Wittig reagents for chemoselective sulfenic acid ligation enables global site stoichiometry analysis and redox-controlled mitochondrial targeting

Yunlong Shi1, Ling Fu2,3,4, Jing Yang2,3,4✉ and Kate S. Carroll1✉

Triphenylphosphonium ylides, known as Wittig reagents, are one of the most commonly used tools in synthetic chemistry. Despite their considerable versatility, Wittig reagents have not yet been explored for their utility in biological applications. Here we introduce a chemoselective ligation reaction that harnesses the reactivity of Wittig reagents and the unique chemical properties of sulfenic acid, a pivotal post-translational cysteine modification in redox biology. The reaction, which generates a covalent bond between the ylide nucleophilic α-carbon and electrophilic γ-sulfur, is highly selective, rapid and affords robust labelling under a range of biocompatible reaction conditions, which includes in living cells. We highlight the broad utility of this conjugation method to enable site-specific proteome-wide stoichiometry analysis of S-sulfenylation and to visualize redox-dependent changes in mitochondrial cysteine oxidation and redox-triggered triphenylphosphonium generation for the controlled delivery of small molecules to mitochondria.

Although modern methods provide access to molecules of increasing complexity, traditional organic transformations remain an essential cornerstone of synthetic chemistry. Their fundamental value is increasingly underlined by their potential to be repurposed as biocompatible reactions, with novel applications in living systems4. Pioneering examples of named reactions that have been broadly employed to discover new biology are the Staudinger reaction8 and the Huisgen 1,3-dipolar cycloaddition9. Application of conventional organic reactions in biological settings also enables us to revisit and expand our understanding of known reactions, which includes the development of new substrates and, by extension, novel products.

The Wittig reaction is a widespread technique employed for the preparation of alkenes through the use of a triphenylphosphonium (TPP) ylide, which holds a highly polarized P–C bond, as illustrated by the zwitterionic ylide form, which highlights the strong nucleophilicity of the carbaneion (Fig. 1a). Anion stabilizing groups (ASG) can further modulate the α-carbon and produce more stable ylides via an electron-withdrawing inductive effect or resonance stabilization, some of which become compatible in aqueous media5. Despite the considerable utility of Wittig reagents in organic synthesis, they have not been mined for use in chemoselective, biocompatible reactions.

In the interest of developing new reactions with applications in cells, we hypothesized that the chemically unique electrophilic sulfur in sulfenic acid, a post-translational modification of protein cysteines (Fig. 1b), could serve as the reaction partner in vitro and in cells. Sulfenic acid is generated through one of two routes: direct oxidation of a thiolate by reactive oxygen species, such as hydrogen peroxide produced during cellular signalling and metabolism, or by the hydrolysis of sulfenyl halides, cyclic sulfenamides and very polarized nitrosothiols and disulfides7 (Supplementary Fig. 1a). Sulfenic acids have a pKa of 6–7 in proteins, which indicates that both protonated and deprotonated states are accessible at physiological pH (ref. 8). If stabilized by the microenvironment, the thiol–sulfenic acid (or sulfinic) pair can operate as a switch triggered by redox changes, which regulate protein function, structure and localization. Alternatively, the electrophilic sulfur in sulfenic acid may condense with a protein or low-molecular-weight thiol to form a disulfide or, under conditions of excess oxidative stress, may be oxidized further to sulfenic and sulfonic acids. In either scenario—as a stabilized or transient intermediate—sulfenic acids are central modifications in the domain of biological redox regulation10,11 (Supplementary Fig. 1b). Furthermore, our group8,12–16 and others17–19 showed that the electrophilic character of sulfur in sulfenic acid is chemically distinct from that in protein electrophiles, including modifications to amino or thiol functional groups, which makes this species an ideal candidate to examine for reactivity with Wittig reagents.

With the unexamined potential of nucleophilic Wittig reagents in biological applications and the advancement of our understanding of electrophilic sulfenic acid modifications in biology and pharmacology in mind, we report the reaction between a Wittig reagent and a sulfenic acid to generate a C–S bond (Fig. 1c). The reaction is highly selective, rapid and affords robust labelling under a range of biocompatible reaction conditions, including in living cells. We highlight the broad utility of this conjugation method to enable site-specific proteome-wide stoichiometry analysis of S-sulfenylation, and visualize redox-dependent changes in mitochondrial cysteine oxidation and redox-triggered TPP generation for the controlled delivery of small molecules to mitochondria (Fig. 1d,e).

Results
Screening Wittig reagent reactivity. We initiated our study with Wittig reagents functionalized with ASGs (1–7). A dipeptide cyclic sulfenamide model compound that generates a sulfenic acid in situ when dissolved in water1 was used to survey the...
reactivity of these reagents (Extended Data Fig. 1). Nitrile- and aldehyde-functionalized Wittig reagents (1 and 2, respectively) underwent hydrolysis in organic aqueous buffer at pH 7.4 and were not further pursued. Monosubstituted derivatives, which include ketone 3, ester 4 and amide 5, and disubstituted Wittig reagents 6 and 7 formed stable products with the sulfenic acid model in quantitative yield. Compounds 3 and 4 showed exceptional reactivity, which required the collection of kinetic data at a lower pH (Supplementary Fig. 2). In general, reaction rates increased with stronger electron-withdrawing substituents, but dramatically decreased with an additional α-substituent, presumably due to steric hindrance. Kinetic data were also in agreement with the measured $p_K$ of the conjugate acids of compounds 3–5 (Supplementary Fig. 3), which strongly suggests that the fraction of Wittig reagent in neutral form is the key factor that controls the reaction rates. The high efficiency of compounds 3–5 was further evident from good isolated yields on scale up and the rapid conversion to their cysteine adducts as monitored by $^{31}$P NMR spectroscopy (Supplementary Fig. 5).

**Preparation and evaluation of Wittig–alkyne reagents in chemical models.** With the success in sulfenic acid trapping using Wittig reagents 3–5, we synthesized the corresponding derivatives with a ‘clickable’ alkyne handle, termed Wittig–alkyne or ‘WYne’ probes (Fig. 2a). WYneC (8) was prepared via alkylation of ketone 3, whereas esterification or amidation of bromoacetyl bromide followed by treatment with triphenylphosphine furnished WYneO (9) or WYneN (10), respectively. All the WYne probes (8–10) were stable in dry, powdered form, and compatible with aqueous media under open air during a typical period of analysis, although amide derivative WYneN (10) was liable under basic conditions and therefore used as a TPP salt (Fig. 2b). WYne probes exhibited a robust reactivity with the sulfenic acid dipeptide model, analogous to the parent Wittig reagents 3–5 (Fig. 2a–d and Supplementary Fig. 6). WYneC exhibited a 10-fold increase in kinetics compared with that of a benzo[c][1,2]thiazine-based sulfenic acid probe (BTD$^+$) and was roughly 1,500-times faster than DYN–2$^+$, an early reagent based on the 1,3-cyclohexanedione scaffold (Fig. 2d and Supplementary Fig. 1c,d). WYneO was nearly as reactive as WYneC, but WYneN exhibited a slower kinetics due to a decrease in nucleophilicity imparted by the amide substituent (Fig. 2a,d). Overall, WYne probes successfully reacted with the dipeptide sulfenic acid model to give the expected S-adduct with rate constants that ranged from 160 to 15,000 M$^{-1}$s$^{-1}$ at physiological pH.

Next, we interrogated the selectivity of WYne probes 8 and 10 against related redox cysteine modifications. WYne probes did not exhibit cross-reactivity with glutathione (GSH), glutathione disulfide (GSSG), S-nitrosothiol (GSNO), cysteine sulfonic acid or glutathione sulfonic acid (Fig. 2d and Supplementary Fig. 7). WYneC underwent a slight, but measurable, reaction with chemically activated disulfides, such as Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB)) or 4,4’-dipyridyl disulfide (4-DPS) (Fig. 2d and Supplementary Fig. 8). However, these polarized disulfides do not naturally occur in proteins and reacted with WYneC at notably decreased reaction rates (~100,000-fold) as compared with that of sulfenic acid. We also investigated the labelling reaction in the presence of electrophilic metabolites. Various aldehydes and other representative electrophiles were surveyed for potential cross-reactions and found not to interfere with the labelling reaction (Supplementary Fig. 9). Lastly, we evaluated the stability of the S-adduct formed between Wittig reagents and the dipeptide (Supplementary Figs. 10 and 11). The S-adducts of Wittig reagents 3–5 or probes 8–10 were generally stable in neutral and acidic buffers, but the WYneN S-adduct was sensitive to basic conditions, which resulted in a loss of the TPP group. The S-adduct of WYneC was stable under millimolar concentrations of reducing
agent, whereas the WYneO and WYneN adducts were labile; therefore, strong reducing environments should be avoided when using these two probes. The S-adducts, although presented as precursors for new Wittig reagents, do not further react with the dipeptide–SOH model due to steric hindrance. Collectively, these data indicate that WYne probes react rapidly and selectively with sulfinic acids under aqueous conditions at neutral pH, which bodes well for the utility of these reagents in more complex systems.

**Profiling WYne reactivity in more complex biological settings.** Bolstered by our success in the chemical models, we moved on to targets with a greater biological relevance and complexity. To this end, WYne probes and various sulfur species in a pH 7.4 aqueous–organic buffer. Owing to the rapid kinetics, the reaction between WYneC and dipeptide–SOH (RSOH) was performed at pH 4.9 and the rate constant extrapolated to pH 7.4 (Supplementary Methods). Reactivity with other biological sulfur species was not observed. Data are represented as best-fit values ± standard errors (n = 10 time points).

**Fig. 2 | WYne-probe reactivity with sulfinic acid in complex biological settings.**

- **a.** Physical properties and kinetic profiles of the WYne probes. **b.** Base-promoted hydrolysis facilitated cleavage of the TPP group from WYne probes. **c.** A small-molecule sulfinic acid model (dipeptide–SOH) was used to kinetically evaluate WYne probes. **d.** Reaction of WYne probes and various sulfur species in a pH 7.4 aqueous–organic buffer. Owing to the rapid kinetics, the reaction between WYneC and dipeptide–SOH (RSOH) was performed at pH 4.9 and the rate constant extrapolated to pH 7.4 (Supplementary Methods). Reactivity with other biological sulfur species was not observed. Data are represented as best-fit values ± standard errors (n = 10 time points).
but reduced Gpx3 (Gpx3-SH), Gpx3 C36 sulfinic acid (Gpx3-SO2H) formed the anticipated adduct with WYne probes in a high yield, analysis demonstrated that Gpx3 C36 sulfenic acid (Gpx3-SOH) WYne adducts by MS, we conjugated the alkyne tags to tetrameth-
ized the resulting products by in-gel fluorescence (Fig. 2g). Signal was detected in WYne-treated Gpx3-SOH but not in Gpx3-SH or Gpx3-SO2H, consistent with the findings in our intact MS analy-
end, we examined WYne-probe reactivity in C64,82S glutathione peroxidase 3 (Gpx3; Fig. 2e), a well-validated model for the study of protein sulfenic acid reactivity [14,15]. Intact mass spectrometry (MS) analysis demonstrated that Gpx3 C36 sulfenic acid (Gpx3-SOH) formed the anticipated adduct with WYne probes in a high yield, but reduced Gpx3 (Gpx3-SH), Gpx3 C36 sulfonic acid (Gpx3-SO2H) and a Gpx3 variant with an intramolecular disulfide bond (Gpx3 C64S-SS) were not modified (Fig. 2f). Having verified the protein–WYne adducts derived from WYne probes. The intact (#) and TPP-cleaved (*) adducts derived from each probe are shown at the same scale. Fig. 3 | Profiling S-sulfenylation dynamics with WYneN in cells. a, Quantitative proteomics provides unequivocal identifications of SOH probe-labelling sites in the A549 proteome. b, Extracted ion chromatograms (XICs) of the isotopically labelled peptide (VPTANVSVDLCR derived from GAPDH) adducts derived from WYne probes. The intact (#) and TPP-cleaved (*) adducts derived from each probe are shown at the same scale. c, Bar chart showing the number of sulfenylated sites profiled by using different SOH-specific probes. d, Plausible mechanism for the formation of TPP-cleaved peptide adducts derived from WYne probes. e, Venn diagram showing the overlap of sulfenylated sites mapped by using WYneN with different doses for in situ labelling. f, Pie charts showing the percentage of functionally annotated cysteine sites from different WYneN-dosing groups. g, Quantitative chemoproteomic profiling of dynamic S-sulfenylation changes in A549 cells on H2O2 treatment. h, Rank order of the determined R2(m) values of WYneN-labelled sites (grey dots) with heavy (H) or light (L) tags from A549 cells treated with or without H2O2, respectively. Sites within CXXC motifs are highlighted in green. i, Representative XICs showing the changes in WYneN-labelled cysteines from TRX1, GLRX1, PDI1 and ATOX1, with the profiles for light and heavy-labelled peptides in red and blue, respectively. j–k, Reduced CXXC motif-containing proteins (10 µM) were treated with WYneN (1mM), followed by H2O2 (15µM) or TCEP (1mM) at r.t. for 1h. Successful labelling was validated by in-gel fluorescence after conjugation with TAMRA-N3 or by intact protein MS with a shift of +95 Da (k), which indicates the loss of TPP. LC, liquid chromatography; mod, modification; RT, run time. Finally, we assessed the ability of WYneN and WYneC probes to enter live cells and directly label endogenous targeted proteins (Fig. 2h). Protein labelling was time and dose dependent; moreover, the high efficiency of WYne probes afforded the opportunity to reduce the concentration applied to cells (≥10-fold) compared to that traditionally required for analysis with BTD, DYn-2 and earlier reagents (≤500 µM versus 5 mM) with a minimal cytotoxic effect (Supplementary Fig. 12). Together, these feasibility studies indicate that the reaction between functionalized Wittig reagents and the electrophilic sulfur in sulfenic acid represents a viable strategy to label proteins in the test tube and cells. Examining WYne-probe reactivity and selectivity through chemoproteomics. Encouraged by the robust reactivity and selectivity...
in sulfenic acid dipeptide and protein models, alongside the cell permeability and lack of cytotoxic effects, we moved forward with a more rigorous evaluation of WYne probes in our well-established chemoproteomics platform\textsuperscript{13,20,21} for site-specific mapping of the S-sulfenylome (Fig. 3a). In brief, native lysates prepared from A549 cells were labelled in separate reactions with WYne probes 8–10. After trypsin digestion, the resulting probe-labelled peptides were conjugated to light or heavy ultraviolet-cleavable azido-biotin reagents (Az-UV-biotin) (1:1) via CuAAC. Light and heavy biotinylated peptides were mixed equally, captured on streptavidin beads and photoreleased for MS–shotgun proteomics for identification and quantification. Probe-labelled peptides covalently conjugated to light or heavy tags yield an isotopic signature in which only peptide assignments with a light/heavy ratio close to 1.0 are recognized as true identifications. Surprisingly, our initial database search that targeted intact modifications derived from WYne probes yielded only 474, 147 and 0 probe-labelled sites for WYneC, WYneO and WYneN, respectively (Fig. 3b and Supplementary Table 1), whereas many unassigned MS1 peaks with the isotopic signature ($R_{1,0} \approx 1.0$) were identified in the raw data. Hence, we conducted a blind search as previously described\textsuperscript{22}, which revealed that all three WYne probes underwent a loss of the TPP moiety, probably caused by base-promoted decomposition during tryptic digestion (Fig. 3d). Interestingly, TPP-cleaved WYneC and WYneO appeared to undergo further oxidation, but the exact mechanism is not clear. The cleaved products exhibited different patterns of MS/MS fragmentation compared with the corresponding ones derived from intact modification, and the intensity of the former was dramatically higher than that of the latter (Fig. 3b and Supplementary Fig. 13). Fortuitously, WYneN-derived cysteine modifications were exclusively transformed into the TPP-cleaved products during the proteomic workflow, which provides a much higher yield of identified sulfenic acid sites (Supplementary Fig. 14) compared with those obtained with WYneC or WYneO as well as two previously reported probes, DYN-2 and BTD\textsuperscript{13,15} (Fig. 3c). Accordingly, we focused subsequent proteomic validation studies on WYneN.

To benchmark the reaction with S-sulfenylome in situ, intact A549 cells were labelled with WYneN and processed with the aforementioned chemoproteomic workflow. The BTD probe was used as control, as it previously provided the largest S-sulfenylome dataset to date\textsuperscript{13,21}. Under identical conditions (500 μM, 37°C, 2 h), WYneN enabled the identification of 2,063 S-sulfenylated sites in A549 cells growing under steady-state redox conditions (that is, no addition of exogenous oxidant), and so drastically outperformed BTD, which yielded only 126 sites (Fig. 3e and Supplementary Table 2). Remarkably, when applied at only 5 μM, 59 S-sulfenylated sites could still be mapped onto 54 proteins using WYneN (Fig. 3f and Supplementary Table 2). Although a dose-dependent increase in identified sulfenic acid sites was not unexpected, we observed that lower concentrations of WYneN correlated with a higher percentage of functionally important cysteine residues (for example, active site, disulfide and metal binding) mapped across sites (Fig. 3f).

Table 1 | Surveying sulfoxides as caged precursors to sulfenic acids and the Wittig reagent trap

| BDP | HOCl | R1 | R2 | R3 |
| --- | --- | --- | --- | --- |
| | | | | |
| BDP | N | N | H | O | S |
| 12–14 | 12, 10 min reaction |
| BDP | N | N | H | O |
| | | | | |
| BDP | N | N | H | O |
| 13, 1 h reaction |
| BDP | N | N | H | O |
| | | | | |
| BDP | N | N | H | O |
| 14, 6 h reaction |
| BDP | N | N | H | O |
| | | | | |
| BDP | N | N | H | O |
| 15, 9% from 12 |
| | | | | |
| BDP | N | N | H | O |
| 16, 43% from 13 |
| | | | | |
| BDP | N | N | H | O |
| 17, 94% from 14 |

BDP = 3-5

Table 1 | Surveying sulfoxides as caged precursors to sulfenic acids and the Wittig reagent trap

| R1 | R2 | R3 |
| --- | --- | --- |
| CH3 | CO2Et | PPh3 |
| 15 |
| | 6% from 12 |
| | 28% from 13 |
| | 95% from 14 |
| | <1% from 12 |
| | 15% from 13 |
| | 17% from 14 |
not perturb the level of reduced protein cysteines in cells and no observable or statistically significant changes in thiol-labelling could be identified (Supplementary Fig. 15c–e). In particular, the reduced form of several hyperreactive redox sites, such as GAPDH C152, PRDX6 C47 and GSTO1 C32, showed little response to the WYneN probe (Supplementary Fig. 15f). Additionally, S-sulfenylome coverage of DYN-2 and BTD1,15 showed a high overlap with WYneN (Supplementary Fig. 15g). These quantitative global proteomic data, together with rigorous studies in small molecules and recombinant protein, demonstrate that WYneN ligates sulfenic acid with a high selectivity and improved performance under a variety of biocompatible conditions.

**Profiling S-sulfenylation dynamics with WYneN in cells.** With validated WYneN probe in hand, we next applied this chemical tool to ratiometrically quantify dynamic changes in S-sulfenylated protein in situ after perturbation with H$_2$O$_2$. Specifically, A549 cells were treated with vehicle or with H$_2$O$_2$, and then treated with WYneN (Fig. 3g). Probe-tagged proteomes with and without oxidant treatment were digested into tryptic peptides, conjugated with light and heavy Az-UV-biotin reagents, respectively, and processed as described above. In this workflow, the light-to-heavy ratio calculated for each WYneN-labelled cysteine residue provided a measure of its relative level in H$_2$O$_2$-treated samples versus unoxidized control samples. In total, we identified and quantified 2,234 probe-modified sites on 1,633 proteins, which included numerous functionally important cysteine residues (Supplementary Fig. 16a and Supplementary Table 3). Of these, 9.9% quantified sites showed ≥1.5-fold dynamic changes after H$_2$O$_2$ treatment. Positively regulated S-sulfenyl lysine residues probably indicate protein-stabilized sulfenic acids, whereas negatively regulated S-sulfenyl lysine residues suggest overoxidation. For example, S-sulfenyl modification of the active sites of PRDX6 (C47) and ASA1H (C43) decreased on H$_2$O$_2$ treatment, indicative of hyperoxidation to sulfenic and sulfonic acid at the active site (Supplementary Table 16b). Moreover, S-sulfenyl modification of the surface-exposed cysteine residue C152 in GAPDH was negatively regulated at a ratio of 0.88; in contrast, the buried cysteine C247 showed a 2.5-fold sulfenyl modification under H$_2$O$_2$-induced stress (Supplementary Fig. 16b). Likewise, three cysteine residues, C90, C152 and C220, of UCHL1 (ubiquitin carboxy-terminal hydrolase L1) were all identified as S-sulfenylated residues, and the most-buried, non-catalytic C220 showed a 2.6-fold increase in modification under stress (Supplementary Fig. 16b). For UCHL1, this residue was previously found to promote the assembly of mTOR complex 2 and phosphorylation of the pro-survival kinase Akt, and is a known S-nitrosylation site as well as a potential alkylation site.22–25 These findings reinforce the concept of dynamic protein S-sulfenylated cysteine residues and offer hypotheses to explain how non-catalytic cysteines may affect enzymatic function via redox regulation.

On a deeper analysis of these data, we noted that WYneN detected a sulfenic acid modification of the key nucleophilic cysteine residue C152 of the 60kDa heat shock protein (HSP60), one of the top redox-modulated targets (Supplementary Table 3), was labelled in vitro by DYN-2, WYneN and WYneN after oxidation with H$_2$O$_2$. Covalent modification on HSP60 by WYneN inhibited its chaperone activity, probably due to the proximity of C442 with the ATP binding site (Supplementary Fig. 17c–e). These findings reveal the CXXC motif in thiol oxidoreductases as a heretofore unrecognized direct targets of oxidation.

**Proteome-wide analysis of cysteine sulfenic acid site stoichiometry.** Although methods have been developed for the relative quantification of sulfenic acid post-translational modifications, it has not been possible to quantify stoichiometry (also referred to as site occupancy) at a global level in cells. Defining the fraction of proteins that contain sulfenic acid at a given site is an essential step towards understanding the mechanistic implications of cysteine oxidation in regulating protein function and targeted drug pharmacology. In the course of our chemoproteomic experiments with WYneN, we recognized that the TPP-cleaved sulfenic acid modification was identical to that resulting from the reaction of a thiol and the alkyne-tagged iodoacetamide probe (IPM) (Fig. 1d). On the basis of this observation, we reasoned that these two probes—WYneN and IPM—could generate chemically identical sites and/or peptides, but that differ in the isotopic mass of their label depending on the original redox state of the cysteine residue. To test this possibility, we synthesized the heavy isotopomer, 13C$_5$ WYneN, and elaborated a chemoproteomic workflow to quantify site-specific S-sulfenyl modification (Fig. 4a). First, sulfenic acids were labelled in situ by incubating cells with 13C$_5$ WYneN. Next, native lysates were generated from the 13C$_5$ WYneN-treated cells and reacted with the thiol-reactive probe, IPM. The isotopically labelled samples were then processed and analysed using the aforementioned chemoproteomic workflow. Site-specific sulfenic acid stoichiometry (%SOH) was calculated using the equation 100/([I(V)/HCSXGXGR][S/T]/G motif in their active site (Fig. 4c). The invariant cysteine is essential for catalysis and can be negatively regulated by oxidation29. Such active sites in the majority of PTPs are identified using targeted MS and quantified, which enabled quantification of the %SOH values on 6,623 unique cysteine residues on 3,372 proteins (Supplementary Table 4). Consistent with the often-transient nature of sulfenic acid in cells, the %SOH values for the majority of the cysteine (73%) were calculated to be lower than 30%, with an average of 21.1% and a median of 14.5% (Fig. 4b). We also found that multiple cysteines on the same protein had considerably different %SOH values. For example, five sulfenylated cysteines were mapped onto NDUFS1, a core subunit of the mitochondrial complex I, with %SOH values that ranged from 13.4 to 74.1% (Fig. 4b), among which were three metal-binding sites (C64, C78 and C92). In another case, the %SOH value of C152 on GAPDH was found to be much higher than that of C247 (Fig. 4b), in accordance with our previous finding based on the spectral counting of sulfenylated peptides that bear these two sites.31 Also of interest, we measured the %SOH values for many protein tyrosine phosphatases (PTPs), which contain a conserved [I/V]HCSXGXGR[S/T/G motif in their active site (Fig. 4c). The invariant cysteine is essential for catalysis and can be negatively regulated by oxidation.29 Such active sites in the majority of PTPs showed a higher %SOH value than the median value for the overall sites (Fig. 4b,c). For example, we detected a 20.0% S-sulfenylation of PTPNI C215 (also known as PTP1B), a value remarkably similar to the percentage reversible oxidation determined using targeted MS approaches.31 In yet another case, we noted that the active site of 1-Cys peroxiredoxin PRDX6 (C47) showed a relatively high %SOH value (36.5%) compared with those of the peroxidatic or resolving cysteines of 2-Cys peroxiredoxins (Fig. 4d). This finding is logical given that PRDX6 is less prone to disulfide bond formation compared with 2-Cys peroxiredoxins.

To investigate the relationship between %SOH and functional activity, we retrieved information about cysteine residues with anno-
tated functions from the UniProt knowledge database. In general, modified cysteines (mainly through S-nitrosylation) tended to be less S-sulfenylated than other annotated or unannotated cysteines (Fig. 4c), as different types of cysteine modifications compete for the same site, and thereby diminish the %SOH. By contrast, active-site cysteines were distributed more broadly in the range of 30–60% S-sulfenylation, compared with those that have other functional annotations or are without annotation (Fig. 4c). In addition to PTPs, other classes of enzymes in which the active-site cysteine is highly prone to oxidation and known to be redox regulated, such as ubiquitinating and deubiquitinating enzymes, were identified (Supplementary Table 4). We also examined the cellular localization and Gene Ontology classification of the proteins with cysteines within the different ranges of %SOH (Fig. 4f,g). Major oxidant-generating cellular compartments, which include the peroxisome, endoplasmic reticulum and mitochondrion, were distinguished as having more highly S-sulfenylated proteins (%SOH ≥ 60) as compared with those of other compartments (Fig. 4f). Likewise, protein cysteines involved in two key oxidative pathways, OP and FA, exhibited a considerably higher overall %SOH relative to those of other compartments (Fig. 4f).

Imaging redox-dependent changes in mitochondrial cysteine oxidation. Organelles play a critical role in cellular function, and...
the detection of location-specific proteins with organelle-selective reagents is an area of intense interest. Mitochondria are unique among cytoplasmic organelles and structurally distinguished by their convoluted double membranes. The inner mitochondrial membrane carries a negative mitochondrial membrane potential (∆Ψm) in the range of 140–180 mV, which allows cations to accumulate in the matrix by a factor of ∼1,000 (ref. 34). Small-molecule lipophilic cations, which exploit the change in membrane potential, are heavily utilized as a vector for cargo delivery into mitochondria, and TPP is one of the most prominent examples (Fig. 1e)35.

Reminiscent of the TPP-targeting moiety, WYneN (pKₐ > 10) is predominantly protonated at physiological pH. Despite its lower abundance, the deprotonated form of WYneN remains highly reactive with sulfenic acid. Given the pH gradient of approximately one unit between the mitochondrial matrix (pH = 8) and the cytosol (pH = 7) (ref. 36), we hypothesized that WYneN would partition to

Fig. 5 | Redox-triggered in situ TPP generation for mitochondrial cargo delivery. a. General principle of a redox-triggered mitochondrial targeting system. b, MPO-mediated activation of the redox-caged sulfenic acids followed by Wittig reagent trapping. Compound 13 or 14 (5 µM) was incubated with MPO (0.25 mg ml⁻¹), pretreated with MPO substrate H₂O₂ (2 mM) for 2 h at 37 °C (b) and then trapped by Wittig reagent 4 (100 µM) for the indicated time. See Table 1 for the structure of BDP. c–e, LPS-activated RAW 264.7 cells promote oxidative activation and trapping of redox-caged sulfenic acid 13. Cells were stimulated with LPS (1 µg ml⁻¹), 24 h) or vehicle, then incubated with compound 13 (0.2 µM), Wittig reagent 4 (5 µM), MPO substrate H₂O₂ (1 mM) and MitoTracker Deep Red (50 nM) in DPBS buffer for 1 h. d, Scale bars, 20 µm; magnified area (right), 20 × 20 µm. Data in e are presented as mean values ± s.d. (n = 5 cells). P values calculated using a two-tailed t-test. **P < 0.0001. f, Redox-caged sulfenic acid 19 (50 µM) was oxidized by HOCl (50 µM, 30 min) and reacted with Wittig reagent 4 (500 µM, 2 h) to afford the mitochondria-targeting compound WittigQ (20), which was actively reduced by complex II to the quinol form. g, WittigQ prevented lipid peroxidation in mitochondria isolated from HeLa cells at a comparable efficacy to that of MitoQ (see Supplementary Methods for detailed procedures). EC₅₀, half-maximum effective concentration.
the mitochondria, become more deprotonated and preferentially label protein sulfenic acids in this compartment (Extended Data Fig. 2a). To test this idea, we functionalized WYneN with a BODIPY tag for fluorescence visualization, but live-cell imaging suggested a poor localization of fluorescence. This finding is consistent with the lack of compartmental bias observed in our chemoproteomic studies and can be explained by the ionic nature of WYneN, which decreases its inherent ability to penetrate phospholipid membranes. To tackle this issue, we synthesized a WYneN derivative with a ten-carbon aliphatic linker, WYneN10 (11; Extended Data Fig. 2b). Satisfyingly, BODIPY-tagged WYneN10 (BDP-WYneN10) exhibited a robust colocalization with the commercial mitochondria indicator, MitoTracker Deep Red, in live A549, HeLa, RKO and NIH3T3 cells, which indicates probe accumulation in this organelle (Extended Data Fig. 2c and Supplementary Fig. 18). In addition, mitochondrial uncouplers, such as FCCP (carboxen cyamide 4-((trifluoromethoxy)phenylhydrazone) and antimycin A dissipated the mitochondrial membrane potential and suppressed the mitochondrial staining pattern (Supplementary Fig. 19). Next, we tested if BDP-WYneN10 could detect redox-dependent changes in mitochondrial cysteine oxidation—using sulfenic acid content as the indicator—under conditions of exogenous oxidative stress. Live A549 cells were imaged after treatment of BDP-WYneN10 and H2O2. Indeed, mitochondrial BODIPY fluorescence intensity increased concomitantly with the oxidant concentration (Extended Data Fig. 2d,e and Supplementary Fig. 20), which can be attributed to increased sulfenic acid modification of the mitochondrial proteins concomitant with the covalent reaction of BDP-WYneN10. To further characterize the effect of WYne probes on the mitochondria function, we performed a mitochondrial stress test and monitored the oxygen consumption rate of A549 cells. Of all the WYne probes, only lipophilic, cationic WYneN10 disrupted the mitochondrial respiration with respect to spare respiratory capacity; maximal respiration and ATP production (Extended Data Fig. 2f and Supplementary Fig. 21). Overall, these data indicate the elevated pK of inherent to the amide-functionalized lipophilic Wittig reagent WYneN10 and its reactivity with sulfenic acid can be exploited to visualize changes in mitochondrial cysteine oxidation.

Redox-triggered in situ TPP generation for mitochondrial delivery. To further showcase the biocompatible, chemoselective reaction between sulfenic acids and Wittig reagents, we considered that the ligation generates a cationic TPP moiety that could be applied for mitochondrial targeting. Specifically, we envisioned a system which, once triggered, generates sulfenic acid available for ligation with functionalized Wittig reagents to deliver cargo inside mitochondria (Fig. 1c). In contrast to existing mitochondrial targeting strategies that are limited to constitutive, uncontrolled delivery, our approach provides a reaction-based switch to sequester non-cationic substrates for mitochondrial enrichment that can respond to cellular redox changes.

To investigate the feasibility of this concept, we utilized sulfenoxides as caged precursors to small-molecule sulfenic acids (Fig. S5)17,18. We prepared several thioether precursors (12–14) and used hypochlorous acid (HOCl)—an oxidant produced by biological systems as an immune response—to convert them into sulfenoxides. The decaging reaction was hindered by substitutions at the C-1 position and promoted by increased C-2 hydrogen acidity via electron-withdrawing groups (EWGs), and offered us a broad range of completion time, from ten minutes to six hours. Next, we employed Wittig reagents 3–5 to capture the nascent sulfenic acids and furnish TPP derivatives 15–17. Notably, the reaction of 14 with highly nucleophilic Wittig reagents 3 or 4 gave 15 or 16 in excellent yields (Table 1). Live-cell images of HeLa cells indicated that the ester derivative 16 exhibited a higher degree of mitochondrial enrichment (R = 0.80, correlation coefficient with MitoTracker) relative to a disulfide control compound 18 (R = 0.39) and the ketone derivative 15 (R = 0.55), owing to the higher pK of the ester19, which leads to a greater degree of protonation at physiological pH (Supplementary Fig. 22). Enzymatically generated HOCl produced by the myeloperoxidase (MPO) enzyme system, also provided the desired product 16 (Fig. 5b and Supplementary Fig. 23). This concept was also evaluated in situ, where redox-caged sulfenic acid 13 was oxidized by HOCl from supplemental MPO to HeLa cells, or endogenous MPO generated by lipopolysaccharide (LPS) stimulation of RAW 264.7 cells, followed by reaction with Wittig reagent 4 to afford mitochondrial vector 16 (Fig. 5c–e and Supplementary Fig. 24). Live-cell imaging showed an elevated mitochondrial localization compared with that of the control, which lacked the HOCl-generating MPO redox trigger. Taken together, this approach offers flexible choices for redox-triggered in situ TPP functionalization and cargo delivery to mitochondria.

As another demonstration of our mitochondria-targeting strategy, we selected the benzoxquinone core of coenzyme Q10 as a functional cargo. A TPP-substituted derivative of Coenzyme Q10, MitoQ, among many other TPP-linked antioxidants, was developed to mitigate oxidative stress in mitochondria20. A coenzyme Q10 derivative 19 with a redox-caged sulfenic acid moiety was prepared and, when exposed to HOCl, reacted with Wittig reagent 4 to give the TPP-linked compound WittigQ (20, Fig. 5f) in good yields. In live Hela cells, a fluorescent derivative of WittigQ showed mitochondrial localization, which can be dissipated by FCCP (Supplementary Fig. 25). Experiments with isolated mitochondria further showed that WittigQ functions similarly to MitoQ with respect to redox cycling and protection against lipid peroxidation (Fig. 5g and Supplementary Fig. 26).

Discussion
Here, we describe Wittig reagents as next-generation probes for protein sulfenic acids—they feature fast reaction kinetics and a high selectivity in biological systems21. Both the probes and their reaction products are compatible with aqueous media, as well as conditions for downstream protein analyses. Owing to the lipophilic nature of the TPP group, WYne probes also displayed a good cell permeability in live-cell experiments. Additionally, the carbon-based nucleophilic probes benefit from the strong C–S bond (dissociation energy D= 712 kJ mol−1), an advantage over heteroatom-based nucleophiles, such as amines or thiols (N–S bond, D= 425 kJ mol−1) (ref. 41). By harnessing this chemistry, we developed a Wittig reagent-based toolbox that offers excellent kinetics and selectivity for the robust sensing of protein cysteine sulfonylation in native environments and, more broadly, opens the door to chemical functionalization of biological targets in living cells.

To date, site-specific chemoproteomic profiling of protein sulfenic acids in intact cells has been predicated almost exclusively on DYN-2, a 1,3-cyclohexadiene-based probe, which is quite selective, but hampered by slow reaction kinetics (Supplementary Fig. 1c). In this regard, a very large amount of protein materials (30–40 mg) is typically required22, thereby greatly precluding many physiologically relevant applications. Notably, as compared to our previous analysis using DYN-23, tenfold fewer starting materials were needed for our chemoproteomics study with WYneN, which achieves an even higher coverage of the sulfonylome. Strikingly, among ~1,000 newly discovered in situ sulfonylated sites were the ‘attacking’ cysteines within CXXC motifs that are prone to rapid thiol–disulfide exchange, which further demonstrates the excellent kinetics of WYne reagents. Moreover, of interest, our dose-dependent labelling experiment (Fig. 3f) reveals that the reactivity of WYneN towards sulfenic acids positively associates with functional importance (for example, active site, disulfide and metal binding). Indeed, S-sulfonylation, as an electrophilic modification
of the cysteine proteome, can effectively reverse the polarity of the nucleophilic sulfur atom and render it more reactive. Therefore, our reaction-based approach to profiling this electrophilic cysteinome directly and reliably discovered functional, redox-regulated cysteines.

Quantification of %SOH may also be essential to assess the potential of drug resistance for those covalent inhibitors that target free thiols. In addition, stoichiometric quantification of %SOH on a particular cysteine site offers another interesting aspect to identify functional nodes through which endogenous reactive oxygen species exert regulatory functions. Existing proteomic approaches often report percent cysteine modification but rely on differential alkylation–reduction strategies to indirectly assess the total reversible oxidation, as they lump all S-modifications together, in contrast with the direct measurement of distinct oxoforms in the cysteinome. Using $^{13}$C$_2$ WYNEN and IPM, we report here the robust approximation of %SOH in cysteines on a proteome-wide scale in cells. We note that %SOH should not be misconstrued as a ‘snapshot’ of absolute stoichiometry; rather, it is the fraction of sulfenic acid trapped by WYNEN during the time course of probe incubation, which is typically one hour. However, the WYNEN trapping of sulfenic acid is rapid compared with the rate of sulfenic acid formation across the majority of the cysteinome (1–10 M$^{-1}$ s$^{-1}$) (ref. 3), which is largely expected to offset this possible issue. As defined here, %SOH provides vital information to compare the extent of the oxidation side-by-side across the cysteinome. Many cysteine residues that play major regulatory roles in redox signalling are S-sulfenylated at relatively low occupancy, including 2-Cys peroxiredoxins. For example, a recent report demonstrates that a redox switch with a low percent oxidation is, indeed, critical for the physiological roles of Akt$^{45}$, consistent with our observations here. Thus, we reason that, as for other post-translational modifications, such as phosphorylation$^{43}$ and acetylation$^{43}$, cysteine S-sulfenylation may not need to reach a high site occupancy to exert regulatory effects.

Another aspect of this study was to investigate the potential of the Wittig reaction as a source of chemical tools with an enhanced organelle specificity, because many oxidants have subcellular distances of diffusion$^{46}$, and certain organelles (for example, mitochondrion, peroxisome and endoplasmic reticulum) produce more oxidants. Of these, cysteine-mediated redox signalling in the mitochondria is key to physiological processes and pathological damage within most mammalian cells$^{47}$. The WYNEN10 probe, engineered to be cationic and lipophilic, enables us to visualize changes in mitochondrial S-sulfenylation levels concomitant with changes in redox homeostasis. The observation that a relatively high level (millimolar range) of exogenously applied H$_2$O$_2$ was required to elicit a notable response is fully consistent with the floodgate hypothesis$^{48}$, in which overoxidation of peroxiredoxins inactivates these antioxidant enzymes and permits the oxidation of other protein targets.

More broadly, we applied Wittig reagents towards the development of controllable tools for redox biology. Existing methods to deliver target small molecules to the mitochondria require the ‘payload’ to be constitutively linked to a cationic group. From a synthetic standpoint, the cationic moiety can present its own challenges for the preparation of mitochondria-targeted compounds. An oxidant-triggered chemical switch for mitochondrial delivery has many additional advantages, which include a bioresponsive targeting mechanism and less cytotoxicity. By tuning the substitutents on the redox-caged substrate, we achieved a wide range of temporal responses for both acute release and prolonged actions. Beyond a proof-of-concept fluorescent cargo, we implemented an antioxidant-based mitochondrial delivery system to counteract the effect of endogenous HOCl. As HOCl is mainly secreted in MPO-rich immune cells, such as neutrophils or macrophages, it causes inflammatory damage to surrounding healthy cells. Thus, an HOCl-triggered antioxidant may alleviate the mitochondrial oxidative damage caused by inflammation. Along these lines, a potential therapeutic application involves the use of this technology to more precisely treat irritable bowel diseases, such as Crohn’s disease and ulcerative colitis. Such pathologies are characterized by chronic intestinal inflammation, HOCl overproduction and oxidation of the membrane lipids, which culminates in mitochondrial dysfunction$^{48–51}$. This bioresponsible or ‘smart delivery’ system may also serve as a springboard to more selective therapies that target cancer, ageing and degenerative diseases, which are often associated with elevated oxidative stress and may increase the efficacy of established mitochondria-targeting drugs functionalized with antioxidant or genetic payloads.

In summary, our findings demonstrate that Wittig reagents, together with a unique form of electrophilic sulfur found in proteins, constitute a new class of highly selective and biocompatible reactions. Projecting forward, we envision several exciting areas wherein this reaction can be applied that should help address fundamental questions about redox modification in the human cysteinome and, more broadly, the sulfenic acid moiety as a target for covalent drugs and chemical ligation handle for mitochondrial targeting.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41557-021-00767-2.

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Methods
Data were obtained from two biologically independent experiments unless otherwise noted. For statistical tests, exact values of null hypothesis significance (P) are defined in Source Data. A detailed Methods section is provided in the Supplementary Information.

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

Data availability
The MS proteomics data have been deposited at the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository52 with the dataset identifier PXD025630. All other data associated with this study are available in the published article and its Supplementary Information. Source data are provided with this paper.

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Author contributions
Y.S., J.Y. and K.S.C. conceived the project, designed experiments and analysed data. Y.S. synthesized and characterized the compounds. Y.S. and L.F. performed intact MS and quantitative proteomic experiments and data analysis. Y.S. performed the probe validation and cell-based experiments.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to J.Y. or K.S.C.

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**Extended Data Fig. 1 | Surveying Wittig reagent reactivity with sulfenic acid.** Wittig reagents 1-7 were screened for reaction with the dipeptide-SOH model compound. Rate constants were obtained in acetonitrile (ACN):25 mM NaOAc (1:2 v/v) pH = 4.9. Isolation yields, literature (in parentheses, reported in DMSO[29]) and experimental $pK_a$ values (in ACN-H$_2$O, see Supplementary Figs. 3-4) are listed if available.
**Extended Data Fig. 2 | Imaging redox-dependent changes in mitochondrial cysteine oxidation.**

**a.** Amide derivative of Wittig reagents exists predominantly in protonated form, setting stage for enrichment and detecting S-sulfenylation in mitochondria. **b.** Structure of the mitochondrial targeting sulfenic acid probe WYneN10 with enhanced lipophilicity. **c.** Live HeLa cells were incubated with BDP-WYneN10 (500 nM) and MitoTracker™ Deep Red FM (100 nM) in DPBS. After 10 min, confocal images were taken. A scale bar of 20 μm is shown. R, Pearson's correlation coefficient. **d.** BDP-WYneN10 tagged S-sulfenylated proteins with fluorescence inside mitochondria. **e.** BDP-WYneN10 fluorescence responded to external oxidative stress (0-5 mM H₂O₂) in live A549 cells (n = 4 areas from one representative experiment). **f.** WYneN10 disrupted mitochondrial respiration in A549 cells to a greater extent than other WYne probes (50 μM) (n = 12 biological replicates). OCR, oxygen consumption rate. Data in **e-f** are presented as box plots (maximum, 75%, median, 25%, minimum). P values were calculated using a two-tailed t-test. ns, not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
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