M. & Lismont, C. Redox signaling from and to peroxisomes: progress, challenges, and prospects. *Antioxid. Redox Signal.* **30**, 95–112 (2019).
45. Schrader, M., Kamoshita, M. & Islinger, M. Organelle interplay–peroxisome interactions in health and disease. *J. Inherit. Metab. Dis.* **43**, 71–89 (2020).
46. Gebicka, L. & Krych-Madej, J. The role of catalases in the prevention/promotion of oxidative stress. *J. Inorg. Biochem.* **197**, 110699 (2019).
47. Fujiki, Y. & Bassik, M. C. A new paradigm in catalase research. *Trends Cell Biol.* **31**, 148–151 (2021).
48. Okumoto, K. et al. The peroxisome counteracts oxidative stresses by suppressing catalase import via Pex14 phosphorylation. *eLife* **9**, e55896 (2020).
49. Roscoe, J. M. & Sevier, C. S. Pathways for sensing and responding to hydrogen peroxide at the endoplasmic reticulum. *Cells* **9**, 2314 (2020).
50. Bestetti, S. et al. Human aquaporin-11 guarantees efficient transport of H_2O_2 across the endoplasmic reticulum membrane. *Redox Biol.* **28**, 101326 (2020).
51. Millare, B., O'Rourke, B. & Trayanova, N. Hydrogen peroxide diffusion and scavenging shapes mitochondrial network instability and failure by sensitizing ROS-induced ROS release. *Sci. Rep.* https://doi.org/10.1038/s41598-020-71308-z (2020).
52. Alshaabi, H. et al. Miro1-mediated mitochondrial positioning supports subcellular redox status. *Redox Biol.* **38**, 101818 (2021).
53. Csordas, G., Weaver, D. & Hajnoczky, G. Endoplasmic reticulum–mitochondrial contactology: structure and signaling functions. *Trends Cell Biol.* **28**, 523–540 (2018).
54. Yoboue, E. D., Sitia, R. & Simmen, T. Redox crosstalk at endoplasmic reticulum (ER) membrane contact sites (MCS) uses toxic waste to deliver messages. *Cell Death. Dis.* **9**, 331 (2018).
55. Tanaka, L. Y., Oliveira, P. V. S. & Laurindo, F. R. M. Peri/epicellular thiol oxidoreductases as mediators of extracellular redox signaling. *Antioxid. Redox Signal.* **33**, 280–307 (2020).
56. Caserta, S. & Ghezzi, P. Release of redox enzymes and micro-RNAs in extracellular vesicles, during infection and inflammation. *Free Radic. Biol. Med.* **169**, 248–257 (2021).
57. Bauer, G. Inhibition of membrane-associated catalase, extracellular ROS/RNS signaling and aquaporin/H_2O_2-mediated intracellular glutathione depletion cooperate during apoptosis induction in the human gastric carcinoma cell line MKN-45. *Antioxid. (Basel)* https://doi.org/10.3390/antiox10101585 (2021).
58. Glorieux, C. & Calderon, P. B. Catalase, a remarkable enzyme: targeting the oldest antioxidant enzyme to find a new cancer treatment approach. *Biol. Chem.* **398**, 1095–1108 (2017).
59. Xiao, H. et al. A quantitative tissue-specific landscape of protein redox regulation during aging. *Cell* **180**, 968–983 (2020).
This paper describes Oximouse, a quantitative mapping data set of the mouse cysteine redox proteome in vivo.
60. Keßler, M., Wittig, I., Ackermann, J. & Koch, I. Prediction and analysis of redox-sensitive cysteines using machine learning and statistical methods. *Biol. Chem.* **402**, 925–935 (2021).
61. Pei, J. F. et al. Diurnal oscillations of endogenous H_2O_2 sustained by $p66^{Shc}$ regulate circadian clocks. *Nat. Cell Biol.* **21**, 1553–1564 (2019).
62. Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **4**, 278–286 (2008).
This work is a comprehensive overview of the chemical principles that underly the biology of ROS.
63. Travasso, R. D. M., Sampaio Dos Aidos, F., Bayani, A., Abranches, P. & Salvador, A. Localized redox relays as a privileged mode of cytoplasmic hydrogen peroxide signaling. *Redox Biol.* **12**, 233–245 (2017).
64. Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J. & Toledano, M. B. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* **111**, 471–481 (2002).
65. Woo, H. A. et al. Inactivation of peroxiredoxin I by phosphorylation allows localized H_2O_2 accumulation for cell signaling. *Cell* **140**, 517–528 (2010).
66. Marinho, H. S., Real, C., Cyrne, L., Soares, H. & Antunes, F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol.* **2**, 535–562 (2014).
67. Held, J. M. Redox systems biology: harnessing the sentinels of the cysteine redoxome. *Antioxid. Redox Signal.* **32**, 659–676 (2020).
68. Yamamoto, M., Kensler, T. W. & Motohashi, H. The KEAP1–NRF2 system: a thiol-based sensor–effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* **98**, 1169–1203 (2018).
This work is a comprehensive review of the mechanism, biology and medical aspects of the KEAP1-NRF2 system that regulates oxidative and electrophilic responses.
69. Mitchell, S., Vargas, J. & Hoffmann, A. Signaling via the NFκB system. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **8**, 227–241 (2016).
70. Lee, P., Chandel, N. S. & Simon, M. C. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. *Nat. Rev. Mol. Cell Biol.* **21**, 268–283 (2020).

71. Scholtes, C. & Giguère, V. Transcriptional regulation of ROS homeostasis by the ERR subfamily of nuclear receptors. *Antioxid. (Basel)* https://doi.org/10.3390/antiox10030437 (2021).
72. Klotz, L. O. et al. Redox regulation of FoxO transcription factors. *Redox Biol.* **6**, 51–72 (2015).
73. Soh, R., Hardy, A. & Zur Nieden, N. I. The FOXO signaling axis displays conjoined functions in redox homeostasis and stemness. *Free Radic. Biol. Med.* **169**, 224–237 (2021).
74. Rius-Pérez, S., Torres-Cuevas, I., Millán, I., Ortega, Å. L. & Pérez, S. PGC-1α, inflammation, and oxidative stress: an integrative view in metabolism. *Oxid. Med. Cell Longev.* **2020**, 1452696 (2020).
75. Shi, T. & Dansen, T. B. Reactive oxygen species induced p53 activation: DNA damage, redox signaling, or both? *Antioxid. Redox Signal.* **33**, 839–859 (2020).
76. Herzig, S. & Shaw, R. J. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat. Rev. Mol. Cell Biol.* **19**, 121–135 (2018).
77. Peralta, D. et al. A proton relay enhances H_2O_2 sensitivity of GAPDH to facilitate metabolic adaptation. *Nat. Chem. Biol.* **11**, 156–163 (2015).
78. Labunskyy, V. M., Hatfield, D. L. & Gladyshev, V. N. Selenoproteins: molecular pathways and physiological roles. *Physiol. Rev.* **94**, 739–777 (2014).
This work is a comprehensive review of the central role of selenoproteins in redox biology.
79. Duarte, T. L., Talbot, N. P. & Drakesmith, H. NRF2 and hypoxia-inducible factors: key players in the redox control of systemic iron homeostasis. *Antioxid. Redox Signal.* **35**, 433–452 (2021).
80. Sies, H., Berndt, C. & Jones, D. P. Oxidative stress. *Annu. Rev. Biochem.* **86**, 715–748 (2017).
This work is a comprehensive review of oxidative stress, eustress and distress.
81. Selye, H. Stress and distress. *Compr. Ther.* **1**, 9–13 (1975).
82. Sies, H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. *Redox. Biol.* **11**, 613–619 (2017).
83. Ursini, F., Maiorino, M. & Forman, H. J. Redox homeostasis: the golden mean of healthy living. *Redox. Biol.* **8**, 205–215 (2016).
84. Lushchak, V. I. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem. Biol. Interact.* **224C**, 164–175 (2014).
85. Handy, D. E. & Loscalzo, J. Responses to reductive stress in the cardiovascular system. *Free Radic. Biol. Med.* **109**, 114–124 (2017).
86. Manford, A. G. et al. A cellular mechanism to detect and alleviate reductive stress. *Cell* **183**, 46–61 (2020).
87. Zhang, L. et al. Biochemical basis and metabolic interplay of redox regulation. *Redox Biol.* **26**, 101284 (2019).
88. Calabrese, E. J. & Kozumbo, W. J. The hormetic dose-response mechanism: Nrf2 activation. *Pharmacol. Res.* **167**, 105526 (2021).
89. Schirrmacher, V. Less can be more: the hormesis theory of stress adaptation in the global biosphere and its implications. *Biomedicines* https://doi.org/10.3390/biomedicines9030293 (2021).
90. Ristow, M. & Schmeisser, K. Mitohormesis: promoting health and lifespan by increased levels of reactive oxygen species (ROS). *Dose. Response* **12**, 288–341 (2014).
91. McEwen, B. S. & Wingfield, J. C. The concept of allostasis in biology and biomedicine. *Horm. Behav.* **43**, 2–15 (2003).
92. Davies, K. J. Adaptive homeostasis. *Mol. Asp. Med.* **49**, 1–7 (2016).
93. Sies, H. Oxidative eustress: on constant alert for redox homeostasis. *Redox Biol.* **41**, 101867 (2021).
94. Sies, H. & Ursini, F. Homeostatic control of redox status and health. *IUBMB Life* https://doi.org/10.1002/iub.2519 (2021).
95. Chouchani, E. T. et al. Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1. *Nature* **532**, 112–116 (2016).
96. Muri, J. & Kopf, M. Redox regulation of immunometabolism. *Nat. Rev. Immunol.* **21**, 363–381 (2020).
97. Mills, E. L. et al. Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell* **167**, 457–470 (2016).
98. Anathy, V. et al. Reducing protein oxidation reverses lung fibrosis. *Nat. Med.* **24**, 1128–1135 (2018).
99. Kim, S. J. et al. Mitochondrial catalase overexpressed transgenic mice are protected against lung fibrosis in part via preventing alveolar epithelial cell mitochondrial DNA damage. *Free Radic. Biol. Med.* **101**, 482–490 (2016).
100. Vicente-Gutierrez, C. et al. Astrocytic mitochondrial ROS modulate brain metabolism and mouse behaviour. *Nat. Metab.* **1**, 201–211 (2019).
101. Margaritelis, N. V., Paschalis, V., Theodorou, A. A., Kyparos, A. & Nikolaidis, M. G. Redox basis of exercise physiology. *Redox Biol.* **35**, 101499 (2020).
102. Chouchani, E. T. et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **515**, 431–435 (2014).
103. Yin, Z. et al. Structural basis for a complex I mutation that blocks pathological ROS production. *Nat. Commun.* **12**, 707 (2021).
104. Hebbar, S. & Knust, E. Reactive oxygen species (ROS) constitute an additional player in regulating epithelial development. *Bioessays* **43**, e2100096 (2021).
105. Rampon, C., Volovitch, M., Joliot, A. & Vriz, S. Hydrogen peroxide and redox regulation of developments. *Antioxidants* **7**, antiox7110159 (2018).
106. Kil, I. S. et al. Feedback control of adrenal steroidogenesis via H_2O_2-dependent, reversible inactivation of peroxiredoxin III in mitochondria. *Mol. Cell* **46**, 584–594 (2012).
107. Harris, I. S. et al. Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. *Cancer Cell* **27**, 211–222 (2015).
108. D'Souza, A. D., Parish, I. A., Krause, D. S., Kaech, S. M. & Shadel, G. S. Reducing mitochondrial ROS improves disease-related pathology in a mouse model of ataxia-telangiectasia. *Mol. Ther.* **21**, 42–48 (2013).
109. Dai, D. F. et al. Mitochondrial-targeted catalase: extended longevity and the roles in various disease models. *Prog. Mol. Biol. Transl. Sci.* **146**, 203–241 (2017).
110. Schriner, S. E. et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* **308**, 1909–1911 (2005).
This paper uses mitochondria-targeted catalase to modulate mitochondrial H_2O_2 levels and, thereby, impact the lifespan in mice.
111. Ngoi, N. Y. L. et al. The redox–senescence axis and its therapeutic targeting. *Redox Biol.* https://doi.org/10.1016/j.redox.2021.102032 (2021).
112. Sommer, N. et al. Bypassing mitochondrial complex III using alternative oxidase inhibits acute pulmonary oxygen sensing. *Sci. Adv.* **6**, eaba0694 (2020).
113. Behring, J. B. et al. Spatial and temporal alterations in protein structure by EGF regulate cryptic cysteine oxidation. *Sci. Signal* https://doi.org/10.1126/scisignal.aay7315 (2020).
114. Wild, C. P. The exposome: from concept to utility. *Int. J. Epidemiol.* **41**, 24–32 (2012).
115. Vermeulen, R., Schymanski, E. L., Barabasi, A. L. & Miller, G. W. The exposome and health: where chemistry meets biology. *Science* **367**, 392–396 (2020).
This work is a comprehensive review of the exposome and health.
116. Peters, A., Nawrot, T. S. & Baccarelli, A. A. Hallmarks of environmental insults. *Cell* **184**, 1455–1468 (2021).
117. Sakaguchi, R. & Mori, Y. Transient receptor potential (TRP) channels: biosensors for redox environmental stimuli and cellular status. *Free Radic. Biol. Med.* **146**, 36–44 (2020).
118. Daiber, A. et al. Effects of air pollution particles (ultrafine and fine particulate matter) on mitochondrial function and oxidative stress — implications for cardiovascular and neurodegenerative diseases. *Arch. Biochem. Biophys.* **696**, 108662 (2020).
119. Al-Kindi, S. G., Brook, R. D., Biswal, S. & Rajagopalan, S. Environmental determinants of cardiovascular disease: lessons learned from air pollution. *Nat. Rev. Cardiol.* **17**, 656–672 (2020).
120. Ansari, M. O. et al. Evaluation of DNA interaction, genotoxicity and oxidative stress induced by iron oxide nanoparticles both in vitro and in vivo: attenuation by thymoquinone. *Sci. Rep.* **9**, 6912 (2019).
121. Münzel, T., Sørensen, M. & Daiber, A. Transportation noise pollution and cardiovascular disease. *Nat. Rev. Cardiol.* **18**, 619–636 (2021).
122. Schuermann, D. & Mevissen, M. Manmade electromagnetic fields and oxidative stress — biological effects and consequences for health. *Int. J. Mol. Sci.* https://doi.org/10.3390/ijms22073772 (2021).
123. Krutmann, J., Schikowski, T., Morita, A. & Berneburg, M. Environmentally-induced (extrinsic) skin aging: exposomal factors and underlying mechanisms. *J. Invest. Dermatol.* **141**, 1096–1103 (2021).
124. Vogel, C. F. A., Van Winkle, L. S., Esser, C. & Haarmann-Stemmann, T. The aryl hydrocarbon receptor as a target of environmental stressors — implications for pollution mediated stress and inflammatory responses. *Redox Biol.* **34**, 101530 (2020).
125. Rothhammer, V. et al. Aryl hydrocarbon receptor activation in astrocytes by laquinimod ameliorates autoimmune inflammation in the CNS. *Neurol. Neuroimmunol. Neuroinflamm.* https://doi.org/10.1212/nxi.0000000000000946 (2021).
126. Bock, K. W. Aryl hydrocarbon receptor (AHR) functions: balancing opposing processes including inflammatory reactions. *Biochem. Pharmacol.* **178**, 114093 (2020).
127. Kubli, S. P. et al. AhR controls redox homeostasis and shapes the tumor microenvironment in BRCA1-associated breast cancer. *Proc. Natl Acad. Sci. USA* **116**, 3604–3613 (2019).
128. Ren, F. et al. AHR-mediated ROS production contributes to the cardiac developmental toxicity of $PM_{2.5}$ in zebrafish embryos. *Sci. Total. Env.* **719**, 135097 (2020).
129. Dumas, A. & Knaus, U. G. Raising the 'good' oxidants for immune protection. *Front. Immunol.* **12**, 698042 (2021).
130. Yardeni, T. et al. Host mitochondria influence gut microbiome diversity: a role for ROS. *Sci. Signal.* https://doi.org/10.1126/scisignal.aaw3159 (2019).
131. Saeedi, B. J. et al. Gut-resident lactobacilli activate hepatic Nrf2 and protect against oxidative liver injury. *Cell Metab.* **31**, 956–968 (2020).
132. Kanner, J. Polyphenols by generating H_2O_2, affect cell eedox signaling, inhibit PTPs and activate Nrf2 axis for adaptation and cell surviving: in vitro, in vivo and human health. *Antioxid. (Basel)* https://doi.org/10.3390/antiox9090797 (2020).
133. Del Rio, D. et al. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **18**, 1818–1892 (2013).
This work is a comprehensive review of the role of polyphenolic phytochemicals in human health.
134. Oteiza, P. I., Fraga, C. G. & Galleano, M. Linking biomarkers of oxidative stress and disease with flavonoid consumption: from experimental models to humans. *Redox Biol.* **42**, 101914 (2021).
135. Woodby, B., Penta, K., Pecorelli, A., Lila, M. A. & Valacchi, G. Skin health from the inside out. *Annu. Rev. Food Sci. Technol.* **11**, 235–254 (2020).
136. Sies, H. & Stahl, W. Nutritional protection against skin damage from sunlight. *Annu. Rev. Nutr.* **24**, 173–200 (2004).
137. Jackson, M. J., Stretton, C. & McArdle, A. Hydrogen peroxide as a signal for skeletal muscle adaptations to exercise: what do concentrations tell us about potential mechanisms? *Redox Biol.* **35**, 101484 (2020).
138. Dimauro, I., Paronetto, M. P. & Caporossi, D. Exercise, redox homeostasis and the epigenetic landscape. *Redox Biol.* **35**, 101477 (2020).
139. Gomez-Cabrera, M. C. et al. Redox modulation of muscle mass and function. *Redox Biol.* **35**, 101531 (2020).
140. Barabasi, A. L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. *Nat. Rev. Genet.* **12**, 56–68 (2011).
141. Dennis, K. K., Go, Y. M. & Jones, D. P. Redox systems biology of nutrition and oxidative stress. *J. Nutr.* **149**, 553–565 (2019).
142. Wang, R. S., Oldham, W. M., Maron, B. A. & Loscalzo, J. Systems biology approaches to redox metabolism in stress and disease states. *Antioxid. Redox Signal.* **29**, 953–972 (2018).
143. Kim, E. et al. Redox probing for chemical information of oxidative stress. *Anal. Chem.* **89**, 1583–1592 (2017).
144. Peake, J. M., Kerr, G. & Sullivan, J. P. A critical review of consumer wearables, mobile applications, and equipment for providing biofeedback, monitoring stress, and sleep in physically active populations. *Front. Physiol.* **9**, 743 (2018).
145. Meng, J. et al. Precision redox: the key for antioxidant pharmacology. *Antioxid. Redox Signal.* **34**, 1069–1082 (2021).
146. Yang, B., Chen, Y. & Shi, J. Reactive oxygen species (ROS)-based nanomedicine. *Chem. Rev.* **119**, 4881–4985 (2019).
147. Elbatreek, M. H., Mucke, H. & Schmidt, H. H. H. W. NOX inhibitors: from bench to naxibs to bedside. *Handb. Exp. Pharmacol.* **264**, 145–168 (2021).

148. Davies, M. J. Myeloperoxidase: mechanisms, reactions and inhibition as a therapeutic strategy in inflammatory diseases. *Pharmacol. Ther.* **218**, 107685 (2021).
149. Casas, A. I. et al. On the clinical pharmacology of reactive oxygen species. *Pharmacol. Rev.* **72**, 801–828 (2020).
This work is a comprehensive overview of applied network pharmacology leading to improved design of redox therapeutics for precision medicine.
150. Taguchi, K. & Yamamoto, M. The KEAP1–NRF2 system as a molecular target of cancer treatment. *Cancers (Basel)* https://doi.org/10.3390/cancers13010046 (2020).
151. Panieri, E. & Saso, L. Inhibition of the NRF2/KEAP1 axis: a promising therapeutic strategy to alter redox balance of cancer cells. *Antioxid. Redox Signal.* **34**, 1428–1483 (2021).
152. Cuadrado, A. et al. Therapeutic targeting of the NRF2 and KEAP1 partnership in chronic diseases. *Nat. Rev. Drug Discov.* **18**, 295–317 (2019).
153. Hitchler, M. J. & Domann, F. E. The epigenetic and morphogenetic effects of molecular oxygen and its derived reactive species in development. *Free Radic. Biol. Med.* **170**, 70–84 (2021).
154. García-Giménez, J. L., Garcés, C., Romá-Mateo, C. & Pallardó, F. V. Oxidative stress-mediated alterations in histone post-translational modifications. *Free Radic. Biol. Med.* **170**, 6–18 (2021).
155. Szypowska, A. A. & Burgering, B. M. The peroxide dilemma: opposing and mediating insulin action. *Antioxid. Redox Signal.* **15**, 219–232 (2011).
156. Keeley, T. P. & Mann, G. E. Defining physiological normoxia for improved translation of cell physiology to animal models and humans. *Physiol. Rev.* **99**, 161–234 (2019).
This work is a comprehensive review of physiological normoxia and the importance of recapitulating it in cell culture experiments.
157. Augusto, O. & Truzzi, D. R. Carbon dioxide redox metabolites in oxidative eustress and oxidative distress. *Biophys. Rev.* https://doi.org/10.1007/s12551-021-00860-3 (2021).
158. Klein, S. G. et al. A prevalent neglect of environmental control in mammalian cell culture calls for best practices. *Nat. Biomed. Eng.* **5**, 787–792 (2021).
159. Wardman, P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med.* **43**, 995–1022 (2007).
160. Winterbourn, C. C. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochim. Biophys. Acta* **1840**, 730–738 (2014).
This paper shows the many artefacts that can arise when using fluorescent probes to assess reactive species.
161. Rezende, F., Brandes, R. P. & Schröder, K. Detection of hydrogen peroxide with fluorescent dyes. *Antioxid. Redox Signal.* **29**, 585–602 (2018).
162. Fuloria, S. et al. Comprehensive review of methodology to detect reactive oxygen species (ROS) in mammalian species and establish its relationship with antioxidants and cancer. *Antioxid. (Basel)* https://doi.org/10.3390/antiox10010128 (2021).
163. Smolyarova, D. D., Podgorny, O. V., Bilan, D. S. & Belousov, V. V. A guide to genetically encoded tools for the study of H_2O_2. *FEBS J.* https://doi.org/10.1111/febs.16088 (2021).
164. Breus, O. & Dickmeis, T. Genetically encoded thiol redox-sensors in the zebrafish model: lessons for embryonic development and regeneration. *Biol. Chem.* **402**, 363–378 (2021).
165. Bazopoulou, D. et al. Developmental ROS individualizes organismal stress resistance and lifespan. *Nature* **576**, 301–305 (2019).
166. Barata, A. G. & Dick, T. P. In vivo imaging of H_2O_2 production in *Drosophila*. *Methods Enzymol.* **526**, 61–82 (2013).
167. Sinenko, S. A., Starkova, T. Y., Kuzmin, A. A. & Tomilin, A. N. Physiological signaling functions of reactive oxygen species in stem cells: from flies to man. *Front. Cell Dev. Biol.* **9**, 714370 (2021).
168. Love, N. R. et al. Amputation-induced reactive oxygen species are required for successful *Xenopus* tadpole tail regeneration. *Nat. Cell Biol.* **15**, 222–228 (2013).
169. Fujikawa, Y. et al. Mouse redox histology using genetically encoded probes. *Sci. Signal.* **9**, rs1 (2016).
170. Kostyuk, A. I. et al. Genetically encoded tools for research of cell signaling and metabolism under brain hypoxia. *Antioxid. (Basel)* https://doi.org/10.3390/antiox9060516 (2020).
171. Belousov, V. V. et al. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods* **3**, 281–286 (2006).
This paper describes HyPer, the first genetically encoded H_2O_2 probe.
172. Gutscher, M. et al. Proximity-based protein thiol oxidation by H_2O_2-scavenging peroxidases. *J. Biol. Chem.* **284**, 31532–31540 (2009).
173. Morgan, B. et al. Real-time monitoring of basal H_2O_2 levels with peroxiredoxin-based probes. *Nat. Chem. Biol.* **12**, 437–443 (2016).
This paper describes the most recent versions of peroxiredoxin-based H_2O_2 probes.
174. Kritsiligkou, P., Shen, T. K. & Dick, T. P. A comparison of Prx- and OxyR-based H_2O_2 probes expressed in *S. cerevisiae*. *J. Biol. Chem.* **297**, 100866 (2021).
175. Steinhorn, B., Eroglu, E. & Michel, T. Chemogenetic approaches to probe redox pathways: implications for cardiovascular pharmacology and toxicology. *Annu. Rev. Pharmacol. Toxicol.* **62**, 551–571 (2021).
176. Steinhorn, B. et al. Chemogenetic generation of hydrogen peroxide in the heart induces severe cardiac dysfunction. *Nat. Commun.* **9**, 4044 (2018).
177. Mishina, N. M. et al. Which antioxidant system shapes intracellular H_2O_2 gradients? *Antioxid. Redox Signal.* **31**, 664–670 (2019).
178. Bogdanova, Y. A., Schultz, C. & Belousov, V. V. Local generation and imaging of hydrogen peroxide in living cells. *Curr. Protoc. Chem. Biol.* **9**, 117–127 (2017).
179. Dickinson, B. C., Huynh, C. & Chang, C. J. A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *J. Am. Chem. Soc.* **132**, 5906–5915 (2010).
180. Maeda, H. Which are you watching, an individual reactive oxygen species or total oxidative stress? *Ann. N. Y. Acad. Sci.* **1130**, 149–156 (2008).
181. Wu, L. et al. Reaction-based fluorescent probes for the detection and imaging of reactive oxygen, nitrogen, and sulfur species. *Acc. Chem. Res.* **52**, 2582–2597 (2019).
182. Iwashita, H., Castillo, E., Messina, M. S., Swanson, R. A. & Chang, C. J. A tandem activity-based sensing and labeling strategy enables imaging of transcellular hydrogen peroxide signaling. *Proc. Natl Acad. Sci. USA* **118**, https://doi.org/10.1073/pnas.2018513118 (2021).
183. Miller, E. W., Dickinson, B. C. & Chang, C. J. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc. Natl Acad. Sci. USA* **107**, 15681–15686 (2010).
184. Zielonka, J. et al. Boronate probes as diagnostic tools for real time monitoring of peroxynitrite and hydroperoxides. *Chem. Res. Toxicol.* **25**, 1793–1799 (2012).
185. Sies, H. Measurement of hydrogen peroxide formation in situ. *Methods Enzymol.* **77**, 15–20 (1981).
186. Oshino, N., Chance, B., Sies, H. & Bücher, T. The role of H_2O_2 generation in perfused rat liver and the reaction of catalase Compound I and hydrogen donors. *Arch. Biochem. Biophys.* **154**, 117–131 (1973).
187. Cox, A. G., Winterbourn, C. C. & Hampton, M. B. Measuring the redox state of cellular peroxiredoxins by immunoblotting. *Methods Enzymol.* **474**, 51–66 (2010).
188. Pastor-Flores, D., Talwar, D., Pedre, B. & Dick, T. P. Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells. *Proc. Natl Acad. Sci. USA* **117**, 16313–16323 (2020).
189. Zielonka, J. & Kalyanaraman, B. Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. *Free Radic. Biol. Med.* **48**, 983–1001 (2010).
190. Zielonka, J. et al. Global profiling of reactive oxygen and nitrogen species in biological systems: high-throughput real-time analyses. *J. Biol. Chem.* **287**, 2984–2995 (2012).
191. Shchepinova, M. M. et al. MitoNeoD: a mitochondria-targeted superoxide probe. *Cell Chem. Biol.* **24**, 1285–1298 (2017).
192. Xiao, H. et al. Versatile fluorescent probes for imaging the superoxide anion in living cells and in vivo. *Angew. Chem. Int. Ed. Engl.* **59**, 4216–4230 (2020).
193. Hu, J. J. et al. Fluorescent probe HKSOX-1 for imaging and detection of endogenous superoxide in live cells and in vivo. *J. Am. Chem. Soc.* **137**, 6837–6843 (2015).
194. Nauseef, W. M. Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. *Biochim. Biophys. Acta* **1840**, 757–767 (2014).
195. Abbas, K., Babic, N. & Peyrot, F. Use of spin traps to detect superoxide production in living cells by electron paramagnetic resonance (EPR) spectroscopy. *Methods* **109**, 31–43 (2016).
196. Mason, R. P. & Ganini, D. Immuno-spin trapping of macromolecules free radicals in vitro and in vivo — one stop shopping for free radical detection. *Free Radic. Biol. Med.* **131**, 318–331 (2019).
197. Dikalov, S. I. & Harrison, D. G. Methods for detection of mitochondrial and cellular reactive oxygen species. *Antioxid. Redox Signal.* **20**, 372–382 (2014).
198. Prolo, C., Rios, N., Piacenza, L., Alvarez, M. N. & Radi, R. Fluorescence and chemiluminescence approaches for peroxynitrite detection. *Free Radic. Biol. Med.* **128**, 59–68 (2018).
199. Ma, C. et al. Recent development of synthetic probes for detection of hypochlorous acid/hypochlorite. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* **240**, 118545 (2020).
200. Kostyuk, A. I. et al. Hypocrates is a genetically encoded fluorescent biosensor for (pseudo)hypohalous acids and their derivatives. *Nat. Commun.* **13**, 171 (2022).
201. Li, P. et al. A new polymer nanoprobe based on chemiluminescence resonance energy transfer for ultrasensitive imaging of intrinsic superoxide anion in mice. *J. Am. Chem. Soc.* **138**, 2893–2896 (2016).
202. Hou, C. et al. Development of a positron emission tomography radiotracer for imaging elevated levels of superoxide in neuroinflammation. *ACS Chem. Neurosci.* **9**, 578–586 (2018).
203. Onukwufor, J. O. et al. Quantification of reactive oxygen species production by the red fluorescent proteins KillerRed, SuperNova and mCherry. *Free Radic. Biol. Med.* **147**, 1–7 (2020).
204. Trewin, A. J. et al. Light-induced oxidant production by fluorescent proteins. *Free Radic. Biol. Med.* **128**, 157–164 (2018).
205. Wojtovich, A. P. & Foster, T. H. Optogenetic control of ROS production. *Redox Biol.* **2**, 368–376 (2014).
206. Zong, C. et al. Surface-enhanced Raman spectroscopy for bioanalysis: reliability and challenges. *Chem. Rev.* **118**, 4946–4980 (2018).
207. Li, X., Duan, X., Yang, P., Li, L. & Tang, B. Accurate in situ monitoring of mitochondrial H_2O_2 by robust SERS nanoprobes with a Au–Se interface. *Anal. Chem.* **93**, 4059–4065 (2021).
208. Kishimoto, S., Krishna, M. C., Khramtsov, V. V., Utsumi, H. & Lurie, D. J. In vivo application of proton–electron double-resonance imaging. *Antioxid. Redox Signal.* **28**, 1345–1364 (2018).
209. Murphy, M. E. & Sies, H. Visible-range low-level chemiluminescence in biological systems. *Methods Enzymol.* **186**, 595–610 (1990).
210. Burgos, R. C. R. et al. Ultra-weak photon emission as a dynamic tool for monitoring oxidative stress metabolism. *Sci. Rep.* **7**, 1229 (2017).
211. Hawkins, C. L. & Davies, M. J. Detection, identification, and quantification of oxidative protein modifications. *J. Biol. Chem.* **294**, 19683–19708 (2019).
This work is a comprehensive review of methods of analysis of oxidative protein modifications.
212. Sampadi, B. et al. Quantitative phosphoproteomics to unravel the cellular response to chemical stressors with different modes of action. *Arch. Toxicol.* **94**, 1655–1671 (2020).
213. Batth, T. S. et al. Protein aggregation capture on microparticles enables multipurpose proteomics sample preparation. *Mol. Cell Proteom.* **18**, 1027–1035 (2019).
214. Brunner, A.-D. et al. Ultra-high sensitivity mass spectrometry quantifies single-cell proteome changes upon perturbation. Preprint at *bioRxiv* https://doi.org/10.1101/2020.12.22.423933 (2021).
215. Schjoldager, K. T., Narimatsu, Y., Joshi, H. J. & Clausen, H. Global view of human protein glycosylation pathways and functions. *Nat. Rev. Mol. Cell Biol.* **21**, 729–749 (2020).
216. Khoder-Agha, F. & Kietzmann, T. The glyco-redox interplay: principles and consequences on the role of reactive oxygen species during protein glycosylation. *Redox Biol.* **42**, 101888 (2021).
This work is a comprehensive review of the role of the redox glycome.
217. Taniguchi, N. et al. Glyco-redox, a link between oxidative stress and changes of glycans: lessons from research on glutathione, reactive oxygen and nitrogen species to glycobiology. *Arch. Biochem. Biophys.* **595**, 72–80 (2016).

218. Rusz, M. et al. Morpho-metabotyping the oxidative stress response. *Sci. Rep.* **11**, 15471 (2021).
219. Hu, X., Go, Y. M. & Jones, D. P. Omics integration for mitochondria systems biology. *Antioxid. Redox Signal.* **32**, 853–872 (2020).
This paper describes multi-omics integration for biologically relevant redox networks.
220. Griffiths, H. R. et al. Biomarkers. *Mol. Asp. Med.* **23**, 101–208 (2002).
221. Pinchuk, I. et al. Gender- and age-dependencies of oxidative stress, as detected based on the steady state concentrations of different biomarkers in the MARK-AGE study. *Redox Biol.* **24**, 101204 (2019).
222. Henning, T. & Weber, D. Redox biomarkers in dietary interventions and nutritional observation studies — from new insights to old problems. *Redox Biol.* **41**, 101922 (2021).
223. Daiber, A. et al. Redox-related biomarkers in human cardiovascular disease — classical footprints and beyond. *Redox Biol.* **42**, 101875 (2021).
224. Frijhoff, J. et al. Clinical relevance of biomarkers of oxidative stress. *Antioxid. Redox. Signal.* **23**, 1144–1170 (2015).
This work is a comprehensive analysis of clinical biomarkers of oxidative stress.
225. Szibor, M. et al. Broad AOX expression in a genetically tractable mouse model does not disturb normal physiology. *Dis. Model. Mech.* **10**, 163–171 (2017).
226. Scialo, F. & Sanz, A. Coenzyme Q redox signalling and longevity. *Free Radic. Biol. Med.* **164**, 187–205 (2021).
227. Fang, J., Sheng, R. & Qin, Z. H. NADPH oxidases in the central nervous system: regional and cellular localization and the possible link to brain diseases. *Antioxid. Redox Signal.* **35**, 951–973 (2021).
228. Helfinger, V. et al. Genetic deletion of Nox4 enhances cancerogen-induced formation of solid tumors. *Proc. Natl Acad. Sci. USA* https://doi.org/10.1073/pnas.2020152118 (2021).
229. Wörsdörfer, P. & Ergün, S. The impact of oxygen availability and multilineage communication on organoid maturation. *Antioxid. Redox Signal.* **35**, 217–233 (2021).
230. Görlach, A. et al. Reactive oxygen species, nutrition, hypoxia and diseases: problems solved? *Redox Biol.* **6**, 372–385 (2015).
231. Ward, J. P. Oxygen sensors in context. *Biochim. Biophys. Acta* **1777**, 1–14 (2008).
232. Warpsinski, G. et al. Nrf2-regulated redox signaling in brain endothelial cells adapted to physiological oxygen levels: consequences for sulforaphane mediated protection against hypoxia–reoxygenation. *Redox Biol.* **37**, 101708 (2020).
233. Ferguson, D. C. J. et al. Altered cellular redox homeostasis and redox responses under standard oxygen cell culture conditions versus physioxia. *Free Radic. Biol. Med.* **126**, 322–333 (2018).
234. Chapple, S. J. et al. Bach1 differentially regulates distinct Nrf2-dependent genes in human venous and coronary artery endothelial cells adapted to physiological oxygen levels. *Free Radic. Biol. Med.* **92**, 152–162 (2016).
235. Pomatto, L. C. D. & Davies, K. J. A. The role of declining adaptive homeostasis in ageing. *J. Physiol.* **595**, 7275–7309 (2017).
236. Place, T. L., Domann, F. E. & Case, A. J. Limitations of oxygen delivery to cells in culture: an underappreciated problem in basic and translational research. *Free Radic. Biol. Med.* **113**, 311–322 (2017).
237. Noll, T., de Groot, H. & Wissemann, P. A computer-supported oxystat system maintaining steady-state O_2 partial pressures and simultaneously monitoring O_2 uptake in biological systems. *Biochem. J.* **236**, 765–769 (1986).
238. Ermakova, Y. G. et al. SypHer3s: a genetically encoded fluorescent ratiometric probe with enhanced brightness and an improved dynamic range. *Chem. Commun.* **54**, 2898–2901 (2018).
239. Mueller, S., Millonig, G. & Waite, G. N. The GOX/CAT system: a novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture. *Adv. Med. Sci.* **54**, 121–135 (2009).
240. Waldeck-Weiermair, M. et al. Dissecting in vivo and in vitro redox responses using chemogenetics. *Free Radic. Biol. Med.* **177**, 360–369 (2021).
241. Bulina, M. E. et al. A genetically encoded photosensitizer. *Nat. Biotechnol.* **24**, 95–99 (2006).
242. Sarkisyan, K. S. et al. KillerOrange, a genetically encoded photosensitizer activated by blue and green light. *PLoS ONE* **10**, e0145287 (2015).
243. Takemoto, K. et al. SuperNova, a monomeric photosensitizing fluorescent protein for chromophore-assisted light inactivation. *Sci. Rep.* **3**, 2629 (2013).
244. Shu, X. et al. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. *PLoS Biol.* **9**, e1001041 (2011).
245. Rabbani, N. & Thornalley, P. J. Protein glycation — biomarkers of metabolic dysfunction and early-stage decline in health in the era of precision medicine. *Redox Biol.* **42**, 101920 (2021).
246. Freeman, B. A., O'Donnell, V. B. & Schopfer, F. J. The discovery of nitro-fatty acids as products of metabolic and inflammatory reactions and mediators of adaptive cell signaling. *Nitric Oxide* **77**, 106–111 (2018).
247. Kagan, V. E. et al. Redox phospholipidomics of enzymatically generated oxygenated phospholipids as specific signals of programmed cell death. *Free Radic. Biol. Med.* **147**, 231–241 (2020).
This paper describes phospholipids in the context of the redox lipidome.
248. Preil, S. A. et al. Quantitative proteome analysis reveals increased content of basement membrane proteins in arteries from patients with type 2 diabetes mellitus and lower levels among metformin users. *Circ. Cardiovasc. Genet.* **8**, 727–735 (2015).
249. Slavov, N. Unpicking the proteome in single cells. *Science* **367**, 512–513 (2020).
250. Shi, Y. & Carroll, K. S. Activity-based sensing for site-specific proteomic analysis of cysteine oxidation. *Acc. Chem. Res.* **53**, 20–31 (2019).
251. Havelund, J. F. et al. A biotin enrichment strategy identifies novel carbonylated amino acids in proteins from human plasma. *J. Proteom.* **156**, 40–51 (2017).
252. van 't Erve, T. J., Kadiiska, M. B., London, S. J. & Mason, R. P. Classifying oxidative stress by F_2-isoprostane levels across human diseases: a meta-analysis. *Redox Biol.* **12**, 582–599 (2017).
253. Cruciani, G., Domingues, P., Fedorova, M., Galli, F. & Spickett, C. M. Redox lipidomics and adductomics — advanced analytical strategies to study oxidized lipids and lipid–protein adducts. *Free Radic. Biol. Med.* **144**, 1–5 (2019).
254. Ravanat, J. L., Cadet, J. & Douki, T. Oxidatively generated DNA lesions as potential biomarkers of in vivo oxidative stress. *Curr. Mol. Med.* **12**, 655–671 (2012).
255. Jørs, A. et al. Urinary markers of nucleic acid oxidation increase with age, obesity and insulin resistance in Danish children and adolescents. *Free Radic. Biol. Med.* **155**, 81–86 (2020).
256. Seifermann, M. & Epe, B. Oxidatively generated base modifications in DNA: not only carcinogenic risk factor but also regulatory mark? *Free Radic. Biol. Med.* **107**, 258–265 (2017).
257. Muruzabal, D., Collins, A. & Azqueta, A. The enzyme-modified comet assay: past, present and future. *Food Chem. Toxicol.* **147**, 111865 (2021).
258. Patel, R. S. et al. Novel biomarker of oxidative stress is associated with risk of death in patients with coronary artery disease. *Circulation* **133**, 361–369 (2016).
259. Abdulle, A. E. et al. Serum free thiols predict cardio-vascular events and all-cause mortality in the general population: a prospective cohort study. *BMC Med.* https://doi.org/10.1186/s12916-020-01587-w (2020).

Acknowledgements
Generous support is acknowledged: G.E.M., British Heart Foundation (FS19/25/34277, FS16/67/32548), Heart Research UK (RG2672) and King's Together Strategic Award; M.J.D., Novo Nordisk Foundation (Laureate grants NNF13OC0004294 and NNF20SA0064214); D.P.J., P30-ES019776, R21-ES031824, R01-ES023485, U2C-ES030163 and RC2-DK118619; M.P.M., Medical Research Council UK (MC_U105663142) and a Wellcome Trust Investigator Award (110159/A/15Z); V.V.B., Ministry of Science and Higher Education, Russian Federation (grant 075-15-2019-1789); and H.S., Deutsche Forschungsgemeinschaft, National Foundation for Cancer Research.

Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Molecular Cell Biology thanks Yimon Aye, Sergey Dikalov, Henry Forman and Ursula Jakob for their contribution to the peer review of this work.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

RELATED LINKS
Gene Expression Omnibus: https://www.ncbi.nlm.nih.gov/geo/
Metabolomics Workbench: https://www.metabolomicsworkbench.org
Oximouse: https://oximouse.hms.harvard.edu/