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

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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 pathological damage. Consequently, the molecular description of oxidants needs to be chemically precise for

rational translation of research on their biological effects into therapeutic benefit in redox medicine. This Expert Recommendation article pinpoints key issues associated with identifying and explaining the physiological roles of oxidants, focusing on H_2O_2 and $O_2^{\cdot-}$. The generic term ROS should not be used as if it were describing a specific molecular agent, and greater precision in measurement of H_2O_2, $O_2^{\cdot-}$ and other oxidants, along with more specific identification of their respective signalling targets are needed. Future work should also focus more on inter-organellar communication in oxidant signalling and on the interactions of redox-sensitive signalling targets within complex biological systems, including whole organisms with their lifetime environmental exposures. To that effect, we recommend that new tools be used which enable site-specific and real-time detection and quantification of individual oxidants in cells and model organisms. 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 more precise definitions and analytical tools will help harmonize research among the many disciplines towards the common goal of understanding redox biology and medicine.

[H1] Introduction

'Reactive Oxygen Species' (ROS) is a generic term for a large family of oxidants derived from molecular oxygen. They are part of a larger family of reactive species, including reactive nitrogen (RNS), sulfur (RSS), carbon (RCS), selenium (RSeS), electrophile (RES) and halogen (RHS) species that can undergo redox (reduction-oxidation) reactions as well as form oxidative modifications on biological macromolecules, thereby contributing to redox signalling. However, it is well-established that supra-physiological concentrations of ROS react non-specifically with proteins, lipids, nucleic acids, 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, 'eustress', and supraphysiological (damaging) oxidative stress, 'distress', is ongoing, as is research to understand how oxidative stress response systems control the cellular redox tone **[G]**. In oxidative eustress, low-level H_2O_2 reaches highly specific targets for physiological redox signalling, whereas in oxidative distress aberrant or disrupted redox signalling ensues when high-level H_2O_2 reacts 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 the research in this field. We also provide recommendations for use of validated analytical

methods and biomarkers, in cell experiments *in vitro* and in widely used animal models (fish, worms, flies, frogs, mice) (key recommendations are summarized in **BOX 1**). As the literature on this topic 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 some secondary oxidants, *e.g.* electrophiles such as 4-hydroxynonenal generated by lipid peroxidation, and peroxynitrite which is 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 research framework for future 'redox medicine' **[G]**. For background we refer readers to the textbook by Halliwell and Gutteridge[4] and to informative reviews[1,5-18].

[H1] 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 molecular oxidant species under investigation and when this is not available, to use term 'oxidant' to refer to these agents[11,19]. H_2O_2 and $O_2^{\cdot-}$, two major oxidants with physiological importance, are continuously produced by mitochondrial NADH-dependent systems and by extramitochondrial NADPH-dependent systems (**FIG.1)** to maintain steady-state intracellular pools, typically being low nanomolar for H_2O_2 and low picomolar for $O_2^{\cdot-}$ [20]. Thus, the H_2O_2 and $O_2^{\cdot-}$ pools are highly responsive to the ways in which they are produced or degraded. Transient changes in H_2O_2 in the nanomolar range behave similarly to the signals generated by Ca^{2+} transients. $O_2^{\cdot-}$, at three orders of magnitude lower concentration than H_2O_2, leads to more localized spatial responses. As with other second messenger systems, signals can be amplified by triggering kinase cascades or transmitted over distances by conversion to more stable species. Signalling outside cells is expected to differ from intracellular signalling due to the higher oxidant concentrations found outside cells, e.g. extracellular fluids or plasma typically have 1-5 μM H_2O_2 [21].

Redox-sensitive targets are sites of high electron density, such as sulfur centres or aromatic rings and/or positions where the resulting species can be stabilized by nearby double bonds, for example at allylic sites in polyunsaturated fatty acids (PUFAs) and aromatic groups in DNA, RNA and proteins. Some oxidative modifications can be reversible (e.g. sulfenic acids, sulfenamides, disulfides, nitrosocysteine, methionine sulfoxide, chloramines), whereas others are essentially irreversible (e.g. organic peroxides, alcohols, carbonyls, ring-chlorinated and nitrated species), with catabolism and excretion the major routes of their removal. For lipid peroxidation and other radical chain reactions, the formation of a single type of initial radical can cascade to multiple different species, so single oxidants will often not act in isolation.

Signalling by H_2O_2 occurs mainly through oxidation of specific protein Cys residues to sulfenic acid and subsequent redox relay mechanisms, often involving peroxiredoxins[22,23], and coupling to metabolism, phosphorylation cascades, transcriptional regulation, cytoskeletal rearrangements, cell replication and other critical cell functions[13,17,24]. Signalling by $O_2^{\cdot-}$ is less well understood, but most probably proceeds by affecting the redox state of transition metal **[G]** complexes in proteins[25].

[H1] Cellular redox signalling

Specificity of redox signalling depends upon spatially-regulated generation and distribution oxidants across subcellular compartments within the cell[26,27]. Considerable knowledge on redox signal generation and transduction at the plasma membrane, nucleus, mitochondria, peroxisomes, and endoplasmic reticulum has accumulated[1]. However, cell research is recommended for critical questions about the mechanisms and orchestration of redox regulation across the cell, 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.

[H2] *Subcellular compartments in redox regulation.* The plasma membrane is an important site of $O_2^{\cdot-}$ and H_2O_2 generation via NADPH oxidases **[G]** (NOXs) [28] under physiological conditions[29] and a lateral concentration profile of oxidants along the membrane surface has been reported[30], providing a potential basis for spatial selectivity such as in areas termed caveolae and at contact sites between organelles. However, the large number of cell types and physiological demands, the existence of polarity across membrane surfaces in tissues, the variable expression of NOXs in different cells, and limited rigorous quantification of site-specific H_2O_2 generation means that each situation 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[31-33]. Complex I generates $O_2^{\cdot-}$ on the matrix side, and complex III produces $O_2^{\cdot-}$ towards the matrix and the intermembrane space[34]. Succinate accumulation, for example during ischaemia **[G]** , can also lead to $O_2^{\cdot-}$ generation from complex I by reverse electron transfer **[G]** [35]. Molecules that selectively suppress $O_2^{\cdot-}$ production[32] can lead to therapeutics to ameliorate mitochondrial ETC-dependent oxidant-linked diseases. It is a widely-held view is that oxidant production by the mitochondrial respiratory chain is unavoidable leakage, but this may actually be a controlled physiological signalling process. Hence, the term “leakage” is discouraged as it implies a non-physiological process. Critical questions that remain are to determine whether site-specific generation of $O_2^{\cdot-}$, H_2O_2 and other oxidants in mitochondria have specific signalling functions. As indicated above, $O_2^{\cdot-}$ may act as a signalling molecule within the matrix, most likely via Fe-S cluster proteins

[G]. Mitochondrial matrix H_2O_2 is reduced by peroxidases, including GSH peroxidases and peroxiredoxins 3 and 5 [36]. Multiple posttranslational modifications of peroxiredoxins may also add to specificity of signalling[37].

Isolated mitochondria can also release H_2O_2 from the matrix to the extramitochondrial space, but the rate at which this occurs physiologically in the intact cell remains unclear. This highlights the problem to identify mechanisms and rates of mitochondrial H_2O_2 release required to exert biological effects. Acute bursts (minutes) of mitochondrial matrix H_2O_2 production such as during reperfusion injury do not appear to be sufficient to reach the cytosol[38]. However, longer term (hours) generation of mitochondrial matrix H_2O_2 such as during immune activation of macrophages can enhance cytosolic levels, perhaps reflecting an eventual override of matrix antioxidant defense systems[39]. To address these key questions, mitochondria should preferentially be studied within their context in intact cells.

Peroxisomes contain multiple H_2O_2-producing and scavenging enzymes and are active in H_2O_2 metabolism[40]. In addition to intraorganellar peroxisomal reactions, there is considerable interaction with extraperoxisomal sites[41,42]. Peroxisomal catalase **[G]** can modulate oxidative stress at the cellular level[43,44], possibly via a mechanism involving suppression of catalase import into the peroxisome[45], allowing for higher extraperoxisomal 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 extraperoxisomal oxidant signalling.

The lumen of the endoplasmic reticulum (ER) is a major source of H_2O_2 production during disulfide bond formation in protein folding, and ER also contains Cyp family enzymes functioning in steroid synthesis and oxidative detoxification of xenobiotics **[G].** ER systems can generate considerable fluxes of $O_2^{\cdot-}$ and H_2O_2, impact protein translation, apoptosis signalling and other pathways for sensing and responding to H_2O_2 [46]. The diversity of ER structures, the functional continuity of the ER with the Golgi and lysosomal compartments and the spatial proximity to mitochondria, nuclei and other subcellular structures emphasizes a need to analyze the redox integration of the ER within different cell types. Aquaporin 11 is of particular interest as a regulator of ER redox homeostasis and signalling, because it serves as a peroxiporin for transport of H_2O_2 through the ER membrane[47]. The function of peroxiporin in H_2O_2 transport highlights a need for more refined studies on the subcellular regulation of oxidants, localized signalling, distribution across the cell and redox crosstalk at specialized membrane contact sites **[G]** between different organelles. In situations where mitochondria are densely-packed, as occur in cardiomyocytes, H_2O_2 diffusion is thought to serve to synchronize responses across the cell[48], and mitochondria can be distributed via the mitochondrial adapter protein Miro1 to regulate oxidant distribution[49]. Further, there is emerging evidence for redox communication between organelles at membrane contact sites **[G]**. Redox nanodomains exist at the ER-mitochondrial interface, and are functionally linked to signalling between these organelles[50]. Mitochondria, ER and peroxisomes further form a 'redox triangle' and focal point in redox messaging[51]. The mitochondrial

fission factor (MFF) is a regulator of peroxisome maturation[52], underlining the close relationship between mitochondria and peroxisomes in H_2O_2 metabolism. Mitochondria form contact sites also with the nucleus to couple pro-survival retrograde responses[53] , so that redox signals can be transmitted directly from mitochondria to the nucleus.

Beyond intracellular redox signalling, it should be noted that oxidants are present and modulated extracellularly. We emphysize the need to integrate the cell biology of redox signalling across organs. The plasma membrane-located NOXs, together with extracellular superoxide dismutase (SOD3), generate a pool of extracellular H_2O_2, the concentration of which is 2-3 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 **[G]** (PDI)[54], thioredoxin **[G]** and peroxiredoxin **[G]** into plasma[55] increases during infection and inflammation. Catalase is associated with the plasma membrane in cancer cells[56,57] and thus becomes an extracellular therapeutic target which requires attention more broadly[2].

[H2] *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 is mostly to be achieved by combined 'omic' approaches and description of the redox interactome **[G]**, including information on interactions between the different reactive species and also with downstream biological targets[58,59]. A major issue is how to identify key redox-sensitive Cys residues in proteins. They can be identified by redox proteomics (e.g. the Oximouse dataset)[60] or potentially predicted using machine-learning methods[61]. Replacement of key Cys with redox insensitive residues, leading to disruption of the signal, can validate their relevance in specific contexts. The considerable circadian (diurnal) variation of oxidant levels needs to be considered in experimental settings[62]. Overall, specificity of the redox signal is given by spatiotemporal control of the oxidant and by the enormous span of reactivity of target thiols (million-fold differences between different protein thiols[8]), and there is a need to develop tools to study this in cell biology.

A second key issue is how oxidants find these key Cys residues on target proteins, as they are orders of magnitude less reactive than oxidant-consuming proteins. This implies that oxidation may involve redox relays[22,63], whereby oxidation signals are passed from one protein to another via Cys residues on each protein, eventually leading to the oxidation of the intended target[64]. These signal-transmitting chains may constitute 'redox networks'. 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 (*i.e.,* a floodgate model in which peroxiredoxins are oxidized to an inactive

state, preventing H_2O_2 degradation, and thus the initial oxidation primes the system for far higher levels of H_2O_2 [65]). Delineation of these possible pathways, each of which may be correct 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 from redox sensors 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 development of 'atlases' of network responses. Using the Cancer Genome Atlas as an example, cumulative assembly of a body of knowledge into resources for data mining has enabled substantial progress in targeted cancer therapeutics; similarly, assembly of redox proteomics data in cell biology models can foster development of targeted redox medicine. Advances have been made in capturing comprehensive readouts from cells in parallel workflow of metabolomics and live cell imaging microscopy[68].

Major redox hubs **[G]** in mammalian cells include NRF2[69], NF-kB[70], HIF[71], ERR[72], FOXO[73], PGC1α[74], p53[75], AMPK[76], GAPDH[77], and UCP[78] **(FIG. 1)**. A notable common feature of the first three of these hubs is the regulation of their turnover and activation by a Cys redox signal on an associated protein, not the transcription factor itself (e.g. KEAP1 for NRF2), which ultimately leads to nuclear accumulation of the active transcription factor. The other hubs typically contain redox-sensitive Cys directly on them. Cell biology methods to visualize these fundamental mechanisms, such as the NRF2-KEAP signalling of responses to oxidative stress, are needed to provide understanding of real-time responses of other signalling systems. Such methods will be especially important to understand how the systems function together within redox networks.

[H2] *The contribution of the antioxidant network*. Antioxidant defences predominantly consist of powerful antioxidant enzymes and, to a lesser degree, low-molecular-mass compounds (LMMC), *i.e.* vitamins, micronutrients, biofactors, that blend in with the physiological redox architecture[79]. A common misconception has been to overemphasise the role of LMMC in redox regulation. While they are essential cofactors for many antioxidant enzymes, LMMC 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 the non-radical (two-electron) oxidants is largely enzyme-catalyzed[8], predominantly mediated by selenocysteine-, Cys-, or heme-containing proteins. Thus, we here emphasize that the major biologically active 'antioxidants' are the antioxidant enzymes, not LMMC. The micronutrient selenium is essential for the activity of several critical antioxidant and repair systems[80]. Other antioxidant enzymes rely on the controlled redox activity of transition metal ions within heme groups (e.g. catalase) or specific metal clusters (e.g. superoxide dismutase). Whereas toxicity of high levels of extraneous transition metal ions is well-documented in environmental and occupational health, activities of low-concentration "labile pools" of redox-active ions within the

physiological redox architecture are not well described. Substantial geographical variations in metal exposure from soil, water, dust and diet warrant additional investigation for their associated effects on oxidant signalling mechanisms. NRF2 and HIF-1α provide pivotal control of systemic iron homeostasis[81], 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.

[H2] *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[82]. A pivotal consideration is the distinction between eustress (good stress) and distress (bad stress)[83]. Oxidative eustress describes physiological deviations from the steady-state redox set point[82,84], which has been called the 'golden mean of healthy living'[85]. At higher levels of oxidative challenge[86], there is the transition to oxidative distress, associated with biomolecular damage. As a corollary, a lack of oxidants manifests as 'reductive stress'[87], and there are cellular mechanisms to detect and counteract reductive stress[88]. Defining the thresholds between eustress and distress in molecular terms is an important task for further research.

[*H2] 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 **[G]** '[89,90] or more specifically as 'mitohormesis'[91], 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 also to promote lifespan and healthspan in a number of organisms. Eventually, as a result of the hormetic response, a new gene expression pattern of enzymes is established in the cell, including increased expression of antioxidant and other stress response pathways This adaptation to altered conditions has been termed 'allostasis'[92] or 'adaptive homeostasis'[93]. Even with eustress, there is constant monitoring and fine-tuning to maintain redox homeostasis[94]. The variety of human endogenous and exogenous exposures (exposome, discussed below) warrants extension of these important response concepts. Key issues in analysis of redox signalling in hormetic responses relate to identifying the spatiotemporal thresholds for induction of a reponse, and to the mechanisms of on- and off-switch of transcription factors, likely with the NRF2 system being most important[89,95]. Appropriate cell culture conditions need to be used to obtain data from cell culture for translation to in *vivo* conditions[96](see below).

[H1] Dissecting oxidant (patho)physiology

Considerable evidence supports the role of oxidants as signals in multiple systems, as has been documented in research on thermogenesis[97], immunity[98,99], fibrosis[100,101], cognition[102], exercise[103], ischemia-reperfusion injury[104,105], development[106,107], steroidogenesis[108], cancer[109,110], ageing[111-113], and oxygen sensing[114] **(FIG. 1)**. Below, we delineate two main research areas that require further study to dissect the impact of oxidants on pathophysiology of entire organisms.

[H2] *Determining oxidant function.* Many of the initial targets of oxidants 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 impossible to determine whether they are regulated by oxidation. An example is provided by glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in which oxidation of the catalytic Cys (C152 in humans) to a sulfenic acid 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. The C156A mutation does, however, attenuate the sensitivity of C152 to H_2O_2-induced oxidation. Another consideration for determining oxidant function is potential spatiotemporal alterations in protein structure, which may expose cryptic Cys residues to the solvent, thereby allowing redox regulation, as reported for the epidermal growth factor (EGF) signalling [115]. Functional *in vivo* testing of oxidant signalling will require development of tissue and cell-specific mouse models for individual target proteins with mutations in specific cysteine residues that prevent that H_2O_2-induced oxidation of the protein.

[H2] *Consideration of lifelong exposures that impact oxidant biology*. The exposome is defined as the cumulative lifelong exposure to external factors which complement the genome as determinants of health and disease[116,117]. Oxidant signalling responses to diet, microbiome, pharmaceuticals, dietary supplements, personal use products and environmental pollutants illustrate that redox systems function as an integrated network responding to environmental resources, such as O_2 and metabolic fuels, and challenges, such as physical demands and threats.

Nutritional components can be essential and beneficial, but also toxic and detrimental, depending on context and amount. The gut is a major interface between nutritive input and the organism, and host-microbiome relationships include redox interactions[118]. For example, oxidants from host mitochondria influence gut microbiome diversity[119], and gut-resident *Lactobacilli* activate hepatic NRF2 to protect against oxidative liver injury[120]. Dietary polyphenols generate H_2O_2, activate NRF2 and affect redox

signalling[121], and polyphenol consumption has been linked to attenuation of oxidative stress and disease[122,123]. Exposure from external sources impinges on the skin, lungs and the gastrointestinal tract, and certain types of radiation affect other organs as well (*e.g.*, the eye). Dietary factors counteract exposomal skin damage[124,125]. Among lifestyle factors, physical activity has a direct relationship to H_2O_2 and adaptive processes in skeletal muscle[126], including epigenetic responses[127] in exercise physiology[128,129].

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[130]. Of note, physiological external stimuli are sensed and processed by the TRP channels **[G]** , whose functional state is subject to modification by H_2O_2 and electrophiles[131]. Supraphysiological oxidant production can occur directly from the agent by non-enzymatic chemical reactions, or from hyperactivation of endogenous pathways. This spectrum of the effects of the exposome on redox balance is complex and illustrated by airborne particulate exposure, which is common in everyday life[132]. These 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 these non-degradable particulates[133]. Oxidant generation can also be enhanced by metal ions bound on particulates. Other oxidant sources are engineered nanoparticles[134], noise[135] and electromagnetic fields[136], indicating potential for widespread exposures from industrial and personal use products, microplastics, and different forms of radiation exposure. It is also established that components of the exposome can induce extrinsic skin aging[137].

A target of environmental stressors is the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that integrates immune responses[138-140]. This receptor controls redox homeostasis and shapes the tumour microenvironment[141]. AHR induces cytochrome P450s which, besides serving to metabolize xenobiotics, contribute to oxidant production, and can cause developmental toxicity as shown by zebrafish embryo exposure to $PM_{2.5}$ particles[142]. We recommend further exploration of the role of AHR in environmental redox biology.

Systems biology and network medicine will enable risks to be stratified within populations and individuals as well as providing a greater understanding of the cellular responses required for stress adaptation and hormesis by the entire organism[143-145]. 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 and other biofactors 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 the redox balance[146,147].

[H1] Applications to redox medicine

With better understanding of the biology of specific oxidants, a horizon is opening for "precision redox medicine"[148], including the recent exploration of the use of nanomaterials with oxidant-modulating properties[149]. 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 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 targeting is focused on selective modulation of enzymatic sources of oxidants, such as NOXs[150] or myeloperoxidase **[G]** [151] focused on an updated mechanism-based definition of disease[152] rather than a non-specific approach. The central redox sensor system involving NRF2 is a prime therapeutic target for cancer[3,153,154] and chronic disease[155]. Several agents known to induce NRF2 are either approved or in Phase III trials[69], but it is not yet established whether NRF2 mediates their therapeutic actions. As with oxidant generation, selective and cell/tissue-specific targeting of the NRF2 system is needed for effective applications[2]. As an additional layer of regulation, the ability of oxidants to alter the epigenetic landscape[156,157] offers perspectives in precision medicine[158].

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[152]. The emerging redox regulation of immunometabolism, with points of control of proliferation, survival and function of T cells, B cells and macrophages via the NRF2 pathway[98], underscores the utility of targeting central regulatory hubs as an approach in redox medicine.

The fine line between beneficial (preventative) or detrimental use of oxidants and antioxidants, is a key challenge. For example, this has been called the 'peroxide dilemma' in describing the concurrent role of H_2O_2 in both mediating and opposing insulin action[159]. Similarly, application of low-molecular-mass antioxidants has been shown to have both beneficial and detrimental effects on the progression of several diseases[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 the central transcriptional regulatory systems, will lead to strategies for enhancing antioxidant defences, re-directing inflammatory signalling, hypoxia adaptation, mitochondrial biogenesis, and other key mechanisms to alleviate disease. Each of these transcriptional systems has multiple positive and negative regulators as well as important targets (such as central kinase signalling systems, as well the glycolytic enzyme GAPDH, mitochondrial UCPs and kinase adaptor proteins), thereby providing different possibilities to enable disease prevention or management. Thus, opportunities exist to

415 develop redox medicine interventions for mitochondrial reprogramming in which feedback from
416 mitochondrial redox and metabolite signals enable mitochondrial function to adapt to conditions, for
417 example by altering mitochondrial dynamics and the transcription of mitochondrial genes,
418 angiogenesis, cell and tissue repair, ageing and cell senescence. In cancer, the therapeutic possibilities
419 are centred on harnessing oxidants for specific killing of malignant cells[160].

420

421 **[H1] Measuring and manipulating 'ROS'**

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

427

428 [H2] *Recapitulating physiological context in cell culture.* Control of environmental factors
429 including ambient O_2 and CO_2, pH and the composition of media is critical to ensure
430 physiologically-relevant cellular function[161,162]. Culture of primary cells and immortalized cell
431 lines *in vitro* is routinely conducted under atmospheric oxygen levels, which means that the
432 cells are exposed to hyperoxia, as their physiological O_2 environment *in vivo* is substantially
433 lower. Such a pro-oxidant environment significantly affects their redox phenotype,
434 upregulates antioxidant defence genes and reduces replicative lifespan. We thus recommend
435 recapitulating physiological O_2 levels in cell culture to enhance the translation of *in vitro*
436 findings (**BOX 3**)[162].

437

438 [H2] *Analytical methods for detecting specific oxidant species*. A major challenge confronting
439 oxidative stress research is to identify what specific reactive oxygen species are produced in living
440 systems and establish when they are produced, in what location and quantity, requiring real-time
441 monitoring with live cells. To date, much of the methodology has relied on redox-active fluorescent
442 and luminescent probes such as dihydrodichlorofluorescein ($DCFH_2$). These are still in widespread use
443 and form the basis of most commercial kits. However, they have significant limitations and give
444 results that are frequently misinterpreted (see **TABLE 1** and numerous reviews for critical
445 assessment[163-166], most recent[167,168]). At best they can provide a preliminary assessment , and we do not
446 recommend using them for analysis of specific oxidants. Small molecule caged fluorophores are less
447 open to artefact (**TABLE 1**). Genetically encoded fluorescent protein probes are available for specific
448 detection of H_2O_2 (**BOX 4**)

Hydrogen peroxide. In systems where expression is possible, genetically encoded fluorescent sensors are recommended for intracellular H_2O_2 detection[169]. They provide spatiotemporal detection and can be expressed in single cells, organoids, organs, and whole organisms (e.g. zebrafish[170], *C. elegans*[171], *Drosophila*[172], mammals[173,174]) as applied to study processes as diverse as embryogenesis, cell proliferation, wound healing and brain hypoxia (**BOX 4)**. HyPer-based[38,175] and roGFP-based sensors[176,177] are available with HyPer7[38] and roGFP2-Tsa2ΔC$_R$ [177] the most sensitive[178]. Enzyme-based (chemogenetic) generation of H_2O_2 has become a powerful approach to study the role of H_2O_2 using D-amino acid oxidase (DAO) or glucose oxidase (GOX) (**BOX 4**). Expression of DAO and targeting to subcellular compartments 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 [179]. Chemogenetic H_2O_2 generators can be co-expressed with genetically-encoded probes to visualize subcellular H_2O_2 production[38,180,181].

Small molecule probes can also be used for detecting intracellular H_2O_2, provided appropriate cautions and caveats are adopted (TABLE 1). Probes that react with H_2O_2 to release a caged fluorophore are strongly recommended over redox-active probes, which are confounded and do not react directly with H_2O_2 [182-184]. Focus has been on boronates **[G]**, and a wide range has been developed that enable detection of localized intracellular or transcellular activity[185]. Low sensitivity to levels of H_2O_2 relevant for signaling can be a limitation [165,186], and H_2O_2 must be distinguished from ONOOH and HOCl, which react much more rapidly[187]. Redox-active probes such as Amplex Red, in combination with horseradish peroxidase to trap the H_2O_2 reliably measure extracellular or released H_2O_2[165].

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. A specific optical readout for H_2O_2 is the charge transfer band of catalase Compound I (near infrared, ~660 nm) in the difference spectrum with the resting enzyme[188]. Using this method, H_2O_2 was first detected in intact cells of perfused liver [189]. Steady-state titration with a hydrogen donor for Compound I, e.g. methanol, permits determination of the rate of H_2O_2 production per gram tissue[190].

Another approach to monitor H_2O_2 is to take advantage of its high reactivity with peroxiredoxins. As mentioned, peroxiredoxins are strongly implicated in redox signalling[22,37], and the distinct locations and binding partners of the five human 2-Cys forms of peroxiredoxins constitute a basis for selectivity of signalling by H_2O_2 [23]. The redox state of peroxiredoxins is monitored by separating the oxidized dimer from the reduced monomer[191], and real-time monitoring is possible using genetically-encoded fluorescent analogues[192].

Superoxide. No genetically encoded probes for $O_2^{\cdot-}$ are currently available. The best validated fluorescent probe is hydroethidine, or its mitochondrially-targeted analogue MitoSOX. Improved

although less available versions based on NeoD do not intercalate with DNA and are more selective[193], An increase in fluorescence alone is not sufficient evidence for $O_2^{\cdot-}$ because hydroethidine is oxidized non-specifically to give fluorescent ethidium [164]. Specificity requires validation by HPLC or mass-spectrometric post-analysis of the $O_2^{\cdot-}$ generated product, 2-hydroxyethidium[194], and interpretation of 2-hydroxyethidium is subject to caveats[164]. Other sensors for detection and imaging of $O_2^{\cdot-}$ [33,195], including-redox probes that show a preference for $O_2^{\cdot-}$ over H_2O_2 and other oxidants[196], have promise but require more testing. Assays using cytochrome c **[G]** or tetrazolium salts **[G]** [197] provide reliable quantification of $O_2^{\cdot-}$ release 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 **[G]**, the assay itself generates $O_2^{\cdot-}$, so is self-defeating [166]. Luciferin analogues such as coelenterazine give less self-generation of $O_2^{\cdot-}$, but are still confounded by reacting with other radicals [166]. The most definitive evidence for $O_2^{\cdot-}$ uses electron paramagnetic (spin) resonance (EPR or ESR) spectroscopy with spin trapping [198]. Signals are highly characteristic, and this method is recommended for unequivocal identification. 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 high sensitivity of immunological techniques[199,200]**,** is useful for detecting stable radical adducts.

Reactive nitrogen (RNS) and halogen species (RHS). These contribute to oxidative eustress and distress and need to be distinguished from other oxidants. Peroxynitrite can be monitored in cells in real time using boronate probes [165,187,201], and it needs to be distinguished from H_2O_2 using appropriate inhibitors or scavengers, such as L-NAME **[G]** to inhibit nitric oxide synthase. HOCl is a major oxidant produced by inflammatory cells, and numerous HOCl-sensitive probes have been described[202]. They are selective rather than specific as they react with all RHS,

To sum up, newer generation genetically encoded sensors and small molecule probes are now available for cellular and organismal studies, and we recommend that they be used rather than the common redox probes and associated kit assays. There are also a number of promising approaches under development that use novel technologies. For $O_2^{\cdot-}$, these include carbon dots with sensitivity in the pM range[203], a nanoprobe based on chemiluminescence resonance energy transfer[204], and a positron emission tomography radiotracer [205]. For studies of $O_2^{\cdot-}$ and/or $^1O_2$ on time scales of seconds, genetically-encoded photosensitizers (optogenetic tools) are recommended [206,207] (**BOX 4**). Examples of their use include inactivation of certain cellular proteins and induction of photoinduced cell death[206,208]. For H_2O_2, surface-enhanced Raman spectroscopy (SERS) shows promise[209], with nanosensors anchored to the outer surface of the cell, or mitochondrial localized, allowing site-specific detection [30, 210]. EPR in combination with low-field magnetic resonance imaging to delineate tissue architecture has been used to obtain information on *in vivo* redox status[211]. Single-photon counting of low-level (ultra-weak) chemiluminescence emitted from electronically excited molecules has been used for dynamic monitoring of oxidative stress metabolism in intact cells and

organs[212,213]. While the photoemitting molecules need to be specified, a correlation between ultraweak photon emission and NADPH oxidase activity has been found[213]. In most cases these need further testing and wider availability before they are ready for general use. We recommend to redox biology researchers that they be aware that new technologies are coming along.

[H2] *Oxidant biomarkers and multi-omics approaches.* Development of reliable oxidant biomarkers is essential, representing oxidation products that are specific to a particular reaction of the oxidant of interest. Ideal techniques provide detection, identification, location and ready quantification of biomarker species, thereby enabling the changes in oxidant level and oxidative damage to be inferred[214]. The most commonly used technique is MS-based proteomics. Here, products generated upon oxidation can be determined directly or chemically derivatized for their detection. The latter increases sensitivity but often prohibits accurate quantification as derivatization is usually not 100% efficient. Novel enrichment techniques, e.g. specific binding columns, immunoprecipitation[215,216], allow MS methods to be readily used on complex systems including *ex vivo* human samples. MS analysis can be carried out (i) at the individual component level after processing to release individual species; (ii) at the peptide level for proteins; or (iii) at the intact molecule level, e.g. phospholipids, cholesterol esters, proteins, lipoproteins, each with advantages and disadvantages (**BOX 5**). MS-based analysis at a single cell level is now within reach[217].

In addition to proteomic analysis, the synthesis of data from multiple different 'omic' methods (proteomics, transcriptomics, metabolomics and epigenomics) is becoming an important tool in redox biology. For example, protein glycosylation pathways enormously amplify the proteome[218], and there are fascinating reciprocal interrelationships between glycan biosynthesis and the redox state[219]. The emerging field of 'glyco-redox'[220] may uncover relations between glycome, glycoproteome and redox proteome. A key advantage of 'omic' methods is that they simultaneously provide information on multiple oxidant targets[68]. The rapid development in 'omic' techniques transforms current understanding of the molecular details of oxidant signalling (FIG. 2, **BOX 5**). Thus, 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, showed that signalling responses are integrated across the 'omic' layers, i.e. they induce effects at the RNA, protein and metabolite levels[221] . Readily-accessible databases, such as the Oximouse (https://oximouse.hms.harvard.edu/) for redox proteomics[60], Metabolomics Workbench (https://www.metabolomicsworkbench.org) for metabolomics, and Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) for transcriptomics, allow mining of pre-existing data to enhance statistical power 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 the effects of oxidative stress [222-225], and can be used as a diagnostic for several diseases associated with excessive generation of oxidants (e.g. cardiovascular, neurodegenerative, diabetes, obesity)[226].

[H2] *Pharmacologic and genetic manipulation of oxidant production*. These can address the site and type of individual oxidant generation, although off-target effects limit both approaches[152]. Pharmacologic interventions can afford specificity, are readily implemented and can probe dose and time-dependencies well as reversibility. A genetic approach to diminish mitochondrial $O_2^{\cdot-}$ is the expression of the alternative oxidase from *C. intestinalis* (AOX)[227], which takes electrons from ubiquinol and transfers them directly to O_2 to form water, limiting electron flow from ubiquinol to Complexes I or III of the ETC for $O_2^{\cdot-}$ generation[228]. AOX expression prevented toxin-induced pathologies or endotoxemia[99] and physiologic responses like acute pulmonary oxygen sensing[114]. The development of molecules that selectively suppress $O_2^{\cdot-}$ production from mitochondrial Complex I_Q or III_{Qo} ,referred to as S1QELs (Suppressors of site I (or III) Electron Leak), provide further evidence that these sites of $O_2^{\cdot-}$ production contribute to pathologies like cardiac ischaemia-reperfusion injury[229,230]. These molecules have advantages over genetic strategies including specificity of suppressing $O_2^{\cdot-}$ from a particular ETC site and relative ease in experimental usage[32]. However, a caveat is that it is not known how they prevent $O_2^{\cdot-}$ production without interfering with normal ETC function.

The use of conditional knockouts to elucidate cell specific effects of NOXs is warranted. 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[231]. Finally, an exciting development in the field has been the translation of NOX1/NOX4 inhibitor setanaxib (initially known as GKT137831) into clinical trials[150].

A direct way to assess the impact of H_2O_2 is to genetically express catalase targeted to different subcellular compartments. Mice were generated that overexpress catalase in either the nucleus (nCAT) or mitochondria (mCAT), as well as the wild-type peroxisomal catalase (pCAT) to study aging and age-related diseases[112]. Strikingly, mCAT mice, unlike pCAT or nCAT mice, displayed a significant increase in maximal and median life span[112]. mCAT overexpression reduced insulin resistance, atherosclerosis, cardiac failure, pulmonary hypertension, muscle atrophy, and various types of cancers[111]. Conditional overexpression of mCAT in adult astrocytes demonstrated that mitochondrial H_2O_2 is necessary to control physiologic cognition[102]. The conditional overexpression of mCAT in different cell types will be essential in elucidating the requirement for H_2O_2 in physiology or pathology.

An downstream effect of H_2O_2 production, in the presence of ferrous irons (Fe^{2+}), is the generation of lipid hydroperoxides (ROOH) from polyunsaturated fatty acids (PUFAs) and subsequent ferroptosis, a form of programmed cell death[232] **(FIG. 1).** A variety of systems including cyst(e)ine/GSH/GPX4, CoQ_{10}/FSP1, squalene, and BH_4/DHFR, prevent ferroptosis by reducing ROOH to ROH[232]. The importance of lipid peroxidation comes from the observation that GPX4 homozygous knockout mice die *in utero*, and neuronal or renal specific loss of GPX4 is lethal. It will be important to compare the effects of organelle-specific conditional overexpression of either catalase or GPX4 in a given cell type to test the importance of H_2O_2-dependent signalling or ROOH-dependent ferroptosis, respectively.

[H1] Concluding Remarks

New opportunities exist for research in cell biology to advance the capability to discriminate oxidative eustress from distress at the cell and molecular level, especially for elucidation of specific oxidant signalling mechanisms in disease. Specifically, it is recognized that pleiotropic functions of oxidant signalling impact master regulators of transcription and cell homeostasis. Thus, we recommend that cell biology studies incorporate redox therapeutics for individual disease processes by focussing on specific cell signalling systems, i.e. to well-defined oxidants, notably H_2O_2 and $O_2^{\cdot-}$, their generation systems as well as their redox targets. Such endeavours require detailed understanding and quantification of the molecular species involved, and sites and rates of production at the cellular level. This requires more precise definitions and improved analytical tools. In principle, antioxidant defences, mitochondrial biogenesis, cell quality control, hypoxia adaptation and other key processes could be adjusted to improve health or counteract disease. It is recommended that real-time imaging tools, which detect localized oxidant generation and signal transduction, be used to more accurately define cellular redox mechanisms in tissues, 3D organoids and cells in culture under well-defined physiological O_2 levels. This cell biological research will provide foundations to help harmonize research among the many disciplines towards a common goal of understanding redox biology and medicine, and allow the design of targeted manipulations to restore beneficial oxidant generation for specific disease phenotypes. It will also provide cell-based tools for high-throughput screening of therapeutics for future personalized redox medicine.

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BOX 1

Key Recommendations for studies in redox biology

General

• Focus research on specific oxidants, e.g. H_2O_2 or $O_2^{\cdot-}$, and only use the generic term "ROS" when referring collectively to multiple species and consider using the term oxidant when the identity of the species is ill-defined.

• Identify specific protein Cys in 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 "by-product" or "leakage";

• Be aware of the intricate relations to other reactive species (RNS, RSS etc), and their interactions within the "redox interactome".

• Define thresholds ("tipping points") between redox eustress and distress in molecular terms

Technical

• Use physiological O_2 atmospheres in cell culture experiments, tailored to the specific cell-type; choose consistent time-of-day for sampling because of circadian (diurnal) fluctuation.

• Use *in vivo* model systems (zebrafish, *C. elegans*, *Drosophila*, *Xenopus,* mammals).

• Use non-invasive genetically encoded probes to identify and quantify oxidant formation.

• Restrict the use of redox-probes, and be aware of non-specificity and other pitfalls.

• Use 'omic' technologies and redox biomarkers to examine clusters of genes, proteins and metabolites that respond together, rather than measure single species.

Cell physiology

• Analyze the dynamics of the cellular "redox landscape", i.e. the subcellular oxidant profile and associated changes in the redox proteome.

• Study mitochondria in the intact cell, not as isolated organelle. Consider redox interactions between organelles, *e.g.* mitochondria, peroxisomes, ER, nucleus.

• Explore patterns of oxidative post-translational modifications.

Organism level

• Interpret pharmacological and genetic intervention at 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.

BOX 2

Terminology of ROS and their characteristics

ROS is a general term that provides no information on the molecular nature of the species being reported and limits the biological information. Whenever possible, designation of specific oxidants such as superoxide ($O_2^{\bullet-}$) or H_2O_2 is preferred. The term "oxidant" is preferred when unspecified reactive species are referred to. ROS are generally divided into radicals (sometimes unnecessarily called a "free" radical) and non-radical species.

Radical (one-electron) ROS:

• **Superoxide anion radical (**$O_2^{\bullet-}$**)**. Commonly called "superoxide", it exists at very low concentrations. Most assay methods are selective but not absolutely specific (see BOX 5). Generated by single electron transfer to O_2 in electron transport chains (mitochondria, ER, plasma membrane) and by enzymes. A weak oxidant (electron or hydrogen atom removal) and also 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 a second $O_2^{\bullet-}$, giving O_2 and H_2O_2. This is catalyzed by superoxide dismutases (SODs). The protonated form (perhydroxyl radical, $HO_2^{\bullet}$) occurs at significant concentrations in acidic environments (pK_a 4.9) and can, unlike $O_2^{\bullet-}$, diffuse through membranes and there can abstract a hydrogen atom from conjugated methylene groups of polyunsaturated fatty acids (PUFAs) to give carbon-centred radicals ($R^{\bullet}$). $O_2^{\bullet-}$ reacts rapidly with other radicals, e.g. nitric oxide ($^{\bullet}NO$) to give peroxynitrite ($ONOO^-$).

• **Hydroxyl radical ($HO^{\cdot}$)**. The most reactive biological oxidant which reacts non-specifically at diffusion-controlled rates ($k \sim 10^9\ M^{-1}\ s^{-1}$) with biomolecules. Products formed from proteins, DNA, RNA and lipids serve as biomarkers of damage. 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 (e.g. by Fe^{2+} or Cu^{+}) may affect other signalling processes.

• **Peroxyl radicals ($ROO^{\cdot}$)**. Formed during lipid and other peroxidation reactions and radical chains, and can also be derived from enzyme-generated lipid hydroperoxides. May serve as signalling agents, as they stable enough to diffuse significant distances.

• **Alkoxyl radical ($RO^{\cdot}$)**. Highly reactive species formed by one-electron reduction of 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, with the radical site providing possible specificity.

Non-radical (two-electron) ROS:

• **Hydrogen peroxide (H_2O_2).** H_2O_2 is a pleiotropic oxidant signalling agent. When validated with appropriate methods, it should be explicitly named and not described as 'ROS'. 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 downstream signalling mechanisms, and the role of redox relays. These can occur via direct thiol oxidation or via heme enzymes (peroxidases), iron–sulfur 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*)**. Excited state species formed by energy transfer from ultraviolet (UV) or visible light with a sensitizer as well as chemical and enzymatic reactions. Important mediators of damage to surface organs (e.g. skin and eye). Can be quenched by dietary carotenoids. Limited information is available concerning 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.

• **Organic peroxides (ROOH and ROOR).** Formed enzymatically by lipoxygenases and cyclooxygenases with specific PUFAs and important in enzyme-mediated and non-enzymatic cell

signalling. Also formed via peroxyl radicals during lipid and protein peroxidation. Can function directly in signalling through the NRF2-KEAP1 system and oxidation of other protein Cys.

• **Hypochlorous (HOCl), hypobromous (HOBr) and hypothiocyanous (HOSCN) acids**. Reactive species generated by myeloperoxidase and related enzymes from H_2O_2 and halide/pseudohalide ions (Cl^-, Br^-, SCN^-) that are 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 alpha,beta-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). 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 ($ONOO^-$).** Peroxynitrite is the anion of peroxynitrous acid (ONOOH; pKa 6.8) formed physiologically from reaction of $NO^{\bullet}$ and $O_2^{\bullet-}$. A powerful two-electron oxidant and nitrating agent studied extensively in pathobiology, warrants examination for signalling activities. Reacts rapidly with CO_2 to form $CO_3^{\bullet-}$ and $NO_2^{\bullet}$, which are responsible for much of its impact.

BOX 3

Achieving physiological normoxia in cell culture.

*Adaptation of cells to physiological normoxia***.** For standard cell culture, as well as co-culture models and 3D-organoids[162,233], we recommend that protocols be employed that provide long-term adaptation to O_2 levels that cells encounter *in vivo*. Standard air incubator O_2 conditions (18% at 75% humidity) results in non-physiological pericellular O_2 for most cell types. For lung and aorta, normoxia is ~13% O_2 but for most others, including microvascular cells, neurons and stem cells, the O_2 level needs to be between 2-7% [162, 234, 235]. To achieve a normoxic phenotype, we recommend adapting cells to a defined O_2 level for 5 or more days. Cellular responses to hypoxic pO_2 levels are mediated via hypoxia-inducible factor (HIF1-α), the stability of which is modulated by oxidant levels[71]. This period of adaptation is critical for establishing a 'normoxic' phenotype in the absence of HIF-1α mediated signaling [96,236,237]. It is preferable to the more common protocol of 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 HIF-1α and HIF1α-mediated hypoxic responses are suppressed [237]. Compared to cells adapted to 5% O_2 culture of endothelial cells at 18% show a ~2-fold increase in NRF2-dependent antioxidant gene expression (HO-

1, NQO1)[71] and their replicative life span is reduced, and embryonic fibroblasts have a decreased ability to upregulate antioxidant defenses in response to H_2O_2[238]. Culture of stem cells under physiological pO_2 (3%) improves cell survival and the potential for tissue repair[168].

Practical considerations. To achieve physiological pericellular (*in vivo* mimetic) pO_2 levels in cell culture, workstations are available (see[162]) in which O_2, CO_2, N_2, and humidity can be carefully regulated in long-term culture. Miniaturized O_2-controlled portable platforms enable short-term maintenance of cells under defined pO_2 for imaging analyses. It is important to consider the permeability of plasticware, volume of medium, and rates of O_2 equilibration. Cellular O_2 consumption, together with limited diffusion of O_2 into solution, generates a gradient between ambient levels and those experienced by cell monolayers[162,239]. Thus, when cultured in the same pO_2 environment, cell types consuming less O_2 experience a greater intracellular pO_2 compared to cells that consume O_2 more rapidly, such as contracting cardiomyocytes. A feedback-controlled Oxystat system **[G]** can be used to maintain pO_2 levels in cell incubations[240]. 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 pre-set pO_2, noting however that subsequent treatments and manipulations would require brief exposure to hyperoxia.

BOX 4

Genetically-encoded techniques 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 (**Scheme, panel A**). Both consist of a modified fluorescent protein linked to H_2O_2 sensing domain (OxyR-RD in HyPer and peroxidases in roGFP-based H_2O_2 probes) that translates oxidation by H_2O_2 into ratiometric changes in the fluorescence spectrum. HyPer7[38] and roGFP2-Tsa2ΔC_R[177] are recent versions that detect H_2O_2 in the low-nanomolar range. The chemical basis of probe function is Cys oxidation to a disulfide, which is reversed by cellular thiol reducing systems. Therefore, in each particular moment the oxidation state of the probe reflects both the presence of H_2O_2 and reducing system activity. Advantages of the probes over synthetic dyes include: (i) subcellular localization to the organelle of interest; (ii) expression in transgenic animals under cell- and tissue-specific promoters; and (iii) greater selectivity and less phototoxicity compared to synthetic dyes. Successful

applications in model organisms were performed *e.g.* in zebrafish[170], *C. elegans*[171], *Drosophila*[172], *Xenopus*[241], mammals[173].

In HyPer probes, OxyR oxidation 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-dependent reducing system. Orp1 can be also reduced by Trx. roGFP alone or fused with non-peroxidase thiol exchange domains (e.g. Grx1-roGFP2) should not be used to detect H_2O_2 or other 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 HyPer 1-3 and HyPerRed are pH-sensitive and should be used only in combination with an appropriate pH control (SypHer)[242]. 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 (e.g. HyPer1-3, HyPerRed, roGFP2-Orp1) should be used.

Chemogenetic tools for generation of oxidants. Synthetic biology approaches to modulating H_2O_2 generation use glucose oxidase (GOX) or D-amino acid oxidase (DAO) (**Scheme, panel B**). GOX oxidizes glucose, releasing H_2O_2[243]. And when added to cell culture medium containing glucose, allows controlled steady-state extracellular H_2O_2 production. The presence of glucose does not allow substrate control but H_2O_2 production can be regulated by enzyme concentration. In contrast, DAO can be expressed intracellularly and regulated by D-alanine concentration. 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. Optogenic probes use fluorescent proteins "open" structures that enable their photoexcited chromophores to donate electrons to O_2, directly or via intermediate redox pairs, generating $O_2^{\cdot-}$ or $^1O_2$ on illumination with appropriate wavelength light (**Scheme, panel B**). These genetically encoded photosensitizers, include GFP-like proteins (e.g. KillerRed, KillerOrange or SuperNova[244-246] or flavin mononucleotide (FMN) binding domain proteins (miniSOG)[247]. A limitation is the low tissue penetration of visible light, mainly restricting their use to cell culture experiments.

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 oxidative damage as surrogate markers (see Insert, derived from data in Ref[226]).

Oxidation products of proteins and peptides (e.g. glutathione (GSH)) can provide information on oxidant signalling:

- Oxidation of Cys in proteins and GSH is highly relevant, with the Oximouse[60] recommended as a key resource.
- Protein carbonyls are formed directly or from hydroperoxides. They provide a general marker of oxidative stress and correlate with some diseases.
- Products formed by particular ROS (3-nitrotyrosine, 6-nitrotryptophan from NO_2/ONOOH; 3-chlorotyrosine from HOCl/myeloperoxidase) can be detected by LC-MS and correlate with some diseases.
- Protein glycation biomarkers include advanced glycation end products (AGE)[248].

Mass spectrometry (MS) allows distinct mass changes induced by specific oxidant species to be detected[249,250]. Peptide mass mapping, either directly or using tagged species, can provide data on modification sites. High sensitivity allows increasingly small samples to be examined, e.g. from laser-dissection of tissues [251], and proteomic analysis of single cells obtained by fluorescence-activated cell sorting [252]. Targeted methods such as Redox Western and biotin-conjugated iodoacetamide (BIAM) blots are available to measure oxidation of specific protein thiols. For instance, 2-Cys peroxiredoxin oxidation by H_2O_2 causes a monomer-dimer conversion detectable by non-reducing SDS-PAGE[191] and has been used along with Redox Western analysis of thioredoxins to measure compartmental H_2O_2 production. Derivatization of Cys sulfenic acids by dimedone-type probes[253] and carbonyls using hydrazines requires significant incubation times, though reagents that allow enrichment[254], and those with higher rate constants are being developed [253].

Oxidized lipids function in signalling as well as occurring as a consequence of cell death. Oxidized lipids can also be derived from the diet. They include: PUFA and phospholipid biomarkers include F_2-isoprostanes[255], 4-hydroxynonenal (HNE) and related aldehydes[256]. F_2-isoprostane levels correlate with disease severity in multiple pathologies.

- Cholesterol oxidation products (7-ketocholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol), also measured by LC-MS, can indicate oxidant production and correlate with disease.

Oxidative products of DNA and RNA are measured by LC-MS. These have been extensively studied for oxidative DNA and RNA damage[257,258]. Oxidatively generated base modifications in DNA have been implicated in redox signalling[259]. 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) are major products due to ease of oxidation of guanosine, but multiple other products are also quantifiable. These are detectable in plasma, urine, cells and tissues. Elevated 8-oxodG and 8-oxoG correlate with ageing and multiple pathologies.

- Chlorinated products are formed by HOCl/myeloperoxidase systems, and together with myeloperoxidase protein, are markers of inflammation and correlate with disease.
- Nitrated products (8-nitro-2′-deoxyguanosine, 8-nitro-guanosine) are generated by peroxynitrous acid and myeloperoxidase and can be detected/quantified by LC-MS.
- DNA damage (strand breaks, DNA adducts, excision repair sites and cross-links) can be detected by the Comet assay (single-cell gel electrophoresis) in eukaryotic cells[260]

Antioxidant products have been extensively studied as biomarkers of oxidative damage, but further research is required to assess their role in oxidant signalling:.

- The glutathione disulfide (GSSG)/GSH ratio (cells or tissues), and cystine/cysteine ratio (plasma) are oxidative stress markers and are perturbed in many diseases. Plasma cystine and GSH concentrations are independent predictors of death in coronary artery disease patients[261].
- Products from α-tocopherol (quinone, hydroquinone), uric acid (allantoin), ascorbic acid (dehydroascorbic acid) and coenzyme Q can be measured by MS-based metabolomic methods.

Table 1: **Advantages and disadvantages of redox-active and caged fluorescent or luminescent probes for detection and quantification of ROS**.

Probe type	**Redox-active probes**	**Caged probes**
Examples	Dihydrodichlorofluorescein ($DCFH_2$), dihydrorhodamine, hydroethidine and (mitoSOX),	Boronates (H_2O_2, $ONOO^-$), sulfonyl fluorescein derivatives ($O_2^{\cdot-}$) thioethers (hypohalous acids)

	luminol, lucigenin and related compounds.	
Mechanism	Oxidized by multi-step 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 readout of cellular redox environment. Used appropriately with a peroxidase, can give sensitive, quantitative detection of extracellular H_2O_2.	Less prone to interference. Improved selectivity. Reaction with H_2O_2 is direct. Standardization possible.
Disadvantages and cautions	React non-specifically with a wide range of ROS 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, independently of the amount of initial oxidant produced. Probe can self-generate ROS 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
Recommendation	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 cautiously.	Preferred as less prone to interference and improved specificity, but assignment to a specific oxidant requires validation.

GLOSSARY

Epiproteome

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.

Hormesis

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

Mitochondrial dynamics

Functional processes associated with changes in shape, distribution and size of mitochondria, especially involving fission, fusion and turnover.

Mitochondrial reprogramming

Functional processes associated with bioenergetic and metabolic changes of mitochondria. These include changes in relative contribution of mitochondrial oxidative phosphorylation and glycolysis to ATP production and concentrations of intermediates impacting regulation of histones by lysine acylation or other cell control system.

Oxystat system

A device or system to maintain a constant O_2 concentration despite fluctuations in 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.

Radical

Atom or molecule with one or more unpaired electrons

Redox hubs

A central node in a redox network through which redox changes can impact multiple downstream components

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 landscape

A topological representation of oxidants or oxidant-sensing systems within subcellular compartments.

Redox tone

Oxidation-reduction steady-states of redox-active elements in cells and tissues

Redox medicine

Use of concepts and strategies of redox biology for applications in diagnosis and therapy

Dismutation reactions
A reaction between two molecules of the same oxidation state in which one becomes oxidized and the second reduced, by equal numbers of electrons

Transition metal ion
A metal ion from the central block (Groups IVB–VIII, IB, and IIB, or 4–12) of the periodic table

NADPH oxidases
A membrane-bound complex assembled from multiple protein components that uses NADPH to reduce O_2 to the superoxide radical ($O_2^{\cdot-}$) and/or hydrogen peroxide (H_2O_2)

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

Reverse electron transfer
A flow of electrons through an electron transport chain in the reverse direction to that which normally occurs

Fe-S cluster proteins
Proteins in which multiple iron and sulfur atoms in the form of geometrical clusters are ligated to a surrounding protein. These occur in multiple forms including [2Fe–2S], [4Fe–3S], [3Fe–4S], and [4Fe–4S] forms and are often involved in electron transfer chains

Catalase

A heme-containing protein that enzymatically dismutates 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

A chemical substance that is not naturally present in an organism

Membrane contact sites

A location where membranes come in close proximity

Protein disulfide isomerase

An enzyme typically, but not exclusively, found in the ER of eukaryotes that catalyzes the formation and breakage of disulfide bonds between cysteine residues.

Thioredoxins

Small ubiquitous redox-active proteins that play a role in redox signalling via the maintenance of cysteine residues in their reduced thiol form.

Peroxiredoxins

A family of six ubiquitous highly reactive thiol-containing enzymes that control H_2O_2 levels and mediate signal transduction.

TRP channels

Transient receptor potential (TRP) channels are a group of ion channels mostly localized on the plasma membrane of animal cells, mediating a variety of sensations such as pain, temperature etc.

Myeloperoxidase

A leukocyte (mainly neutrophil and monocyte)-derived heme enzyme that catalyzes the conversion of 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.

Glutathione

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

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

Cytochrome c

A small (~12 kDa) heme protein usually found loosely associated with the inner mitochondrial membrane where it functions to transfer electrons between complex III and complex IV of the ETC via cycling between the Fe^{3+} and Fe^{2+} states. Release into the cell 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 substrates for active cellular dehydrogenases and reductases. In the presence of NADH/NADPH, they are

reduced to formazan which have strong, distinct optical absorption spectra. Widely used as a means of distinguishing metabolically-active cells from metabolically-inactive (dead).

Lucigenin

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

L-NAME

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

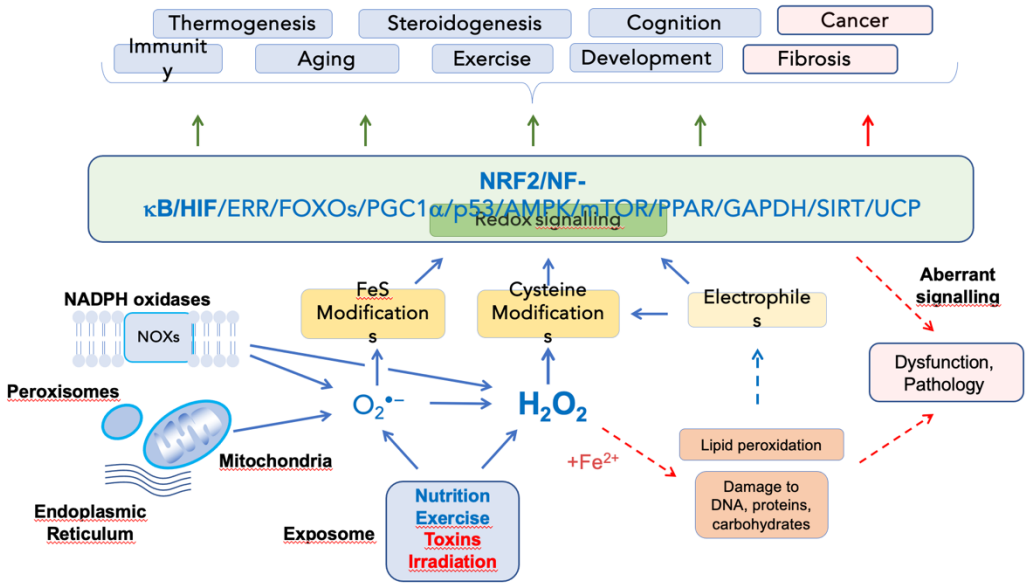

FIG. 1**. Simplified scheme of generation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and their relation to redox signalling**. $O_2^{\cdot-}$ and H_2O_2 (highlighted in red) are generated by multiple enzymatic and non-enzymatic processes including NAPDH oxidases (NOXs), mitochondria, endoplasmic reticulum, peroxisomes and external stimuli (‘exposome’)(blue boxes, lower left). Reaction with iron-sulfur cluster proteins (FeS), or the ionized form of cysteine residues (thiolate) on multiple redox sensitive proteins (yellow boxes), results in modifications that induce redox signalling (green box) and downstream biological effects (solid blue arrows). This includes signalling between the subcellular compartments (lower left). Endogenous or exposome-derived electrophiles (light yellow box) also induce redox signalling. Alternative reactions of oxidants (dotted red arrows) induce damage to biomolecules than can generate additional electrophiles and secondary species implicated in aberrant signalling and pathology (red boxes). Cys modifications predominantly include Cys-SOH and

Cys-SO_2H. Modifications not discussed in detail include Cys-SNO (nitrosothiol formation), Cys-SSH (persulfidation), and Cys-SSG (glutathionylation) as well as Tyr-nitration by peroxynitrite, which is formed from $O_2^{\cdot-}$ with nitric oxide. Molecular signalling systems (Center, green box) include NRF2, nuclear factor erythroid 2-related factor 2; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; HIF, hypoxia-inducible factor-1 alpha; ERR, estrogen-related receptor; FOXOs, forkhead box O transcription factors; PGC1a, peroxisome proliferator-activated receptor gamma co-activator 1 alpha; p53, cellular tumour antigen p53; AMPK, 5'-adenosine monophosphate-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIRTs, sirtuin protein family; UCP1, uncoupling protein 1. These signalling systems contribute to multiple biologic processes, including disease (green arrows, top boxes),

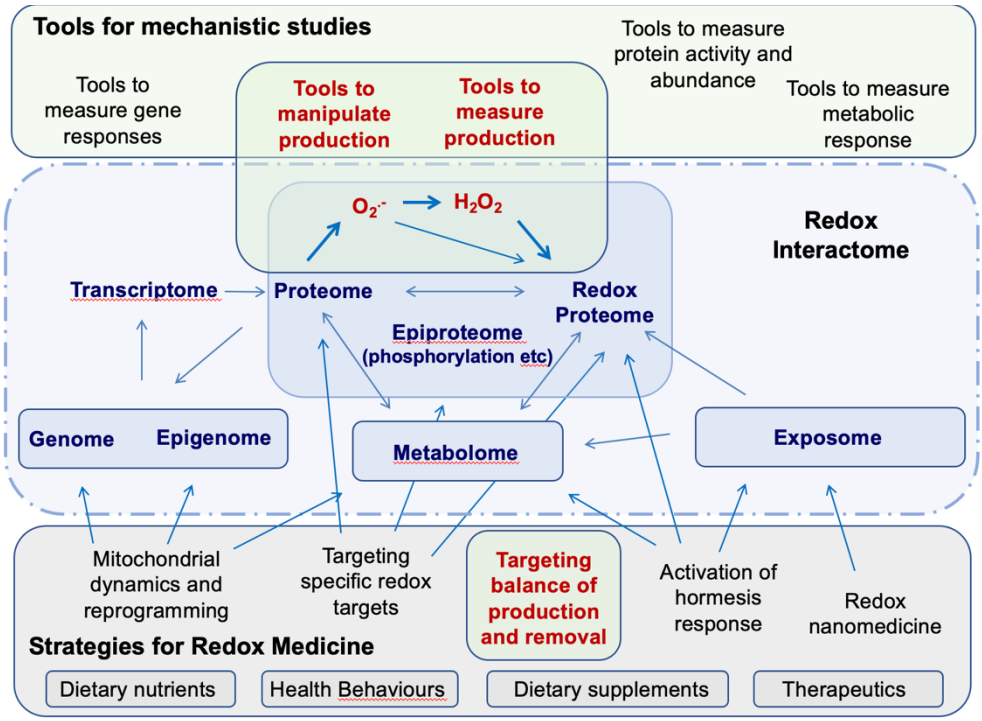

FIG. 2. **Redox interactome of H_2O_2 and $O_2^{\cdot-}$.** (***Center***) The redox interactome (blue box) is a functional network linking H_2O_2 and $O_2^{\cdot-}$ (highlighted in red) with all 'omics' domains (blue text). These omics domains are often measured as scalar entities with little or no spatial reference. New cell biological methods with spatial and temporal resolution are becoming available to enhance mechanistic understanding (red box, top). In this depiction, "redox interactome" is a collective term for the 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". The proteome includes many types of post-translational modifications, termed the "epiproteome **[G]** ", which interact with the redox proteome and also are involved in the 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. (***Top***) Advanced tools for mechanistic studies are available to manipulate and measure H_2O_2 and $O_2^{\cdot-}$ (highlighted in red), and these 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 the 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. (***Bottom***) Strategies for redox medicine (green box) should build upon understanding of the redox interactome (blue box) and the new tools used for mechanistic studies (red box). Prior research has focused extensively upon the balance of production and removal of oxidants (highlighted in red, center). While this balance remains important, new tools for mechanistic studies and omics capabilities to measure the redox interactome enable more targeted manipulations of dietary nutrients, micronutrients, dietary supplements, health behaviours and therapeutics to impact redox mechanisms underlying physiologic regulation and disease. These can include mechanisms such as mitochondrial dynamics **[G]** and mitochondrial reprogramming **[G]** and hormesis. Ongoing advances in pharmacologic approaches and redox nanomedicine will enable the 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.

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