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1 **Reaction-based fluorogenic probes for selective detection of cysteinyl oxidation in living cells**

2

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9

10 Measuring reactive oxygen, nitrogen and sulfur species in cells is established technology, but turn-on
11 fluorescence tools for detecting the products of their reaction with protein cysteines remain essentially
12 unknown. Toward this goal, here we describe fluorogenic probes for sulfenic acid, a redox modification
13 of protein cysteines inextricably linked to signaling and oxidative stress. The probes, called CysOx1 and
14 CysOx2, are reaction-based, exhibit excellent cell permeability, rapid reactivity, and high selectivity with
15 minimal cytotoxicity. We applied CysOx2 in a cell-based 96-well plate assay to determine whether kinase
16 inhibitors modulate protein S-sulfenylation as well as O-phosphorylation. Analysis of these data revealed
17 an unexpected positive association of S-sulfenylation and inhibition of select kinases within the TK, AGC,
18 and CMGC families including GSK3, a multitasking Ser/Thr kinase and emerging therapeutic target for
19 neurodegenerative and mood disorders. Chemoproteomic mapping of sulfenic acid-modified cysteines
20 in GSK3 inhibitor-treated cells shows that sites of S-oxidation localize to regulatory cysteines within key
21 components of antioxidant defense systems. Our studies with CysOx probes offer up new insights into
22 kinase-inhibitor dependent modulation of sulfenylome dynamics and should accelerate future efforts in
23 the modern era of translational redox medicine.

24

25 Main

26 Redox reactions and oxidative stress have been implied in the etiology of numerous diseases as well as
27 in the aging process¹. The modern era in translational redox medicine seeks to identify types, sources,
28 metabolizers, and targets of oxidants in order to develop effective drugs and therapies for ROSopathies².
29 In this context, turn-on fluorescence probes also referred to as fluorogenic probes that measure oxidative
30 stress in living cells have proven invaluable for redox-related biomedical research^{3,4}. Analogous tools for
31 detecting the reaction products between biological oxidants and proteins are grossly underdeveloped, a
32 point exemplified by the lack of fluorogenic probes for detecting protein cysteine (*i.e.*, cysteinyl) oxidation.
33 The latter issue is especially striking since protein cysteines are the major target of oxidants originating
34 from both endogenous and exogenous sources⁵.

35

36 Sulfenic acid (Cys-SOH) is a central redox modification of protein cysteines and is inextricably linked to
37 oxidant signaling and stress⁶. Sulfenic acid is generated by oxidation of a thiolate by reactive oxygen
38 species (ROS) such as hydrogen peroxide (H₂O₂) produced during cellular signaling and metabolism or
39 by hydrolysis of sulfenyl halides, and very polarized nitrosothiols and disulfides⁷. If stabilized by the
40 protein microenvironment, the thiol-sulfenic acid pair can operate as a switch that is triggered by redox
41 changes to regulate protein function, structure, and localization⁸⁻¹¹. The electrophilic sulfur atom in
42 sulfenic acid can also react with a protein or low-molecular-weight thiol to form a disulfide^{12,13} or, under
43 conditions of excess oxidative stress, can be oxidized further to sulfinic and sulfonic acids¹⁴. In either
44 scenario, stabilized or as transient intermediate, sulfenic acids are key modifications in the domain of
45 biological redox regulation.

46

47 Selective chemical detection of sulfenic acid is predicated on the chemical nature of this moiety in which
48 the sulfur can behave as both a nucleophile and electrophile. The latter reactivity has been exploited by
49 numerous groups to develop carbon-based nucleophilic probes for detecting sulfenic acid in proteins and
50 cells¹⁵. The first fluorescent probe for sulfenic acid was reported in 2007¹⁶, consisting of fluorescein

attached at the C-4 position on 1,3-cyclohexanedione (Supplementary Fig. 1a). The nucleophilic C-2 carbon reacts selectively with sulfenic acid, but its application has been limited by the absence of “turn-on” fluorescence or fluorogenic response. In 2016, our group identified phenaline-1,3-dione as a chemical scaffold having fluorogenic potential vis-à-vis its reaction with sulfenic acid¹⁷ that was subsequently adapted in the design of a ratiometric fluorescent probe¹⁸ (Fig. 1a and Supplementary Fig. 1b). However, exceedingly slow reaction rates of less than 0.01 M⁻¹s⁻¹ and the absence of turn-on fluorescence signal precluded any practical application.

To address the significant gap in chemical tools available that report on cysteinyl oxidation in real-time in living cells, here, we describe fluorogenic probes for detecting sulfenic acid (Fig. 1b and Supplementary Fig. 1c). The probes are reaction-based, exhibit excellent cell permeability, rapid reactivity, and high selectivity with minimal cytotoxicity. The chemical tools have enabled live-cell imaging studies to detect redox-dependent changes in cysteinyl oxidation. Adaptation to a 96-well plate format enabled high-throughput analysis of sulfenic acid in cells and this platform was used to screen a curated inhibitor library, targeting major families of the human kinome, as potential modulators of cysteinyl oxidation. These studies identified a cohort of GSK3 kinase inhibitors that elicited significantly increased sulfenic acid modifications that localized to the regulatory cysteines of proteins involved in the response to oxidative stress. Collectively, these studies report on first-in-class tools for facile fluorogenic detection of cysteinyl oxidation in a variety of formats with broad application in future studies of redox biology and drug pharmacology.

Results

To create a fluorogenic probe for sulfenic acid, three key alterations of the phenaline-1,3-dione scaffold were envisioned: (i) replacement of a carbonyl group with the more electron withdrawing sulfonyl group to increase the rate of reaction; (ii) appendage of an amino group to create an electron “push-pull system” system, and (iii) fluorination of the aromatic core to enhance absorption and fluorescence wavelengths

(Fig. 1b). Optimized synthetic schemes for the resulting compounds is described under [Synthetic Procedures](#). In brief, 6-bromo-2H-naphtho[1,8-bc]thiophen-2-one was prepared from naphthalene-1-thiol in three steps as previously reported. The thiophenone core was hydrolyzed and dimethylated to produce both thioether and ester functional groups and the thioether was oxidized to a sulfone group with mCPBA. The brominated sulfone intermediate was then used in amination reactions, followed by condensation in presence of NaH to produce the C-nucleophile center. Finally, a fluorination step provided the desired compounds.

Fig. 1 | Design strategy for developing fluorogenic probes for detecting sulfenic acid. **a**, General structure of phenaline-1,3-dione scaffold and the tautomerization between enol and keto forms. Reaction between the nucleophilic C-2 and electrophilic sulfenic acid sulfur shifts the keto-enol equilibrium toward the keto form, which has fluorogenic potential, but is slow and poorly soluble. **b**, Colored circles denote the position for introducing electron-withdrawing groups to favor fluorogenic reaction with sulfenic acid (left). The resulting fluorogenic reaction-based probes are rapid, selective, and water soluble.

Kinetic and fluorescence characterization of phenaline-1,3-dione derivatives. We first sought to assess the reactivity of phenaline-1,3-dione (**1**) and its analogs with an established small-molecule model for cysteine sulfenic acid, known as CSA^{19,20} (Fig. 2a). Pseudo first-order rate constants were obtained for compound **1** and related analogs designed to evaluate features that modulate reactivity and fluorescence properties (Fig. 2b and Supplementary Fig. 2). Compound **1** exhibited modest kinetics 0.002 s^{-1} and fluorination of this scaffold at the C-2 position (**2** and **3**) decreased reactivity by more than two orders of magnitude. Replacement of one carbonyl group with a sulfonyl moiety (**4**) increased the reactivity of the phenaline-1,3-dione core by more than 1000-fold. C-2 fluorination (**5**) of the parent sulfonyl compound enhanced reactivity while introduction of a 7-amino group (**6**) decreased reaction rate. Although less reactive, the amino group enhanced the water solubility as compared to non-aminated analogs. Further modification by fluorination at C-6 (**7**) retarded kinetics by 10-fold. Finally, the 7-amino

group was replaced by the cyclic amine, azetidine (**8**) affording a compound with an observed reaction rate of approximately 0.1 s^{-1} . Overall, the sulfonyl group dramatically increased nucleophilic reactivity towards the sulfenic acid electrophile, which was tempered somewhat by the installation of amino and fluorine groups, as expected.

In subsequent experiments, we evaluated the fluorescence properties of sulfonyl derivatives **4** through **8**. For this purpose, the reaction product between each analog and CSA was prepared and isolated from milligram-scale reactions. Fluorescence spectra of the resulting adducts were then recorded in organic solvent or aqueous solution and compared to that of non-adducted compounds alone (Fig. 2c-d, and Supplementary Fig. 3). CSA adducts of **4** and **5** exhibited weak fluorescence signal and were essentially nonfluorogenic. Furthermore, compounds **4** and **5** were only sparingly soluble in water. Compound **6** showed improved solubility but gave only weak fluorescence signal before and after reaction with CSA. By contrast, CSA adducts of compounds **7** and **8** were fully soluble in PBS, exhibited bathochromic shifts of excitation and emission maxima as well as increases in fluorescence intensity compared to **7** or **8** alone, indicating that these compounds are fluorogenic for sulfenic acid (Fig. 2c-d). Specifically, the CSA-**7** adduct gave a slight bathochromic shift of excitation and emission maxima of 3 nm with no change in the Stokes shift. The bathochromic shift was more pronounced for the CSA-**8** adduct (53 and 75 nm for excitation and emission maxima, respectively) accompanied by an increase in the Stokes shift of 22 nm. The increase in fluorescence intensity was 2.3- for CSA-**7** and 11.4-fold for CSA-**8** (quantum yields increased by 2.1- and 2.6-fold, respectively) compared to **7** and **8** alone. UV-Vis spectroscopy analysis of **7** and **8** with their respective CSA adducts showed an increase in extinction coefficient of 1.7- and 6.4-fold, respectively (Supplementary Table 1 and Supplementary Fig. 4-5). Finally, a bathochromic shift in the absorption maxima of CSA-**8** was also observed, in agreement with the shift observed in the fluorescence spectra. Comparison of data obtained for compounds **7** and **8** to compound **6** demonstrates that fluorination is essential for their fluorogenic response.

Fig. 2 | Chemical modification of the phenaline-1,3-dione scaffold yields fluorogenic probes for detecting sulfenic acid. **a**, Rate constants were measured under pseudo-first-order conditions with small-molecule sulfenic acid model, CSA, in excess over phenaline-1,3-dione nucleophiles as described in Supplementary Methods. **b**, k_{obs} (s⁻¹) for compounds **1** through **8** were determined by curve fitting to a single exponential function. Second-order rate constant, k is extrapolated from k_{obs} divided by the concentration of the limiting agent. **c**, Absorption and emission spectra of **7** and isolated CSA-**7** (250 μM) in PBS:ACN pH 7.4 (9:1). **d**, Absorption and emission spectra of **8** and isolated CSA-**8** (250 μM) in PBS:ACN pH 7.4 (9:1). **e**, Fluorescence intensity monitored over time for the reaction between **7** (5 to 100 μM) and CSA (1 mM) after excitation at 443 nm (left). Fluorescence intensity at 15 min plotted versus the concentration of **7** (right). $n = 3$. **f**, Fluorescence intensity monitored over time for the reaction between **8** (5 to 100 μM) and CSA (1 mM) after excitation at 447 nm (left). Fluorescence intensity at 15 min plotted versus the concentration of **8** (right); $n = 3$. **g-h**, Rate constants were measured under pseudo-first-order conditions with CSA in excess over phenaline-1,3-dione nucleophiles as described in Supplementary Methods. A representative trace from these collective reactions is shown. Linear fit of k_{obs} (min⁻¹) plotted versus CSA concentration gives 91 ± 3 and 105 ± 2 M⁻¹ s⁻¹ for **7** and **8**, respectively (inset). $n = 3$. **i-j**, Box and whisker plot of normalized fluorescence intensity (F/F_0) for reaction between **7** or **8** (50 μM) and various analytes (1 mM) after 1 h; $n = 4$.

Next, we measured the fluorescence intensity of **7** and **8** in real-time reactions with CSA. Reactions containing **7** or **8** were excited at 443 nm and 447 nm, respectively. Fluorescence emission intensity increased over time and was linear with probe concentration (Fig. 2e,f). Pseudo first-order rate constants were then obtained at different probe concentrations to obtain second-order rate constants, which were

126 91 ± 3 and $105 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$ for **7** and **8**, respectively (Fig. 2g,h). To screen for potential side reactions, we
127 evaluated the reactivity of **7** and **8** with a panel of potentially reactive biomolecules, such as aldehyde
128 and disulfide electrophiles as well as related sulfur species including reduced glutathione, glutathione
129 nitrosothiol, glutathione sulfinic and sulfonic acid. No significant reaction took place between **7** or **8** and
130 any of the aforementioned compounds (Fig. i,j). Taken together, these studies indicate that compounds
131 **7** and **8**, referred to hereafter as **CysOx1** and **CysOx2**, exhibit reaction-based turn-on fluorescence, rapid
132 reactivity, and high selectivity when evaluated in a small-molecule model of cysteine sulfinic acid.

133

134 **Evaluation of CysOx reactivity and fluorescence in complex biological settings.** Encouraged by
135 our success in the CSA model, we then moved on to more targets with greater biological relevance and
136 complexity. Toward this end, we examined CysOx probe reactivity in C64,82S glutathione peroxidase 3
137 (Gpx3; Fig. 3). This Gpx3 variant has one redox-sensitive cysteine, C36 that is readily oxidized to sulfinic
138 acid using stoichiometric amounts of hydrogen peroxide (H_2O_2) and has been well-validated as a model
139 for the study of protein sulfinic acid reactivity^{19,20}. Intact mass spectrometry (MS) analysis demonstrated
140 that Gpx3 C36 sulfinic acid (Gpx3-SOH) formed the anticipated adduct with CysOx probes in high yield,
141 while reduced Gpx3 (Gpx3-SH) was not modified (Fig. 3b,c). Having verified the correct protein adducts
142 by MS, we next evaluated the fluorescence spectra of isolated Gpx3-CysOx adducts compared to that of
143 free CysOx probes. Satisfyingly, **CysOx1** and **CysOx2** exhibited a “turn-on” fluorescence response after
144 respective excitation at 357 nm and 394 nm (Fig. 3d,e). Of the two probes, **CysOx2** showed a larger
145 fluorescence enhancement of up to 4-fold. Fluorescence spectra of analogous real-time or *in-situ*
146 reactions gave similar results and also demonstrate that fluorogenic response is only observed when all
147 three components – CysOx, H_2O_2 , and Gpx3 – are present in the reaction (Fig. 3f,g). Compared to data
148 obtained with CSA, the fluorescence spectra of Gpx3-CysOx adducts were blue-shifted by about 50 nm.
149 Finally, the reaction products of CysOx probes and Gpx3-SOH were visualized by in-gel fluorescence
150 (Fig. 3h,i). Intense signal was detected in CysOx-treated Gpx3-SOH but not in Gpx3-SH, consistent with
151 the findings in our intact MS analysis.

Fig. 3 | CysOx1 and CysOx2 are reaction-based fluorogenic probes for protein cysteine (cysteiny) sulfenic acid. **a**, Structures of **CysOx1** and **CysOx2**. **b-c** Intact MS analysis of the reaction products of reduced or sulfenic acid forms of C64,82S Gpx3 (10 μM) with **CysOx1** or **CysOx2** (1 mM) after 1 h in 50 mM HEPES pH 7.4. **d-e**, Emission spectra of **CysOx1** or **CysOx2** alone (10 μM) compared to purified Gpx3-**CysOx1** or Gpx3-**CysOx2** (10 μM). Reactions with **CysOx1** or **CysOx2** were excited at 357 nm and 394 nm, respectively. Spectra were recorded in 50 mM HEPES pH 7.4. **f-g**, Emission spectra of **CysOx1** or **CysOx2** alone (10 μM) or in combination with H₂O₂ (15 μM) or Gpx3 (10 μM) or H₂O₂ (15 μM) and Gpx3 (10 μM). Only the reaction containing all three components gives a fluorogenic product. Reactions with **CysOx1** or **CysOx2** were excited at 357 nm and 394 nm, respectively. Spectra were recorded in 50 mM HEPES pH 7.4. **h-i**, In-gel fluorescence analysis of reaction products between Gpx3 (10 μM) and **CysOx1** or **CysOx2** (1 mM) with or without H₂O₂ (15 μM) or TCEP (1 mM) after 1 h in 50 mM HEPES pH 7.4.

152

153 Having established that CysOx probes are fluorogenic and selective in small-molecule and protein
 154 models of sulfenic acid, we next assessed their ability to enter live cells and provide a fluorogenic readout
 155 of endogenous S-sulfenylated proteins. For these experiments we employed glucose oxidase (GOX) at
 156 0.2, 2, and 20 U/mL to provide continuous production of H₂O₂ in glucose-containing cell culture media²¹.
 157 HeLa cells incubated with **CysOx1** or **CysOx2** showed significant time- and GOX-dependent increases
 158 in fluorescence intensity (Fig. 3a,b and Supplementary Fig. 6). Fluorescence was distributed throughout
 159 the cytoplasm with no apparent co-localization and minimal cytotoxicity (Supplementary Fig. 7-8). Live-
 160 cell labeling by non-fluorogenic analog **6** was also investigated. In contrast to CysOx probes, compound
 161 **6** failed to promote oxidant-dependent “turn-on” fluorescence, instead giving weak signal throughout the
 162 duration of the experiment (Supplementary Fig. 9), consistent with our CSA studies. In-gel fluorescence
 163 analysis of lysates derived from HeLa cells incubated with CysOx probes showed concentration- and
 164 redox-dependent protein labeling by **CysOx2** (Fig. 4c); however, signal from **CysOx1** was much weaker
 165 (Supplementary Fig. 6c), reflecting the inherent difference in their fluorogenic intensities. Collectively,

these studies indicate that the reaction between CysOx probes and the electrophilic sulfur in sulfenic acid represents a viable strategy for fluorogenic detection of cysteinyl oxidation in the test tube and in real-time in living cells.

Fig. 4 | Cell permeable CysOx probes for no-wash live-cell microscopy of cysteine sulfenic acid in cells. **a**, No-wash live-cell confocal images of HeLa cells at different time points after the addition of **CysOx2** (10 μ M) and GOX at the indicated concentrations (0-20 U/mL). λ_{ex} = 458 nm; scale bar = 50 μ M. **b**, Box and whisker plot of the average pixel intensities from panel **a**. Data are representative of ten independent readings from five different frames. Error bars are $\pm$ SEM. Variance was analyzed by two-way ANOVA test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ when compared against cells treated with probe only. **c**, In-gel fluorescence analysis of lysates derived from HeLa cells incubated with **CysOx2** and GOX (20 U/mL) or *t*-BOOH (500 μ M).

169

170 Application of CysOx2 to identify kinase inhibitors that modulate cysteinyl oxidation in cells.

171 Crosstalk between cysteine oxidation and other post-translational modifications such as phosphorylation
172 had been reported^{22,23} but the interplay between kinase inhibition and cysteinyl oxidation remains unclear.
173 To further showcase the utility of fluorogenic probes in the detection of cysteinyl oxidation and address
174 the question above, we subsequently adapted **CysOx2** for use in a cell-based 96-well plate assay. After
175 probe concentration and treatment times were optimized for this scale (Supplementary Fig. 10) we
176 screened a curated library of kinase inhibitors was in HeLa cells. Analysis of the resulting data revealed
177 a 3-fold or greater increase in fluorescence for 8% (12 out of 154 compounds) of kinase inhibitors as
178 compared to vehicle-treated cells (Fig. 4a and Supplementary Table 2). Protein S-sulfenylation was
179 modulated by inhibition of RTKs as well as select kinases from the AGC and CMGC families, including
180 the multitasking Ser/Thr kinase GSK3 (Fig. 4b). Upon closer inspection, three of the ten compounds
181 associated with the greatest increase in fluorescence intensity (**SB-216763**, **BIM-IX**, and **BIO**) were
182 identified as GSK3 inhibitors (Fig. 4a-c), suggesting a relationship between this idiosyncratic kinase and
183 protein S-sulfenylation. Underscoring this point, application of the most effective inhibitor identified in this
184 screen, **SB-216763** promoted a fluorescence increase equivalent to that of exogenously applied peroxide

(Fig. 4c). The ability of **SB-216763** to increase cellular protein S-sulfenylation was also confirmed through fluorescence imaging microscopy (Fig. e,f) and in-gel fluorescence analysis (Supplementary Fig. 11).

Fig. 5 | Multi-well based screening identifies kinase inhibitors that elevate cysteinyl oxidation in cells. **a**, Relative fluorescence intensity of HeLa cells treated with various kinase inhibitors (10 μ M) and **CysOx2** (50 μ M) after 1 h at 37 $^{\circ}$ C. **b**, Twelve compounds demonstrated a relative increase in fluorescence intensity of 3-fold or greater. The primary target and kinase family for each compound is listed. **c**, Three GSK3 inhibitors were identified among the top ten hits: BIO, BIM-IV (BIM) and SB-216763 (SB). These inhibitors were purchased, their purity verified by LC-MS, and rescreened in HeLa cells at 1 μ M in the presence or absence of **CysOx2** (50 μ M). The concentration of *t*-BOOH control was 2 mM. **d**, General structure of disubstituted maleimide-based GSK3 inhibitors. **e**, No-wash live-cell confocal images of HeLa cells at different time points after the addition of **CysOx2** (5 μ M) in the presence or absence of SB-216763 (0.1 μ M). λ_{ex} = 458 nm; scale bar = 50 μ M. **f**, Box and whisker plot of the average pixel intensities from panel **e**. Data are representative of ten independent readings from five different frames. Error bars are $\pm$ SEM. Variance was analyzed by two-way ANOVA test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ when compared against cells treated with probe only.

Site-specific proteomic ID of cysteines that undergo S-sulfenylation in GSK3 inhibitor-treated cells. Having identified an interesting connection between GSK inhibition and cysteinyl oxidation, we next sought to site-specifically and quantitatively profile dynamic changes in the HeLa S-sulfenylome induced by GSK-3 inhibitors using **BTD**-based chemoproteomics (Fig. 5a). **BTD** is a clickable chemical probe for sulfenic acid detection²⁴. In agreement with fluorescence screening data, **SB-216763** treatment resulted in the most significant perturbation to the S-sulfenylome, with 56.3% measured S-sulfenylation events exhibiting at least a 1.5-fold change (Fig. 5b). Moreover, S-sulfenylation levels in nearly half of quantified sites with Uniprot functional annotations were changed by **SB-216763** (Fig. 5c), implying broad regulation. Gene ontology classification further revealed that the sub-sulfenylome perturbed by GSK3 inhibition ($R_{L/H}^{SB-216763} \geq 1.5$, $R_{L/H}^{BIM-IX} \geq 1.0$, $R_{L/H}^{Bio} \geq 1.0$) was enriched in many biological processes involved in antioxidant response, including regulatory cysteines of thioredoxin, thioredoxin reductase, glutaredoxin and glutaredoxin reductase (Fig. 5d,e). GSK3 inhibition also increased oxidation of PRDX6 C47 whose

200 catalytic cysteine is notoriously susceptible to overoxidation under oxidative stress, whereas exogenous
 201 H_2O_2 downregulates this redox event in the same cell line¹⁴. GSK3 inhibition and H_2O_2 both decreased
 202 sulfenylation at C152 of GAPDH, which is also well-known redox sensitive site (Supplementary Fig. 12).
 203 These findings suggest that redox changes induced by GSK3 inhibition might be more specific as
 204 opposed to simple diffusion of H_2O_2 .

Fig. 6 | GSK3 inhibition selectively perturbs the S-sulfenylome. **a**, Workflow for the BTD-based quantitative S-sulfenylome analysis. HeLa cells were treated with vehicle or with GSK3 inhibitors as indicated, and then labeled with 5 mM BTD *in vitro*. BTD-labeled proteomes with and without inhibitor treatment were digested into tryptic peptides, conjugated with heavy and light azido biotin reagents with a photocleavable linker, respectively. Light and heavy labeled samples then were combined, clicked, enriched with streptavidin beads. The photoreleased peptides were analyzed by LC-MS/MS for identification and quantitation. Heavy to light ratio calculated for each BTD-labeled cysteine residue provided a measure of its relative level in inhibitor-treated samples versus controls. **b**, Box plot showing the side-by-side comparison of the quantitation results from three GSK3 inhibitors. **c**, Ranking plots showing the distribution of Log₂ R_{H/L} values for each GSK3 inhibitor. **d**, GO enrichment of the sub-sulfenylome perturbed by GSK3 inhibition. **e**, Representative extracted ion chromatograms (XICs) showing changes in BTD-labeled peptides from the proteins as indicated. The profiles for light- and heavy-labeled peptide are shown in red and blue, respectively. The average R_{H/L} values calculated from two biological replicates are displayed below each individual XIC.

205 Discussion

206 In contrast to cysteinyl oxidation products, turn-on fluorescent probes for the detection of low-molecular-
 207 weight thiols like cysteine and glutathione have enjoyed a lengthy history of development and research²⁵.

208 Such reagents have utility in cells and often show a degree of discrimination among different biothiols.
209 Despite such advances, the degree to which these tools report on cysteine residues in proteins is not
210 clear. Furthermore, this general class of reagents only detects biothiol oxidation as a loss of fluorescence
211 signal. To address this significant gap, this study presents the first instance of fluorogenic tools for direct
212 detection of protein cysteine or cysteinyl oxidation, specifically monitoring the formation of sulfenic acid.

213

214 The fluorogenic character of CysOx probes stems, at least in part, from the fluorescence quenching effect
215 of the phenaline-1,3-dione keto tautomer, in which nearly parallel carbonyl groups gives the largest large
216 dipole moment²⁶. Reaction of the phenaline-1,3-dione scaffold at nucleophilic C-2 with the electrophilic
217 sulfur atom in sulfenic acid serves to stabilize the fluorogenic enol tautomer. A second key contributor to
218 the fluorescence of these probes is the electron-withdrawing fluorine atom attached to the naphthalene
219 core, which serves to stabilize the excited state²⁷. A final feature of CysOx probes is the push-pull system
220 wherein the electron-donating amino group is in conjugation with fluorine *via* the intervening π -system²⁸.

221

222 Another aspect of this study was to apply CysOx technology to identify kinase inhibitors that modulate S-
223 sulfenylation in cells. The curated screening library used in this study includes inhibitors of lipid, receptor
224 and non-receptor tyrosine, serine/threonine, and dual specificity kinases²⁹. Several interesting patterns
225 emerged from this screen. For instance, among inhibitors associated with a significant increase in cellular
226 S-sulfenylation, about 40% targeted kinases in the RTK family. This is consistent with reports from our
227 lab and others indicating that growth factor signaling is intimately linked to ROS metabolism in cells^{5,8}.
228 The balance of “hits” within this cohort target members of the AGC and CMGC families and, of special
229 interest, three of these inhibit GSK3 function³⁰. GSK3 is an unusual kinase in that it is constitutively
230 active and its substrates (over 100 are known) need to be pre-phosphorylated by another kinase, and it
231 is inhibited as opposed to activated by the two pathways known to converge on GSK3, the insulin and
232 Wnt pathways³¹. The elucidation of such patterns highlights an important strength of CysOx probes for

233 identifying agents or conditions that modulate protein S-sulfenylation, an area of central importance to
234 many fields including covalent targeting of semi-conserved cysteines which is a growing strategy in drug
235 design^{32,33}.

236

237 To independently verify the relationship between GSK3 inhibition and cysteinyl oxidation we performed
238 chemoproteomic analysis using the sulfenic acid-selective probe, **BTD**. We found that GSK3 inhibitors
239 did indeed increase cellular S-sulfenylation with **SB-216763** exhibiting the greatest effect, followed by
240 **BIM-IX** and **BIO**, consistent with the findings from our kinase inhibitor screen. In this regard, we note
241 that GSK3 inhibition activates Nrf2 for the transcription of antioxidant machinery³⁴ and recent studies
242 suggest that the Nrf2 pathway can also be triggered by inactivation of thioredoxin and glutathione-
243 glutaredoxin systems, which are key reducing mechanisms in eukaryotes³⁵. Consistent with this idea, S-
244 sulfenylation of regulatory redox-active cysteines in thioredoxin, thioredoxin reductase, glutathione
245 reductase and glutaredoxin, all increased with GSK3 inhibition and may represent a KEAP1-independent
246 means to activate Nrf2. Future research will be required to dissect this intriguing link in greater molecular
247 detail.

248

249 As with all technologies, it is worth noting current limitations. At present, the CysOx fluorophore presents
250 low brightness and quantum yields when compared to other dyes, such as fluorescein and BODIPY. This
251 is reflected in the low quantum yields (0.9-2.4%) observed for the reaction product of sulfenic acid and
252 CysOx probes. This limitation does not hinder application of CysOx probes in the studies presented in
253 this work. However, attempts to further miniaturize the cell-based assay to a 384-well plate using **CysOx2**
254 failed, which may be addressed in future iterations of these tools with brighter probes and enhanced
255 dynamic range.

256

257 In summary, we have introduced a general chemical strategy for reaction-based fluorogenic detection of
258 cysteinyl sulfenic acid. Using this strategy we created two chemical probes, **CysOx1** and **CysOx2** that

259 have excellent cell permeability, rapid reactivity, and high selectivity with minimal cytotoxicity. The kinetic
260 and spectroscopic properties of CysOx probes make them powerful first-in-class tools for live-cell
261 fluorescence imaging studies of redox-regulated biology in physiology and disease.

262

263 **Methods**

264 **General Spectroscopy.** Fluorescence spectra for studies not in cell culture were recorded on an Agilent
265 Technologies Cary Eclipse Fluorescence Spectrophotometer running the Cary Eclipse v1.2 software.
266 Absorbance spectra were recorded on a Varian Cary 300 Bio UV-Visible Spectrophotometer using Cary
267 WinUV v4.20 software. Stock solutions of chemical probes were prepared in DMSO and then diluted in
268 PBS (pH 7.4) at the indicated concentrations.

269

270 **Preparation of probe-CSA adducts.** To a solution of dipeptide cyclic sulfonamide CSA (36.6 mg, 0.100
271 mmol) in DCM (3.0 mL) was added a C-nucleophile probe (0.100 mmol, 1 equiv), followed by Et₃N (13.9
272 μ L, 0.100 mmol, 1 equiv). The reaction mixture was stirred at rt overnight. LC-MS analysis of the reaction
273 mixture indicated complete consumption of the starting materials and the formation of the desired CSA-
274 probe adduct as a single product. The reaction mixture was neutralized with TFA, diluted in DCM (20
275 mL), and washed with water (20 mL) and brine (20 mL). The organic phase was dried over MgSO₄,
276 filtered and concentrated. The obtained products were purified via column chromatography (SiO₂, 0-
277 100% EtOAc in hexanes) and used as is in subsequent experiments.

278

279 **C64,82S Gpx3 labeling with CysOx1 or CysOx2.** C64,82S Gpx3-SH was spin-filtered into labeling
280 buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4) using pre-equilibrated P30 columns (BioRad). The
281 concentration of C64,82S Gpx3-SH was determined by measuring the absorptivity at 280 nm (ϵ = 24,410
282 M⁻¹cm⁻¹). Solutions of C64,82S Gpx3-SH (20 μ M) and **CysOx1 or CysOx2** (20 μ M) were prepared in

283 labeling buffer and kept in ice. The Cary Eclipse software was set to Kinetics mode using the fluorescence
284 excitation and emission maxima observed for Gpx3-CysOx adducts (357/498 nm for **Gpx3-CysOx1** and
285 394/535 nm for **Gpx3-CysOx2**). C64,82S Gpx3-SH (50 μ L) and CyxOx probe (50 μ L) were mixed in a
286 cuvette. Following sample insertion into the fluorimeter, H₂O₂ (100 μ L of 30 μ M made up in labeling
287 buffer) was fluorescence measurements were acquired. Three reactions were analyzed per chemical
288 probe. For control reactions, labeling buffer was added instead of selected components of the reaction.

289

290 **In-gel fluorescence analysis of CysOx-labeled proteins.** For Gpx3: C64,82S Gpx3 incubated with
291 **CysOx1** or **CysOx2** (21 μ L) were boiled for 5 min in reducing loading buffer (7 μ L). For HeLa lysates
292 derived from **CysOx**-treated cells: Lysate (20 μ g protein) was diluted to a final volume of 30 μ L and boiled
293 for 5 min in non-reducing loading buffer (10 μ L). SDS-PAGE precast 4-20% or 4-15% gels (BioRad)
294 were electrophoresed in Tris-Glycine-SDS running buffer at 80 V for 10 min and then at 120 V for 60 min.
295 Gels were then washed in H₂O and imaged using the ChemiDoc MP imaging system (BioRad). Settings
296 were as follows: Fluorescein (epi-blue 460-490 nm excitation and 577-613 nm emission filter). After
297 fluorescence imaging, gels were stained in Coomassie Brilliant Blue solution for 5 min at rt, washed in
298 destaining solution (H₂O/MeOH/AcOH 5:4:1) overnight, and then scanned by ChemiDoc MP to visualize
299 Coomassie staining.

300

301 **Fluorescence cell imaging of live cells treated with CysOx probes.** HeLa cells (seeding population:
302 8.0×10^4 cells/well) were incubated in EMEM (+10% FBS) in 6-well glass bottom plates at 37 °C. At 70-
303 80% confluence, media was aspirated, and cells were washed twice with DPBS and serum-free EMEM
304 was added to wells. After incubation for 16 h at 37 °C media was aspirated, and cells were washed twice
305 with DPBS. For GOX treatment: Cells were treated with CysOx probe and GOX at the indicated
306 concentrations in DPBS containing 0.1% DMSO and glucose (1 mg/mL). At the indicated time points,
307 well were directly analyzed in an Olympus FluoView IX81 confocal microscope using the FV10-ASW 3.0

308 software. For *t*BOOH treatment: Oxidant was added at the indicated concentrations in EMEM for 10 min
309 at 37 °C followed by the addition of CysOx probe at the indicated concentration for additional 15 min at
310 37 °C. The media was then aspirated, cells washed twice with DPBS, and fresh DPBS added. The wells
311 were then analyzed in an Olympus FluoView IX81 confocal microscope using FV10-ASW 3.0 software.
312 The 458 nm laser channel was used to excite incorporated CysOx probes. Fluorescence signal was
313 filtered using a SDM560 dichroic mirror, followed by BA505-605 band pass filter to observe the CysOx
314 fluorescence. Five frames were recorded per condition. Images were analyzed using ImageJ software,
315 where the pixel intensity of the cellular cytoplasmic regions was measured with at least 10 measurements
316 per condition.

317

318 **Multi-well cell-based screening assay to identify kinase inhibitors that modulate S-sulfenylation.**

319 HeLa cells were incubated in EMEM supplemented with 10% FBS in black 96-well plates with clear
320 bottoms (seeding population: 2×10^4 cells/well) at 37 °C. At 90% confluence (48 h), media was aspirated,
321 cells washed twice with PBS and serum-free EMEM was added to the wells. After incubation for 16 h at
322 37 °C media was aspirated, cells were washed twice with PBS, and then DPBS (90 µL) was added to
323 each well. **CysOx2** (50 µM) and kinase inhibitor (10 µM, Kinase Screening Library - Cayman Chemical,
324 item no. 10505) in DPBS containing 1% DMSO was added each well for a total volume per well of 100
325 µL. Cells were then incubated at 37 °C for 60 min. Selected control wells did not receive kinase inhibitor
326 and were instead treated with either PBS (negative control) or *t*BOOH (200 µM, positive control).
327 Following the incubation period, DPBS (100 µL) and extracellular fluorescence quencher (20 µL, Solution
328 C of Beta-Lactamase Loading Solutions Kit from Life Technologies, part number K1048) were added to
329 each well prior to measurement in a Molecular Devices SpectraMax M5 plate reader and analyzed using
330 SoftMax Pro v5.4 software. The fluorescence parameters of **CysOx2** were selected according to the
331 maxima measured in the fluorescence spectra (excitation/emission: 447/ 606 nm). Preliminary screening
332 to identify hits was performed in duplicate and compounds exhibiting more than a 2.5-fold increase in
333 fluorescence were subsequently confirmed in quintuplicate according to the same procedure.

334

335 **Reporting Summary**

336 Further information on research design is available in the Nature Research Reporting Summary linked to
337 this article.

338

339 **Data availability**

340 All data associated with this study are available in the published article and its Supplementary
341 Information.

342

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417

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428

429 **Author Contributions**

430 R.B.F, K.S.C. and J.Y. designed the experiments, analyzed data and all authors wrote the
431 manuscript and provided input. R.B.F. synthesized probes, performed rate studies, compound
432 characterization, acquisition of UV-Vis and fluorescence spectra, fluorescence microscopy for
433 live-cell imaging, fluorescence microplate assays, intact MS, SDS-PAGE, and in-gel
434 fluorescence analysis of CysOx-Gpx3 adducts. J.Y. and L.F. performed chemoproteomic
435 experiments and analyzed the data.

436

437 **Competing Interests statement**

438 The authors declare no competing financial interests.

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