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Defining the Cell Surface Cysteinome Using Two-Step Enrichment Proteomics

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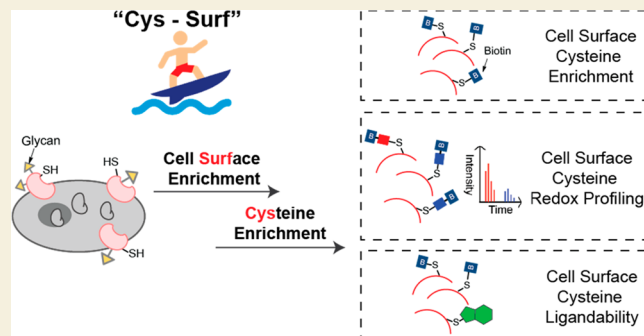
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ABSTRACT: The plasma membrane proteome is a rich resource of functionally important and therapeutically relevant protein targets. Distinguished by high hydrophobicity, heavy glycosylation, disulfide-rich sequences, and low overall abundance, the cell surface proteome remains undersampled in established proteomic pipelines, including our own cysteine chemoproteomics platforms. Here, we paired cell surface glycoprotein capture with cysteine chemoproteomics to establish a two-stage enrichment method that enables chemoproteomic profiling of cell Surface Cysteinome. Our “Cys-Surf” platform captures >2,800 total membrane protein cysteines in 1,046 proteins, including 1,907 residues not previously captured by bulk proteomic analysis. By pairing Cys-Surf with an isotopic chemoproteomic readout, we uncovered 821 total ligandable cysteines, including known and novel sites. Cys-Surf also robustly delineates redox-sensitive cysteines, including cysteines prone to activation-dependent changes to cysteine oxidation state and residues sensitive to addition of exogenous reductants. Exemplifying the capacity of Cys-Surf to delineate functionally important cysteines, we identified a redox sensitive cysteine in the low-density lipoprotein receptor (LDLR) that impacts both the protein localization and uptake of low-density lipoprotein (LDL) particles. Taken together, the Cys-Surf platform, distinguished by its two-stage enrichment paradigm, represents a tailored approach to delineate the functional and therapeutic potential of the plasma membrane cysteinome.

KEYWORDS: Cell surface, Cysteines, Chemoproteomics, Oxidation states, T cell activation



INTRODUCTION

The cell surface proteome regulates nearly all aspects of cellular function, spanning, cell–cell communication,^{1–3} signal transduction,^{4–6} and changes to cell state,⁷ including activation,^{8–10} proliferation,^{11,12} and death.¹³ Consequently, nearly 50% of modern drug targets are found in the membrane proteome, which is also rich in biomarkers indicative of disease.^{14–18} Notably, over 200 cell surface proteins have been reported as overexpressed in human cancers.¹⁹ Despite their prominence as high value therapeutic targets, the hydrophobicity and low abundance of most cell surface proteins complicates functional characterization and therapeutic targeting of this important fraction of the proteome.^{20–23}

A number of high value biochemical and mass spectrometry-based proteomic techniques have been developed to improve capture and identification of the cell surface proteome, most notably cell surface biotinylation^{24–29} and cell surface capture (CSC).^{30–37} As the *N*-hydroxysuccinimidobiotin (NHS-biotin) reagent used in cell surface biotinylation has been shown to label intracellular proteins,^{38,39} CSC has emerged as a favored method for specifically enriching membrane bound glycoproteins. CSC leverages the heavy glycosylation of nearly all membrane proteins—90% of the proteins on the cell surface

have been reported to be glycosylated⁴⁰—to afford high specificity capture of the cell surface proteome. In the CSC method, cis-diols found on all glycans are first oxidized to aldehyde groups in the presence of sodium periodate. Subsequently, these aldehyde moieties are trapped with aminoxy- or hydrazide-biotin reagents, which afford robust biotinylation of cell surface proteins via formation of a stable oxime or hydrozone. Biotinylated protein groups are then identified via established enrichment-based proteomic workflows. Showcasing the widespread utility and adoption of the CSC, recent studies have implemented this chemistry to identify and quantify surfaceome alterations in cancer cell lines,^{18,41–44} such as overexpression of CD166 in head and neck squamous cell carcinoma⁴⁵ and carbonic anhydrase 12 in colon cancer.⁴⁶

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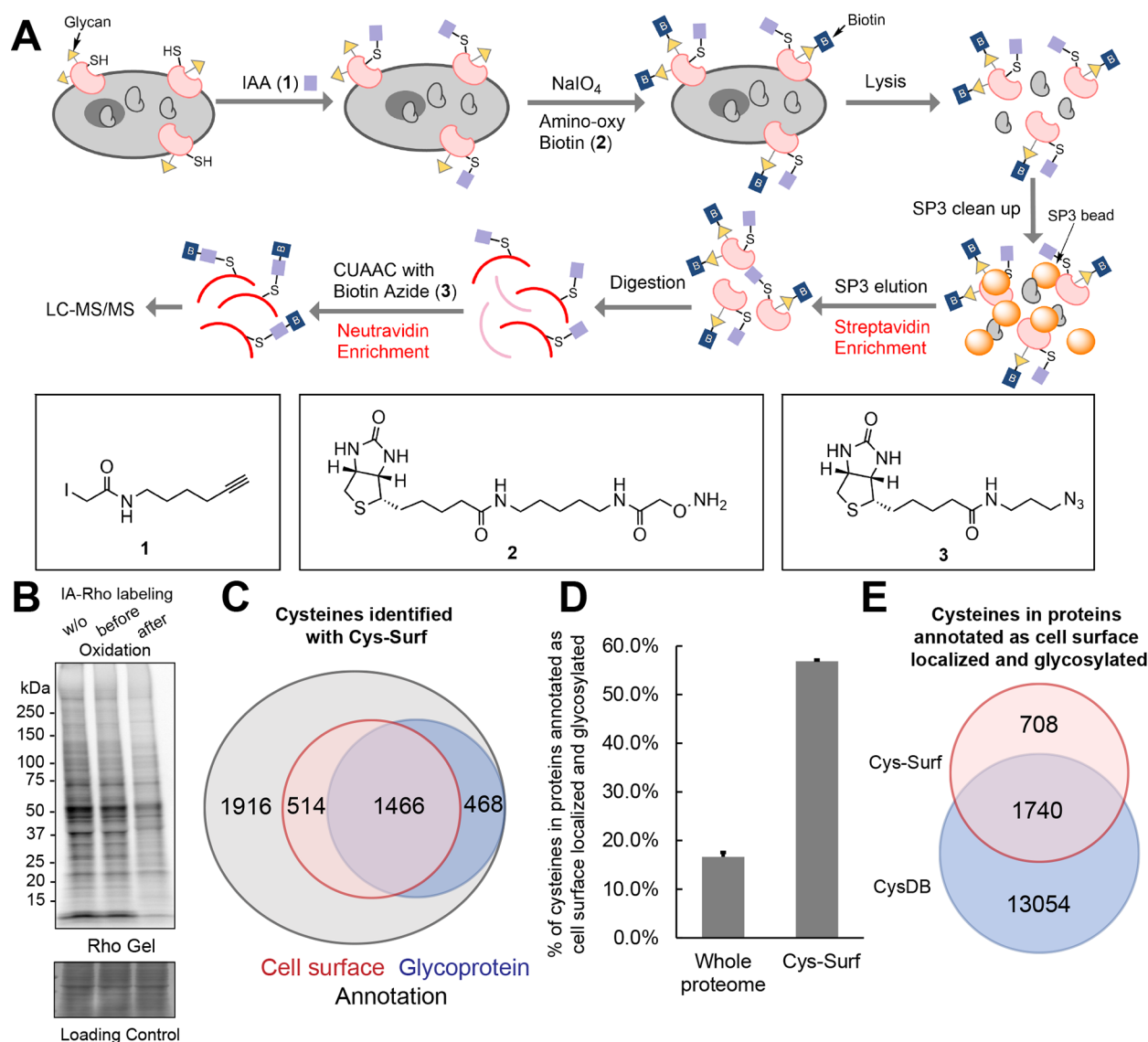


Figure 1. Cell surface cysteine enrichment enabled with cell surface capture and two-step biotinylation. (A) The schematic workflow of Cys-Surf. (B) Iodoacetamide-Rhodamine (IA-Rho) gel of Jurkats cells labeled with $5 \mu\text{M}$ IA-Rho without oxidation or before and after oxidation with NaIO_4 . (C) Venn diagram of cysteines identified with Cys-Surf annotated as on the cell surface or from glycoproteins. (D) Percentage of cysteines in proteins annotated as cell surface localized and glycosylated identified from MS analyses of trypsin digests from whole cells and with Cys-Surf. Data is represented as mean \pm stdev. (E) Venn diagram of cysteines in proteins annotated as cell surface localized and glycosylated identified with Cys-Surf and in CysDB. MS experiments were conducted in 3 replicates in Jurkats cells. All data can be found in Table S1.

Cysteine residues are a ubiquitous, functionally important, and therapeutically relevant feature of cell surface proteins.⁴⁷ Membrane proteins are particularly rich in structural disulfides, and redox-active cysteines are widely known to regulate membrane protein function.^{48–52} Multiple cell surface cysteines are reported to be redox active, including C53 of aquaporin-8 AQP8, persulfidation of which is known to gate H_2O_2 to regulate cell stress,⁵³ and C23, C45, and C106 of high mobility group box 1 protein HMGB1, reduction of which are known to be crucial for anti-inflammatory processes.^{54,55} Activation-induced changes to cysteine oxidation have been linked to enhanced T cell activation,^{56–58} and cysteines on CD4 and GP120 have been implicated in the entry of HIV in T cells and in the regulation of HIV infection of T cells.^{59,60} Cell surface cysteines have also been targeted by drugs and drug-like molecules, as showcased by Afatinib and Ibrutinib, blockbuster anticancer agents, that function by selectively and

irreversibly labeling conserved noncatalytic cysteines found within kinase active sites.^{61,62} Consequently, identifying redox sensitive and potentially druggable cysteine residues in membrane proteins is of high value for expanding the scope of the druggable proteome.

Mass spectrometry-based cysteine chemoproteomics is one such method that is well positioned to pinpoint such residues in membrane proteins. As shown by previous reports,^{63–67} including our own, the human proteome harbors thousands of cysteines amenable to modification by druglike molecules—each cysteine–ligand pair represents the potential starting point for a drug development campaign. Similar to small molecule screening platforms, redox-directed chemoproteomic methods, including biotin-switch,⁶⁸ OxICAT,⁶⁹ SP3-Rox,⁷⁰ QTRP,⁷¹ and Oximouse,⁷² robustly report the relative and absolute oxidation states of cysteines analyzed from bulk proteomes. By pairing these methods with proximity labeling,

recent methods including our own Cys-LOx have begun to shed light on subcellular compartment-specific cysteine oxidation states, which can vary markedly depending on protein localization.^{73,74} Whether analogous approaches can be extended to the cell surface proteome remains to be seen.

Here, we establish cell Surface Cysteine enrichment (Cys-Surf), which pairs cell surface capture (CSC) with cysteine chemoproteomics to reveal the redox and ligandable cell surface cysteinome. Cys-Surf analysis of Jurkat and primary T cells identified 2,836 reactive cell surface cysteines on 1,046 proteins, which represents a >4-fold improvement of cell surface specificity as compared to bulk proteome analysis. Of these residues, 821 were not previously identified by cysteine chemoproteomics as reported by CysDB,⁶³ and we find that 211 proteins are ligandable by cysteine reactive compounds. By pairing Cys-Surf with our previously reported cysteine redox proteomic method SP3-Rox,⁷⁰ we quantify the absolute oxidation states of 1,246 cell surface cysteines on 489 proteins, reveal marked cysteine reduction during T cell activation, and stratify membrane cysteines sensitive to addition of exogenous oxidants and reductants. Guided by this Cys-Surf analysis, we pinpoint a heretofore uncharacterized reduction-sensitive cysteine in an EGF repeat of the low-density lipoprotein receptor (LDLR), which impacts receptor localization and lipoprotein uptake.

RESULTS

Establishing an SP3-Enabled Aminooxy-Biotin Cell Surface Platform

To establish our cell Surface Cysteine enrichment platform (Cys-Surf) (Figure 1A), we envisioned pairing cell surface capture (CSC)^{30,31} with cysteine peptide enrichment. Guided by our prior study, which paired TurboID proximity labeling with similar cysteine peptide enrichment to achieve subcellular redox proteomics,⁷³ we expected that efficient protein recovery throughout the Cys-Surf workflow would be essential to achieve high cysteine coverage.

Therefore, we first prioritized establishing and enhancing the CSC portion of our method. We observed saturable labeling for aminooxy-biotin with maximal labeling achieved with 1 mM treatment conditions (Figure S1A). As we had previously found that the single-pot, solid-phase-enhanced sample preparation (SP3) method⁷⁵ afforded enhanced recovery of biotinylated samples,^{64,76} we opted to incorporate SP3 into the CSC workflow. We established an SP3-based protein cleanup method in which the SP3 resin is employed to decontaminate samples prior to protein enrichment on streptavidin resin (Figure S1B). By comparing wash and protein elution conditions, we found that EtOH outperformed MeCN as the washing solvent and that addition of detergent (e.g., 0.2% SDS, which is not required for peptide elution^{64,77}) is essential for protein recovery from SP3 resin (Figure S1C,D). Addition of SP3 to the CSC method afforded a modest yet significant improvement in the peptide coverage (Figure S1E). As further confirmation that our implementation of CSC was robustly capturing cell surface glycoproteins, we additionally subjected enriched peptides to the PNGase F digest (Figure S2A). We used the unique mass shift (0.98 Da) that remains on glycosylated asparagine residues after digest to identify the glycosylation sites. ~53% of detected peptides feature putative glycosylation sites, and of these, ~93% belong to proteins annotated as localized to the cell surface (Figure S2B).

Establishing Cell Surface Cysteine Enrichment (Cys-Surf)

We next paired our established CSC platform with cysteine labeling to establish our Cys-Surf workflow, shown in Figure 1A. We expected that the sodium periodate used to oxidize glycans in the CSC workflow likely would also oxidize exposed cysteine thiols. Confirming this likelihood, we observed a decrease in cysteine reactivity toward iodoacetamide rhodamine (IA-Rho) following treatment with sodium periodate; in contrast, the IA-Rho signal remained unchanged when sodium periodate was applied after cysteine capping (Figure 1B). To circumvent this oxidation, we opted to first cap all cysteines *in situ* with the pan-cysteine reactive probe iodoacetamide alkyne (IAA; 1).

The subsequent two-stage Cys-Surf capture workflow then proceeded smoothly. Following streptavidin-based CSC and on-resin digestion, cell surface peptides were collected and subjected to peptide-level biotinylation via click conjugation. After a second round of enrichment on neutravidin resin, the biotinylated peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In aggregate, Cys-Surf analysis of Jurkat cells captured a total of 4,364 unique cysteines in 1,889 total proteins. This coverage compares favorably to our previously reported two-stage enrichment platform using TurboID, in which we typically captured 500–1,500 total cysteines found on 300–800 proteins.⁷³

To facilitate evaluation of the cell surface specificity of the Cys-Surf platform, specifically the fraction of captured cysteines that are localized to cell surface proteins, we generated a comprehensive cell surface protein database aggregating the localization information from the Human Protein Atlas,⁷⁸ UniProtKB,⁷⁹ and CellWhere⁸⁰ (Table S1). In aggregate, 99,811 cysteines found in 7,018 proteins were annotated as localized on the cell surface by one or more databases analyzed. Consistent with our previous study in which we performed similar localization analysis for intracellular compartments,⁷³ we observed that a substantial fraction of the proteins with cell surface localization are also annotated as localizing to one or more additional subcellular compartments (4,339 total). As these multilocalized proteins could confound our analyses, we generated an additional data set of glycosylated proteins generated from UniProtKB annotations as orthogonal evaluation of the method, which includes 85,933 cysteines on 5,139 proteins (Table S1). In total, 3,929 proteins are shared between both data sets.

Overall, Cys-Surf identified 1,980 cysteines in 700 proteins with annotated cell surface localization and 1,934 cysteines in 633 proteins annotated as glycoproteins (Figures 1C and S3). ~75% of the glycoprotein cysteines (1,466/1,934) are also annotated as cell surface cysteines (1,466/1,980) (Figure 1C), which is consistent with the CSC enrichment of glycoproteins on the cell surface. Altogether, 56% (2,448/4,364) of cysteines identified with Cys-Surf are annotated either on the cell surface or in glycoproteins. By comparison, only ~15% of cysteines are annotated as on the cell surface in prior cysteinome data sets generated from whole cell proteomes⁶⁴ (Figure 1D). Further showcasing the value of this dual capture method for identifying tough-to-detect residues, when compared with our CysDB database,⁶³ which aggregated a total of 62,888 cysteines in nine high coverage cysteine chemoproteomic data sets, 708 cysteines in proteins annotated as cell surface or glycosylated identified by Cys-Surf have not been previously identified (Figure 1E).

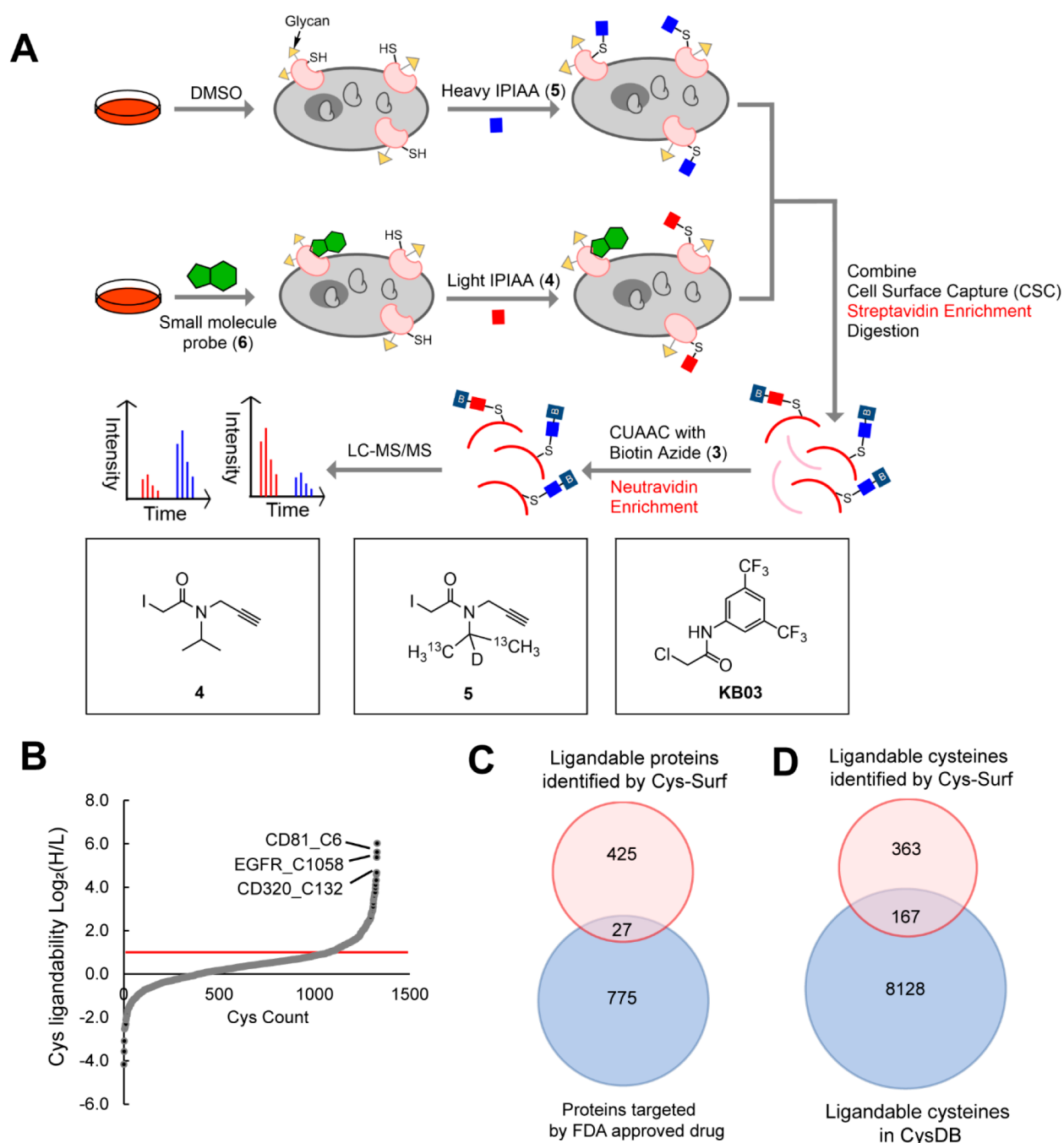


Figure 2. Application of Cys-Surf to identify ligandable cell surface cysteines. (A) The schematic workflow to identify ligandable cell surface cysteines with Cys-Surf. (B) Ligandability of cysteines quantified with Cys-Surf. (C) Ligandable proteins identified by Cys-Surf compared with FDA approved drug targets. (D) Ligandable cysteines identified by Cys-Surf compared to those in CysDB. Compound treatment was 50 μM for 1 h at 37 $^{\circ}\text{C}$. MS experiments were conducted in 3 replicates in Jurkats cells. All MS data can be found in Table S2.

Cys-Surf Captures Ligandable Cell Surface Cysteines

Motivated by the robust capture of cell surface cysteines, we next set out to enable quantitative measurements of subcellular cysteine ligandability by incorporating isotopic labeling into the Cys-Surf workflow. We envisioned that our previously reported isotopically labeled light- and heavy-isopropyl iodoacetamide alkyne (IPIAA-L 4 and IPIAA-H 5), which had enabled high coverage bulk redox proteomics,⁷⁰ would prove compatible with competitive electrophilic small molecule screening. In a manner highly analogous to the widely utilized competitive chemoproteomic profiling platform, isotopic

Tandem Orthogonal Proteolysis–Activity-Based Protein Profiling (isoTOP-ABPP),^{66,81} we treated samples in a pairwise manner with either scout fragment 3,5-bis-(trifluoromethyl)aniline chloroacetamide **KB03** or vehicle (DMSO) followed by either light- or heavy-IPIAA (Figure 2A). After CSC and peptide level cysteine enrichment, FragPipe search and IonQuant⁸² were utilized to calculate the competition ratio (ratio (*R*) of IPIAA-H over IPIAA-L).

Out of the 2,194 cysteines quantified, 530 cysteines belonging to 452 proteins were found in peptides that showed elevated MS1 ratios ($\text{Log}_2(\text{H/L}) > 1$), indicating covalent labeling by **KB03** (Figure 2B). Out of the 530 ligandable

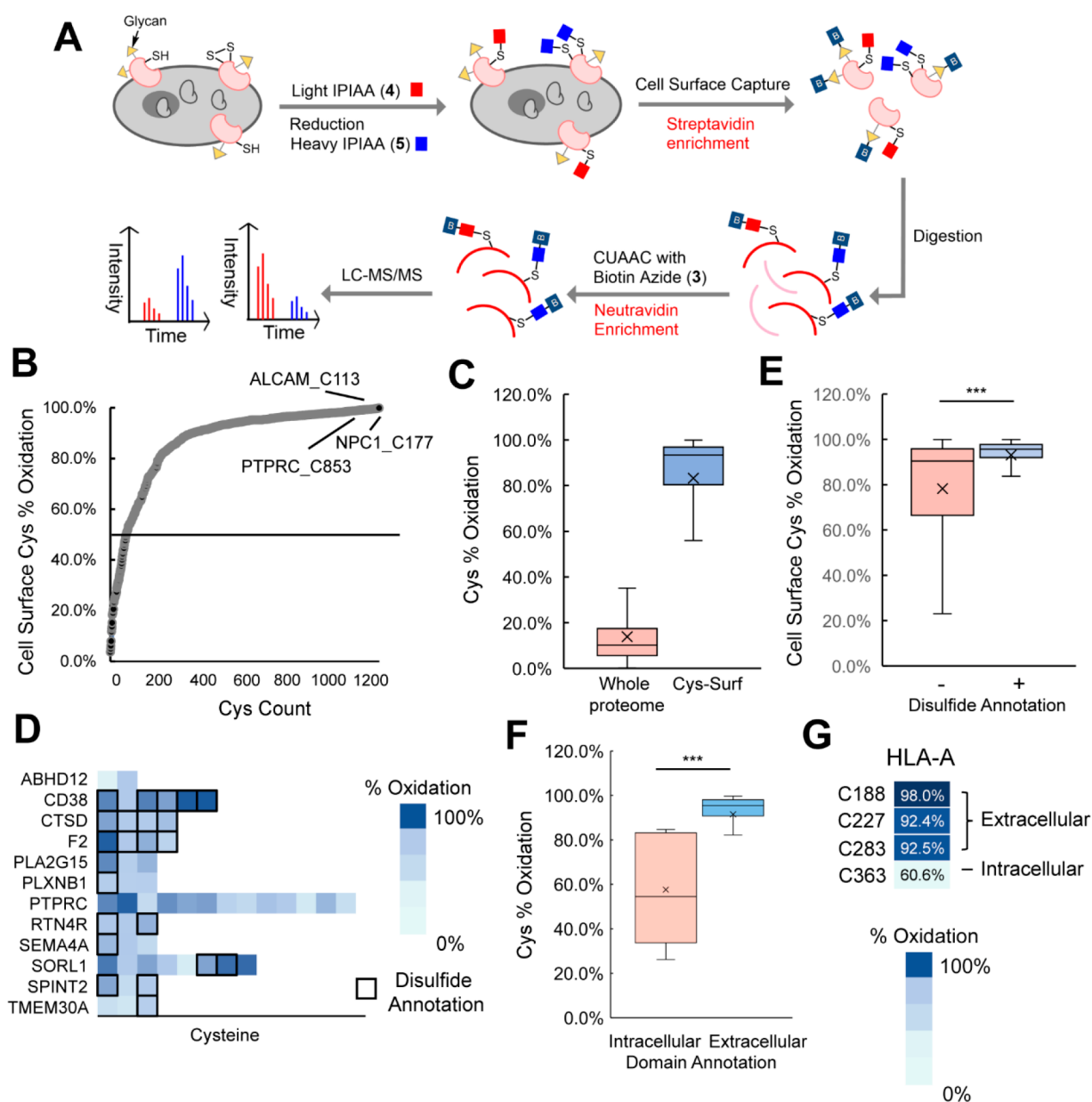


Figure 3. Application of Cys-Surf to quantify oxidation states of cell surface cysteines. (A) The schematic workflow of quantification of oxidation states of cell surface cysteines with Cys-Surf. Reduced cysteines were first labeled using our custom isotopically light isopropyl iodoacetamide alkyne (IPIAA-L) probe. Subsequently, the samples were subjected to reduction and labeled by using our isotopically differentiated heavy (IPIAA-H) probe. After CSC and peptide level cysteine enrichment, Frappipe IonQuant reported the ratio (R) of IPIAA-H over IPIAA-L labeled cysteine peptides, and (B) percentage oxidation of cysteines quantified with Cys-Surf calculated using the formula $(R/(1 + R)) \times 100$ as reported previously.^{70,73} Average percentage oxidation of 4 biological replicates is reported. (C) Percentage oxidation of cysteines quantified with Cys-Surf compared with that quantified with whole proteome. (D) Percentage oxidation of representative cysteines quantified with Cys-Surf. (E) Percentage oxidation of cysteines quantified with Cys-Surf with or without disulfide annotation. (F) Percentage oxidation of cysteines quantified with Cys-Surf with domain analysis of extracellular or intracellular. (G) Percentage oxidation of 4 cysteines in HLA-A quantified with Cys-Surf. For panel E and F, statistical significance was calculated with unpaired student's t tests, $***p < 0.001$. For panels C, E, and F, the mean is represented by "x". MS experiments were conducted in 4 biological replicates in Jurkats cells. All MS data can be found in Table S3.

cysteines, 211 cysteines are annotated as from proteins on the cell surface or glycosylated. Cys-Surf identified unique ligandable cell surface proteins that differ from those already targeted by FDA approved drugs (Figure 2C). More than 350 ligandable cell surface cysteines identified by our study are unique and have not been previously identified in CysDB⁶³ (Figure 3D). While we do not identify C797 in EGFR—this is the cysteine labeled by targeted tyrosine kinase (TKIs) inhibitors (e.g., afatinib)⁶¹—we do observe an elevated ratio

($\text{Log}_2(\text{H}/\text{L}) = 2.74$) for C1058 in EGFR. This cysteine has been previously identified as a palmitoylation site located in a disordered loop, which has been implicated in the palmitoylation-dependent regulation of EGFR activity.^{83,84} Gene ontology (GO) analysis⁸⁵ of proteins that harbor ligandable cysteines demonstrated an enrichment of proteins involved in processes including integrin-mediated signaling pathway, membrane organization, and biosynthesis (Figure S4). We expect that many of these ligandable cysteines,

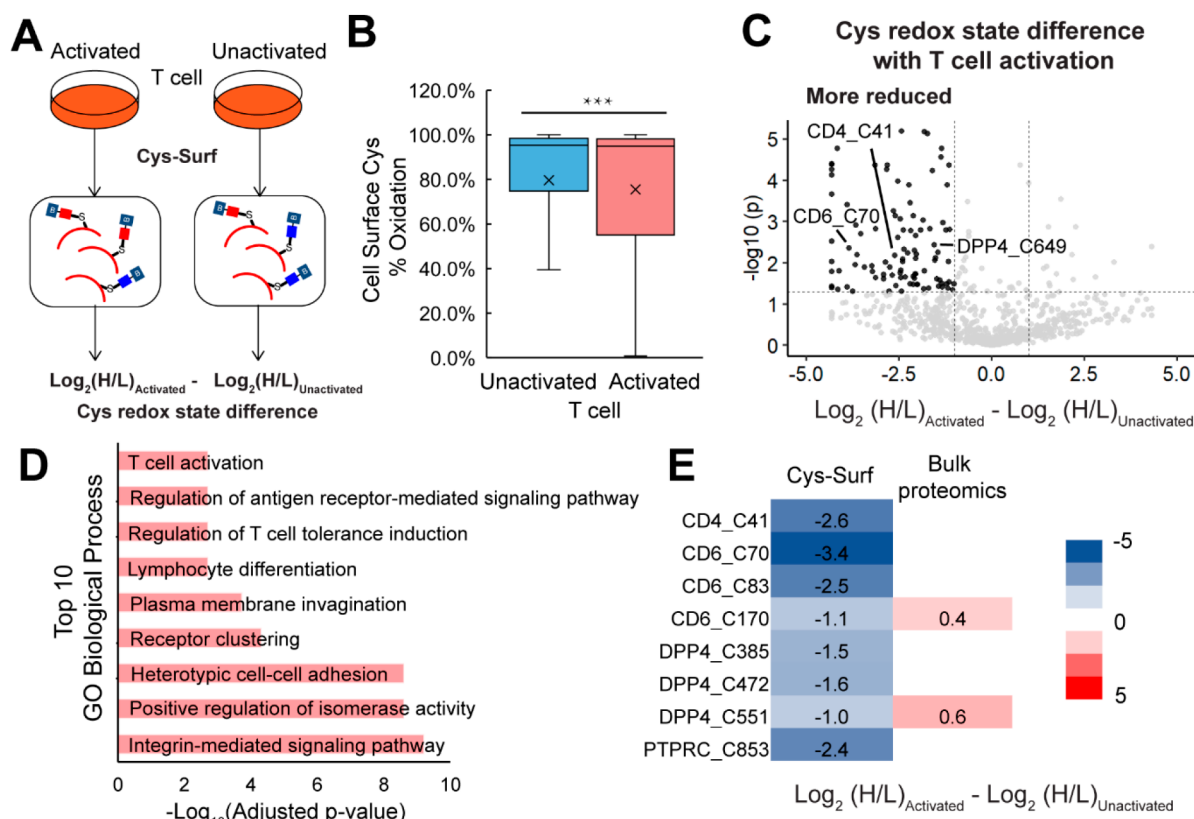


Figure 4. Application of Cys-Surf to quantify redox changes of cell surface cysteines during T cell activation. (A) The schematic workflow to identify redox sensitive cell surface cysteines during T cell activation with Cys-Surf. (B) Redox states of cell surface cysteines quantified with Cys-Surf in naive and activated T cells. The mean is represented by “x”. Statistical significance was calculated with unpaired student’s *t* tests, ****p* < 0.001. (C) Difference of redox states for cysteines quantified with Cys-Surf during T cell activation. (D) Top 10 GO biological processes enriched with proteins harboring reduced cell surface cysteines during T cell activation. (E) Difference in redox states of representative cysteines quantified with Cys-Surf or bulk proteomics in unactivated or activated T cells. MS experiments were conducted in 3 replicates in human T cells. All MS data can be found in Table S4.

including C6 of CD81⁸⁶ and C132 of CD320,⁸⁷ represent intriguing potential targets for immunomodulation.³⁴

Among the integrin-related ligandable cysteines, C363 from HLA-A (HLA class I histocompatibility antigen; A-3 alpha chain) was identified as labeled by KB03 ($\text{Log}_2(\text{H/L}) = 1.26$). Inspection of the AlphaFold^{88,89} structure of HLA-A (AF-P04439-F1) revealed that C363 is located in a putatively unstructured c-terminal portion of the HLA protein (Figure S5A). Given the potential opportunities for targeting MHC complexes for immunomodulatory applications,^{90,91} we opted to further assess the ligandability of this cysteine through a gel-based activity-based protein profiling (ABPP) assay.⁹² We established an immunoprecipitation-based ABPP assay in which cells were first treated in situ for 1 h at 50 μM compound with one of three compounds, chloroacetamide (KB03), acrylamide (KB14), or propynamide (6). After lysis, lysates were labeled with IA-rhodamine and HLA-A protein was enriched by immunoprecipitation, which revealed that 6 labeled HLA-A, as indicated by the near-complete blockade of IA-Rho-labeling; more modest partial competition was observed of KB03 and KB14 (Figure S5B). Taken together, these findings support the idea that Cys-Surf can faithfully identify ligandable cysteines within membrane-associated proteins.

Quantitative MS Analysis of Oxidation States of Cysteines on the Cell Surface with Cys-Surf

As the HLA-A Cys363 cysteine had been previously reported as redox active,^{93,94} we next asked whether Cys-Surf would extend to quantification of site-specific cysteine oxidation. Building upon our previously reported SP3-Rox⁷⁰ and Cys-LOx⁷³ redox proteomics platforms, we modified our Cys-Surf platform to capture the cell surface redox proteome (Figure 3A). Similar to many redox proteomic methods,^{68,69,95,96} we calculated the cysteine percentage oxidation state based on the relative cysteine labeling by our isotopically enriched IPIAA reagents, with IPIAA-L labeling reduced cysteines and IPIAA-H labeling oxidized cysteines following global reduction with DTT. In support of the robustness of our method, we also generated data sets in which the cysteine capping reagents were swapped, with IPIAA-H capping reduced cysteines and IPIAA-L capping the oxidatively modified cysteines after reduction. A high concordance ($R^2 = 0.8942$, $n = 1,538$ unique cysteines identified in both data sets) was revealed for the precursor ion intensity ratios calculated by FragPipe IonQuant⁸² (Figure S6).

Demonstrating the uniquely oxidizing environment of the cell surface cysteinome, 1,173 out of the 1,246 cysteines (89% of cysteines) with cell surface annotations quantified from 489 proteins were found to have elevated ratios ($\text{Log}_2(\text{H/L}) > 1$), corresponding to >50% percent oxidation (Figure 3B). In aggregate, cysteines in cell surface proteins showed an average

oxidation state of 83%; this high degree of overall oxidation is in stark contrast to the 10% mean cysteine oxidation state previously reported for whole cell proteome,^{70,72,81,97–100} which supports Cys-Surf's capacity to assay the subcellular oxidation state of cysteines in the plasma membrane (Figure 3C).

Given the markedly oxidized nature of most cysteines identified, we next asked whether these residues were known to be involved in the formation of disulfide bonds. We find that 495 of the identified cysteines from 192 total proteins were annotated as disulfide sites by UniProtKB. This coverage (40% of residues identified) represents a marked enrichment for cysteines involved in disulfides when compared to bulk proteome analysis—in CysDB, 17% ($n = 1,077$) of identified cysteines are annotated as involved in disulfides. Exemplary cysteines involved in disulfide bonds include Cys177 of NPC intracellular cholesterol transporter 1 (NPC1), Cys296 of cluster of differentiation (CD) 38 antigen, and Cys113 of CD166 antigen ALCAM (Figure 3B,D).

Looking beyond these anecdotal examples, we find that the cysteines with annotated disulfide involvement showed a 93% mean oxidation, which reflects increased oxidation compared with all identified cell surface cysteines and those lacking disulfide annotations (83% and 78% mean oxidation, respectively; Figure 3E). Notably, 453 additional cysteines, which lacked UniProtKB disulfide annotations, were also detected with >90% percent oxidation (Table S3). We expect that a number of these sites are likely cysteines involved in heretofore unannotated disulfides.

Given the predominance of highly oxidized cysteines in our data set, we next asked whether Cys-Surf would have sufficient sensitivity to identify domain specific differences in cysteine oxidation. Using UniProtKB topological domain feature annotations, we identified that our data set contained 175 cysteines located within annotated intracellular protein domains and 589 cysteines in annotated extracellular domains. Consistent with a high degree of oxidation of cysteines in the plasma membrane, we observe mean oxidation states of 92% for extracellular and 58% for intracellular domain cysteines (Figure 3F). For the aforementioned ligandable cysteine Cys363 in HLA-A, which is located in an annotated intracellular topological domain, we observe substantially reduced oxidation of 61% when compared with the three HLA-A extracellular cysteines (Cys188, Cys227, and Cys283; Figure 3G).

Cys-Surf Identifies Adaptive Immune Cell State-Dependent Changes to Cysteine Oxidation

During activation, T cells are exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS), derived from both intracellular mitochondrial ROS production and extracellular neighboring phagocytes. Intriguingly, this oxidative environment has been connected with both T cell hypo-responsiveness and T cell activation, including increased IL2 and NFAT expression.^{101–103} These seemingly antithetical activities of ROS as both pro- and antiproliferative have been ascribed to both dose-dependent activity (low ROS is pro-proliferative and higher ROS-negatively impacting growth) and compartmentalized redox signaling. Intriguingly, while bulk proteome redox analysis revealed increased cysteine oxidation during T cell activation for a number of immunomodulatory targets,^{70,104,105} an increase in cell surface cysteine free thiols is strongly associated with immunological stimuli.^{56,57,106–109}

Inspired by these intriguing observations, we deployed Cys-Surf to pinpoint T cell activation-dependent changes to the plasma membrane-associated cysteine oxidation state. Healthy donor T cells (bulk CD4+ and CD8+) were subjected to the Cys-Surf workflow (Figure 4A) pre- and poststimulation (anti-CD3 and anti-CD28). In aggregate, the oxidation states of 2,402 cysteines on 1,190 proteins were quantified, in which 1,466 cysteines from 638 proteins are annotated as on the cell surface or are glycosylated (Table S4). Consistent with the aforementioned reports of activation-dependent increases in plasma membrane free thiol content, T cell stimulation afforded a modest, yet significant, decrease in the measured mean percentage cysteine oxidation state (Figure 4B). Out of 821 cysteines from 471 proteins quantified in both unstimulated and stimulated T cell proteomes, 100 cysteines were significantly reduced (difference < -1, p -value < 0.5) upon T cell stimulation (Figure 4C). GO analysis⁸⁵ of genes that harbor significantly reduced cysteines revealed an enrichment of proteins involved in processes including regulation of T cell tolerance induction, lymphocyte differentiation, and integrin-mediated signaling, which are all closely related to T cell activation (Figure 4D). Exemplary reduced cell surface cysteines involved in adaptive immune response include a number of disulfide-bonded cysteines (Figures 4E and S7), including C41 from the T cell surface glycoprotein (CD4), which functions as a coreceptor for the MHC class II molecule:peptide complex,^{57,110} C70, C83, and C170 from the T cell differentiation antigen (CD6),^{111,112} and C385, C472, and C551 from the dipeptidyl peptidase 4 (DPP4), which is a known positive regulator of T cell activation.^{113–116} For the receptor-type tyrosine-protein phosphatase C (PTPRC or CD45), which is a DPP4 interactor that also acts as a positive regulator of T cell coactivation, active site C853 that forms a phosphocysteine intermediate¹¹⁷ was markedly reduced. Notably, the disulfide-rich extracellular domains of CD45 have been implicated in increased domain rigidity and formation of heterodimers.^{118,119} For CD4^{120,121} (Cys41) and Tapasin (TAPBP)¹²² (Cys91), the reduced disulfides are potential RHStaple bonds—these are allosteric disulfides that link adjacent strands in the same β sheet,^{123,124} which aligns with the comparatively labile nature of these bonds.^{125,126} Exemplifying the value of subcellular redox measurements, for C551 of DPP4 and C170 of CD3, which were identified by both our previous bulk proteomic⁷⁰ and our cys-Surf analysis of T cell activation, we observe markedly divergent state-dependent changes to thiol oxidation (Figure 4E).

Cys-Surf Identifies Cysteines Sensitive to Exogenous Reductants and Oxidants

Inspired by the seeming ubiquity of labile disulfides, we opted to broaden the scope of redox sensitive cell surface cysteines by pairing Cys-Surf with cell treatments with exogenous oxidoreductants. For oxidants, we chose the exemplary oxidizing agents H₂O₂ and S-Nitrosoglutathione (GSNO), with the goal of pinpointing cell surface cysteines sensitive to ROS and RNS-mediated cysteine oxidative modifications. For reductants, we selected both the pan-cysteine reactive Tris(2-carboxyethyl)phosphine (TCEP) reducing agents as well as N-acetylcysteine (NAC), which is a clinically administered reductant that is also widely taken as a dietary supplement.^{127,128}

We subjected Jurkat cells to in-gel fluorescence analysis using the cell-impermeable cysteine-reactive Alexa Fluor 594

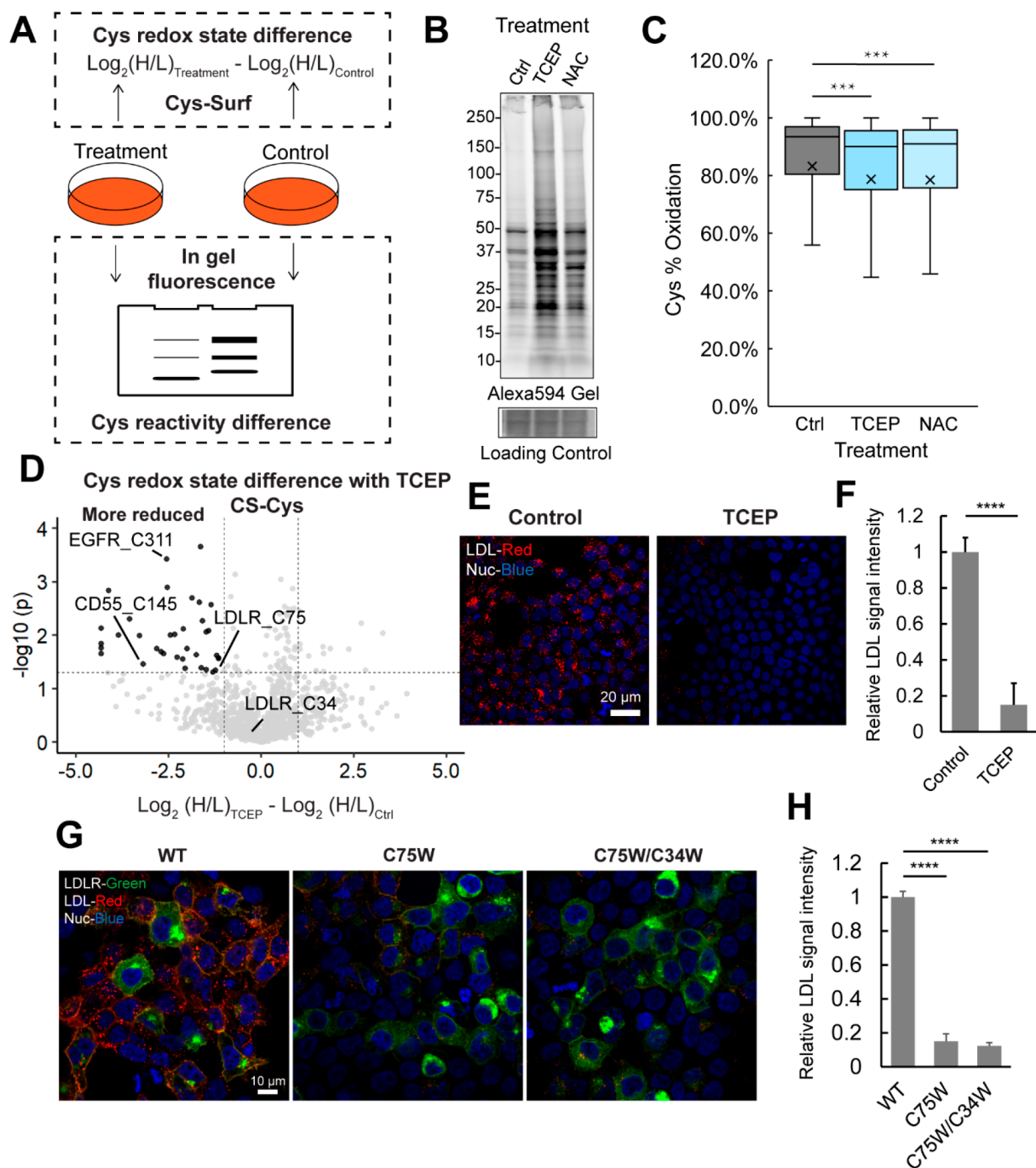


Figure 5. Application of Cys-Surf to identify redox sensitive cell surface cysteines. (A) The schematic workflow to identify redox sensitive cell surface cysteines with Cys-Surf and in-gel fluorescence. (B) Gel of cell surface cysteines labeled with Maleimide-Alexa594 treated with DMSO as control, tris(2-carboxyethyl)phosphine (TCEP), or N-acetylcysteine (NAC). (C) Redox states of cell surface cysteines quantified with Cys-Surf treated with DMSO, TCEP or NAC. The mean is represented by “x”. (D) Difference of redox states for cysteines quantified with Cys-Surf with or without TCEP treatment. (E) LDL uptake (signals in red) in HEK293T cells with or without the treatment of TCEP. (F) Quantification of relative LDL uptake signal intensities in HEK293T cells with or without the treatment of TCEP. (G) LDL uptake (signals in red) in HEK293T cells with the expression of LDLR^{WT}, LDLR^{C75W}, and LDLR^{C75W/C34W} (signals in green). (H) Quantification of relative LDL uptake signal intensities in HEK293T cells with the expression of LDLR^{WT}, LDLR^{C75W}, and LDLR^{C75W/C34W}. TCEP treatment was 5 mM for 20 min at 37 °C. NAC treatment was 10 mM for 1 h at 37 °C. For panels C, F, and H, statistical significance was calculated with unpaired student’s *t* tests, ****p* < 0.001, *****p* < 0.0005. For panels F and H, data are represented as mean ± stdev. MS experiments were conducted in 3 replicates in Jurkats cells. All MS data can be found in Table S5.

C₅ Maleimide (Maleimide-Alexa594)^{129,130} to visualize treatment-dependent changes in cell surface cysteine reactivity (Figure 5A, top). For the H₂O₂- and GSNO-treated cells, we observed a modest decrease in the reactivity of free thiols

toward Maleimide-Alexa594 (Figure S8A). In contrast, lysates derived from TCEP- and NAC-treated cells exhibited increased Maleimide-Alexa594 labeling, consistent with reduction-dependent increased availability of free thiols. When

compared to NAC, samples treated with TCEP exhibited a more substantial increase in Maleimide-Alexa594 reactivity, consistent with TCEP's greater reduction potential (Figure 5B).

As our gel-based analyses confirmed treatment-dependent changes to cysteine oxidation, we next extended these analyses to Cys-Surf characterization of the oxidation- and reduction-sensitive cell surface proteomes. Following the workflow shown in Figure 5A (bottom), we identified treatment-dependent changes to cysteine oxidation state based on the difference in MS1 intensity ratios comparing additive- and vehicle-treated samples. Consistent with the comparatively small decrease in Maleimide-Alexa594 labeling observed for oxidant treated samples (Figure S8A), Cys-Surf analysis of H₂O₂- and GSNO-treated cells revealed no significant treatment-dependent changes in the aggregated mean percentage of oxidation of the cysteines identified (Figure S8B). These findings are consistent with our aforementioned observation (Figure 2B,C) that, at basal state, most cell surface cysteines are already highly (mean >80%) oxidized and thus insensitive to additional oxidant. This observed insensitivity is in stark contrast to that observed for whole proteome,⁷⁰ where we found that >51.0% of cysteines were significantly oxidized with GSNO. Out of 1,310 cysteines identified in both samples treated with vehicle and H₂O₂, 16 cysteines showed significant ratio changes (difference > 1, *p*-value < 0.05) sensitive to H₂O₂, including C590 of integrin beta-2 ITGB2, which has been reported as ligandable in human T cells¹⁰⁴ (Figure S8C). Out of 305 cell surface cysteines identified in both samples with control and GSNO treatment, 3 cysteines showed significant GSNO induced oxidation (difference > 1, *p*-value < 0.05), including C109 of platelet endothelial cell adhesion molecule PECAM1, a protein that is required for leukocyte transendothelial migration (TEM) under most inflammatory conditions^{131,132} (Figure S8D).

Guided by the observed comparative insensitivity to exogenous oxidations and high degree of baseline oxidation state of cell surface cysteines, we next asked whether a more substantial fraction of the cell surface cysteinome would be sensitive to exogenous reductants TCEP and NAC. Consistent with our in-gel fluorescence analysis (Figure 5B), Cys-Surf analysis revealed a modest yet significant decrease in the measured bulk mean cysteine oxidation state after TCEP and NAC treatment, decreasing from 93.4% to 90.0% and 91.0%, respectively (Figure 5C). Out of the 916 cysteines from 618 proteins identified in both samples with control and NAC treatment, 15 cysteines showed significant NAC induced reduction (difference < -1, *p*-value < 0.05), including C173 of signaling threshold-regulating transmembrane adapter 1 SIT1, which has been reported as immune-enriched and hyper-reactive in activated human T cells¹⁰⁴ (Figure S9). Out of the 1,189 cysteines from 698 proteins identified in both samples treated with control and TCEP, 39 cysteines were sensitive to TCEP-induced reduction (difference < -1, *p*-value < 0.05), of which 32 has cell surface annotation. We initially were surprised by the low overlap between the NAC and TCEP data sets, with 534 shared total cysteines. Together with the difference in reducing potentials for TCEP and NAC, we expect that the comparatively modest coverage (1,573 total cysteines) together with the stochastic nature of data dependent acquisition (DDA) likely rationalizes the modest overlap between data sets.

Several intriguing reduction labile cysteines were identified only in the TCEP data sets. Annotated disulfides in multiple CD antigens were identified as reducible, including C145 of CD55, which has been identified as significantly reduced during T cell activation in our previous bulk proteome data set⁷⁰ (Figures S10 and S11). C311 of the epidermal growth factor receptor kinase (EGFR) was found to be markedly sensitive to TCEP (difference = -2.55). This cysteine is localized to a disulfide bridge (C311-C326) in a cysteine-rich region of EGFR that is required for kinase activation.¹³³ Notably, disulfide breaking mutations at C326 were recently reported to be gain-of-function for Lhermitte-Duclos Disease.¹³⁴

Similarly, a large number of mutations at disulfide bonded extracellular cysteines in the low-density lipoprotein receptor (LDLR) have been implicated in Familial Hypercholesterolemia (FH), one of the most common genetic disorders.^{135,136} In our TCEP data set, we identified two cysteines from LDLR, highly reduced C75 (difference = -1.5) and comparatively reduction insensitive C34 (difference = -0.3). While variants of these two cysteines have been reported in ClinVar,¹³⁷ with clinical significance as likely pathogenic and associations with familial hypercholesterolemia, the impact of these variants on low-density lipoprotein (LDL) uptake is, to our knowledge, unknown. Notably, mutation at the proximal C46 has already been demonstrated to reduce LDL uptake, which inspired us to further characterize Cys75.¹³⁸

Using fluorescently labeled LDL with GFP tagged on the C-term of LDL, we next assayed the impact of TCEP and C75 mutations on LDL uptake and LDLR activity. TCEP treatment attenuated both basal HEK-293T cellular LDL uptake and LDLR-dependent uptake with heterologously overexpressed LDLR (Figures 5E,F and S11). Guided by these findings, we then generated point mutations in our LDLR-GFP construct, including at C75 (LDLR^{C75W}) and the double mutant (LDLR^{C75W/C34W}), with the latter chosen to assess whether C75 mutations would synergize with the reduction-insensitive C34 residue. Of note, we chose the comparatively bulky tryptophan mutation both because it is reported in ClinVar and is a mutation that mimics small molecule labeling at this site, which could help guide future efforts for the development of labile-disulfide-directed covalent modifiers.

HEK293T cells overexpressing the LDLR^{C75W} mutant showed markedly attenuated LDL uptake. In contrast to the substantial plasma membrane localization observed for the wild-type LDLR construct, we observed increased intracellularly localized LDLR^{C75W}, consistent with mutation-induced changes in protein translocation to the plasma membrane. The double mutant (LDLR^{C75W/C34W}) afforded a similar decrease in LDL uptake and intracellular protein retention (Figures 5G,H and S12). The lack of synergy between these two mutations supports that the single C75W mutation is sufficient to block LDL uptake. Taken together, our findings support the pairing of Cys-Surf with exogenous reductant treatments to pinpoint functional and reduction-labile disulfides. They also point toward future opportunities for therapeutic targeting of this comparatively underexplored portion of the cysteinome.

DISCUSSION

By establishing the cell Surface Cysteine enrichment (Cys-Surf) platform, here we achieved unprecedented local cysteinome analysis, including the identification of ligandable

and redox sensitive membrane cysteines. Cys-Surf features a unique two-step biotinylation method: after in situ IAA capping to protect cysteines from oxidation, cell surface glycoproteins are then subjected to CSC on streptavidin followed by peptide-level click conjugation and neutravidin capture of cell surface-associated cysteine peptides. While our method draws inspiration from our recent pairing of TurboID proximity labeling with cysteine chemoproteomics,⁷³ to our knowledge, such sequential rounds of enrichment for cell surface proteins is unprecedented. Application of Cys-Surf to primary immune cells and immortalized cell lines identified in aggregate 2,836 total cell surface cysteines from 1,046 proteins, including 211 ligandable cysteines and 153 redox sensitive cysteines. For more translational applications, we expect that our rich data set of ligandable cell surface cysteines will be of particular interest, as demonstrated by the blockbuster status of several covalent kinase inhibitors that target cell surface-associated kinases (e.g., EGFR and HER2).

Enabled by our pairing of Cys-Surf with isotopically enriched cysteine capping reagents,⁷⁰ we stratified the cell surface redoxome. Consistent with prior studies, we find that the redox state of membrane associated cysteinome differs markedly from bulk proteomic analysis, both at the basal state and for cells subjected to stimuli and exogenous oxidants and reductants. We find a mean oxidation state of ~90% for the 1,246 cell surface cysteines for which quantitative measures of absolute oxidation were achieved. This striking oxidation contrasts with the previously reported 10% median cellular cysteine oxidation state, as measured by bulk redox proteomics.^{70,139,140} Consistent with this marked oxidation and the highly oxidizing extracellular environment, we find that comparatively few cell surface cysteines are prone to oxidation, both with the addition of exogenous oxidants and under cellular activation conditions. This latter finding is in contrast with the marked activation-induced oxidation observed for bulk proteomes.⁷⁰ These findings are consistent with previous reports of compartmentalization of redox-mediated signaling^{141,142} and highlight the value of subcellular measures of cysteine oxidation, as bulk proteome analysis will likely mask compartment-specific changes.

In contrast with the comparative insensitivity of the plasma membrane cysteine to oxidation, Cys-Surf revealed a prevalence of reduced labile cysteines, including many sensitive to T cell activation. While the plasma membrane is known to become more reduced during T cell activation,^{56,57,106,108} the specific cysteines sensitive to this cell state change remain overwhelmingly uncharacterized. As such, our study provides a roadmap to identify functional cysteines involved in T cell activation. Further showcasing Cys-Surf's capacity to pinpoint high value reduction-labile cysteines, we also identify 53 total cysteines sensitive to exogenous reductants TCEP and NAC. A number of noteworthy residues stood out, including those found in -RHStaple bonds. In total, we identified 21 disulfides sensitive to the exogenous reductants TCEP and NAC. Noteworthy examples of labile bonds included those found in multiple clusters of differentiation proteins implicated in cellular activation, a cysteine (C311) within an activation-associated region of EGFR, and a cysteine (C75) within the LDLR protein. While this cysteine (C75) of LDLR has been reported to be mutated in cases of Familial Hypercholesterolemia (FH), the functional impact of these mutations has not, to our knowledge, been probed previously. Providing compelling proof-of-concept evidence of the likely functional

importance of these reduction-labile disulfide bonds, here, by combining mutational analysis and LDL uptake studies, we reveal that C75W mutations lead to retention of LDLR in the cytoplasm and decreased LDL uptake. As C75W has been reported in ClinVar, we expect that our finding should help to aid in interpretation of the clinical significance of this variant and, more broadly, will add to the growing body of deleterious genetic variants in the cysteine-rich extracellular domain of LDLR that impact LDLR activity.^{136,138} Given that both the TCEP treatment and C75W mutation block LDL uptake, we expect that the C75–C95 disulfide is likely important for LDLR activity. However, as the point mutation also causes decreased LDLR plasma membrane localization, we cannot rule out the possibility that the TCEP-induced decreased lipoprotein uptake could also be due to a reduction of additional functionally important plasma membrane cysteines. Looking beyond LDLR, we anticipate that our work will help guide ongoing and future efforts to characterize the functional significance of labile disulfides, particularly in the context of receptor-mediated cell signaling. We expect that many of the residues identified here may prove sensitive to reductive stress, which is an area of emerging interest and a critical modulator of many human diseases.^{143–147} Thus, we expect our work to help inform disease pathogenesis for reductive-stress-sensitive conditions, spanning metabolic disorders to cancer.

Achieving a more comprehensive analysis of this intriguing fraction of the cysteinome will likely require improvements in the coverage for future iterations of Cys-Surf. While the Cys-Surf coverage exceeded that of our TurboID-based platforms,⁷³ Cys-Surf cysteine peptide identification remained comparatively modest, with ~1,000–2,000 cysteines captured per experiment. Thus, when compared with the typical >10,000 cysteines captured from bulk proteomic analysis, Cys-Surf is likely still substantially undersampling plasma membrane cysteines. Exemplifying this limitation, while we do identify cysteines from EGFR, we fail to capture C797, which is the cysteine modified by EGFR-directed covalent kinase inhibitors. Analysis of additional cell lines, including EGFR over-expressing cells, together with implementation of Cys-Surf with additional sequence specific proteases to improve coverage of cleave site poor transmembrane regions should afford enhanced coverage. Notably, despite this modest coverage, across the study, ~30% (821/2836) of the cysteines identified by Cys-Surf were not found previously in CysDB, our database of high coverage cysteine chemoproteomic data sets.

Looking beyond the current study, we expect that Cys-Surf analysis should synergize with genetic¹⁴⁸ and proteogenomic¹⁴⁹ approaches aimed at pinpointing functional and therapeutically relevant cysteines, including those impacted by genetic variation. Given the seeming ubiquity of reduction-labile disulfide bonds identified by Cys-Surf, we expect that a subset of these cysteines should be susceptible to covalent inhibitor development efforts, including a fraction of the >500 ligandable cysteines identified here. While covalent inhibition of labile-disulfide-bonds is predated by molecules targeting oxidoreductase enzymes,^{150,151} the scope and functional impact of targeting redox environment-dependent cysteine proteoforms remain to be realized. The accessibility of extracellular cysteines identified by Cys-Surf to biologic agents with poor cell penetration, including peptides and antibodies, offers a unique opportunity for the future development of

cysteine-directed biological agents as well as proteoform-specific therapies.

METHODS

Cell Culture

Cell culture reagents including Dulbecco's phosphate-buffered saline (DPBS), Roswell Park Memorial Institute (RPMI) media, Dulbecco's Modified Eagle Medium (DMEM) media, and penicillin/streptomycin (Pen/Strep) were purchased from Fisher Scientific. Fetal Bovine Serum (FBS) was purchased from Avantor Seradigm (lot # 214B17). All cell lines were obtained from ATCC, routinely tested for mycoplasma, and maintained at a low passage number (<20 passages). Jurkat (ATCC: TIB-152) cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). HEK293T (ATCC: CRL-3216) cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (Penn/Strep, 100 U/mL). Media were filtered (0.22 μ m) prior to use. Cells were maintained in a humidified incubator at 37 °C with 5% CO₂.

Blood from a healthy donor was obtained from UCLA/CFAR Virology Core (SP30 AI028697) after informed consent. After Trima filter isolation, peripheral blood mononuclear cells (PBMCs) were purified over Ficoll–Hypaque gradients (Sigma-Aldrich) and T cells were purified via negative selection with magnetic beads (EasySep Human T Cell Iso Kit, 17951, STEMCELL). The purified T cells were washed with sterile PBS. Unstimulated cells were harvested by centrifugation. The remaining cells were then resuspended in RPMI-1640 supplemented with FBS, penicillin, streptomycin, and glutamine (2 million cells per mL), and 200,000 cells per well were seeded on nontreated tissue culture, 96-well transparent plates that had been coated with anti-CD3 (1:200, BioXcell) and anti-CD28 (1:500, Biologend) in PBS (100 μ L per well). After 72 h, the cells were harvested by centrifugation (4500g, 5 min, 4 °C), washed 3 times with cold DPBS, and prepared as described below.

Proteomic Sample Preparation for Cys-Surf, Protein Level Enrichment

After cells were treated as indicated, harvested by centrifugation (4500g, 5 min, 4 °C), and washed with DPBS 3 times, they were resuspended in DPBS and labeled with 2 mM IAA for 1 h at room temperature (RT). For cysteine oxidation quantification, cells were labeled with 2 mM L-IPIAA for 1 h at RT, reduced with 1 mM DTT for 15 min at 37 °C, and labeled with 2 mM H-IPIAA for 1 h at RT, with a PBS wash between the IPIAA labeling. For cysteine ligandability quantification, cells were labeled with either compound or vehicle for 1 h at RT, followed by treatment of 2 mM L-IPIAA or H-IPIAA for 1 h at RT, respectively, and then combined. Following cysteine capture, cells were oxidized with 1.6 mM NaIO₄ in 1 mM NaAc (pH = 6.5) for 20 min at RT in the dark. After washing with DPBS, cells were labeled with 1 mM aminoxy-biotin in PBS for 1 h at 4 °C. Cells were then washed with DPBS, lysed in RIPA buffer (Fisher, Cat# AAJ6288SAE) for 30 min at 4 °C, and clarified by centrifuging (21,000g, 10 min, 4 °C). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher, catalog no. 23227), and the lysate was normalized to 500 μ L of 1.5 mg/mL. 50 μ L of Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic (GE Healthcare, 65152105050250), and 50 μ L of Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic (GE Healthcare, 45152105050250), were mixed and washed with water three times. The bead slurries were then transferred to the lysate and incubated for 5 min at RT with shaking (1000 rpm). EtOH (1 mL) was added to each sample, and the mixtures were incubated for 10 min at RT with shaking (1000 rpm). The beads were then washed (3 \times 1 mL of 80% EtOH) with a magnetic rack. Proteins were eluted from SP3 beads with 500 μ L of 0.2% SDS in PBS for 30 min at 37 °C while being shaken (1000 rpm). 50 μ L of Pierce streptavidin agarose beads were washed with 0.2% SDS/PBS and incubated with the eluted lysates for 2 h at RT. The proteins bound to beads were washed once with 1 mL of 0.2% SDS/PBS, 3 times with 1 mL of PBS, and 3 times with 1 mL of H₂O. The beads were resuspended in 200 μ L of 6 M urea, either

reduced with 1 mM DTT for 15 min at 65 °C and labeled with 2 mM IAA for 1 h at RT for Cys-CS identification or reduced with 10 mM DTT for 15 min at 65 °C and labeled with 20 mM IA for 30 min at 37 °C for Cys-CS oxidation and ligandability quantification. Then, beads were washed with PBS and resuspended in 200 μ L of 2 M urea. 3 μ L of 1 mg/mL trypsin solution (Washington) was added. Proteins were digested off the beads overnight at 37 °C with shaking.

Proteomic Sample Preparation for Cys-Surf, Peptide Level Enrichment

After digestion, CuAAC was performed with biotin-azide (4 μ L of 200 mM stock in DMSO, final concentration = 4 mM), TCEP (4 μ L of fresh 50 mM stock in water, final concentration = 1 mM), TBTA (12 μ L of 1.7 mM stock in DMSO/*t*-butanol 1:4, final concentration = 100 μ M), and CuSO₄ (4 μ L of 50 mM stock in water, final concentration = 1 mM) for 1 h at RT. 20 μ L of Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic, and 20 μ L of Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophilic, were mixed and washed with water three times. The bead slurries were then transferred to the CuAAC samples, incubated for 5 min at RT with shaking (1000 rpm). Approximately 4 mL of acetonitrile (>95% of the final volume) was added to each sample, and the mixtures were incubated for 10 min at RT with shaking (1000 rpm). The beads were then washed (3 \times 1 mL acetonitrile) with a magnetic rack. Peptides were eluted from SP3 beads with 100 μ L of 2% DMSO in MB water for 30 min at 37 °C with shaking (1000 rpm). The elution was repeated again with 100 μ L of 2% DMSO in MB water. For each sample, 50 μ L of NeutrAvidin Agarose resin slurry (Pierce, 29200) was washed three times in 10 mL of IAP buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, and 50 mM NaCl buffer) and then resuspended in 800 μ L of IAP buffer. Peptide solutions eluted from SP3 beads were then transferred to the NeutrAvidin Agarose resin suspension, and the samples were rotated for 2 h at RT. After incubation, the beads were pelleted by centrifugation (2000g, 1 min) and washed (3 \times 1 mL of PBS, 3 \times 1 mL water). Bound peptides were eluted twice with 60 μ L of 80% acetonitrile in MB water containing 0.1% FA. The first 10 min incubation was at RT and the second one, at 72 °C. The combined eluants were dried (SpeedVac), then reconstituted with 5% acetonitrile and 1% FA in MB water, and analyzed by LC-MS/MS.

Database Construction

Subcellular location annotations and glycosylation annotations from CellWhere Atlas (accessed 2208), Human Protein Atlas (HPA) version 21.1, and UniProtKB/Swiss-Prot (2208_release) were aggregated. Unique proteins were established by using UniProt protein identifiers. Aggregated annotations were mined for specific keywords (ex. "Cell Surface" or "Glycosylation"). Proteins containing these keywords are reported in Table S1.

Gene Enrichment Analysis

Enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) 2021 and Gene Ontology (GO) Biological Processes 2021 gene set library terms was performed using the GSEapy package.¹⁵² Proteins identified by our chemoproteomics studies were used as the background protein set. UniProtKB protein identifiers were mapped to Entrez gene symbols as inputs for Enrichr. *p*-values were computed from Fisher's exact test to determine the significance of each enriched term. The negative logarithm of these *p*-values was calculated using R.

Cell Treatment

The treatments were as follows. H₂O₂: Cells were treated with 500 μ M H₂O₂ for 1 h at 37 °C. S-Nitrosoglutathione (GSNO): Cells were treated with 500 μ M GSNO for 1 h at 37 °C. Tris(2-carboxyethyl)-phosphine (TCEP): Cells were treated with 5 mM TCEP for 20 min at 37 °C. N-Acetylcysteine (NAC): Cells were treated with 10 mM NAC for 1 h at 37 °C. 3,5-Bis(trifluoromethyl)aniline chloroacetamide (KB03), acrylamide (KB14), and propynamide (6): Cells were treated with 50 μ M compound for 1 h at 37 °C.

Gel and Western Blot

For streptavidin blot, lysates after Cell Surface Capture (CSC) were normalized to 2 mg/mL and separated on a 4–20% SDS-PAGE gel. Gels were transferred to nitrocellulose membrane (Bio-Rad) and blocked in 2% (w/v) BSA in TBS-T (Tris-buffered saline, 0.1% Tween 20) for 1 h at RT. Membranes were incubated with a streptavidin-fluorophore conjugate overnight at 4 °C. Membranes were imaged on a Bio-Rad ChemiDoc. For fluorescent gel, cells were treated as described and labeled with 5 μ M IA-Rho or 5 μ M Maleimide-Alexa594. After lysis, lysates were normalized to 2 mg/mL and separated on a 4–20% SDS-PAGE gel. Gels were imaged on Bio-Rad ChemiDoc. Reagents can be found in Table S6.

Immunoprecipitation

Cell lysates were incubated with the antibody overnight at 4 °C. 25 μ L of Protein G beads was washed, added to the lysate, and incubated at 4 °C for 2 h with rotation. After washing the beads with lysis buffer 3 times, 25 μ L of SDS loading dye was added to the sample and incubated at 95 °C for 5 min. Supernatant was collected for gel analysis after centrifugation at 12 000g for 2 min.

Construction of LDLR Plasmids

Human LDLR gene was cloned from HepG2 cell cDNA and was then sequentially subcloned into pEGFP-N1 using Gateway technology (Invitrogen). The LDLR mutations for the pEGFP-N1-LDLR construct were introduced via site-directed mutagenesis.

LDL Uptake Assay and Microscopy

HEK293T cells were plated in a standard 24-well plate, with each well containing a poly D-lysine-coated glass coverslip (NC0672873, Fisher Scientific). Cells were transfected with LDLR-GFP (WT, C75W, and C75W/C34W) for 48 h with Fugene 6 (Promega). Cells were starved in 5% LPDS medium (MilliporeSigma, S5519) for 12 h. Cells were then incubated with 10 μ g/mL Dil-LDL (Kalen Biomedical, 770230-9) for 50 min. After Dil-LDL incubation, cells were washed three times with DPBS and fixed with 4% PFA for 20 min at room temperature. After fixation, the cells were washed three times with DPBS and incubated with Hoechst 33342 (H3570, Fisher Scientific). Cells were mounted on slides with Prolong Diamond Antifade Mountant (P36961, ThermoFisher). Images were acquired using an Inverted Leica TCS-SP8 Confocal Microscope (CNSI) and analyzed by ImageJ.

Liquid-Chromatography Tandem Mass-Spectrometry (LC-MS/MS) Analysis

The samples were analyzed by liquid chromatography tandem mass spectrometry using a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer. Peptides were fractionated online using an 18 cm long, 100 μ M inner diameter (ID) fused silica capillary packed in-house with bulk C18 reversed phase resin (particle size, 1.9 μ m; pore size, 100 Å; Dr. Maisch GmbH). The 70 min water–acetonitrile gradient was delivered using a Thermo Scientific EASY-nLC 1200 system at different flow rates (Buffer A, water with 3% DMSO and 0.1% formic acid; buffer B, 80% acetonitrile with 3% DMSO and 0.1% formic acid). The detailed gradient includes 0–5 min from 3% to 10% at 300 nL/min, 5–64 min from 10% to 50% at 220 nL/min, and 64–70 min from 50% to 95% at 250 nL/min buffer B in buffer A (Table S7). Data was collected with charge exclusion (1, 8, >8). Data was acquired using a Data-Dependent Acquisition (DDA) method consisting of a full MS1 scan (resolution = 120,000) followed by sequential MS2 scans (resolution = 15,000) to utilize the remainder of the 1 s cycle time. Precursor isolation window was set as 1.6, and normalized collision energy was set as 30%. The MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository^{153,154} with the data set identifier PXD042403. File details can be found in Table S8.

Protein, Peptide, and Cysteine Identification

Raw data collected by LC-MS/MS were converted to mzML and searched with MSFragger (v3.3) and FragPipe (v19.0).¹⁵⁵ The proteomic workflow and its collection of tools was set as default, and

PTMprophet was enabled.¹⁵⁶ Precursor and fragment mass tolerance was set at 20 ppm. Missed cleavages were allowed up to 1. Peptide length was set at 7–50, and peptide mass range was set at 500–5000. For Cys-Surf identification, cysteine residues were searched with differential modification C+463.2366. For Cys-Surf oxidation and ligandability quantification, MS1 labeling quantification was enabled with the Light set as C+463.2366 and the Heavy set as C+467.2529. MS1 intensity ratio of heavy and light labeled cysteine peptides was reported with Ionquant (v1.8.9). Calibrated and deisotoped spectral files produced by FragPipe were retained and reused for this analysis. The MS search data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD042403. File details can be found in Table S8. Custom python scripts were implemented to compile labeled peptide data sets. Unique proteins, unique cysteines, and unique peptides were quantified for each data set. Unique proteins were established based on UniProt protein IDs. Unique peptides were found based on sequences containing a modified cysteine residue. Unique cysteines were classified by an identifier consisting of a UniProt protein ID and the amino acid number of the modified cysteine (ProteinID_C#); residue numbers were found by aligning the peptide sequence with the corresponding UniProt protein sequence. When there are multiple cysteines in one peptide, all of the modified cysteine residue numbers will be reported as ProteinID_C#_C#.

Data Analysis

For the cell surface annotation, our customized localization database was used to cross-reference with the proteins or cysteines identified. For isotopical quantification, the medium of heavy over light ratios for the same cysteine residue from cysteine peptides of different charges and miscleavages in the same sample was calculated. Means of reported logged ratio values for each condition (\pm H₂O₂ or \pm GSNO or \pm TCEP or \pm NAC or activated/naive T cells) were calculated for all replicates per condition. Percentage oxidation for a cysteine was calculated based on heavy to light ratio via the following formula: $(R/(1 + R)) \times 100$, using unlogged ratios. When calculating the oxidation difference, relative oxidation changes between two cellular conditions were reported by calculating the change of heavy to light ratios between treated and untreated samples.

UniProtKB Disulfide and Domain Analysis

Counts of how many identified proteins had UniProtKB annotations for disulfide bonds and topological domain annotations were calculated based on matches between the position of the identified residue and UniProtKB functional region. Further parsing of UniProtKB disulfide bond site annotations was extracted to obtain specific residues and amino acid numbers. Exact amino acid positions of UniProtKB cysteines involved in disulfide bonds or within regions of annotated domains were cross-referenced with those of our cysteine identifiers. Cysteines with “extracellular” topological domain annotations were classified as cysteines in extracellular domains, while cysteines with “cytoplasmic”, “mitochondrial intermembrane”, “mitochondrial matrix”, and “perinuclear space” were classified as cysteines in intracellular domains.

Statistics

For box plots in Figures 3C,E,F, 4B, 5C, and S8B, the average of replicates was reported as indicated. Statistical significance was calculated with unpaired Student's *t* tests using R stats (v 3.6.2) if applicable. For bar plots in Figures 1D, 5F,H, S1E, S2B, and S3, error bars were calculated using standard deviation. Statistical significance was calculated with unpaired Student's *t* tests using R stats (v 3.6.2) if applicable. For volcano plots in Figures 4C, 5D, S8C,D, and S9A, variances were calculated for each sample–condition pairing, and a corresponding two-sample *t* test was performed using R stats (v 3.6.2) to generate *p*-values. *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

■ ASSOCIATED CONTENT

Data Availability Statement

We have submitted all data sets associated with this manuscript to the PRIDE repository with the following identifying information: ID: PXD042403

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacsau.3c00707>.

Streptavidin blot of cell surface capture (CSC), schematic workflow of protein level SP3 clean up and CSC, stain free blot of the input and eluted lysates of SP3 clean up with different conditions, number of cell surface peptides captured with or without SP3 clean up, unique counts of proteins, peptides, PSMs, and cysteines, top 10 GO biological processes enriched with proteins harboring ligandable cell surface cysteines, predicted structure of HLA-A, IA-Rho gel and western blot of HLA-A, Log₂(H/L) data, difference of redox states for representative cysteines quantified with Cys-Surf during T cell activation, gel of cell surface cysteines, redox state data, LDL uptake, antibodies and fluorescent probes used in this study, LC conditions, and PRIDE data sets (PDF)

Table S1: Aggregated data set for Figure 1 (XLSX)

Table S2: Aggregated data set for Figure 2 (XLSX)

Table S3: Aggregated data set for Figure 3 (XLSX)

Table S4: Aggregated data set for Figure 4 (XLSX)

Table S5: Aggregated data set for Figure 5 (XLSX)

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T.Y. and K.M.B. conceived of the project. T.Y. and L.C. collected data. T.Y. and L.M.B. performed data analysis. L.M.B. wrote software. T.Y. contributed to the figures. T.Y. and K.M.B. wrote the manuscript with assistance from all authors.

Notes

The authors declare no competing financial interest.

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