REVIEW

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Orchestrating Redox Signaling Networks through Regulatory Cysteine Switches

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ABSTRACT Hydrogen peroxide (H_2O_2) acts as a second messenger that can mediate intracellular signal transduction *via* chemoselective oxidation of cysteine residues in signaling proteins. This Review presents current mechanistic insights into signal-mediated H_2O_2 production and highlights recent advances in methods to detect reactive oxygen species (ROS) and cysteine oxidation both *in vitro* and in cells. Selected examples from the recent literature are used to illustrate the diverse mechanisms by which H_2O_2 can regulate protein function. The continued development of methods to detect and quantify discrete cysteine oxoforms should further our mechanistic understanding of redox regulation of protein function and may lead to the development of new therapeutic strategies.

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and the hydroxyl radical ($^{\bullet}OH$) are generally deemed toxic consequences of aerobic life that are swiftly eradicated to maintain cellular homeostasis. If left unchecked, ROS can indiscriminately damage biomolecules and contribute to aging and pathologies such as cancer, diabetes, and neurodegenerative disorders (*1*–*3*). However, studies performed over the past decade also indicate that a diverse array of external signals (Table 1) stimulate the controlled production of ROS in healthy cells and have uncovered a role for oxidants as essential second messengers in intracellular signaling pathways. An important cellular target or "sensor" of ROS is the thiol (RSH) functional group of the amino acid cysteine, which can exist in a number of oxidation states such as disulfides (RSSR) or sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) acids (*4*). Such oxidative cysteine modifications can constitute a facile switch for modulating protein function, akin to phosphorylation. In this Review, we present current mechanistic insights into signal-mediated H_2O_2 production and highlight recent advances in methods to detect ROS and cysteine oxidation both *in vitro* and in cells. Selected examples from the recent literature of proteins that form disulfides, SOH, and SO_2H are discussed, underscoring the variety of mechanisms by which ROS can modulate protein function and signal transduction cascades.

H_2O_2 as a Signaling Molecule. $O_2^{\bullet -}$ spontaneously dismutates to H_2O_2, a process that is enhanced at least 1,000-fold by a class of enzymes known as superoxide dismutases (SOD) (*5*). In the presence of metal ions (iron or copper), H_2O_2 can be decomposed through the Fenton reaction to form $^{\bullet}OH$. Among these, H_2O_2 is the most abundant ROS (*in vivo* concentration of 10^{-7} M)

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TABLE 1. External stimulants that induce ROS production

Stimulant	Organism[a]	ROS source[b]	Effect of stimulant	Reference
		Peptide Growth Factors		
Epidermal growth factor (EGF)	Hs,M,R	NOX[c]	Proliferation	*40, 127–129*
Platelet-derived growth factor (PDGF)	Hs,M,R	NOX	Proliferation/migration	*129–132*
Basic fibroblast growth factor (bFGF)	B	NOX	Proliferation	*133*
Vascular endothelial growth factor (VEGF)	P	L	Angiogenesis/proliferation	*134*
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	H	ND	Proliferation/migration	*135*
Insulin	M,R	NOX	Glucose uptake/transport	*136, 137*
		Cytokines		
Lipopolysaccharide (LPS)	M	NOX	Induction of immune response	*39, 42, 138*
Interleukin-1β (IL-1β)	Hs,M	NOX,L	Induction of immune response	*129, 139*
Interleukin-3 (IL-3)	Hs	ND	Induction of immune response	*135*
Interleukin-4 (IL-4)	Hs	NOX	Induction of immune response	*27*
CD28 stimulation	Hs	L	Induction of immune response/ proliferation	*140*
Tumor necrosis factor α (TNFα)	B,M,Hs	NOX	Apoptosis	*26, 28, 129, 133*
Transforming growth factor-β1 (TGF-β1)	M	ND	Cell cycle arrest	*141*
		Agonists of GPCRs[d]		
Angiotensin II (AngII)	R	NOX	Hypertrophy	*22, 142–144*
Lysophosphatidic acid (LPA)	Hs	NOX,L	Proliferation	*145, 146*
Thrombin	Hs	NOX	Proliferation	*130*
Serotonin	Ha	NOX	Proliferation	*147*
		Other stimulants		
Wounding	Z	NOX	Leukocyte recruitment	*9*
Oxidative stress	D	MT	Differentiation	*33*
Reoxygenation after hypoxia	R	MT	$O_2^{\bullet -}$ burst	*43*

[a]B, bovine; D, *Drosophila melanogaster*; Ha, hamster; Hs, human; M, mouse; P, pig; R, rat; Z, zebrafish. [b]NOX, NADPH oxidase; M, mitochondria; L, lipoxygenase; ND, not determined. [c]For many of these cases, the specific NOX isoform activated is unknown. Each NOX isoform demonstrates disparate tissue expression, and continued studies will be required to elucidate the regulation of each NOX isoform in response to diverse external signals. [d]Guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors (GPCRs).

with the longest half-life ($t_{1/2} = 10^{-5}$ s) (*6, 7*). The relative stability and uncharged nature of H_2O_2 permits its enhanced diffusion across long distances and membranes, though it is likely that this oxidant is less membrane permeant than a gas such as nitric oxide. Interestingly, recent evidence indicates that $O_2^{\bullet -}$ may also cross membranes through anion channels (*5*). Owing to its highly diffusible nature, H_2O_2 has been shown to act as a paracrine signal both in plant cell differentiation (*8*) and more recently in the recruitment of immune cells to wound sites in zebrafish larvae (*9*). As will be discussed below, H_2O_2 can be quickly generated in cells, selectively perceived by downstream proteins, and undergo degradation by cellular antioxidant defense systems. Collectively, these properties make H_2O_2 an ideal mediator of signal transduction processes.

Signal-Mediated ROS Production. The mitochondrial electron transport chain (ETC) funnels electrons from reduced matrix substrates through four protein complexes (I–IV) to molecular oxygen producing water and establishing a proton gradient across the inner mitochondrial membrane. The energy from this gradient is then harnessed to drive the production of the primary cellular energy source, adenosine triphosphate (ATP). The final complex in this pathway, complex IV, delivers electrons to molecular oxygen to generate water; however, electrons can leak prematurely from the ETC upstream of complex IV to cause the univalent reduction of oxygen to $O_2^{\bullet -}$ (*6*). The accidental production of $O_2^{\bullet -}$ by the ETC is thought to be the primary intracellular source of this oxidant, though cellular signals can also stimulate $O_2^{\bullet -}$ generation in the mitochondria. This process is strictly dependent upon the redox enzyme $p66^{Shc}$, which has been shown to be a genetic determinant of lifespan in mammals (*10*). In response to signals that include growth factor deprivation, oxidative stress, or UV irradiation, $p66^{Shc}$ translocates to the mitochondria where it generates ROS (either H_2O_2 or $O_2^{\bullet -}$) by delivering electrons from the ETC to molecular oxygen (Figure 1, panel a) (*11, 12*).

P66Shc-derived ROS can diffuse into the cytoplasm where it down-regulates the activity of FoxO3, a transcription factor implicated in the expression of mitochondrial antioxidant enzymes including manganese

SOD (MnSOD) and catalase (*13, 14*). The resulting decrease in the mitochondrial antioxidant capacity renders the organelle more susceptible to oxidative stress. This may enhance the pro-apoptotic effect of $p66^{Shc}$ through increased permeability of the mitochondrial inner membrane, ultimately resulting in apoptosis (*15*). Mice lacking $p66^{Shc}$ accumulate significantly less ROS over time and exhibit extended life spans and reduced incidence of aging-associated degenerative diseases without an increase in tumor frequency (*10, 11, 16–18*). Therefore, $p66^{Shc}$ has recently been deemed a potential therapeutic target for treating diseases such as neurodegenerative disorders that are associated with ROS accumulation and induction of apoptosis (*6, 19, 20*).

A variety of extracellular signals have also been shown to stimulate ROS production by activating NADPH oxidase (NOX) enzymes, which translocate an electron from reduced nicotinamide adenine dinucleotide phosphate (NAPDH) across the cell membrane to generate H_2O_2 (Table 1) (*6, 9, 21–23*). ROS production by these enzymes requires a catalytic subunit, of which there are seven known human isoforms (Nox1–5, Duox1, and Duox2) that show disparate cell- and tissue-specific expression patterns. Full activity of these multicomponent enzymes also requires the binding of flavin adenine dinucleotide (FAD) and the association of either a distinct set of cytoplasmic coactivator proteins or calcium to the intracellular domain (Figure 1, panel b) (*24*). Recent work indicates that receptor-mediated NOX activation occurs through the recruitment of these additional proteins (*25–27*) and cofactors (*27*), though the precise mechanistic details appear to be pathway- and isoform-specific. For example, NOX1 and NOX2 activation by tumor necrosis factor (TNF) requires riboflavin kinase (RFK). This association may promote enzyme activation by increasing local levels of the FAD prosthetic group (*28*). Future studies on the mechanism of NOX activation are likely to reveal additional biochemical features that could conceivably lead to the identification of potential therapeutic targets. Lastly, it is important to note that intracellular and extracellular signals can also initiate ROS production through $p66^{Shc}$- and NOX-independent mechanisms (Table 1) (*29–31*).

Regardless of the specific cellular source, the H_2O_2 signal diffuses into the cytoplasm where it can induce distinct physiological responses including proliferation, differentiation, and apoptosis/necrosis (*6, 32–34*). However, the high diffusability of H_2O_2 also raises the specter of aberrant signaling. To circumvent this problem, NOX complexes appear to be targeted to distinct regions of the plasma membrane *via* lipid rafts (*24*) and assemble at focal adhesions (*35*) to direct H_2O_2 production to specific cellular microdomains. The precise mechanisms that prevent H_2O_2 diffusion from such microenvironments are unknown (*36, 37*). One possibility is that antioxidant enzymes including glutathione peroxidases, catalase, and peroxiredoxins co-localize with NOX complexes to limit extraneous ROS dissemination (*24*).

Cellular ROS Detection. The subcellular location and relative ROS concentration produced in response to external signals can have a dramatic impact on the cellular outcome (*e.g.*, proliferation or apoptosis). Chemical probes for oxidant detection have emerged as essential tools to probe signal-mediated ROS production in cells (*38*). Compounds such as dihydrodichlorofluorescin (DCFH), dihydrorhodamine-123 (DHR), and more recently dihydrocyanines (*39*) are routinely used to visualize intracellular ROS. Often times, however, these reagents exhibit high background fluorescence resulting from auto- and photo-oxidation. An innovative, new generation of reagents employs a caged boronate switch and provides chemoselective detection of cellular H_2O_2 (*40*). Ratiometric sensors (*41*), nanoparticles (*42*), and protein-based (*43*) systems have also been developed for ROS detection. Continued improvement in the reaction kinetics and dynamic range of these reagents should facilitate detection of intracellular ROS at subcellular resolution (*44*).

Sensing H_2O_2 through Cysteine Oxidation. The reaction of H_2O_2 with biomolecules provides a mechanism for how cells can "sense" changes in redox balance. In proteins, the thiol side chain of the amino acid cysteine is particularly sensitive to oxidation (*45*). Some cysteines are more

KEYWORDS

Chemoselective/chemospecific probes: Small molecules that react specifically with one chemical moiety to either remove a protecting group or to generate a covalent adduct. These probes are an attractive means by which to chemically trap reversible/transient PTMs, such as sulfenic acids.

NADPH oxidases (NOX): A family of heme/flavin-containing protein complexes of which there are seven human isoforms that generate superoxide and hydrogen peroxide by translocating electrons from NADPH to molecular oxygen. These enzymes are an inducible source of ROS production for cellular signaling events.

Oxidative stress: A condition wherein the production of ROS exceeds the biological system's ability to readily detoxify these intermediates with its peroxiredoxin, peroxidase, Trx/TrxR, and GSH/GSR systems. This condition can result in oxidative damage of proteins, lipids, and DNA.

Oxoform: A general term referring to an oxidized form of the thiol side chain of a protein cysteine residue such as a disulfide or sulfenic acid.

Posttranslational modification (PTM): The chemical modification of a protein after its translation. Examples of PTMs include O-phosphorylation, acetylation, SUMOylation, and cysteine oxidation.

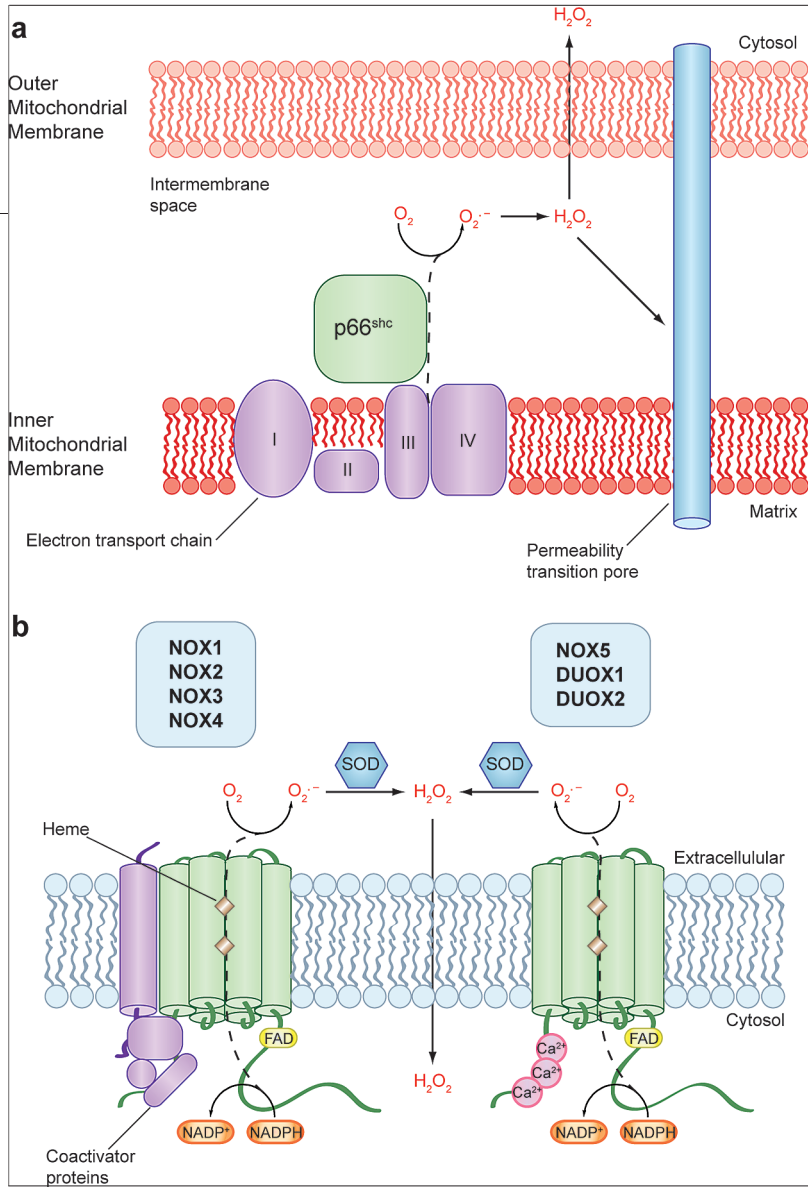

Figure 1. Signaling-derived sources of intracellular reactive oxygen species (ROS). a) p66^{Shc} generates pro-apoptotic ROS in the mitochondria. In response to oxidative stress, UV irradiation, or growth factor deprivation, p66^{Shc} localizes to the mitochondria where it generates ROS ($O_2^{\bullet -}$ or H_2O_2). H_2O_2 ultimately produced can diffuse across the outer mitochondrial membrane to the cytosol where it can modulate the activity of diverse proteins. P66^{Shc}-derived H_2O_2 also stimulates the opening of the permeability transition pore causing mitochondrial swelling and apoptosis. b) NOX enzymes assemble at discrete locations in the cell such as the plasma membrane and at focal adhesions to generate ROS in response to diverse extracellular signals. The catalytic subunit of each NOX isoform (NOX1–5, DUOX1–2) has a conserved domain structure of six transmembrane α-helices and binding sites for two heme prosthetic groups. The C-terminal intracellular domain binds the FAD and NADPH cofactors, and electrons from NADPH are translocated across the membrane through the heme prosthetic groups to generate $O_2^{\bullet -}$ (NOX1–5) or H_2O_2 (DUOX1–2). Full enzymatic activity of these enzymes requires the association of coactivator proteins (NOX1–4) or Ca^{2+} (NOX5, DUOX1–2) to the N-terminal intracellular domain. The $O_2^{\bullet -}$ produced is dismutated by SOD to H_2O_2, which can freely diffuse across the membrane to the cytosol to regulate protein activity and signaling cascades.

susceptible to oxidation than others, and this provides a basis for specificity in ROS-mediated signaling. Thiolate anions (RS^-) are intrinsically better nucleophiles and show enhanced reactivity with H_2O_2, compared to the thiol form (*46*). Thus, the pK_a value of the thiol group can modulate cysteine reactivity. In proteins, a typical cysteine residue has a pK_a of ~8.5. However, the presence of polar or positively charged amino acids can stabilize the thiolate form through electrostatic interactions and decrease the pK_a to as low as 3.5 (*45, 47*).

Other determinants of cysteine reactivity toward H_2O_2 include access of the oxidant to its target and the presence of specific binding sites. For example, peroxiredoxins have low pK_a catalytic cysteines (4.5–5.9) (*48–50*) that react with H_2O_2 with second-order rate constants of 10^5–10^8 M^{-1} s^{-1} (*51, 52*). The catalytic cysteine of protein tyrosine phosphatases (PTPs) is also characterized by a low pK_a value (4.6–5.5) (*53, 54*). However, H_2O_2 reacts with PTPs at second-order rate constants between 10 and 160 M^{-1} s^{-1} (*46, 55, 56*). This difference in reactivity is likely due to the unique architecture of the peroxiredoxin active site and facilitates rapid reaction with low, endogenous levels of H_2O_2 (*57*). Importantly, the decreased reactivity of nonperoxiredoxin thiolates with H_2O_2 provides a potential mechanism to modulate protein activity only after robust changes in oxidant concentration (*e.g.*, in response to external signals).

The initial reaction of a cysteine thiolate with H_2O_2 yields a sulfenic acid (SOH), which is implicated in a number of important biochemical transformations. Once formed, a SOH lies at a crossroad and can lead to formation of additional posttranslational modifications (PTMs) (Figure 2). The stability of a SOH is influenced, in part, by the presence of nearby cysteine residues and by the accessibility of the modification site to the low molecular weight thiol, glutathione (GSH) (*4*). The reaction of SOH with either a neighboring cysteine or GSH will generate a disulfide bond that, in the case of GSH, is known as *S*-glutathiolation (*58*). Both disulfide products can be reduced back to the thiol by the action of either the GSH/glutathione reductase (GSH/GSR) or the thioredoxin/thioredoxin reductase (Trx/TrxR) systems (*59*). Cysteine thiolates can also react with reactive nitrogen species (RNS) including nitric oxide (NO) to generate *S*-nitrosothiols (S-NO) that can hydrolyze to form SOH or react with a second cysteine to form a disulfide (*60, 61*).

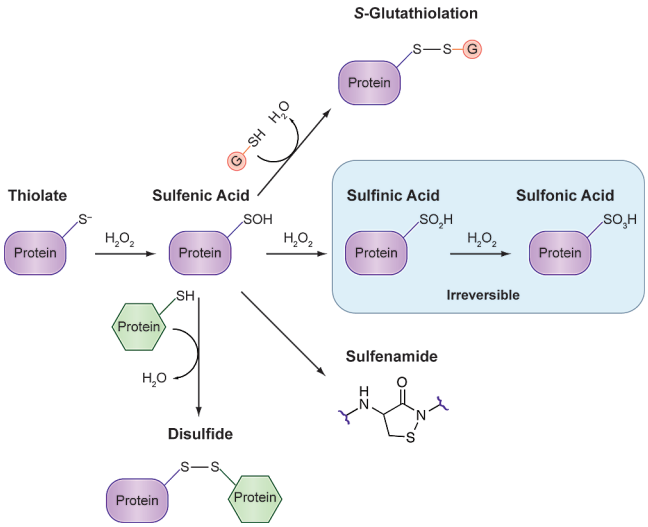

Figure 2. Oxidative modifications of protein cysteine residues. Low pK_a cysteines are present in the cell as thiolates and form a sulfenic acid (SOH) upon reaction with H_2O_2. Once formed, the SOH can react with a second cysteine either in the same or a second protein to yield a disulfide. Alternatively, a SOH can react with the low molecular weight thiol glutathione (GSH) (pink circle) to form a special disulfide known as *S*-glutathiolation. In the event that a neighboring cysteine or glutathione are absent, the amide nitrogen of the neighboring residue can attack the SOH to form a sulfenamide. Each of these oxoforms can be reduced by the GSH/glutathione reductase or thioredoxin/thioredoxin reductase systems to regenerate the thiols (not depicted). The SOH can also further react with H_2O_2 to generate the irreversible SO_2H and SO_3H oxoforms.

SOH can undergo further reaction with H_2O_2 to generate the SO_2H and SO_3H oxoforms (Figure 2), though the rate of these reactions is slower than observed for a thiolate (*49*). With the exception of one protein family, both the SO_2H and SO_3H modifications are considered irreversible, and the latter is deemed a hallmark of diseases such as cancer, diabetes, and neurodegenerative disorders that are associated with oxidative stress (*1–3*). To prevent overoxidation of critical cysteine residues, SOH may be converted to a disulfide or be *S*-glutathiolated. Sulfenamide (*62–65*) and hypervalent sulfur (*66*) species also form through SOH intermediates and may also safeguard against overoxidation (Figure 2).

The switch-like nature of the disulfide and SOH highlights their ability to function as a reversible means to regulate protein function, analogous to phosphorylation. The SO_2H oxoform has also emerged as an important PTM. For these reasons, efforts have been aimed at identifying proteins with redox-active cysteine residues and to elucidate the biological roles of these cysteine oxoforms. To highlight the progress in this area over the past few years, the remainder of this Review will focus on recent examples from the literature that demonstrate the diverse ways in which these PTMs regulate vital cellular processes.

Disulfide Bonds. Disulfide bond formation in proteins is a widely recognized cysteine modification and, under normal conditions, occurs predominately in the endoplasmic reticulum (ER). This organelle provides an oxidizing environment to facilitate disulfide bond formation in nascent proteins destined for export to the extracellular milieu (*67*). By contrast, the cytoplasm, nucleus, and mitochondrial matrix are reducing environments. In these compartments, cysteines are maintained in their thiol form by the combined activity of the GSH/GSR and Trx/TrxR systems (*59, 67*), though protein disulfides can be generated by the action of the Erv family of sulfhydryl oxidases (*68*). In response to external signals and under stress conditions the cytoplasm becomes more oxidizing, which allows protein disulfides to accumulate until redox balance is restored.

Disulfide bond formation can influence the catalytic activity, protein–protein interactions, and subcellular localization. Underscoring the importance of this oxoform, a number of methods have been developed to identify proteins that undergo this modification (*69, 70*). These approaches

KEYWORDS

Ratiometric labeling: The use of isotopically labeled small molecules to derivatize unmodified (*e.g.*, thiol) versus modified (*e.g.*, disulfide) proteins to obtain quantitative information about the fraction of modified protein in terms of total protein available in a given sample. This method facilitates the direct comparison of the percentage of modified protein between different samples (*e.g.*, ± stimulus) since fluctuations in protein expression are compensated for in the ratio.

Reactive oxygen species (ROS): Reduced forms of oxygen that are ions, radicals, or peroxides. These species are reactive as a result of the presence of unpaired valance shell electrons or a labile peroxide bond.

Redox signaling: The regulation of protein activity and the transduction of signals to downstream proteins through oxidative modification of reactive cysteine residues by ROS.

Second messenger: A diffusible molecule produced in cellular signal transduction pathways that modulates the activity of effector proteins thereby propagating the signaling event. Examples of second messengers are cAMP, phosphoinositols, and more recently hydrogen peroxide.

Trx/TrxR and GSH/GSR: The buffering systems of the cell that use electrons from NADPH to reduce protein disulfides and thereby act to maintain cysteine residues in their reduced thiol form.

TABLE 2. Examples of Redox-Regulated Proteins and Complexes

Protein	Oxoform[a,b]	Effect of oxidation on protein	Reference
		Phosphatases	
LMW-PTPs	A,B	Inactivates	*90*
PTEN	A,B	Inactivates	*92, 95*
Cdc25	A,B	Inactivates	*56, 91*
PTP1B	A,B,C	Inactivates	*63, 64*
PTP2α	A,B,C	Inactivates	*65*
SHP-1/SHP-2	A,B	Inactivates	*94*
		Kinases	
Sty1/Tpx1	A	Activates	*148*
PKA RI	A	Activates	*149*
Src tyrosine kinase	A	Activates/inactivates	*150, 151*
PKG-1α	A	Enhances affinity for substrates	*76*
ASK1	A	Initiates oligomerization/activates	*123*
		Transcription factors	
AP-1 (Fos/Jun)	A	Inhibits DNA binding	*77*
Hsf1	A	Activates	*152, 153*
Nrf-2/Keap-1	A	Enhances Nrf-2 stability	*121*
FoxO4/p300/CBP	A	Acetylates/inactivates	*82*
OxyR	A,B	Activates	*78, 79*
Yap1/Gpx3	A,B	Activates	*106, 126, 154*
OhrR	A,B	Inhibits DNA binding	*62, 155*
SarZ	A,B	Inhibits DNA binding	*86*
		Other	
Hsp33	A	Activates	*75*
HDAC4/DnaJb5	A	Inactivates/inhibits complex formation	*80*
GDE2	A	Inactivates	*96*
DJ-1	D	Locates to mitochondria/active as a cytoprotectant	*114, 115*
MMP-7	D	Activates	*113*

[a]The significance of oxidation for many of these proteins in live cells remains to be determined. [b]A, inter/intramolecular disulfide; B, sulfenic acid; C, sulfenamide; D, sulfinic acid.

are typically based on loss of reactivity with thiol-modifying reagents or restoration of labeling by reducing agents such as dithiothreitol (DTT) with subsequent analysis by mass spectrometry (MS). To enable quantitative analysis of redox-sensitive cysteines, Cohen and colleagues have employed isotope-coded affinity tag (ICAT) methodology (*71*). This differential isotopic labeling method uses a subtractive approach to monitor fluctuations in levels of reduced protein thiols under different conditions (*e.g.*, ± oxidant). Jakob and co-workers have expanded the application of ICAT to develop a ratiometric labeling approach, termed OxICAT (*72*). This approach permits direct identification and quantitative evaluation of proteins that form disulfides under different cellular conditions.

Global studies to identify proteins that undergo disulfide bond formation implicate this modification in the regulation of numerous biological processes including redox homeostasis, chaperone activity, metabolism, transcriptional regulation, and protein translation (Table 2) (*72, 73*). Once formed, a disulfide can have divergent effects on protein function, which are central to the ability of H_2O_2 to orchestrate cellular signaling events, which can lead to diverse biological outcomes

(Table 2). For example, starvation-induced autophagy is associated with a temporary increase in ROS production that inactivates a key cysteine protease, Atg4, by forming a disulfide bond involving the catalytic cysteine (*74*). In contrast, survival of bacteria such as *Escherichia coli* under conditions of both oxidative and heat stresses requires activation of the molecular chaperone Hsp33 *via* intramolecular disulfide bond formation (*75*).

H_2O_2 can also regulate the activity of protein tyrosine phosphatases (PTPs) by inducing intramolecular disulfide bond formation, which inactivates the phosphatases to permit prolonged flux through the corresponding signaling pathways (Table 2). Protein kinases are also believed to undergo redox control; however, the evidence for this is less direct since increased activity may also be attributed to inhibition of the opposing phosphatase. Recently, the serine/threonine kinase PKGIα was shown to undergo intermolecular disulfide formation between monomers, and this modification appears to enhance its affinity for target proteins (*76*).

The terminal targets of signal transduction cascades are transcription factors that regulate gene expression. Some transcription factors, such as AP-1 (*77*) and OxyR (*78, 79*), appear to be regulated by direct oxidative modification (Table 2). The activity of transcription factors can also be regulated by changes in the accessibility of their target genes, for example, by PTM of histones. The class II histone deacetylases (HDACs) function as transcriptional corepressors of various developmental and differentiation processes. The activity of one isoform, HDAC4, is regulated by its interaction with the small molecular chaperone DnaJb5 (*80*). This chaperone forms a multiprotein complex with thioredoxin (Trx1) and importin α (Imp), a component of the nuclear import machinery, through the adapter protein Trx binding protein-2 (TBP-2) (Figure 3) (*81*). In a recent study, Sadoshima and colleagues demonstrated that cysteine residues in DnaJb5 can form a disulfide, preventing its interaction with HDAC4. Dissociation from the DnaJb5 multiprotein complex coupled with disulfide bond formation in HDAC4 exposed the nuclear export signal (NES) resulting in cytoplasmic localization of HDAC4 and derepression of its target genes (*80*). Sadoshima and co-workers proposed a model whereby Trx1 reduces intramolecular disulfides in DnaJb5 and HDAC to restore complex formation and nuclear accumulation (Figure 3). This model presents a mechanism for how signal-mediated H_2O_2 production may promote developmental defects such as cardiac hypertrophy and highlights this pathway as a potential target for therapeutic intervention.

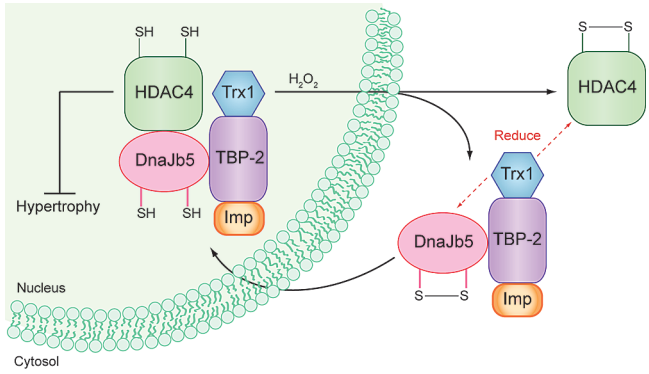

Figure 3. Model for redox-regulation of cardiac hypertrophy by HDAC4. The type-II histone deacetylase HDAC4 normally modifies histones to repress the expression of genes involved in hypertrophy. Nuclear localization of HDAC4 is mediated by its association with importin α (Imp) through a multiprotein complex consisting of the molecular chaperone DnaJb5, TBP-2, and Trx1. In the presence of H_2O_2, intramolecular disulfide bonds form within HDCA4 and DnaJb5, which stimulates dissociation and nuclear export of the complex. Upon removal of H_2O_2, Trx1 reduces the disulfides in both HDAC4 and DnaJb5 to restore formation and nuclear localization of the complex.

Disulfide bond formation can also lead to additional PTM of oxidized proteins and represents another important mechanism to modulate activity. An example of such a regulatory mechanism was recently demonstrated for the FoxO4 transcription factor, which is inactivated by forming an intermolecular disulfide with either the p300 or CREB-binding protein (CBP) acetyltransferases (*82*). Caspase-9, the initial caspase in the mitochondrial apoptotic cascade, also appears to be regulated in this manner since formation of an intermolecular disulfide with apoptotic protease-activating factor 1 (Apaf-1) stimulates autocleavage of caspase-9 and initiation of the apoptotic cascade (*83*).

Sulfenic Acids. Sulfenic acids are relatively unstable and reactive groups that have traditionally been viewed as intermediates en route to other oxidation states (Figure 2). In recent years, however, stable SOH have been identified in a growing list of proteins and have received intense interest for their roles in cell signaling (Table 2) (*4, 36, 84*). Indeed, the appropriate protein microenvironment can lead to stable SOH formation. For example, SOH modification of human serum albumin

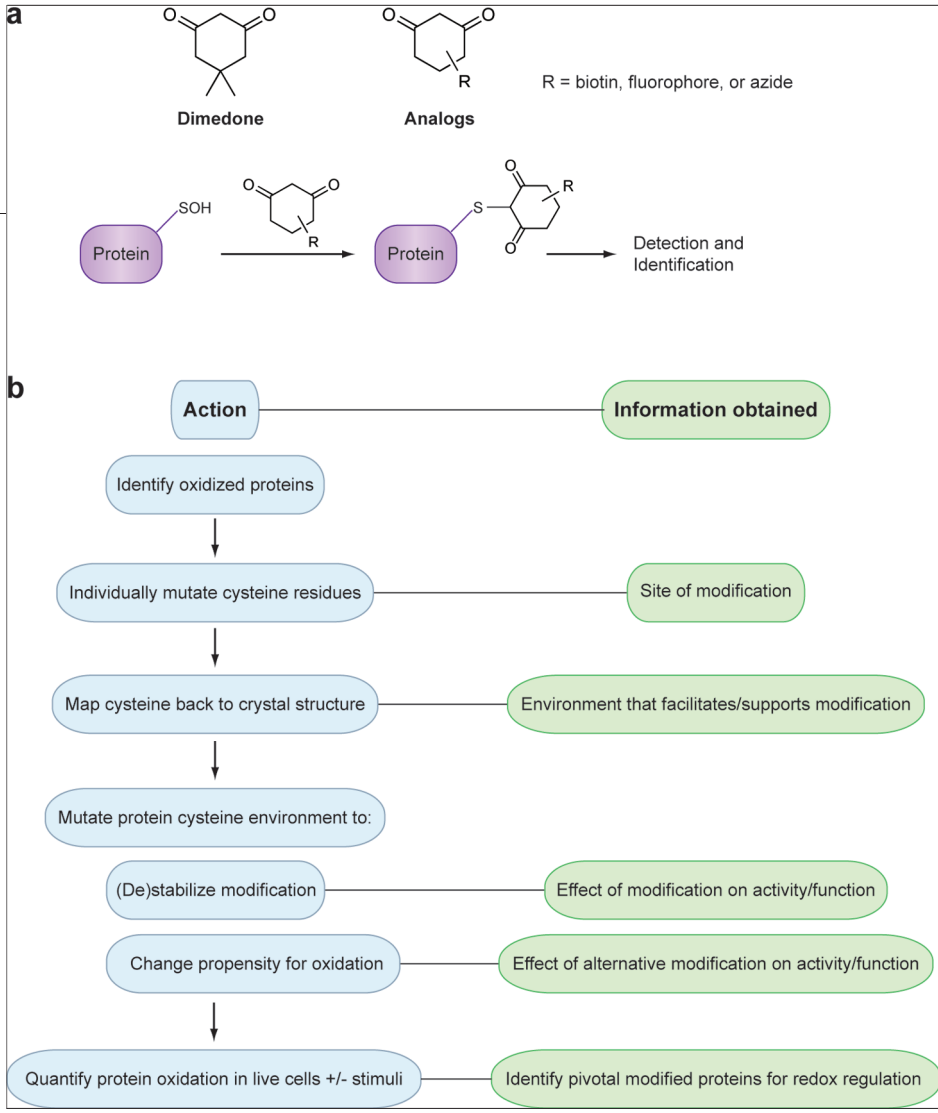

Figure 4. Detection and characterization of oxidized proteins. a) Structures and reaction scheme for chemoselective tools used to detect protein SOH *in vitro* and *in vivo*. b) Flowchart of steps that can be undertaken and the corresponding information obtained to elucidate the significance and prevalence of protein oxidation *in vivo*.

can persist for hours (*85*) and has been observed in more than 40 crystal structures (*47, 86*).

The PTP family of phosphatases is another commonly cited example of SOH-mediated regulation of activity (*87–89*). In these enzymes, the low pK_a catalytic cysteine can oxidize to SOH with concomitant inactivation. Crystal structures of PTP1B and PTPα demonstrate that the SOH modification can react with the backbone amide nitrogen of a neighboring amino acid to form a cyclic sulfenamide (*63–65*). However, the rate of sulfenamide formation is slow relative to reaction of the SOH intermediate with thiols such as GSH or cysteine (*62*). Alternatively, the SOH intermediate in PTPs can condense with a proximal "backdoor" cysteine to generate an intramolecular disulfide, as has been observed for low molecular weight (LMW) (*90*), Cdc25 (*56, 91*), and PTEN phosphatases (*92*). Two members of the tandem Src homology 2 (SH2) domain-containing PTPs (SHPs) also undergo oxidative modification in activated T cells (*93*). Interestingly, SHPs possess two "backdoor" cysteines that comprise a unique regulatory mechanism (*94*). Sequential reaction of these proximal cysteines with the SOH intermediate and subsequent disulfide exchange generates a disulfide between the "backdoor" cysteines that inactivates the enzyme.

Peroxidases and peroxiredoxins also form SOH intermediates as part of their catalytic cycle (*36*). The primary role of these enzymes is to metabolize peroxides and maintain the reducing environment of the cell. Recent studies, however, reveal additional regulatory functions for these antioxidant enzymes. For example, peroxiredoxin 1 (Prdx1) was shown to promote PTEN tumor suppressor activity by protecting against oxidative inactivation (*95*). A molecular mechanism was not provided in this study; however, it is possible that Prdx1 either neutralizes local H_2O_2 to prevent PTEN oxidation or acts as a reductase to reduce the PTEN disulfide. The latter activity is analogous to the newly elucidated role for Prdx1 in promoting neuronal cell differentiation (*96*).

Small molecule probes that recognize specific cysteine oxoforms over similar species represent promising new tools for elucidating signaling pathways and regulatory mechanisms that involve redox signaling and thiol oxidation. To this end, approaches have been developed that allow for the detection of sulfenic acid modifications on proteins that exploit the unique chemical reactivity of this species (*97–102*). Although SOH are often metastable species, the direct detection of SOH formation has several advantages including the identification of the reactive site where the oxidation chemistry was initiated (*36*).

All recently developed reagents for sulfenic acid detection are based on 5,5-dimethyl-1,3-cyclohexanedione, also known as dimedone (Figure 4, panel a). The chemoselective reaction between dime-

done and a protein SOH was first reported by Benitez and Allison in 1974 (*103, 104*). Since then, this reaction has been exploited to detect SOH modifications by MS and through direct conjugation to fluorophores and biotin (*97, 99*). More recently, azide analogues of dimedone known as DAz-1 (*100, 101*) and DAz-2 (*98*) have been developed that can be used to label sulfenic acid-containing proteins in live cells, thereby minimizing the potential for oxidative artifacts during cell lysis. Proteins tagged by the azidodimedone analogues can be conjugated to biotin or fluorophores *via* chemical ligation techniques such as the Staudinger ligation or click chemistry (Figure 4, panel a) (*101, 105*). Application of azidodimedone probes to discover protein targets of oxidation in human cell lines has shown that as many as 200 different cellular proteins undergo SOH modification (*98*). The newly identified proteins have roles in signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control. Azidodimedone probes have also been used to identify a functional role for SOH modifications in the yeast peroxide-sensing system comprising the peroxidase Gpx3 and the transcription factor Yap1 (*106*).

Sulfinic Acids. The SO_2H modification has been best characterized in peroxiredoxins and forms through reaction of H_2O_2 with the SOH intermediate. Notably, only the eukaryotic homologues of the peroxiredoxins are susceptible to SO_2H formation (*107, 108*). For a subset of eukaryotic peroxiredoxins, the SO_2H modification can be reversed by an enzyme termed sulfiredoxin (*109*). Recent studies indicate that SO_2H repair proceeds through a sulfinic acid phosphoryl ester intermediate formed by the direct transfer of the γ-phosphate from ATP to peroxiredoxin (*110–112*). The reversibility of SO_2H in peroxiredoxins suggests that this modification may also function as a controllable redox switch in proteins. Indeed, Poole and co-workers have proposed the floodgate model of signaling, which posits that SO_2H modification of peroxiredoxin permits a temporary increase in cellular H_2O_2 (*108*). In addition to peroxiredoxins, important biological functions for SO_2H modifications have been demonstrated in matrix metalloproteases (*113*) and the Parkinson's disease protein DJ-1 (*114, 115*). Although oxidation of cysteine to SO_2H is gaining acceptance as an important regulatory mechanism as well as a marker of protein damage, the full scope of these modifications remain unknown. The development of chemical tools for SO_2H detection may afford new opportunities to elucidate the role of this modification in human health and disease.

Regulation of Protein Signaling Complexes. H_2O_2 can also influence protein activity through oxidative modification of regulatory protein complexes, as illustrated by the mammalian NRF2/KEAP1 system. NRF2 is a basic leucine zipper (bZIP) transcription factor that regulates the expression of enzymes involved in oxidant and xenobiotic detoxification (*116*). This transcription factor has a nuclear localization sequence (NLS); however, it is held in the cytoplasm under nonstress conditions by KEAP1, which functions as a homodimer and interacts with the DLG and ETGE sites of NRF2 (Figure 5, panel a) (*117, 118*). KEAP1 serves as an adaptor for a ubiquitin ligase complex, and binding of KEAP1 to both the DLG and ETGE sites optimally orients NRF2 lysine residues for ubiquitination, which targets it for degradation (*119*). Nuclear accumulation and activation of NRF2 in response to oxidative stress is associated with increased NRF2 stability and is dependent upon oxidative modification of three cysteine residues in KEAP1, which weakens its interaction with the DLG motif in NRF2 (*117–120*). Until recently, it was not clear how KEAP1 oxidation enhances the stability of NRF2 since oxidized KEAP1 still interacts fully with the ETGE site and weakly with the DLG site. A new study demonstrated that $p21^{Cip1/WAF1}$, a protein involved in numerous cellular processes including cell-cycle arrest and apoptosis, could compete with KEAP1 for binding to the DLG site of NRF2. Displacement of KEAP1 by $p21^{CIP1/WAF1}$ inhibits KEAP1-mediated ubiquitination of NRF2 and provides a unique regulatory role for $p21^{Cip1/WAF1}$ (Figure 5, panel a) (*121*).

The apoptosis signal-regulating kinase (ASK1)/Trx1 system represents another H_2O_2-sensitive protein complex (Figure 5, panel b). Two models have been proposed to explain H_2O_2-mediated activation of ASK1. One model posits that Trx1 sequesters ASK1 in an inactive complex and, upon treatment of cells with TNF or H_2O_2, undergoes intramolecular disulfide formation. In subsequent steps, ASK1 is released, which permits oligomerization to form the active kinase complex (Figure 5, panel b, left) (*122*). A recent study, however, demonstrated that stable ASK1 oligomerization and activation in response to H_2O_2 is mediated by disulfide bond formation between ASK1 monomers (*123*). Hence, an alternative regulatory model was presented whereby

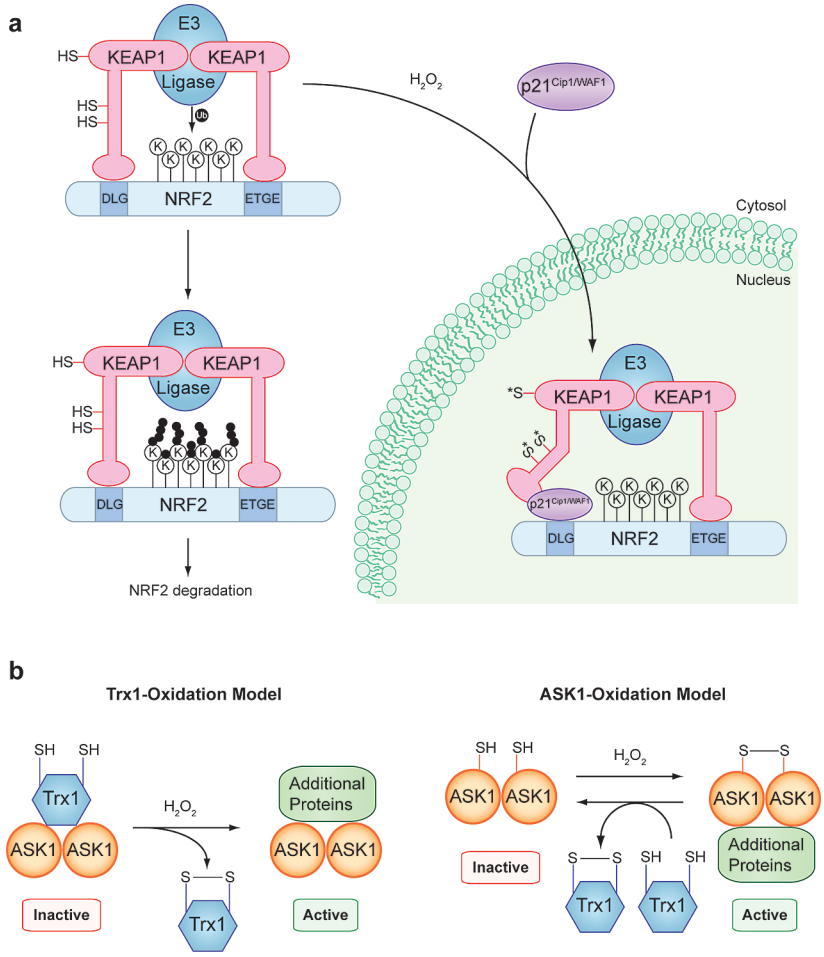

Figure 5. Redox-regulation of protein complexes influences gene transcription and signaling cascades. a) Proposed mechanism for redox-regulation of NRF2 stability and activity by KEAP1 and $p21^{CIP1/WAF1}$. Binding of KEAP1 to the DLG and ETGE sites in NRF2 optimally orients lysine residues in NRF2 for ubiquitination (black circles) leading to degradation. In the presence of H_2O_2, three cysteine residues in KEAP1 are oxidatively modified (oxoform unknown, S*), which induces a conformational change in KEAP1 that decreases its affinity for the DLG site. Additionally, KEAP1 oxidation may mask its NES, leading to nuclear accumulation of the complex and activation of NRF2. $p21^{CIP1/WAF1}$ can compete with oxidized KEAP1 for binding to the NRF2 DLG site to enhance the stability of the transcription factor. b) Two proposed models for H_2O_2-mediated activation of ASK1. ASK1 assembles into multimers in the cell that interact with Trx1. Association of Trx1 with ASK1 sequesters the kinase in an inactive conformation. Upon oxidation of Trx1 by H_2O_2, ASK1 is released to interact with additional proteins forming the active signaling complex (Trx1-oxidation model). Alternatively, H_2O_2 induces intermolecular disulfide bond formation between ASK1 monomers to facilitate the interaction with additional proteins forming the activate kinase complex (ASK1-oxidation model). In this second model, Trx1 negatively regulates ASK1 by maintaining the kinase in a reduced and inactivate state.

Trx1 negatively regulates ASK1 signaling under resting conditions by maintaining it in a reduced state (Figure 5, panel b, right). This alternative model is attractive since it is consistent with the known disulfide reductase activity of Trx1.

Prolonged activation of ASK1 by TNF signaling induces apoptosis, which is also associated with ROS production from the NOX1 complex (*26*). ASK1 activates the Jun N-terminal kinase (JNK) and $p38^{MAPK}$-signaling pathways. The latter is required for induction of mitochondrial apoptosis during oxidative stress by enhancing the stability of p53 (*124*). Interestingly, p53 regulates the expression of $p66^{Shc}$, which is required for stress-activated p53 to stimulate mitochondrial ROS production and apoptosis (*19*). This apoptotic signaling pathway provides an attractive mechanistic link between NOX activation and the initiation of $p66^{Shc}$-dependent mitochondrial ROS production, though further studies will be required to evaluate this potential connection.

Cysteine Oxidation in Disease. To date, a number of proteins have been identified wherein chemoselective oxidation of cysteine residues serves as a mechanism to regulate normal cellular functions (Table 2). It is important to note, however, that excessive H_2O_2 production, through either aberrant receptor

activation or mitochondrial dysfunction, can lead to spurious modification and hyperoxidation of cysteines. This would be expected, for example, in disease states that are associated with excessive ROS production such as cancer, diabetes, or neurodegenerative disorders (*1–3*). Consistent with this proposal, a recent study found that SOH modification of proteins is enhanced in malignant breast cell lines using an antibody that recognizes the protein-dimedone adduct (*102*). Although Trx/TrxR, GSH/GSR, and the recently identified bacterial sulfenate reductase (*125*) can repair reversible forms of thiol oxidation, persistent oxidative stress can overpower these systems and lead to aberrant protein oxidation that may contribute to disease pathogenesis.

Future Perspectives. The recent development of chemical tools to detect cellular ROS as well as mechanistic studies into NOX enzymes activation and $p66^{Shc}$ have greatly expanded our understanding of how ROS are produced in response to diverse external signals. Continued development of ROS-sensing reagents should facilitate the temporal and spatial resolution of signal-mediated ROS production. Once formed, ROS can modulate the activity of proteins and regulate signaling pathways involved in cell proliferation, cell differentiation, and apoptosis *via* chemoselective oxidation of cysteine residues. The recent development of methods to detect disulfides and SOH has expanded the inventory of protein cysteine residues known to undergo oxidation modifications, though probes for SO_2H are lacking. Such proteins targets of oxidation are implicated in a wide array of cellular processes including signal transduction, DNA repair, metabolism, protein synthesis, redox homeostasis, nuclear transport, vesicle trafficking, and ER quality control. Though some reactive cysteines are susceptible to numerous modifications, the majority of thiols appear to undergo specific oxidative PTMs, which suggests that there are fundamental differences in the chemical and biological basis for target specificity (*98*).

Profiling oxidized proteins (*i.e.*, inventory mapping) serves as the first step to elucidating the biological roles of these cysteine PTMs (Figure 4, panel b). Mapping sites of cysteine modification can be used to expand our understanding of features within a protein microenvironment that facilitate the oxidation process. The transition from inventory mapping to the mapping of functional cellular context will be greatly facilitated by genetic and biochemical experiments. For example, site-directed mutagenesis can be employed to remove the modified cysteine or alter the protein environment in order to influence the redox sensitivity, as in DJ-1 (*114*) and Gpx3 (*126*). Another important step toward evaluating the physiological significance of oxidative cysteine modifications will be to quantify redox-dependent changes in the extent of protein oxidation. To this end, the OxICAT method (*72*) should facilitate such analysis for disulfide bond formation. Since increased H_2O_2 concentrations can lead to aberrant SOH formation (*102*), similar ratiometric methods should be developed for SOH to hone in on the modified proteins that are pivotal for regulation of cellular signaling.

Studies reported in the past three years have expanded our knowledge regarding mechanisms of signal-mediated ROS production and the means by which ROS regulate cellular signaling networks. The continued emergence of methods to detect and quantify discrete cysteine oxoforms should further our mechanistic understanding of redox regulation of protein function and could lead to the development of new therapeutics.

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