Defining and refining the cysteine redoxome with sulfur chemical biology

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Posttranslational changes in the redox state of cysteine residues can rapidly and reversibly alter protein function, modulating biological processes and drug pharmacology. Recent innovations in organosulfur chemistry, small-molecule tools, and computational methods for proteomic analysis have dramatically improved selectivity, cellular application, and site-specific quantitation of the cysteine redoxome. In this perspective, we start with a brief overview of cysteine sulfur chemistry and factors affecting thiol reactivity, followed by a critical discussion of similarities and differences between reactive and redox-sensitive cysteine residues. Next, we address mechanisms of post-translational cysteine oxidation and methods to quantify redoxome site-stoichiometry to prioritize follow-up study. Finally, we highlight recent chemoproteomic studies of the cysteine redoxome that offer new insights into the regulation of physiological processes and provide a framework for development of novel redox-based targeted therapeutic strategies.

Main

Second messengers transmit information along the cellular signal transduction highway. Such messengers include ions, gaseous molecules, hydrophilic and hydrophobic molecules and, as beneficial adaptations to increasing oxygen content in the earth's atmosphere, signals, which are predicated on electron transfer during oxidation-reduction (redox) reactions. Methionine, histidine, tryptophan, and tyrosine can undergo oxidation, however, the unique thiol (–SH) containing side chain of cysteine make it a favored broker for protein redox transactions in biological systems[1]. In this paradigm, individual cysteine residues directly react with endogenous or exogenous oxidants under physiological or pathophysiological conditions with ensuant formation of largely reversible *S*-modifications that can and do modulate protein and cellular function.

From a chemical perspective, redox signals such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide ($^{\bullet}NO$) are converted into an unparalleled space of proteomic reactivity centered at cysteine sulfur (**Fig. 1a**). Symmetrical or mixed disulfides and *S*-nitrosothiols behave like weak electrophiles, while thiols, persulfides, sulfinic and sulfonic acids exhibit varying degrees of nucleophilic character, dictating OxiPTM reactivity toward biomolecules and exogenous agents. Sulfenic acids, distinguished by dual nucleophilic and electrophilic chemical reactivity, populate the vast wilderness between these two groups. Additional boutique OxiPTMs have been identified in a handful of proteins and contribute further to the complexity of cysteine sulfur. Examples include the cyclic sulfenamide observed in select tyrosine phosphatases[2,3], as well as thiosulfonate and thiosulfinate intermediates generated during the catalytic cycle of sulfonucleotide reductase (SR)[4] and sulfiredoxin (SRX)[5] enzymes, respectively.

Among the 20 common amino acids, cysteine is generally the least abundant in organisms, yet is most often present in sites associated with catalytic, regulatory, structural, metal ion coordination and other essential roles, defined as "functional cysteines". The central function of cysteine and

OxiPTMs is also evident from increased utilization during evolution (from 0.5% in archaebacteria to 2.2% in mammals)[6]. Bioinformatic analysis of the location and distribution of cysteine among homologous proteins indicates highly polarized conservation patterns[7]. For example, the cysteine-cysteine pair in structural disulfide bonds are more conserved (96.9% in humans) than any other amino acid[8]. On the other hand, unpaired cysteines located near the protein surface are less well conserved, but are more likely to have a functional role[9].

The nucleophilic thiol side chain of cysteine reacts with electrophiles *via* its deprotonated, thiolate form. In the absence of significant steric considerations, intrinsic cysteine reactivity is dictated by thiol pK_a and, in proteins, this value varies between three and 14[10]. Compared to a pK_a of 8.3 for free cysteine, the pK_a range in proteins underscores the profound influence of microenvironment on thiol ionization. Another basic, but often overlooked, factor in reactivity is thiol nucleophilicity, which decreases as pH increases due to greater thiolate anion stability. A plot of reactivity versus pH for a typical thiol with a Bronsted coefficient (β_{nuc}) of ~0.5 is bell-shaped with a maximum rate when the solution pH is equal to thiol pK_a.

Reactive, redox-sensitive, and regulatory cysteines. Experimental and computational approaches have been applied to parse reactive-cysteines based on thiolate reactivity with alkyl halides or unsaturated carbonyl electrophiles[10] but datasets that compare and contrast biochemical versus *in situ* findings suggest a far more complex reality[11]. Whether a cysteine is considered redox-sensitive is also open to debate. For example, protein tyrosine phosphatases (PTPs) and peroxiredoxins (PRXs) have active site cysteines with similarly low pK_as but thiolate attack on hydrogen peroxide (H_2O_2) is orders of magnitude faster for PRXs due to superior transition state stabilization[12]. Many in the field consider both enzyme families to be redox-sensitive albeit with differing reactivity, while others argue that PRXs are the sole target of biological H_2O_2. Recent chemoproteomic and intrabody studies demonstrate that PTP oxidation

occurs in cells. Clearly then, a complete understanding of biological H_2O_2 targets these issues requires consideration of additional factors, like PRX inactivation by phosphorylation or hyper-oxidation of the catalytic cysteine, target localization, protein-protein interactions, and protein turnover. Equally important, but often ignored is that PRX must recycling back to their active thiol form, which is rate-limiting and consistent with high levels of oxidized PRX in cells[13]. Going one layer deeper, another question can be asked: Of the subset of cysteines that are redox-sensitive, which of these are regulatory? Indeed, not all cysteine residues identified as redox-sensitive in chemproteomic studies are associated with significant functional effects in cells. Answering this question for any given protein is generally painstaking work, even at a basic biochemical level. Key parameters including the precise mechanism and kinetics of OxiPTM reduction, identity and concentration of reactive species are critical to resolving this question but often lie just outside of our experimental reach at the cellular level.

Based on site-specific chemoproteomic data[14–17], the hierarchical relationships between different classes of cysteines can be conceptualized as a Venn diagram (**Fig. 1b**). The human genome encodes about 214,000 protein cysteines, constituting the “cysteinome”. Hyper-nucleophilic or reactive-cysteines make up the largest subset, followed by redox-sensitive, and redox-regulatory thiols. Over the last decade, rigorous chemoproteomics shows that a significant population of cysteines identified as being reactive do not, in fact, respond to changes in cellular redox balance (**Fig. 1c**). Furthermore, not all redox-sensitive cysteine residues serve a functional or regulatory role though there is significantly greater overlap between these populations. Herein, our goal is to define the cysteine redoxome as seen through the lens of empirical data and highlight recent advancements that reveal hitherto unknown regulatory roles within.

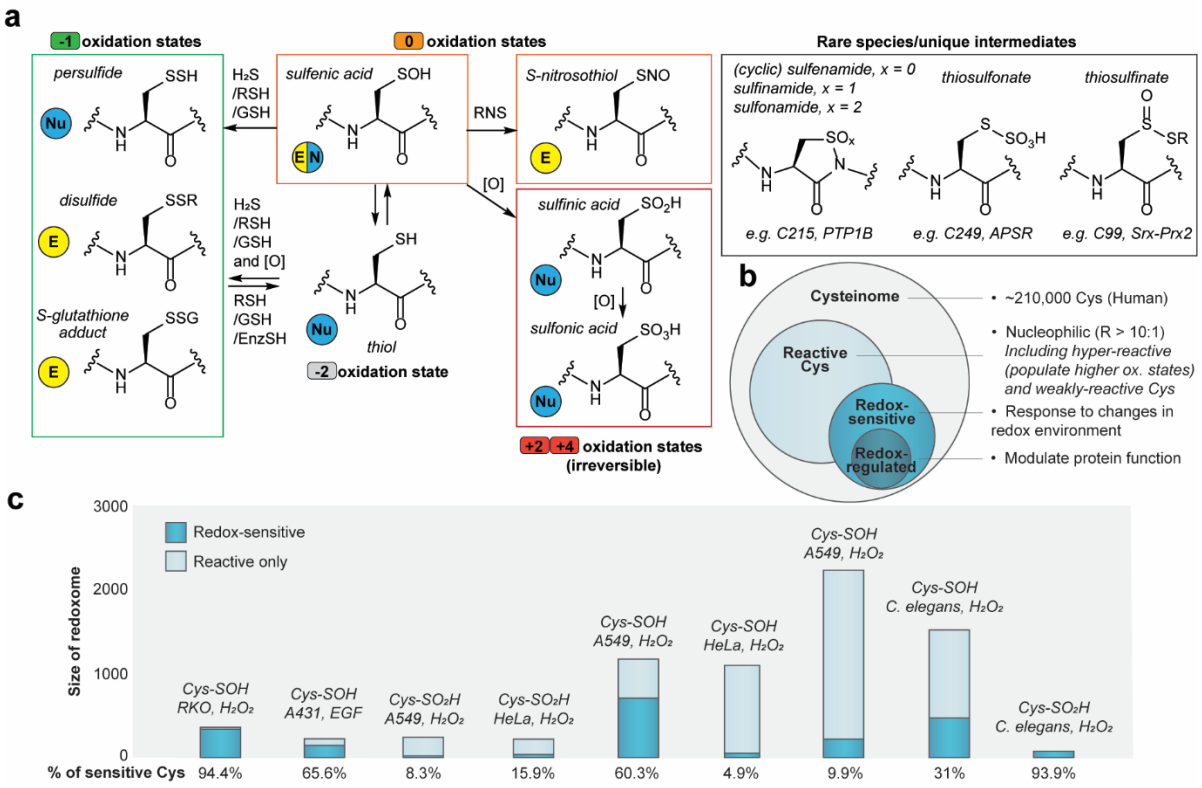

Fig. 1 Cysteine oxidation and reactivity a Oxidative post-translational cysteine modifications. **b** Venn diagram illustrating the relationship of the cysteinome, reactive, redox-sensitive, and redox-regulated cysteine residues. **c** Number of redox-sensitive (dark blue) and reactive cysteine (light blue) sites identified by chemoproteomics among various cell lines and in *C. elegans*.

The cysteinome in redox-sensing and homeostasis. The first step in defining the cysteine redoxome is to delineate kinetic reactivity between individual biological oxidants and thiol targets. Two-electron oxidation of cysteine thiol(ate)s by H_2O_2 is a fundamental reaction in biology and serves as an excellent illustration of the broad spectrum of rates spanning eight orders of magnitude[18]. Figure 2 lists second-order rate constants for the reaction of cysteine with H_2O_2 measured in biochemical studies for representative proteins. What emerges from this analysis are three “bins” or categories of cysteines: i) “redox hypersensitive” peroxidases such as 2-Cys PRXs and glutathione peroxidases (GPXs), which are efficient ROS scavengers and control cysteine oxidation in less reactive targets; ii) “redox-sensitive” thiol switches in transcription

factors and metabolic enzymes (*e.g.,* OxyR, GAPDH) that elicit rapid responses to changes in cellular redox balance; iii) “weakly reactive” signaling targets like epidermal growth factor receptor (EGFR) and PTPs that enable longer-term temporal control of cellular functions as diffusion rates limit the impact of highly reactive oxidant species, while redox modifications such as PTP1B-C215 sulfenamide and EGFR-C797 sulfenic acid can persist on signaling time-scales[19]. Other reactive species like peroxynitrite (ONOO), hydrogen sulfide (H_2S) or hypochlorous acid (HOCl) with distinct reactivity profiles and cellular half-lives can be similarly conceptualized.

As with any reaction, chemical transformation among the cysteine redoxome is controlled by both thermodynamic and kinetic factors. The redox potential of a given thiol/disulfide pair relates to the thermodynamic parameter of thiol-disulfide exchange. The redox potential for the half-reaction ($2\ RSH - 2e = RSSR + 2H^+$) merely reflects the equilibrium of an isolated system over a long period of time. However, the cysteine redoxome exists heterogeneously in cells at a dynamic steady state, underscoring the importance of kinetic rate. For example, reactions of GSH-dependent enzymes are kinetically controlled by the concentration of GSH, but not the ratio of GSH/GSSG[20]. In other words, the GSH/GSSG ratio is an indicator, rather than a regulator, of local redox poise.

Since GSH is the most abundant low-molecular-weight (LMW) thiol in cells (~10 mM), it was traditionally considered to be the major, if not exclusive, redox buffer in cells[21]. However, mounting evidence indicates that the cysteinome constitutes the majority of the pool of reduced thiols (*e.g.,* up to 70% in whole cells[22] or ~25-fold more concentrated (~90 mM) than GSH in mitochondria[23]). Additionally, LMW thiols like GSH are much less reactive toward peroxides (less than 30 $M^{-1}s^{-1}$) compared to peroxidatic cysteines in proteins like PRXs and GPXs (10^7-10^8 $M^{-1}s^{-1}$). Consequently, protein cysteine thiols (*i.e.,* cysteinyl thiols) often represent the dominant oxidation target in the cellular milleu. Within the cysteinome itself, redox homeostasis is regulated by thioredoxin (TRX) or glutaredoxin (GRX) oxidoreductase systems where, depending on kinetic rate and metabolic

demand, reduction of oxidized protein cysteines can be rate-limiting. This balance permits weakly reactive cysteines in signaling proteins to robustly populate the oxidized state.

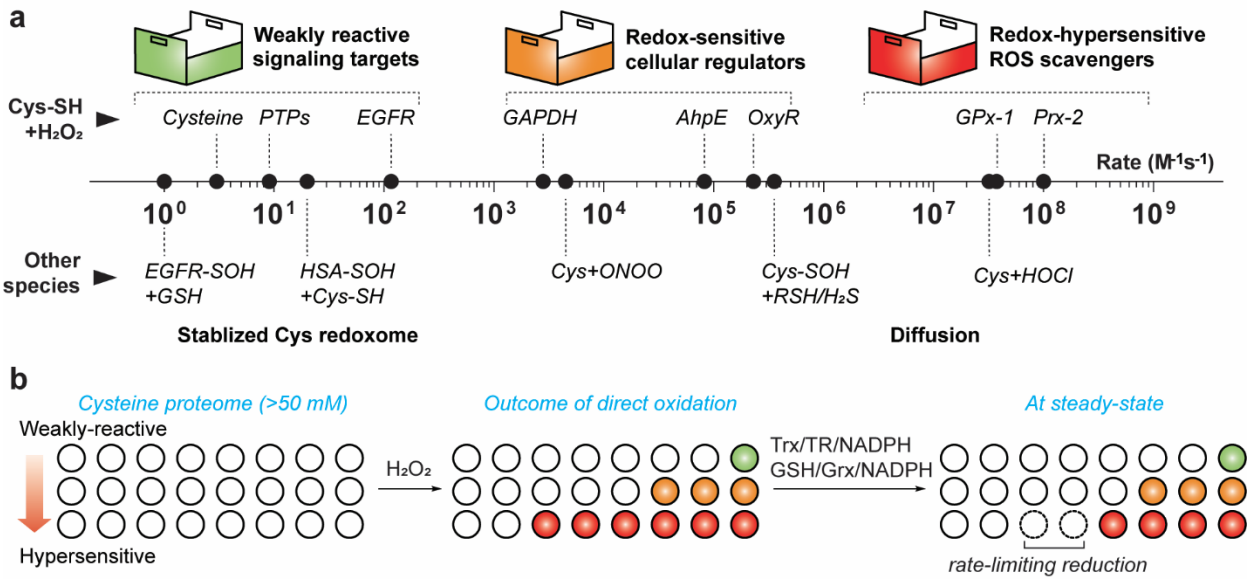

Fig. 2 Redox reactivity across the cysteinome. a Sorting cysteine thiol(ates) into different "bins" according to H_2O_2 reactivity (top). Related reactive species and associated cysteine OxiPTMs are also listed for reference (below). **b** Kinetic control of cysteine oxidation. The cysteinome (>50 mM in cells) can be conceptualized as a redox buffer regulated by TRX and GRX oxidoreductases. Reduction of weakly reactive cysteines (*e.g.,* PTPs) can be fully or partially rate-limiting, therefore the oxidized form of these proteins can be robustly populated at steady state.

Enzymatic and non-enzymatic mechanisms of post-translational cysteine oxidation. Among the more than 400 known types of post-translational modifications (PTMs) to proteins, the majority are mediated by enzymes which, by virtue of binding affinity, imparts a level of target specificity. Non-enzymatic post-translational modifications occur when a nucleophilic or redox-sensitive amino acid side chain encounters a reactive metabolite. Non-enzymatic PTMs, especially those that are reversible, also contribute to a distinct mechanism of cellular regulation[24]. In the context of cysteine oxidation, both enzymatic and non-enzymatic mechanisms of PTM are operative. The mechanism of enzymatic cysteine oxidation that has been best elucidated to date

is referred to as a “redox relay” system wherein H_2O_2 reacts with a hypersensitive peroxidase conduit followed by a transfer of the oxidizing equivalent to a second protein through thiol-disulfide exchange[25]. One such example of an enzymatic cysteine oxiPTM is the GPx3-Yap1 redox relay found in yeast (**Fig. 3a**)[26,27]. While this model, characterized in a handful of cases, can provide convenient answers to nagging questions surrounding target selectivity for a diffusible second messenger like H_2O_2 an equally troubling new set of selectivity issues arise, which cannot be satisfactorily addressed in target identification experiments that employ so-called “trap mutants” in which the peroxidase lacks the resolving cysteine. Furthermore, positing the redox relay as the exclusive mechanism of cysteine OxiPTM contradicts the proteome-wide discovery[28] of cysteine sulfenic and sulfinic acids OxiPTMs, known respectively as *S*-sulfenylation and *S*-sulfinylation, using reaction-based probes whose chemoselectivity has been shown in numerous reports[15,29].

Nonenzymatic cysteine oxidation is predicated on proximity- (**Fig. 3b**) or concentration-driven (**Fig. 3c**) mechanisms. In the latter, inactivation of PRXs by *S*-sulfinylation of the catalytic thiol or by *O*-phosphorylation enables transient accumulation of H_2O_2[30,31] for direct oxidation of weakly reactive cysteine signaling targets. A recent report indicates that PRX hyperoxidation is facilitated by bicarbonate, a natural buffer in living cells[32]. In addition to PRX inactivation, receptor-mediated bursts in H_2O_2 can elevate basal low nanomolar levels of the oxidant to the mid or high micromolar range[33]. Redox signals can also be directly transmitted through proximity of the cysteine target with the oxidant source, such as the direct oxidation of EGFR[19,34] and Src-family kinases (SFKs)[35] by NOX at the plasma membrane. Clearly, neither enzymatic nor non-enzymatic mechanisms can exclusively account for all cysteine oxidation, instead, they mutually exist and weave layers of redox signaling together with distinct driving forces.

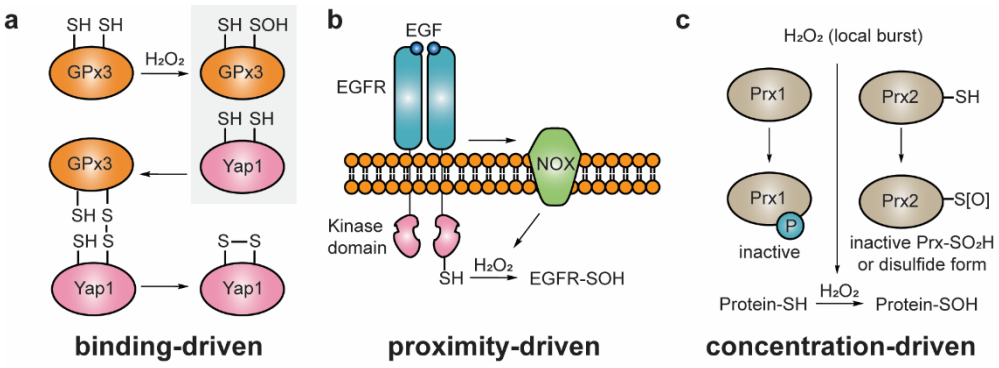

Fig. 3 Enzymatic and nonenzymatic mechanisms of cysteine oxidation.

Chemoproteomic platforms to discover the cysteine redoxome. Chemoproteomic methods using tandem mass spectrometry (MS) have become the standard in redox biology due to their incomparable ability to generate “big data” compared to earlier gel-based techniques, largely relegated to validation studies. Advances in proteomics through innovation in instrumentation, bioinfomatics and chemical tools have progressively expanded the scope of the redoxome. For example, the size of the *S*-sulfenylome has grown from hundreds to thousands of sites of dynamic OxiPTM[36]; targets of the sulfinic acid reductase, sulfiredoxin have increased from PRXs to greater than 60 proteins[16], sites of persulfidation, known as *S*-sulfhydration, have grown 10-fold[37].

The discovery of cysteine redoxome relies on two different approaches. One approach is tracks cysteine thiol(ate) redox activity in a differential alkylation-based workflows (**Fig. 4a**). Application of such methods are limited to cell lysates where oxidation artifacts occurring during lysis limit sensitivity. Thiol-reactive alkylating agents are the foundation of such approaches, but also react with cysteine OxiPTMs such as sulfenic acids, nitrosothiols and persulfides[38]. Such side reactions and incomplete alkylation or reduction occur during the three-step process and can propagate false positive or negative identifications. The second approach is predicated on selective reaction between a unique cysteine OxiPTM, such as sulfenic acid, and a small-molecule probe (**Fig. 4b**). In essence, redox proteomics has been reframed as a problem of biorthogonal chemistry and has

rapidly become the gold standard in the field[1,28]. The advantages of this approach are many including *in situ* detection of cysteine oxidation and important molecular information regarding the identity of the OxiPTM. At the same time, the selectivity of the underlying chemical reaction must be rigorously scrutinized a task recently made infinitely more feasible with the introduction of unbiased computational search approaches[39]. For ease of discussion, we refer to the two different approaches as "activity-based" and "reaction-based", respectively and outline modern platforms from both categories below. Although previous platforms/methods like oxidative isotope-coded affinity tags (OxICAT), resin-assisted capture (RAC) and biotin switch technique (BST) serve historical importance in the redox biology field, they are considered inefficient by today's standard and their pitfalls have been thoroughly discussed[40–42].

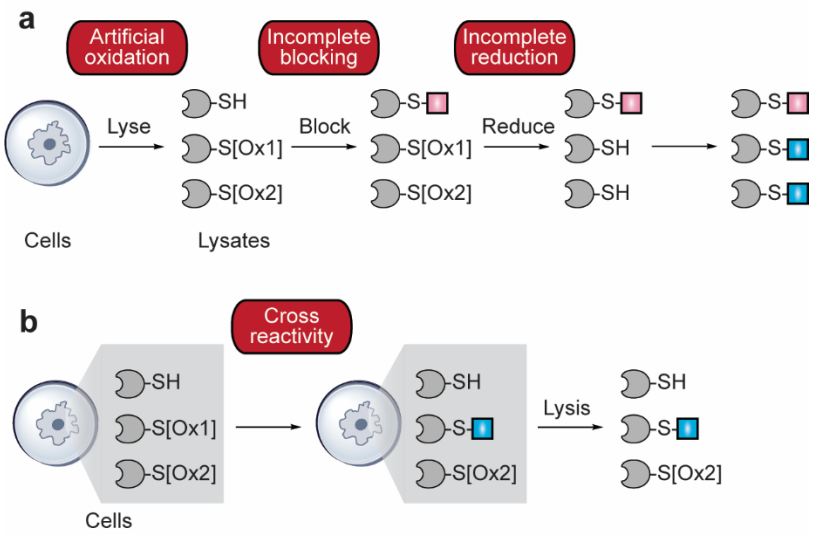

Fig. 4 Chemical biology workflows used to profile redox-sensitive cysteines. a Differential alkylation-reduction also termed activity-based. **b** Reaction-based**.** Ostensible issues are listed in red boxes.

The isotopic tandem orthogonal proteolysis–activity-based protein profiling (ISOTOP-ABBP) platform utilizes an "IA-alkyne" probe, consisting of a thiol-reactive iodoacetamide moiety and a "clickable" alkyne handle for enrichment[43]. In addition to its applications in fragment-based ligand

discovery, ISOTOP-ABPP has been adapted to investigate the cysteine redoxome, including as H_2O_2-sensitive cysteines in bacteria[44], and *S*-nitrosation sites in MCF-7 cell lysates[45]. ISOTOP-ABPP has also been used to identify redox-active cysteines in isolated organelles such as mitochondria and the endoplasmic reticulum[46,47]. A UV-caged version of the IA-alkyne probe minimizes cytotoxicity of the alkylating agent enabling *in situ* cysteine labeling[48]. More recently, an optimized workflow termed SLC-ABPP reduces instrument time and input material, achieving a depth of >8,000 reactive cysteine sites[49]. Future implementation of SLC-ABPP in redox biology is greatly anticipated; however, it is essential to note that ISOTOP-ABBP and related derivatives identify redox activity as a loss of thiolate reactivity and provide no information regarding OxiPTM identity.

Quantitative thiol reactivity profiling (QTRP) is a variant of ISOTOP-ABPP with three notable differences: i) The “click” reaction is performed at peptide level instead of protein level, allowing better enrichment for labeled peptides; ii) A UV-cleavable linker is used instead of a TEV-protease cleavable linker; iii) Excess click chemistry reagents are removed by strong cation exchange (SCX) instead of chloroform/methanol precipitation. The QTRP workflow has been used to identify and quantify more than 5,000 unique cysteine modifications[42]. Moreover, cysteine thiol(ate) reactivity is blunted when QTRP is carried at a low pH, but persulfide reactivity persists due to its lower pK_a. Therefore, low-pH QTRP can be employed as a direct method to profile cysteine *S*-sulfhydration[37].

The SulfenM and SulfenQ platforms were specifically designed for the discovery of cysteine *S*-sulfenylome using carbon nucleophiles[17], which remain the most reliable bioorthogonal probes for sulfenic acid detection with respect to kinetics and chemoselectivity[29]. Both platforms enable site-level identification of *S*-sulfenylation, and the depth of the discovered sulfenylome correlates well with the molecular reactivity of the probes[50]. While the “first generation” dimedone-based probes like DYn-2 can identify hundreds of *S*-sulfenylation sites, thousands of sites have been

discovered by more recently developed chemical tools, such as BTD and the Wittig phosphine, WYneN[15,36]. Additionally, SulfenQ can quantify the relative levels of *S*-sulfenylation from two samples (control/stimulus) *via* isotope-coded probes and bioconjugation handles. In the recently profiled WYneN-*S*-sulfenylome, the majority of sulfenic acids remained static during a short burst of oxidative stress, controlled by antioxidant systems, while other sites increased dramatically, which is indicative of regulatory function. Another methodology termed iTORC[51] coupled sulfenic acid probe BTD with differential alkylation strategy, and quantified relative cysteine thiol, sulfenic acid and disulfide levels.

Electrophilic nitrogen species (ENS) ligation refers to the chemical reaction in which the weakly nucleophilic sulfur in cysteine sulfinic acid attacks an electron-deficient nitrogen[52]. Mitsonubo diazenes form stable sulfonamide adducts with sulfinic acids and have been developed as probes for detection and chemoproteomics[16]. In this study, a probe called DiaAlk was used to identify and quantify 387 *S*-sulfinylated sites, revealing significant overlap with the sulfenylome. Under oxidative stress, the sulfinylome was less dynamic compared to sulfenic acids, underscoring the weaker nucleophilicity of sulfinic acid cysteine sulfur and hinting at an accumulation of sulfinic acids over longer times. Application of ENS ligation revealed new substrates of the cysteine sulfinic acid reductase, SRX previously considered to be specific to 2-Cys PRXs.

Total protein level is also crucial parameter to consider when defining the cysteine redoxome. Several MS platforms can reveal protein abundance while reporting on redox status *via* differential alkylation. Such methods remain incompatible with affinity enrichment, which negatively impacts proteome coverage. OxSWATH[53] is a data independent acquisition method for a comprehensive peptide quantification with deep coverage and enhanced accuracy. However, the success of this approach requires that alkylating agents have a relatively large mass difference. CysTMTRAQ[54] combines two isobaric tags cysTMT and iTRAQ. When applied in *E. coli* cells, 33 peptides were

identified as redox-sensitive, five of these also underwent changes in protein level. GELSILOX[55] utilizes $^{16}O/^{18}O$ proteolytic labeling to determine protein abundance changes, and was used to analyze the redoxome in heart mitochondria. SILAC-iodoTMT[56] reports on protein levels through metabolic labeling in the SILAC channel, and percentages of reversibly oxidized cysteines in the iodoTMT channel. This workflow was used to quantify global protein and redox changes with H_2O_2 treatment and revealed differential response to stress in PRXs cysteines. Together these studies point to changes in protein levels over time, which are subtle compared to changes in their redox status but remain an important parameter when evaluating the effect of long-term oxidative stress.

Table 1. Recent platforms used to define the cysteine redoxome.

Platform (year)	Target redoxome	Labeling in-situ?	Affinity enriched?	Size of redoxome	Quantifications performed
ISOTOP-ABPP (2010)	Reversible OxiPTMs	No	Yes	500-1000	Relative fold-changes
SLC-ABPP (2021)	N/A	Yes	Yes	8000	Not yet applied in mapping redoxome
SulfenM and SulfenQ (2014-2021)	Cys-SOH	Yes	Yes	50 (DYn-2) 1200 (BTD) 2000 (WYneN)	-SOH fold-changes and site occupancy
Low-pH QTRP (2020)	Cys-SSH	No	Yes	1500	Relative fold-changes
Improved QTRP (2020)	Reversible OxiPTMs	No	Yes	5000	Relative fold-changes
ITORC (2019)	Cys-SOH and other reversible	No	No	600 (-SOH) 2800 (-SH) 1000 (reversible)	Relative levels of -SH, -SOH and other reversible oxoforms
oxSWATH (2019)	Reversible OxiPTMs	No	No	1691 proteins, 250 with redox changes	All peptides in label-free SWATH
SILAC-iodoTMT (2019)	Reversible OxiPTMs	No	No	~4000 peptides	Protein levels (SILAC) and oxidation (TMT)
CysTMTRAQ (2015)	Reversible OxiPTMs	No	No	912 peptides	Protein levels in iTRAQ channel

GELSILOX	Reversible OxiPTMs	No	No	388 peptides	Protein levels in $^{16/18}O$ proteolytic channel
ENS ligation (DiaAlk)	Cys-SO_2H	No	Yes	387 sites on 296 proteins	-SO_2H fold-changes

Cysteine redoxome site stoichiometry. Site-level ratiometric quantification of relative cysteine oxidation (also referred to as "fold-change") effectively addresses the question of "who's active in the cysteine redoxome", while another aspect of quantitative analysis focuses on modification stoichiometry (also referred to as "site-occupancy") under steady-state or oxidative stress and addresses the question of "how much of the cysteine redoxome" is involved in redox homeostasis. Although highly-sensitive or heavily-modified sites are more likely to have regulatory roles, neither fold-change nor stoichiometry should be considered an absolute indicator of functional relevance. For example, a redox-sensitive target (high fold-change) with low site-occupancy that is inactivated by cysteine modification may not exert major biological impact (**Fig. 5a**). On the other hand, OxiPTMs that switch, or augment protein function can affect signaling pathways even at low stoichiometric levels (**Fig. 5b**).

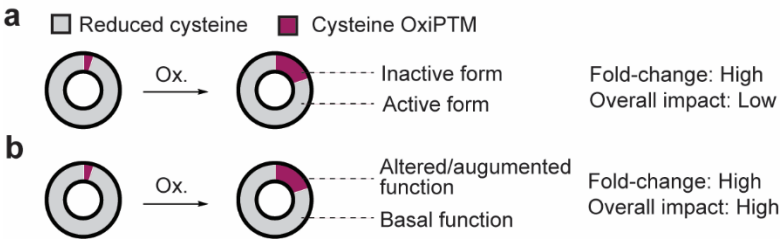

Fig. 5 Conceptual examples for the outcome of substoichiometric cysteine oxidation.

MS-based proteomics has been used in absolute stoichiometry measurements of various PTMs, including "gold-standards" such as acetylation and phosphorylation.[57] However, determination of redoxome stoichiometry faces greater challenges, due in large part to the chemical stability of sulfur modifications. Differential and competitive labeling (**Fig. 4a**) remain the most common

approaches to quantify the stoichiometry of cysteine oxidation. However, an identical thiol-alkylating agent or isotopomers are required for consistency during labeling and the LC-MS/MS workflow; ICAT and iodoTMT are most often employed. Although ICAT reagents are equipped with an affinity handle for enrichment, they are bulky and typically provide low coverage of the cysteinome. Nevertheless, when coupled with differential labeling, oxidative ICAT (OxICAT) can report on the stoichiometry of reversibly oxidized cysteines. Analyses of the *Saccharomyces cerevisiae* and *Drosophila melanogaster* cysteinome reported respective oxidation of 7.4% and 22% at steady-state[58,59]. H_2O_2 treatment, even at lethal levels, only mildly increased the average oxidation. These findings are intriguing and have been used to support the redox relay hypothesis in these organisms. These data are also fully consistent with the cysteinome functioning as the major redox buffer. By switching nonselective reductants for reagents with greater selectivity, the stoichiometry of defined OxiPTMs can also been measured. Two such modifications that have been addressed are *S*-nitrosation and *S*-glutathionylation site-occupancy ascertained through copper/ascorbate and glutaredoxin reduction, respectively[60,61].

Isobaric tags generate a single MS1 peak and are quantified at the MS2 level for reduction of interference, or at the MS3 level to further eliminate ratio distortion. Several studies have used cysteine-reactive iodoTMT probes to quantify cysteine oxidation in mouse tissues or mammalian cells but provided unsatisfactory cysteinome coverage even with affinity enrichment steps. To tackle this issue, a platform named OxiMouse was applied to define the cysteine redox landscape across ten mouse tissues[62]. OxiMouse featured a phosphate-tagged iodoacetamide probe CPT for cysteinome enrichment *via* immobilized metal affinity chromatography (IMAC), followed by MS3-based TMT quantification. This dataset covered ~171,000 cysteine sites (~34,000 unique) across ~9,400 proteins. Cysteine thiol stoichiometry was mapped onto an interactome database to highlight ostensible tissue- and age-specific redox networks that are valuable in future studies of tissue-related processes and aging.

Concerns regarding artifactual oxidation and incomplete alklyation-reduction (**Fig. 4a**) also apply to the quantification of cysteine redoxome stoichiometry. Direct labeling through bioorthogonal chemical reactions is the superior approach, but its application in site-stoichiometry requires chemically identical protein/peptides for accurate quantification (*i.e.,* the probes for parent thiol and its OxiPTM must have identical chemical structure). This challenge has recently been solved[15] for sulfenic acid, using a combination of $^{13}C_5$ WYneN and alkyne-tagged iodoacetamide (IPM). In this chemical strategy, the electrophilic sulfur in sulfenic acid is attacked by an ylide carbon nucleophile followed by elimination of the triphenylphosphonium group. These sequential reactions fortuitously result in identical modified protein/peptides for the thiol functional group and sulfenic acid modification. This workflow was used to measure *S*-sulfenylation site-stoichiometry in more than 7,000 cysteine residues[15]. Consistent with the often-transient nature of sulfenic acid in cells, the site-stoichiometry for the majority of the cysteinome (73%) was lower than 30%, with an average of 21.1% and a median of 14.5%. On the other hand, intriguing examples of near stoichiometric *S*-sulfenylation were identified in non-PRX targets. Additional interesting findings from this study are that multiple cysteines on the same protein can have significantly different oxidation stoichiometry, modified cysteines as reported by the UniProt database were less S-sulfenylated than other annotated or unannotated cysteines (consistent with diminished sulfur nucleophilicity in sulfenic acid), S-sulfenylation was more prevalent in oxidant-generating organelles such as the mitochondria and robust oxidation of the PTP1B catalytic cysteine was identified at steady-state. Going forward, quantification of sulfenic acid site-stoichiometry using the $^{13}C_5$ WYneN probe should greatly facilitate prioritization of sites for functional analyses and in defining mechanistic models of thiol-based redox regulation.

As with all methods, limitations must be kept in mind. For example, reaction-based approaches to quantify site-stoichiometry trap dynamic modifications over time and could, therefore, report higher occupancy values due to a shift in equilibrium. At the same time, the accumulative effect is expected to be trivial since most oxidation occurs on a time-scale that is slower than the trapping reaction. In addition, unlike canonical PTMs characterized by two discrete states (*i.e.,* hydroxyl or phosphoryl) redox modification of cysteine can lead to many additional states. Indeed, cysteine sulfur is protean in complexity compared to any other biofunctional group as recently highlighted by profiling thiol, sulfenic and sulfinic acid states in *Caenorhabditis elegans*[13].

Table 2. Measurement of cysteine redoxome stoichiometry in recent literature.

Probe or Platform	Target redoxome	Size of redoxome	Organism	Workflow	Stoichiometry finding
OxICAT[58]	Reversible OxiPTMs	4,457 peptides in 2,243 proteins	*S. cerevisiae*	Differential alkylation	7.4% average oxidation
OxICAT[59]	Reversible OxiPTMs	1,082 peptides in 424 proteins	*Drosophila melanogaster*	Differential alkylation	22% average oxidation
RAC-TMT[60]	Reversible OxiPTMs and -SSG	4099 sites in 1959 proteins	RAW 264.7 cells	Competitive alkylation	4.0% *S*-glutathionylation 11.9 total
SNOxICAT[61]	-SNO	~1,500 peptides	Mouse heart	Differential alkylation	7.0% average *S*-nitrosation
OxiMouse[62] **(CPT+TMT)**	Reversible OxiPTMs	171,000 sites (34,000 unique) in 9400 proteins	Mouse tissue	Competitive alkylation	10-20% average, up to 60% in secreted proteins
IodoTMT[63]	Reversible OxiPTMs	~1,700 peptides	Mouse tissue	Differential alkylation	20-30% average oxidation
IodoTMT[64]	Reversible OxiPTMs	846 sites in 403 proteins	Mouse liver	Differential alkylation	40% average oxidation
WYneN[15]	-SOH	6,623 sites in 3,372 proteins	Live A549 cells	Reaction-based protein profiling	21.1% average *S*-sulfenylation (14.5% median)

The cysteine redoxome in disease and drug discovery. It has long been recognized that unmitigated production of reactive species is closely associated with a variety of diseases and disorders. Oxidants represent both the origin and aftermath of biomolecule damage and studies focused on the detection of reactive species, or markers of protein oxidation, have been extensively reviewed[65–68]. Using sulfur chemical biology approaches, scientists can now mine the dynamic cysteine redoxome for OxiPTMs that "drive" the pathological state. Of the myriad studies that have recently emerged in this scope, several key advances are selected below for discussion for their therapeutic potential as redox-targeted approaches.

Our understanding of the biology of aging has evolved from random biomolecule oxidation to defined changes in cell signaling[69]. While elevated total cysteine oxidation is a hallmark of aging supported by many redox proteomics studies[70], the gaseous signaling molecule, hydrogen sulfide (H_2S) appears to extend lifetime in *C. elegans*. Persulfides are rapidly formed by the reaction of H_2S with cysteine sulfenic acids, a process that essentially converts a thiol electrophile (-SOH, oxidative equivalent) to a nucleophile (-SSH, reductive equivalent), thereby reversing oxidative damage (**Fig. 6a**). A unifying mechanism where H_2S-mediated protein *S*-sulfhydration regulates signaling events has recently been proposed[71]. These studies elegantly show that grow factor stimulation results in a transient burst of protein *S*-sulfenylation that declines with concurrent upregulation of *S*-sulfhydration, including of downstream kinases in growth factor signaling.

S-nitrosation mediates the pathogenesis and progression of many neurodegenerative diseases through formation of aggregates of damaged proteins, along with other mechanisms (**Fig. 6b**). For example, *S*-nitrosation of ubiquitin ligase, Parkin and disulfide isomerase, PDI inhibits their activity, causing accumulation of misfolded proteins and eventually leading to neuron damage[72]. In addition to several indirect methods for their detection in neurodegenerative disease models, Tannenbaum and coworkers recently reported the chemoproteomic technique, SNOTRAP[73],

which directly labels nitrosothiols. Increased levels of *S*-nitrosation in mouse hippocampus and cortex were indicative of early neurodegeneration.

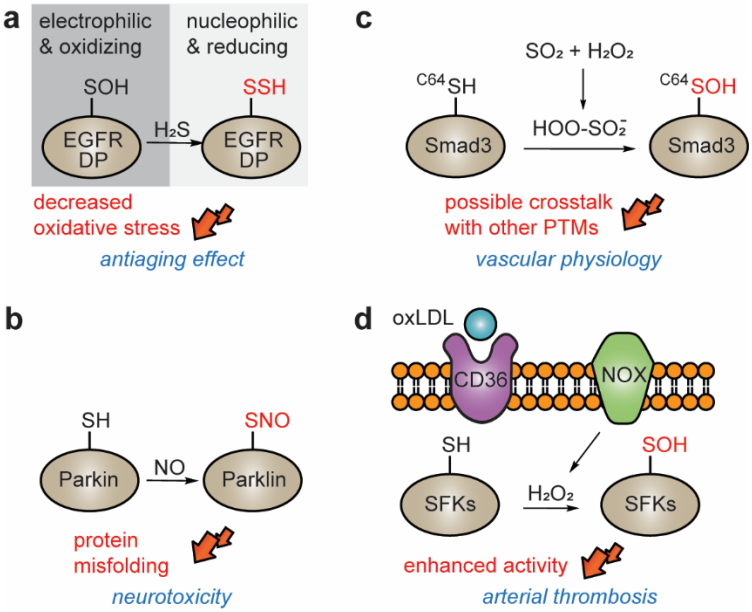

Fig. 6 Mining the cysteine redoxome for new therapeutic targets.

Sulfur dioxide (SO_2), endogenously produced *via* metabolism of L-cysteine, is another emerging gasotransmitter and appears to have a regulatory role in many physiological and pathological cardiovascular events[74]. The mechanism of SO_2-dependent protection of vascular remodeling and hypertension has been linked to conversion of H_2O_2 to the potent oxidant, peroxymonosulfite ($HOO\text{-}SO_2^-$) and concomitant *S*-sulfenylation of functional protein targets (**Fig. 6c**)[75]. Of these targets, Smad3 is essential to vascular physiology and *S*-sulfenylation of this transcription factor at cysteine 64 mediates interactions with other PTMs and has drawn major therapeutic interest.

Cluster of differentiation 36 (CD36) is a scavenger receptor for multiple types of endogenous ligands. Binding of CD36 to oxidized lipoproteins promotes NOX-mediated ROS generation and leads to arterial thrombosis. During CD36 signal transduction, SFKs are recruited and activated

through direct *S*-sulfenylation by H_2O_2 (**Fig. 6d**)[35]. Formation of this OxiPTM specifically affects the CD36 pathway and is required for platelet aggregation in pathophysiological thrombosis, opening up the possibility of targeting CD36 in cardiovascular diseases[76].

Summary and perspective. Key paradigm shifts have taken place in recent years: i) detecting different forms of biological sulfur has been reframed terms of chemoselectivity; ii) a conceptual shift from GSH as the major cellular redox buffer to the cysteinome and associated redoxome; iii) selectivity in cysteine modification is achieved through non-enzymatic and enzymatic mechanisms; iv) as in acetylation and phosphorylation, cysteine OxiPTMs can exert regulatory roles at a stoichiometry of less than one. Development of reaction-based chemical probes and advancement of analytical methods have led to significant changes in our understanding of cysteine redox-regulation. Although coverage of the cysteine redox continues to improve, reaction-based chemical tools for critical modifications including nitrosothiols, persulfides, and disulfides still have a long way to go. As we continue to define and refine the cysteine redoxome, exciting new challenges emerge. Namely, how can we explore the functional consequences of cysteine oxidation with greater ease? From the perspective of validation, methods such as site-directed mutagenesis and CRISPR can be employed for follow-up and functional studies; however, such approaches are subject to their set of limitations. In this regard, the development of methods for genetic incorporation of cysteine OxiPTMs would be a game changing. To this end, our lab has reported a caged derivative of cysteine sulfenic acid[77] and has been working to evolve a suite of synthetases for OxiPTM incorporation. Recent success has been made in fragment-based ligand discovery campaigns to target nucleophilic cysteine thiols[78]. OxiPTMs, particularly those that confer alternate or augmented activities, can be similarly exploited in drug discovery by targeting electrophilic sulfur[79,80]. These and other avenues of research indicate that the future of sulfur chemical biology is bright, indeed.

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