Global profiling of phosphorylation-dependent changes in cysteine reactivity

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Proteomics has revealed that the ~20,000 human genes engender a far greater number of proteins, or proteoforms, that are diversified in large part by post-translational modifications (PTMs). How such PTMs affect protein structure and function is an active area of research but remains technically challenging to assess on a proteome-wide scale. Here, we describe a chemical proteomic method to quantitatively relate serine/threonine phosphorylation to changes in the reactivity of cysteine residues, a parameter that can affect the potential for cysteines to be post-translationally modified or engaged by covalent drugs. Leveraging the extensive high-stoichiometry phosphorylation occurring in mitotic cells, we discover numerous cysteines that exhibit phosphorylation-dependent changes in reactivity on diverse proteins enriched in cell cycle regulatory pathways. The discovery of bidirectional changes in cysteine reactivity often occurring in proximity to serine/threonine phosphorylation events points to the broad impact of phosphorylation on the chemical reactivity of proteins and the future potential to create small-molecule probes that differentially target proteoforms with PTMs.

large-scale genomic and transcriptomic profiling methods have transformed our understanding of the molecular composition of biological systems and their regulation by physiological and pathological stimuli, as well as by pharmacological perturbation. Much of the biochemistry of human cells is dedicated, however, to shaping the proteome in a contextual and temporal manner through post-translational events that are poorly captured by genomic and transcriptomic profiling. Indeed, it has been estimated that the ~20,000 human genes in the human genome produce a much larger number of unique proteins (~10^5), or proteoforms, due to splice variations and PTMs.

Understanding PTMs and their impact on protein function is an intense area of research, but also a daunting task because, as the number of proteoforms originating from a single gene increases, so does their potential for combinatorial representation in a given cellular state, and methods to resolve each proteoform remain limited. One effective strategy is top-down proteomics, which enables independent analysis of individual proteoforms, but, despite recent advances, it is a challenge for this method to analyze proteins of larger size (>50 kDa), especially in complex biological systems. Bottom-up proteomics, on the other hand, in which cell lysates are digested by a protease such as trypsin before analysis by liquid chromatography–mass spectrometry (LC–MS), typically assesses all proteoforms of a given protein as a single integrated signal. Various strategies have been introduced to enrich specific PTM forms of proteins, such as phosphorylated, glycosylated, or lipided proteins, and recent studies have shown how such PTMs can be related to the thermal stability of proteins in cells. However, the impact of PTMs on many other features of protein structure and function remains unknown. Addressing this important topic, especially on a global scale, would facilitate identification of PTM events that create proteoforms with distinct chemical properties, biochemical activities, and druggability, thereby refining our understanding of, and potential to pharmacologically control, cellular processes.

Mitosis is the biological process responsible for faithfully segregating the duplicated genome to two daughter cells for proper cell division. Failure to regulate cell division has severe consequences, including the uncontrolled proliferation state that is a hallmark of cancer. Proper cell cycle progression is temporally controlled through a complex network of PTMs that influence protein activity, stability, localization, and biomolecular interactions. Phosphorylation plays a major role in regulating the cell cycle, and phosphoproteomic studies have revealed a massive burst of protein phosphorylation during mitosis involving over 30,000 distinct mitotic phosphosites, with 20% of these phosphosites being exclusively found in mitosis. These phosphorylation events mainly occur on serine and threonine residues (referred to hereafter as S/T phosphorylation events) and conform to a consensus sequence in which the phosphorylated residue is followed by proline (S/T/P motif), reflecting the robust activation of cyclin-dependent kinases (CDKs) during mitosis. Notably, quantitative proteomic methods have also revealed that many mitotic phosphorylation events occur with high stoichiometry, meaning that the majority of a given protein undergoing mitotic phosphorylation has been converted into its phosphorylated proteoform.

Because mitosis is rich in unique proteoform content, in part through PTMs like phosphorylation, it has been intensively investigated by large-scale methods aimed at globally dissecting protein state and activity relationships. These approaches include not only phosphoproteomics, but also complementary proteogenomic methods, such as cellular thermal shift assays (MS-CETSA) that give insight into biophysical changes in proteins that may arise, for instance, from cell-cycle-specific PTMs or protein–protein metabolite interactions, size-exclusion chromatography (MS-SEC), which can identify mitotic protein complex reorganization, and single-cell immunofluorescence microscopy to map proteins with temporal or spatial changes throughout the cell cycle.

While the aforementioned proteomic approaches have revealed striking changes in protein modification state, structure, localization, and function during mitosis, it remains unclear how unique mitotic proteoforms, or phosphorylated proteoforms more generally, may differ in their chemical properties compared with...
Cysteine residues are dynamic sensors that coordinate diverse cellular signaling events. The reactivity of cysteines is sensitive to changes in the environment surrounding these reactive residues, enabling real-time monitoring of redox signaling and redox balance. These attributes make cysteines particularly interesting targets in post-translational modification analysis, given their critical roles in enzyme catalysis and metal coordination, as well as their potential for modification by other proteinaceous amino acids such as phosphorylation on TMT–ABPP experiments can be used to assess changes in cysteine reactivity, which is a dynamic property that can change in the presence of a variety of chemical and biological modifications. The chemical reactivity of cysteines, for instance, is a dynamic property that can influence the potential for covalent modification by endogenous or exogenous small molecules. Cysteine is the most intrinsically nucleophilic amino acid in the proteome and plays important roles in enzyme catalysis and metal coordination, as well as other important redox signaling pathways. These characteristics enable cysteine residues to serve as sensors of changes in cell and protein states, as well as primary targets for covalent chemical probe and drug development. We have previously described an activity-based protein profiling (ABPP) platform that leverages broad, cysteine-directed iodoacetamide (IA) chemical probes coupled with multiplexed (tandem mass tagging, or TMT) proteomic analysis to quantitatively monitor changes in the reactivity of many thousands of cysteines in native human proteomes. We have leveraged this platform to establish a baseline understanding of changes in cysteine reactivity in mitotic cells. HeLa cells were synchronized in early mitosis by a thymidine-noctadazole block, and corresponding asynchronous HeLa cells were seeded and collected in parallel. Proteomic lysates from each cell state were then analyzed by TMT–ABPP: the lysates were treated with the cysteine-reactive probe iodoacetamide-deshiobiotin (IA-DTB, 100 µM, 1 h) and trypsinized, and biotinylated cysteine-containing peptides were isolated and comparatively quantified by streptavidin enrichment.

**Results**

**Cysteine reactivity profiling of mitotic cells.** We first set out to establish a baseline understanding of changes in cysteine reactivity in mitotic cells. HeLa cells were synchronized in early mitosis by a thymidine-noctadazole block, and corresponding asynchronous HeLa cells were seeded and collected in parallel (Extended Data Fig. 1a). Mitotic lysates from each cell state were then analyzed by TMT–ABPP: the lysates were treated with the cysteine-reactive probe iodoacetamide-deshiobiotin (IA-DTB, 100 µM, 1 h) and trypsinized, and biotinylated cysteine-containing peptides were isolated and comparatively quantified by streptavidin enrichment, TMT labeling, and LC–MS using an Orbitrap Fusion instrument. Across ~17,000 cysteines from ~5,800 proteins, we identified >3,000 cysteines displaying a twofold or greater change in reactivity between mitotic and asynchronous HeLa cells (Fig. 1a and Supplementary Dataset 1). Though there have been reported increases in reactive oxygen species in mitosis, we did not observe changes in highly oxidation-sensitive cysteines in mitotic HeLa cells (for example, GAPDH C152 and PARK7 C106 (ref. 41); Extended Data Fig. 1b), which may indicate that such oxidation events occur with stoichiometry that is too low to alter the relative IA-DTB reactivity signals of cysteines in mitotic and asynchronous cell states. In parallel with our TMT–ABPP approach, we performed unenriched proteomics (TMT-exp, Extended Data Fig. 1c), allowing us to distinguish cysteines with authentic changes in reactivity versus those showing signal differences caused by alterations in protein abundance. More specifically, cysteines with authentic reactivity changes were expected to show substantial differences in IA-DTB reactivity (≥twofold change) in mitotic cells against a backdrop of unaltered protein expression (<1.5-fold change). While we observed a generally good overlap in proteins quantified by unenriched proteomics versus TMT–ABPP (Extended Data Fig. 1d), each approach quantified ~20–30% of proteins not detected by the other method. We therefore included an additional filter: cysteine changes were assigned as reactivity-based if additional cysteines quantified in the same protein were unaltered between mitotic and asynchronous cell states (<1.6-fold change). These analyses identified many instances of cysteines that showed striking apparent increases or decreases in reactivity in mitotic cells (Fig. 1b,c). In total, ~1,800 proteins were found to display cysteine changes, and of the >1,000 proteins confidently assigned to reactivity or expression change groups, ~60% reflected reactivity changes (Fig. 1d and Supplementary Dataset 1). Representative proteins with cysteine reactivity or expression changes are shown in Fig. 1e (upper and lower graphs, respectively). Proteins with reactivity-based cysteine changes were enriched in cellular processes involved in the cell cycle, as determined by GO analysis (Extended Data Fig. 1e).

In comparing our cysteine reactivity changes to proteome alterations mapped in mitotic cells by complementary methods, we noted interesting examples of convergent findings. For instance, two cysteines in thymidylate kinase (DTYMK)—C117 and C163—showed substantial reductions in reactivity in mitotic cells (Extended Data Fig. 1f). DTYMK was also shown to exhibit impaired thermal stability in S and prometaphase states by MS-CETSA analysis, a feature that was interpreted to reflect the removal of the thymidine block as part of the protocol to induce mitosis. Interestingly, we found that gel filtration of mitotic cell lysates restored DTYMK C117 reactivity, while no such change in the reactivity of this cysteine was observed in gel-filtered asynchronous cell lysates (Extended Data Fig. 1g,h). Considering that C117 is distal (>10 angstroms away) to the ATP and TMP binding sites of DTYMK, we propose that the disruption of the thymidine block associates with changes in reactivity of cysteines in the enzyme catalytic site to enable the removal of the thymidine block as part of the protocol to induce mitosis. We also identified >3,000 cysteines displaying a twofold or greater change in reactivity between mitotic and asynchronous HeLa cells (Fig. 1a and Supplementary Dataset 1). Though there have been reported increases in reactive oxygen species in mitosis, we did not observe changes in highly oxidation-sensitive cysteines in mitotic HeLa cells (for example, GAPDH C152 and PARK7 C106 (ref. 41); Extended Data Fig. 1b), which may indicate that such oxidation events occur with stoichiometry that is too low to alter the relative IA-DTB reactivity signals of cysteines in mitotic and asynchronous cell states.

**Cysteine reactivity profiling of mitotic and asynchronous cells.** a. Cysteine reactivity ratio values of mitotic versus asynchronous (Mitosis/Asynch) HeLa cell proteomes. Blue and red data points mark cysteine reactivity values that are ≥twofold higher (dotted lines) in mitotic or asynchronous proteomes, respectively. Each data point is median value for n = 2 (or more) independent experiments. b. Comparison of cysteine reactivity values to the expression values for corresponding proteins harboring these cysteines in scatter plot (b) or heat map (c) format. Ratio values are presented as Mitosis/Asynch (log transformed). For b, blue and red mark cysteine reactivity values that are ≥twofold higher in mitotic or asynchronous proteomes, respectively, where the corresponding protein expression value is unchanged (<1.6-fold difference). For c, blue and red mark cysteine reactivity or protein expression values that are ≥twofold higher in mitotic or asynchronous cell proteomes, respectively. Data shown are median values for n = 2 (or more) independent TMT-ABPP experiments and n = 1 (or more) independent unenriched proteomic experiments. d. Distribution of proteins with cysteine reactivity changes assigned as reactivity-based (blue) or expression-based (green) in the mitotic proteome. Proteins with unassigned cysteine changes are not shown. e. Representative reactivity-based (top) and expression-based (bottom) cysteine changes with corresponding protein expression values (Mitosis/Asynch). Dotted lines designate changes that are ≥twofold. Data shown are median values for n = 2 (or more) independent TMT–ABPP experiments and n = 1 (or more) independent unenriched proteomics experiments. f. Cysteine reactivity changes in proteins DTWD1 (left), NOL8 (middle), and RRPI5 (right) that localize to mitotic chromosomes (top). Bottom, box plots showing cysteine reactivity profiles for each protein, where blue and red colors mark cysteines with greater reactivity in the mitotic and asynchronous proteomes, respectively. The horizontal black line for each cysteine marks the median value, boxes mark upper and lower quartiles and whiskers mark the 1.5 x interquartile range. Dotted lines designate boundaries that are ≥twofold changes for n = 2 (or more) independent experiments (circles).
Data Fig. 1i), we interpret the change in reactivity of this cysteine to potentially reflect an allosteric effect caused by altered metabolite interactions with DTYMK in the mitotic cell state. Changes in cysteine reactivity in mitotic cells might also reflect alterations in protein–protein interactions, which may in turn affect protein localization. Consistent with this hypothesis, a multitude of proteins...
exhibiting cysteine reactivity changes, including the transfer RNA aminocarboxypropyltransferase DTWD1, the nucleolar protein NOL8, and the ribosomal RNA processing protein RRP15, were recently shown to redistribute to chromosomes during mitosis by single-cell immunofluorescence microscopy16 (Fig. 1f and Extended Data Fig. 1j). These findings indicate that integrating TMT–ABPP with other large-scale proteomic analyses can reveal how cell-state-dependent biomolecular interactions may promote site-specific changes in the chemical reactivity of proteins.

Mapping phosphorylation-dependent cysteine reactivity changes. We next set out to determine what fraction of cysteine reactivity changes in the mitotic cell proteome may be caused by S/T phosphorylation, which has been shown to be globally increased in the mitotic cell state to produce many high-stoichiometry phosphorylation sites51–54. We first showed that the majority of S/T phosphorylation events in the HeLa mitotic proteome could be removed by treatment with lambda phosphatase (LPP) by both immunoblot analysis (Fig. 2a) and MS-based phosphoproteomic analysis (Fig. 2b and Supplementary Dataset 1), without affecting protein abundances (Supplementary Dataset 1). We quantified over 11,000 phosphorylation sites, which had good overlap with previously quantified mitotic phosphorylation sites51–54 (Extended Data Fig. 2a). LPP treatment of the mitotic proteome generally did not affect protein abundance (Supplementary Dataset 1). The effective in vitro dephosphorylation by LPP enabled a robust and convenient protocol for a pairwise comparison of conditions—high phosphorylation (LPP-untreated (LPP(–)) mitotic proteome) and low phosphorylation (LPP-treated (LPP(+) mitotic proteome)—for cysteine reactivity profiling (Fig. 2c). With this method, we comparatively quantified >16,000 cysteines on ~5,600 proteins between LPP(–) and LPP(+) mitotic proteomes, an extent of coverage that compares favorably to previous TMT–ABPP studies55, indicating that the incorporation of an LPP treatment step did not substantially alter sensitivity. Across this global landscape of quantified cysteines, we found ~1,000 cysteines showing substantial (>twofold) differences in reactivity in the LPP(+) proteome (Fig. 2d and Supplementary Dataset 1).

To verify that the observed phosphorylation-dependent cysteine reactivity changes were not indirectly reflective of protein misfolding potentially caused by the LPP protocol, we also evaluated cysteine reactivity profiles in mitotic cell proteomes denatured with high molarity urea. Consistent with past studies, which have shown that protein misfolding radically alters cysteine reactivity profiles56, we found that ~75% of the ~15,500 quantified cysteines exhibited reactivity changes upon denaturation (Extended Data Fig. 2b and Supplementary Dataset 1). Importantly however, these denaturation-induced changes in cysteine reactivity did not correlate with LPP-mediated cysteine reactivity changes (Extended Data Fig. 2c–f). On the contrary, we observed a population of LPP-induced increases in cysteine reactivity that were anti-correlated with the effects of denaturation (Extended Data Fig. 2c). Such cysteines showing decreased reactivity in the denatured proteome were also more likely than unchanging cysteines to reside in predicted disordered regions (blue, Extended Data Fig. 2g), which might indicate that solvent-exposed cysteines in disordered regions become aggregated or solvated by urea and less accessible to reaction with IA-DTB upon denaturation. In contrast, cysteines with increased reactivity in the denatured mitotic proteome were much less likely than unchanging cysteines to reside in predicted disordered regions56 (orange, Extended Data Fig. 2g), possibly indicating that these residues are buried in structured regions of proteins and consequently shielded from IA-DTB labeling until denaturation, which exposes them to reaction with the IA-DTB probe. We believe that these data, taken together, support that our LPP treatment protocol achieves global dephosphorylation while preserving most proteins in their folded state, allowing us to profile the effects of S/T phosphorylation on cysteine reactivity in native proteomes.

Most of the cysteine reactivity changes caused by LPP treatment were also observed as reactivity differences between mitotic and asynchronous cells (Fig. 2c,f), consistent with the much greater overall S/T phosphorylation in the mitotic cell state (Fig. 2a,b). The vast majority of LPP-induced changes in cysteine reactivity were site-specific in that other quantified cysteines within the same protein were unchanged (Fig. 2g). As one example, we show the cysteine reactivity profile for the actin-binding protein filamin B (FLNB), in which a single cysteine, C991, was substantially increased in reactivity in the mitotic proteome compared with the asynchronous proteome, as well as in the LPP(–) versus LPP(+) mitotic proteomes, whereas the many other quantified cysteines in this protein were unaltered across the comparison groups (Fig. 2b). As a converse case study, a site-specific reduction in reactivity was observed for C481 in the E3 SUMO-ligase PIAS1 in the mitotic/ LPP(–) proteome compared with the asynchronous or LPP(+) proteomes (Fig. 2i). The directionality of change for these examples is interpreted to reflect phosphorylation-dependent increases (for example, FLNB C991) or decreases (PIAS1 C481) in cysteine reactivity in the mitotic cell proteome.

While many of the differences in cysteine reactivity between LPP(–) and LPP(+) proteomes directionally aligned with the changes observed in mitotic versus asynchronous cells (Fig. 2c), there were instances of anti-correlated cysteine reactivity changes,
such as C246 in the stress-activated protein kinase MAP2K4, which displayed elevated reactivity in the asynchronous compared with the mitotic proteome, but decreased cysteine reactivity in the LPP(+) versus LPP(−) mitotic proteome (Fig. 2j). Other cysteines in MAP2K4 were generally unchanged across the comparison groups (Fig. 2j), indicating bidirectional, site-specific effects...
of phosphorylation and the mitotic cell state on C246 reactivity. In contemplating potential mechanisms for the observed reactivity profile of MAP2K4 C246, we noted that this residue is located in the ATP-binding pocket of the kinase (Fig. 2k) and that early mitotic cells have been reported to display elevated ATP content.[43,44] We furthermore found that gel filtration to remove small-molecule metabolites resulted in a site-selective increase in MAP2K4 C246 reactivity in mitotic, but not asynchronous, cell proteomes (Extended Data Fig. 2h,i). From these data, we propose a model in which MAP2K4 C246 reactivity is suppressed in mitotic cells by greater occupancy of the kinase active site by ATP (or ADP), and mitotic S/T phosphorylation may then counteract ATP/ADP binding to MAP2K4 (by, for instance, lowering the affinity of this interaction) such that LPP treatment leads to even greater ATP/ADP engagement, further decreasing MAP2K4 C246 reactivity. We also note that MAP2K4 has been shown to display an unusually high affinity for ATP among protein kinases (dissociation constant \(K_d = 1.5 \mu M\))[45], indicating that it could be particularly susceptible to high-occupancy nucleotide binding based on cell state-dependent changes in ATP/ADP concentration, though we cannot rule out that other metabolite interactions may also contribute to the altered MAP2K4 C246 reactivity in mitotic proteomes. Interestingly, the high-stoichiometry mitotic phosphorylation sites found in MAP2K4—S26, S90, and S394—differ from the canonical stress-activated phosphorylation sites (S257 and T261)[46,47]. Consistent with a model in which mitosis produces functionally distinct proteoforms of MAP2K4, we found that ultraviolet-irradiation-induced phosphorylation and activation of MAP2K4 were disrupted in mitotic, but not asynchronous HeLa cells (Fig. 2j). Finally, we noted that active-site proximal cysteines in other MAP2K proteins were also altered in mitotic and/or LPP-treated cell proteomes (Extended Data Fig. 2), suggesting a more generalized impact of S/T phosphorylation on altering the reactivity of ATP pocket cysteines in this kinase family.

Interpreting proximal phosphorylation-cysteine interactions. In analyzing the cysteine reactivity changes caused by LPP treatment in mitotic proteomes, we noted a strong enrichment in S/T-P sequences on tryptic peptides containing cysteines with apparent increases in reactivity following exposure to LPP (Fig. 3a,b). This outcome pointed to a potential technical problem with the original protocol for cysteine reactivity profiling because high-stoichiometry phosphorylation of a S/T residue on the same tryptic peptide as the quantified cysteine would produce a loss in MS-based proteomic signal that may be unrelated to a change in cysteine reactivity (Fig. 3c, top). We therefore sought to adapt the TMT–ABPP method to distinguish authentic versus artifactual changes in cysteine reactivity caused by proximal S/T phosphorylation events (Fig. 3c). We initially considered directly quantifying the phosphorylated peptides in our TMT–ABPP datasets, but very few peptides bearing both differential modifications (S/T phosphorylation and IA-DTB-labeled cysteines) were quantified in the search outputs (Supplementary Dataset 1), consistent with the low overall coverage of phosphorylated peptides in proteomic experiments that lack a specific enrichment step[48]. We therefore instead established an adapted TMT–ABPP protocol that identifies artifactual differences in cysteine reactivity by removing proximal S/T phosphorylation after IA-DTB probe labeling (Fig. 3c, bottom). Importantly, treatment with LPP after probe labeling does not affect IA-DTB–cysteine interactions, since they represent irreversible adducts. Specifically, mitotic cell proteomes were exposed to LPP or control buffer (LPP(+) or LPP(−)), followed by IA-DTB treatment and streptavidin-mediated enrichment of IA-DTB-labeled proteins; then, both samples were once again dephosphorylated with LPP (Fig. 3c,d), and proteins were digested with trypsin and processed as normal for MS analysis (Fig. 3c). We reasoned that, in this adapted protocol, authentic cysteine reactivity changes would be preserved following the second LPP treatment (Fig. 3c, red) and thus distinguished from artifactual cysteine reactivity changes (Fig. 3c, yellow), which would be removed by the second LPP treatment. We refer hereafter to the four different proteomic groups on the basis of their LPP treatment (− or +) relative to IA-DTB treatment (before, after): LPP(−,−), LPP(−,+), LPP(+,−), LPP(+,+), with the second position discerning the original protocol (LPP(−,−) and LPP(+,+)) from the adapted protocol (LPP(−,+) and LPP(+,+)).

The cysteine reactivity profiles were generally similar between the original and adapted TMT–ABPP protocols, as reflected in the reactivity ratios for LPP(−,−)/(+) (original) versus LPP(−,+)/ (+,+) (adapted) treatment groups (Fig. 3e). The most striking pattern of change was found for cysteines exhibiting LPP-induced increases in apparent reactivity using the original protocol (that is, low LPP(−,−)/(+) ratios), which split into two nearly equal subsets displaying either preservation (red dots, Fig. 3e) or loss (yellow dots, Fig. 3e) of this increased reactivity as reflected in their LPP(−,−)/(+) (+,+) ratios using the adapted protocol (Fig. 3f). We interpret the preservation of LPP-dependent increases in reactivity using the adapted protocol, as exemplified by PIAS1 C481 (Fig. 3g), as indica-
tive of authentic decreases in cysteine reactivity caused by S/T phosphorylation. In contrast, cysteines that no longer showed a change in reactivity using the adapted protocol, as exemplified by MAP2K1 C277, were considered artifactual events presumed to be associated with high-stoichiometry S/T phosphorylation sites on the same tryptic peptide as the quantified (and unchanging) cysteine.
reactivity (for example, MAP2K1 T286, Fig. 3b; also see Extended Data Fig. 3a,b, for other examples in which the proximal S/T phosphorylation sites were directly mapped by phosphoproteomics). We found that immunoblotting of IA-DTB-enriched proteins with a monoclonal antibody no. 9146 that recognizes an epitope on MAP2K1 containing D282 mirrored our chemical proteomic results, showing suppression of MAP2K1 signal in the LPP(−,−) group that is recovered in the other three groups exposed to LPP (Fig. 3i), consistent with loss of epitope binding due to T286 phosphorylation during mitosis (also see Extended Data Fig. 3c). Strikingly, immunoblotting with monoclonal antibody no. 12671, which recognizes a different epitope on MAP2K1, confirmed expression of MAP2K1 in LPP(−,−) groups and even supported high-stoichiometry phosphorylation through a band shift that is lost upon LPP treatment (Fig. 3i and Extended Data Fig. 3c).

Notably, we found that not only artifactual, but also authentic changes in reactivity were frequently associated with cysteines on the same tryptic peptide as S/T phosphorylation sites (Fig. 3j), pointing to the potential for proximal phosphorylation events to perturb the nucleophilicity of neighboring cysteines. PIAS1 C481, for instance, is found on a tryptic peptide with two S/P sequences, the phosphorylation of which may account for the decreased reactivity of C481 in mitotic cells (Fig. 3g). Even more surprising, using the adapted protocol, we also discovered masked phosphorylation-induced increases in cysteine reactivity, such as FLNA C1453, which resides on a tryptic peptide along with S1459, a high-stoichiometry mitotic phosphorylation site (Fig. 3k and Extended Data Fig. 3d). The profile of FLNA C1453 across different conditions underscored how its reactivity change would have been overlooked without the adapted protocol. For instance, C1453 shows an apparent decrease in reactivity in mitotic versus asynchronous cells and unchanged reactivity using the original LPP protocol. The adapted protocol clarifies this type of unexpected profile as reflecting phosphorylation-induced increases in cysteine reactivity (C1453 in FLNA; or see Extended Data Fig. 3e,f for SLAIN2 C152 as another example), counterbalanced by a proximal mitotic phosphorylation event on the same tryptic peptide (S1459 in FLNA; see Supplementary Dataset 1) that prevents visualization of heightened cysteine reactivity until the proteome is exposed to LPP both before and after treatment with IA-DTB.

Features of proteins with cysteine reactivity changes. Phosphorylation-dependent cysteine reactivity changes were found to be enriched on proteins with high-stoichiometry phosphorylation events mapped previously in phosphoproteomic studies of mitotic cells41 (Fig. 4a and Extended Data Fig. 4a), as well as on proteins with LPP-sensitive phosphorylation sites identified in this study (Extended Data Fig. 4b). These relationships support a model in which the observed cysteine reactivity changes are often caused by phosphorylation events on the same parent protein (versus being indirectly modulated by phosphorylation events on other proteins). Cysteines with phosphorylation-dependent changes in reactivity, as well as those changing in reactivity in mitotic versus asynchronous cells, were found on a diverse array of cell-cycle-related proteins43 (Fig. 4b and Extended Data Fig. 4c), and GO analysis44,46 revealed that these proteins were also enriched in cellular processes related to mitosis, such as microtubule formation and chromosome condensation, even after removing artifactual cysteine reactivity changes caused by proximal phosphorylation events on the same tryptic peptide (Fig. 4c).

High-stoichiometry phosphorylation events have been shown to frequently occur at intrinsically disordered regions of proteins47,48. Consistent with this past work, we observed that >50% of cysteines with authentic phosphorylation-dependent decreases in reactivity were located in predicted disordered regions49, which was a much greater fraction than all cysteines quantified in our chemical proteomic studies mapping cysteine interactions with electrophilic small molecules39,40,42,90,91 (Fig. 4c). Features of proteins with mitotic phosphorylation-dependent changes in cysteine reactivity. a. Proteins with phosphorylation-dependent cysteine reactivity changes (bottom) are enriched for high-stoichiometry mitotic phosphorylation sites7 (>50% occupancy; blue) compared with all quantified proteins (top). Proteins that only contained artifactual phosphorylation-dependent cysteine reactivity changes were excluded from the bottom pie chart. Proteins lacking sufficient data for phosphorylation stoichiometry calculation or exhibiting low stoichiometry (<50% occupancy) sites in phosphoproteomics datasets7 were grouped and labeled as ‘Low or unquantified stoichiometry’ (orange). b. KEGG cell cycle pathway (HA04110) diagram73 marking proteins with (1) cell state- and phosphorylation-dependent cysteine reactivity changes in light blue; (2) only cell state-dependent cysteine reactivity changes in dark blue; (3) only phosphorylation-dependent cell state reactivity changes in green, and (4) unchanged cysteine reactivities in gray. Proteins not quantified in our proteomic experiments are in white. c. GO cellular enrichment analysis of proteins with cell state- and phosphorylation-dependent cysteine reactivity changes.41 Proteins with only artifactual phosphorylation-dependent cysteine reactivity changes were omitted from analysis. d. Percentage of cysteines from the indicated categories that reside in predicted disordered domains (IUPreD score > 0.5) based on IUPreD24 analysis.92,93 Proteins with only artifactual phosphorylation-dependent cysteine reactivity changes were omitted from analysis. e. Percentage of cysteines from the indicated categories that are ligandable by cysteine-reactive electrophilic small molecules; >80% engagement, as determined in refs. 39,45,47. f. Fraction of ligandable cysteines from the indicated categories showing phosphorylation-dependent reactivity changes within the specified amino acid distances from an S/T-P site. For d-f, artifactual phosphorylation-dependent cysteine reactivity changes were omitted from analysis. g. X-ray crystal structure of KLC2 (PDB: 3EDT)90 with the phosphorylation-dependent cysteine reactivity change (C441) and the proximal serine in an S/T-P site (S445) highlighted. Bottom, tryptic peptide from KLC2 containing C441 (asterisk) and S445 (red). h. Box plot showing cysteine reactivity values across indicated comparison groups for quantified cysteines in KLC2. The horizontal black line for each cysteine marks the median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5× interquartile range for n = 2 (or more) independent experiments (circles). Dotted lines designate boundaries for 2-twowold changes.
ated by local changes in structure or formation of protein–protein interactions that are caused by proximal phosphorylation events. No such enrichment was observed for ligandable cysteines showing either phosphorylation-dependent increases in reactivity or phosphorylation-independent reactivity in the mitotic proteome (Fig. 4f). A similar profile was also observed for all cysteines showing phosphorylation-dependent increases in reactivity or phosphorylation-independent reactivity in the mitotic proteome (Fig. 4f).
ing authentic phosphorylation-dependent changes in reactivity (Extended Data Fig. 4d). Many other cysteines, however, were unaffected in reactivity by proximal phosphorylation events, as exemplified by the artificial category uncovered using the adapted protocol (Extended Data Fig. 4e), indicating that the potential impact of phosphorylation events on cysteine reactivity cannot be simply predicted by sequence proximity relationships.

Very few of the phosphorylation-dependent decreases in reactivity for ligandable cysteines occurred in proteins (or regions of proteins) with three-dimensional structures. One exception was C441 in the kinesin light chain KLC2, which has been found to react with electrophilic fragments in human T cell proteomes39 and resides in close proximity to S-P site S445–P446 (Fig. 4g). This CXXXSP sequence is conserved in other KLCs (for example, KLC1; see Extended Data Fig. 4f) and is located at a junction region of the carboxy-terminal TRP domain involved in binding cellular cargo for transport along microtubules35. Interestingly, ERK-mediated phosphorylation of S460 of KLC1 (corresponding to S445 of KLC2) increases affinity for the cargo protein calsthenin-1 (ref. 39), which has been suggested to reflect a phosphorylation-induced change in TRP conformation42. In mitotic cells, we only observed phosphorylation-dependent decreases in reactivity for KLC2 C441, while KLC1 C456 was unchanged (Fig. 4h and Extended Data Fig. 4g). These findings suggest that the C-terminal domains of KLC1 and KLC2 may be regulated by distinct kinases, and, consistent with a possible role for KLC2 in the cell cycle, this protein has been shown to bind to SMAD2 through its C-terminal domain during mitosis40. We also note the presence of additional phosphorylation-dependent changes in the reactivity of ligandable cysteines that were not obviously associated with proximal mitotic phosphorylation events, such as the decreases observed for the shared cysteine in the ATP-binding pocket of MAP2Ks (Extended Data Fig. 2). Taken together, our findings point to cysteines in proteins that, due to mitotic phosphorylation-dependent alterations in their reactivity, might show distinct susceptibility to covalent modification in actively dividing cells.

Finally, while we focused our efforts on mapping phosphorylation-dependent changes in cysteine reactivity that occurred specifically in mitotic cells, we noted rare examples of LPP-sensitive cysteines showing equivalent reactivity in asynchronous and mitotic cell proteomes. One such instance was C270 on the RNA-binding protein FXR2, which interestingly also possessed an LPP-sensitive phosphorylation event on S450 that was similar in magnitude between asynchronous and mitotic cell proteomes (Extended Data Fig. 4h,i). Considering that asynchronous cells exhibit a much less extensively phosphorylated proteome compared with mitotic cells, these findings indicate the potential of our method to discover phosphorylation-dependent cysteine reactivity changes across diverse landscapes of global protein phosphorylation.

Discussion

The molecular characterization of proteoforms presents a major challenge for the field of proteomics, as these protein variants are often dynamically regulated and produced at low concentrations in cells4. Enrichment strategies to isolate specific post-translationally modified states of proteins (for example, phosphopeptide enrichment) have proven effective at addressing, at least in part, this challenge44, but are still typically performed with bottom-up protocols, which are not well-suited to readout the effect of PTMs on many aspects of protein structure/function. Top-down proteomics, while generally limited at present to proteins of smaller size (<50 kDa)45,46, has underscored the importance of PTM cross-talk in regulating protein structure and function46,47,48. More generally, how individual proteoforms may differ in their chemical reactivity, a feature that impacts the potential for post-translational regulation49 and covalent engagement by small molecules50, remains poorly understood.

Here, we have addressed this question by developing a chemical proteomic method to globally assess the impact of S/T phosphorylation on cysteine reactivity in biological systems.

Our method leverages the broad substrate scope of LPP, which we found to globally strip proteomes of S/T phosphorylation events while maintaining proteins in a folded state, providing a convenient compare-and-contrast system for mapping phosphorylation-dependent changes in cysteine reactivity. Furthermore, by studying mitosis—a cell state rich in high-stoichiometry S/T phosphorylation events—we increased the probability of identifying such cysteine reactivity changes. When considering extending our method to additional biological systems in which fewer high-stoichiometry phosphorylation events may exist, we note that phosphorylation-induced increases in cysteine reactivity should be less dependent on stoichiometry due to the technical ease in detecting gain-of-signal changes. Future studies could also map tyrosine phosphorylation effects on cysteine reactivity by enriching proteins with anti-phosphotyrosine antibodies prior to cysteine reactivity profiling or investigate the effect of other PTMs (for example, N-linked/O-linked glycosylation, K acetylation, K/R methylation) on cysteine reactivity. Finally, LPP should find broader use as a tool for studying phosphorylation effects on other properties of proteins, such as protein complexation states48.

One limitation of our method is that the LPP treatment occurs in vitro, and therefore some phosphorylation-dependent changes in cysteine reactivity may have been lost after cell lysis. To the extent that the phosphorylated proteome can be experimentally controlled in cells (for example, by treatment with kinase or phosphatase inhibitors), in situ cysteine reactivity profiling using, for instance, caged electrophilic probes49, may provide a means to identify phosphorylation-dependent cysteine reactivity changes that are caused, for instance, by dynamic interactions or localization in the cell. As a bottom-up approach, our strategy also does not specifically assign the phosphorylation events responsible for causing changes in cysteine reactivity. Nonetheless, integrating our data with phosphoproteomic information suggests that the observed cysteine reactivity changes are frequently caused by phosphorylation events occurring on the same proteoform of a protein (versus being indirectly caused by phosphorylation events on other interacting proteins). Future studies in which candidate S/T sites are mutated to prevent (for example, alanine mutants) or mimic (for example, glutamate mutants) phosphorylation should allow for more conclusive assignment of phosphorylation events that cause cysteine reactivity changes. We additionally note the importance of relating phosphorylation-dependent changes in cysteine reactivity to potential effects on protein function, which could be explored in the future using cysteine-directed ligands and/or cysteine mutagenesis.

Some of the most striking phosphorylation-dependent changes in cysteine reactivity occurred on proteins with important roles in cell division. Filamin A (FLNA), for instance, which possesses two cysteines showing phosphorylation-dependent increases in reactivity (C1260, C1453; Extended Data Fig. 3d)—one of which (C1453) was only revealed using the adapted TMT–ABPP protocol—forms a complex with CDC25C to stimulate the activity of this phosphatase during mitosis45, and mutation of mitotic phosphorylation sites in FLNA, including S1459, impairs daughter-cell separation46. SLAIN2, another protein found to possess a phosphorylation-induced increase in reactivity (C152) regulates processive microtubule depolymerization in interphase cells48. Other cysteine reactivity changes may point to alternative functions for proteins in mitosis, such as for MAP2K4, where high-stoichiometry mitotic phosphorylation events at sites different from those regulated by canonical stress-activation pathways may create proteoforms with altered cysteine reactivity (C246) and sensitivity to stressors (for example, ultraviolet irradiation; see Fig. 2i).
We believe that proteomic methods relating the dynamic PTM-modified states of proteins to changes in cysteine reactivity have the potential to identify opportunities for proteoform-selective chemical probe development. Considering that the reactivity of cysteines can influence their covalent ligandability, an increase or decrease in cysteine reactivity associated with a specific phosphorylated proteoform of a protein may point to greater or lesser potential for targeting this cysteine with electrophilic small molecules. Phosphorylation may also affect reversible ligand binding to proteins, as was recently shown for the HPK1 kinase. We further speculate that the cysteine reactivity changes discovered herein may facilitate PTM crosstalk in cells, by, for instance, enabling phosphorylation events to influence the introduction of cysteine-related PTMs (for example, nitrosylation, sulfenylation, electrophilic ligation). Finally, we point out that the IA-DTBP probe used herein to map cysteine reactivity changes is a structurally simple compound and may overlook phosphorylation-dependent changes in the environment surrounding cysteines that could positively or negatively influence modification by more elaborated endogenous or exogenous small molecules. This concept could be explored in the future by using different types of cysteine-reactive probes or even probes targeting modified forms of cysteine (for example, sulfenyl-ation probes). In this way, the impact of dynamic phosphorylation events on cysteine modification and ligandability can be fully realized to understand and control proteoform function.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgments, 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/s41592-022-01398-2.

Received: 4 June 2021; Accepted: 14 January 2022;
Published online: 28 February 2022

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Methods

Cell culture and cell synchronization. HeLa cells were obtained from ATCC and were maintained at 37 °C with 5% CO₂. HeLa cells were cultured and synchronized in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS, Omega Scientific), penicillin (100 U/mL), streptomycin (100 μg/mL) and l-glutamine (2 mM). Cells were synchronized by a thymidine-nocodazole block. 2.2 × 10⁻² × 2.4 × 10⁹ cells were plated in a 15-cm dish. The next day, the medium was replaced with fresh medium with or without thymidine (2 mM thymidine, 15–16 mL) for mitotic and asynchronous cells, respectively. After 24 hours, the cells were rinsed twice with warm DPBS and resuspended in fresh medium (14 mL). After 14 hours, 0.5–0.6 × 10⁹ cells, nocodazole (100 mg/mL) or DMSO was added to the cells (1:10,000 vol/vol). After 13 hours, asynchronous cells were rinsed 1× with warm DPBS and resuspended in 13 mL of warm medium. Mitotic cells were loosely attached after 13 hours. Medium and floating cells were separated by centrifugation (400 g, 3 min), and the cells were rinsed 1× with warm DPBS, separated by centrifugation (400 g, 3 min), and resuspended in 1 mL of warm medium. After 30 minutes at 37 °C, cells were scraped on ice, rinsed 1× with ice-cold DPBS with phosphatase inhibitors (1 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate), then two plates of cells were transferred in 1 mL ice-cold DPBS with phosphatase inhibitors to an Eppendorf tube. Cells were centrifuged (8000 g, 5 min). DPBS was aspirated, and cell pellets were flash frozen in liquid nitrogen until further use.

Proteomics: asynchronous versus mitosis whole-proteome tandem mass tagging. TMT-experiment: sample preparation. For whole-proteome tandem mass tagging (TMT-experiment), all comparison groups were run in either duplicate or triplicate. Cell pellets from 2 × 15-cm plates were resuspended in 700 μL of DPBS with EDTA-free protease inhibitors (Sigma) and PhosStop (Sigma). All samples were lysed by using a Branson Ultrasonics Sonifier S-250A cell disruptor (~2 × 8 pulses, 35% duty cycle, output setting = 3.5). Following lysis, cell lysates were centrifuged at 9000 g for 10 min. Protein concentration was determined with the DC Protein Assay (Bio-Rad), and the absorbance was measured using a CLARIOstar microplate reader following manufacturer’s instructions. Protein concentration was diluted to 2 mg/mL in 100 μL. Next, 400 μL of ice-cold 1:3 methanol:CHCl₃ was added to each sample followed by 200 μL of water. The mixture was vortexed and centrifuged (9000 g, 3 min) to afford a protein disc at the interface of CHCl₃ and aqueous layers. Both layers were aspirated without perturbing the disk, which was gently rinsed 1× with cold methanol (300 μL) and then resuspended in cold methanol (300 μL) by brief sonication. The proteins were pelleted (9000 g, 5 min, 4 °C), and the resulting pellets were air dried.

TMT-experiment: trypsin digestion and TMT-labeled. Dried protein pellets were resuspended in 100 μL EPPS buffer (200 mM, pH 8) containing proteomics-grade urea (8 M) and DTT (10 mM) by either vortexing or bath sonication for 5 minutes, 4 °C), and the resulting pellets were air dried. samples were diluted with wash buffer (350 μL, 500 mM solution in H₂O) as previously described.

TMT-experiment: LC-MS analysis. Samples were desalted and fractionated as previously described. In brief, samples were desalted in buffer A (1 mL, 95% H₂O, 5% CH₃CN, 0.1% formic acid) by bath sonication (5 minutes) and desalted by passing through Sep-Pak C18 cartridges (55–105 μm, Waters). After eluted peptides were dried by vacuum centrifugation, peptides were resuspended in buffer A (510 μL) by bath sonication and fractionated into 96 deep-well plate using HPLC (Agilent) using a gradient between 10 mM sodium bicarbonate in water and 100% acetonitrile, at a flow rate of 0.2 μL/min. Samples were then collected and analyzed by liquid chromatography tandem mass spectrometry using an Xcalibur v4.0 software on an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000 Series Rapid Separation LC system and autosampler (Thermo Scientific Dionex), using the same capillary column, flow rate, and gradient as previously detailed. Methods of data acquisition are as previously described.

Cysteine residues were searched with a static modification for carboxymethylmethionine (+57.02146 Da). Amino-termini and lysine residues were also searched with a static modification corresponding to the TMT tag (+229.1629 Da). All other relevant settings are detailed in Vinogradova et al. Proteins were required to have at least two unique quantified peptides in an independent experiment for quantification. Quantification in at least one experimental replicate was required for interpretation.

Proteomics: asynchronous versus mitosis cysteine reactivity profiling (TMT-ABPP). TMT-ABPP: sample preparation. All comparison groups were run in either duplicate or triplicate. Cell pellets from 2 × 15-cm plates were resuspended in 700 μL of DPBS with EDTA-free protease inhibitors (Sigma) and PhosStop (Sigma). Samples were lysed, centrifuged, and protein concentration measured as described in ‘TMT-experiment: sample preparation’. Protein concentration was diluted to 2 mg/mL in 500 μL, and samples were then treated with iodoacetamide polychloroethylene dinitrophenyl hydrazine (5 μL of 5% solution in DMSO, final concentration: 100 μM) or iodoacetamide alkyne (IA-alkyne, synthesized in house as previously described) 5 μL of 10 mM stock in DMSO, final concentration: 100 μM, for 1 hour at ambient temperature with end-over-end rotation. For samples treated with IA-alkyne, alkylated proteins were then conjugated to deshiothiobiotin-PEG3-azide (N3-DTβ BroadPharm) using copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC). Reagents for the CuAAC reaction were pre-mixed prior to their addition to the proteome samples: 100 μM N3-DTβ (5 μL of 10 mM in DMSO), 100 μM tris(benzyltriazolylmethyl)amine ligand (TBTA; 30 μL of 1.7 mM stock in DMSO for tert-butanol 1:1), 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCPP; 50 μL of fresh 50 mM stock in H₂O), 10 μL of 50% M N₃-DTβ stock in water) were combined, vortexed and added to the proteomes (55 μL/sample), vortexed, and incubated at RT for 1 h. For all samples, 600 μL of ice-cold 4:1 methanol:CHCl₃ was then added, and the mixture was vortexed and centrifuged (9000 g, 3 min) to yield a protein disc at the interface of CHCl₃ and aqueous layers. Both layers were aspirated without perturbing the disk, which was gently rinsed 1× with cold methanol (1 mL) and aspirated. Pellets were flash frozen at −80 °C until the following day.

TMT-ABPP: trypsin digestion and streptavidin enrichment. Samples were resuspended in cold methanol (300 μL) by sonication, centrifuged (10,000 g, 10 minutes, 4 °C), and the resulting pellets were allowed to air dry. Samples were then resuspended in 90 μL of buffer containing 9 M urea, 10 mM DTT in triethylammonium bicarbonate (TEAB) buffer (50 mM, pH 8.5), and cysteines were alkylated with iodoacetamide (100 μL, 500 mM solution in H₂O) as previously described. Following alkylation, samples were diluted with 300 μL of triethylammonium bicarbonate buffer (50 mM, 1/20 dilution of 1.0 M stock, pH 8.5) and vortexed. If precipitate was present in samples, samples were probe sonicated until minimal precipitation (typically 10 pulses, 40% and output 4). Trypsin (8 μL of 0.25 μg/μL trypsin in trypsin buffer, containing 25 mM CaCl₂) was then added, and the digestion proceeded at 37 °C overnight. The following day, samples were diluted with wash buffer (350 μL, 50 mM TEAB, 150 mM NaCl, 0.2% NNP, containing streptavidin beads (40 μL) for 10 minutes per sample) and the bead mixture was rotated for 2 hours at room temperature or overnight at 4 °C. Beads were transferred to a BioSpin column affixed to a vacuum, and washed (2 × 1 mL wash buffer, 5 × 1 mL PBS, 5 × 1 mL H₂O). Peptides were eluted into a Protein LoBind Eppendorf tube (VWR) by the addition of 300 μL of 50% aqueous acetonitrile containing 0.1% formic acid. The eluate was then evaporated to dryness using SpeedVac vacuum concentrator.

TMT-ABPP: TMT labeling. TMT labeling and mass spectrometry were performed as previously described. Briefly, peptides were resuspended in 100 μL EPPS/
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TMT–ABPP: LC–MS analysis. Peptides were analyzed by MS and assigned as described in ‘TMT-exp: LC–MS analysis,’ in addition to searching cysteine residues with a differential TMT phosphopeptide modification (IA-DTB). Cysteine residues with static modifications were searched as described in ‘TMT–ABPP: trypsin digestion and streptavidin enrichment’ with the following changes. instead of searching cysteine residues with static modifications, all cysteine residues were searched as predicted to form 1 LPP (+) (collection of all LPP (+) peptides), 1 LPP (−) (all LPP (−) peptides), 1 LPP (+) + LPP (−) (both LPP (+) and LPP (−) peptides), 1 LPP (+) − LPP (−) (only peptides with differential reactivity), and 0 LPP (+) and 0 LPP (−) (no differential reactivity). The median value of all its peptides was also 1.5-fold changing or the protein expression was changing 1.5-fold in the same direction. For proteins with 1–2 quantified peptides, a cysteine was considered to have a change in expression if its peptide ratio differed more than twofold and the corresponding protein expression changed at least 1.5-fold.

Proteomics: LPP (+) versus LPP (−) (original LPP TMT–ABPP protocol). Original LPP TMT–ABPP: sample preparation. All comparison groups were run in either duplicate or triplicate. Cell pellets from 2 × 15-cm plates of mitotic and asynchronous cells were resuspended in 700 μL of lysis buffer (DPSB with EDTA-free protease inhibitors (Sigma)). Cell pellets were split in half, 10 μL of PhosStop in DPBS (Sigma) was added to a final concentration of 1 × to all LPP (+) samples, and the same volume of DPBS was added to all LPP (−) samples. Samples were lysed, clarified, and protein concentration measured as described in ‘TMT–ABPP: sample preparation.’ Mitotic and asynchronous samples were then washed three times with DPBS by centrifugation (15,000 × g, 30 seconds) and were capped and suspended peptides were added to the column and gently flicked to mix. For a total of 30 minutes of incubation at room temperature, every 10 minutes, samples were gently mixed. Samples were then washed 3x with wash buffer, 1x with water, 1x with 500 μL of lysis buffer, and samples were eluted into fresh Eppendorf tubes with Elution buffer. Samples were then dried immediately by speed vacuum. Note that the elution buffer contains ammonium hydroxide, so samples were dried thoroughly.

TMT–ABPP: cysteine reactivity ratio calculation. At the individual TMT experiment level, the following filters were applied to remove low-quality peptides: removal of half-tryptic peptides with the exception of those at the beginning and end of protein sequences; removal of peptides with more than one internal missed cleavage site, allowing for missed tryptic sites if they are immediately followed/ preceded by another charged amino acid (such as different charge states, tryptic termini, high pH fractionation fractions) were grouped together, and the median ratio value was reported for a given TMT experiment. Representative median ratio values for a given peptide from each TMT experiment was computed. If the median ratio value for that given peptide were inverted for s.d. and mean calculation only. The median value was reported unless the s.d. was greater than 60% of the mean. For peptides quantified in fewer than 4 replicates, peptides that had standard deviations greater than 60% of the mean were not interpreted unless at least 80% of ratios changed by 1.5-fold. The overall median of all TMT replicates was reported alongside the shortest quantified tryptic peptide sequence. All ratios were log-transformed for presentation. For interpretation in Fig. 1 and Extended Data Fig. 1, cysteine-containing peptides were required to be quantified in at least two replicates.

TMT–ABPP: categorization of cysteine reactivity changes. For Fig. 1d, categorization of cysteine changes was determined as previously described with minor changes. For proteins with 5 or more quantified peptides, cysteine changes were considered reactivity-based if (1) the peptide ratio (Mitosis/Asynch) value differed more than twofold from both the median ratio value of all quantified cysteines on the same protein and, if available, the protein expression ratio (Mitosis/Asynch) measured in TMT-exp experiments and (2) the protein had at least 1 unchanging cysteine (<1.5-fold). Unchanging cysteines (<1.5-fold change) were considered to have a reactivity change if (1) they differed twofold in both the median ratio value of all quantified cysteines on the same proteins or protein expression level measured in TMT-exp experiments (if available) and (2) the median ratio value of all quantified cysteines also changed by at least twofold. For proteins with 3–4 quantified peptides, cysteine changes were considered reactivity-based if (1) the peptide ratio (Mitosis/Asynch) value differed more than twofold from both the median ratio value of all quantified cysteines on the same protein or from the protein expression level measured in unenriched proteomics or was two times greater from the other peptide that was also required to be unchanging or changing in the opposite direction. For proteins with 3–4 peptides, a cysteine was considered to have a change in expression if its peptide ratio value differed more than twofold from the protein expression level measured in unenriched proteomics or was two times greater from the other peptide that was also required to be unchanging or changing in the opposite direction. For proteins with 3–4 peptides, a cysteine was considered to have a change in expression if at least one cysteine was changing twofold and the median value of all its peptides was also 1.5-fold changing or the protein expression was changing 1.5-fold in the same direction. For proteins with 1–2 quantified peptides, a cysteine was considered to have a change in expression if its peptide ratio differed more than twofold and the corresponding protein expression changed at least 1.5-fold.

Proteomics: asynchronous, mitosis LPP (+), and mitosis LPP (−) TMT phosphopeptide enrichment. TMT phosphoproteomics: sample preparation. All comparison groups were run in duplicate. Cells were lysed, protein concentrations were determined, and appropriate samples were dephosphorylated as described in ‘Original LPP TMT–ABPP: sample preparation.’ Proteins were then precipitated as described in ‘Original LPP TMT–ABPP: sample preparation.’ Whole protein expression TMT-exp was performed for all samples in parallel as described in ‘TMT-exp: sample preparation,’ ‘TMT-exp: trypsin digestion and TMT labeling,’ ‘TMT-exp: high pH offline fractionation,’ ‘TMT-exp: LC–MS analysis,’ and ‘TMT-exp: protein ratios calculation.’

**TMT phosphoproteomics: phosphopeptide enrichment.** Samples were processed and digested with trypsin according to the protocol described in ‘TMT–ABPP: trypsin digestion and streptavidin enrichment’ with the following changes. Instead of proceeding to streptavidin enrichment, samples were diluted with 450 μL of buffer A (1 mM, 95% H2O, 5% acetonitrile, 0.1% formic acid) by bath sonication (~5 minutes). Formic acid was added until a final pH of < 3. Some precipitate was present upon acidification. Samples were centrifuged and supernatant was desalted by passing through Sep-Pak C18 cartridges (55–105 μm, Waters), eluted and dried into a Protein LoBind Eppendorf tube (VWR) as described in ‘TMT-exp: high pH offline fractionation and LC–MS analysis.’

Phosphopeptides were enriched using High-Select Fe–NTA Phosphopeptide Enrichment Kit (Thermo Scientific) using manufacturer’s instructions. In brief, dried peptides were resuspended in binding wash buffer (200 μL) by bath sonication for 5 minutes. Columns were equilibrated twice with Binding/Wash buffer (1,000 g, 30 seconds). Columns were capped and suspended peptides were added to the column and gently flicked to mix. For a total of 30 minutes of incubation at room temperature, every 10 minutes, samples were gently mixed. Columns were washed 3x with wash buffer, 1x with water, 1x with 500 μL of lysis buffer, and samples were eluted into fresh Eppendorf tubes with Elution buffer. Samples were then dried immediately by speed vacuum. Note that the elution buffer contains ammonium hydroxide, so samples were dried thoroughly.

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TMT phosphoproteomics: TMT labeling and high pH offline fractionation. Peptides were TMT-labeled as described in TMT-exp – trypsin digestion and TMT labeling with the following exceptions. Peptides were resuspended in 100µL EPPS/CH3CN buffer – 200 mM, pH 10 (with CH3CN), LPP-phosphorylation, and TMT Tags (10 µL channel in dry CH3CN, 20 µg/µL) were added to the corresponding tubes and the reaction was allowed to proceed at room temperature for 60 minutes. The reaction was quenched by the addition of 5% hydroxylamine (5µL per sample), acidified with formic acid (5 µL), and then samples were combined and dried in a SpeedVac vacuum concentrator and kept at −80 °C until the high pH fractionation step. Samples were fractionated as described in 'TMT-exp: high pH offline fractionation'.

TMT phosphoproteomics: LC–MS analysis. Peptides were analyzed by MS and assigned as described in 'TMT-exp: LC–MS analysis', in addition to searching cysteine residues with a static modification for carboxymethylation (+57.0255 Da) and cysteine residues with a differential modification corresponding to the TMT tag (+2,296.1269 Da), serine and threonine residues were also searched with a differential modification for phosphorylation (+97.9663 Da), with a total of two differential modifications allowed for quantification. Peptides were required to be at least six amino acids long, and to have at least one tryptic terminus. ProLuCID data was filtered through DTASelect (version 2.0) to achieve a spectrum false positive rate below 1%. The MS3-based peptide quantification was performed with reporter ion mass tolerance set to 20 ppm with Integrated Proteomics Pipeline (IP2).

Peptide ratios were calculated for Mitosis/Asynch and LPP(−)/LPP(+) as described in 'TMT-ABPP: cysteine reactivity ratio calculation'. For interpretation in Fig. 1, Extended Data Figs. 3 and 4, and Table 1, all comparison groups were standardized as described in 'TMT–ABPP: sample preparation' . Denatured samples were brought to room temperature, then were treated with IA-DTB, followed by protein precipitation as described in 'TMT-ABPP: sample preparation'. Denatured TMT-ABPP: LC–MS analysis. Samples were processed according to the protocol described in 'TMT-ABPP: sample preparation'. Following the labeling with iodoacetamide, samples were re-suspended in 300 µL of lysis buffer (DPBS with EDTA-free protease inhibitors (Sigma)). Samples were lysed and clarified, and protein concentration was determined as described above. Then, 1 mg of proteome was brought up in 300 µL of lysis buffer (DPBS with 1% CH3CN) and 150 µM NaCl, then heated to 65 °C for 15 minutes. Native samples were kept on ice. All samples were then allowed to come to room temperature, then were treated with IA-DTB, followed by protein precipitation as described in 'TMT-ABPP: sample preparation'. Denatured samples had fragile protein disks, or protein pellets at the bottom of the tube. Solvent was aspirated carefully, re-suspended in 1 mL methanol, and vortexed. Precipitated proteins were pelleted (16,000 g for 10 minutes at 4 °C) and methanol was aspirated. Protein pellets were allowed to air dry.

Denatured TMT-ABPP: LC–MS analysis. Samples were processed according to the protocol described in 'TMT-ABPP: trypsin digestion and streptavidin enrichment'. TMT–ABPP: tag removal and TMT high pH offline fractionation. Samples were processed according to the protocol described in 'TMT-ABPP: cysteine reactivity ratio calculation'. For interpretation in Supplementary Fig. 2, cysteine-containing peptides were required to be quantified in one replicate.

Proteomics: mitotic native versus denatured cysteine reactivity profiling (TMT–ABPP). Denatured TMT-ABPP: sample preparation. All comparison groups were run in triplicate. Cell pellets from 1 × 15-cm plates of mitotic and asynchronous cells were re-suspended in 300 µL of lysis buffer (DPBS with EDTA-free protease inhibitors (Sigma)). Samples were lysed and clarified, and protein concentration was determined as described above. Then, 1 mg of proteome was brought up in 320 µL of lysis buffer, and 240 µg of urea was added to samples to be denatured, and 180 µL of DPBS was added to samples preserved in native state (final volume 500 µL). Denatured samples were vortexed and heated to 65 °C for 15 minutes. Native samples were kept on ice. All samples were then allowed to come to room temperature, then were treated with IA-DTB, followed by protein precipitation as described in 'TMT-ABPP: sample preparation'. Denatured samples had fragile protein disks, or protein pellets at the bottom of the tube. Solvent was aspirated carefully, re-suspended in 1 mL methanol, and vortexed. Precipitated proteins were pelleted (16,000 g for 10 minutes at 4 °C) and methanol was aspirated. Protein pellets were allowed to air dry.

Denatured TMT-ABPP: LC–MS analysis. Samples were processed according to the protocol described in 'TMT-ABPP: trypsin digestion and streptavidin enrichment'. TMT–ABPP: tag removal and TMT high pH offline fractionation. Samples were processed according to the protocol described in 'TMT-ABPP: cysteine reactivity ratio calculation'. For interpretation in Supplementary Fig. 2, cysteine-containing peptides were required to be quantified in one replicate.

Proteomics: LPP(−)/LPP(+) versus LPP(−,−)/LPP(−,+) and LPP(−,−)/LPP(−,−) (adapted LPP TMT–ABPP) protocol. Adapted LPP TMT-ABPP: sample preparation. Samples were processed as described in Original LPP TMT–ABPP: sample preparation. Samples were then resolubilized as described in Original LPP TMT–ABPP: sample preparation, but also included the addition 25 µL SDS (10% in water). Samples were reduced and alkylated as described in Original LPP TMT–ABPP: sample preparation. Following the labeling with iodoacetamide, samples were diluted with 350 µL of wash buffer (50 mM TEAR, 150 mM NaCl, 0.1% NP–40). If precipitate was present in samples, samples were probe sonicated until minimal precipitation (typically 10 pulses, 35% and output 3). Samples were diluted with wash buffer (300 µL, 50 mM TEAR, 150 mM NaCl, 0.1% NP–40) containing streptavidin-agarose beads (20 µL/compact bead per sample), and the bead mixture was rotated for 2 hours at room temperature or overnight at 4 °C. After incubation, the beads were pelleted by centrifugation (2,000g, 1 min), and washed with wash buffer (2 × 0.75 mL 50 mM TEAB containing 1% NP–40, 150 mM NaCl and 0.5% Triton X-100) and DPBS (2 × 0.75 mL). Samples were rinsed one time with 1× PMP buffer (NER, 100 µL) and re-suspended in 100 µL of 1× PMP buffer containing 1 mM Cre and 30% (v/v) PEG 6000 to both solubilize and enrich LPP(−) samples, respectively. Samples were flicked gently, taking care to ensure beads did not get stuck on the Eppendorf tube walls. Samples were laid flat at 30 °C for 1 hour, then washed with DPBS (2 × 1 mL) and re-suspended in 150 µL of TEAR (50 mM). Tryptsin (4 µL of 0.25 µg/µL trypsin in trypsin buffer, containing 25 mM CaCl2) was then added and the samples were digested at 37°C overnight. Following digestion, samples were diluted with wash buffer (500 µL, 50 mM TEAR, 150 mM NaCl, 0.2% NP–40) transferred to a BioSpin column affixed to a vacuum. Streptavidin enrichments were washed and peptides eluted as described in 'TMT-ABPP: trypsin digestion and streptavidin enrichment'. Samples were processed according to the protocol described in 'TMT-ABPP: TMT labeling', 'TMT-ABPP: LC–MS analysis', and 'TMT-ABPP: cysteine reactivity ratio calculation'.

Adapted LPP TMT–ABPP: data filtering and processing. The adapted protocol processed four groups simultaneously, LPP(−), LPP(+), LPP(+,−), and LPP(+,−). While the LPP(−)/(+) ratio (LPP after IA-DTB labeling) can differ legitimately from the original protocol (no LPP after IA-DTB labeling) (detailed above), the LPP(−)/LPP(+) (LPP before IA-DTB labeling) should, in principle, be equivalent to the LPP(−)/(+) ratios of the original protocol, with any differences pointing to technical or biological variation. If the LPP(−)/(+) ratio was not quantified or it varied by more than twice the original LPP(−)/(+) ratio, the LPP(−)/(+) ratio (LPP after IA-DTB labeling) was flagged for manual review. For a flagged peptide had an original LPP(−)/(+) ratio (LPP after IA-DTB labeling) that exhibited phosphorylation-dependent reduction in cysteine reactivity (1.6-fold change in reactivity in LPP(−,−) versus LPP(−,+) and 2) artificial changes were defined as those that had less than 1.6-fold change in reactivity in LPP(−,−) versus LPP(−,+) and LPP(−,−)/LPP(−,+) replicates. All other flagged peptides were manually inspected.

Adapted LPP TMT–ABPP: categorization of cysteine reactivity changes. For cysteines that exhibited phosphorylation-dependent reduction in cysteine reactivity (twofold), reactivity changes were categorized as follows: (1) authentic changes were defined as those that maintained at least a 1.8-fold decrease in reactivity in LPP(−,−)/LPP(−,+) and (2) artificial changes were defined as those that had less than 1.6-fold change in reactivity in LPP(−,−) versus LPP(−,+) and LPP(−,−)/LPP(−,+) replicates.
over LPP(–,−) but less than 1.6-fold reduction in LPP(−,+) versus LPP(+,+) were removed from analysis. The proteins containing cysteines that passed these filters were used for enrichment of GO cellular process terms. The background list represented proteins that had at least one cysteine that was quantified in at least two LPP(−,−), TMT–ABPP replicates (from either the original or adapted protocol).

For Extended Data Fig. 1, proteins with cysteine reactivity changes in mitotic versus asynchronous cell proteomes that were reactivity-based (as opposed to expression-based, as defined in TMT–ABPP) were categorized as reactivity changes based on the reactivity change in LPP versus asynchronous cell proteomes. The background list represented proteins that had at least one cysteine that was quantified in at least two Mitosis/Asynchronous TMT–ABPP replicates.

All GO analyses were performed with Webgestalt (http://www.webgestalt.org) using the significance level (FDR < 0.05)45. The minimum and maximum number of genes for a given category were set at 5 and 2,000, respectively. Benjamini–Hochberg was used for q-value adjustment. Enriched terms were then passed through REVIGO (http://revigo.irb.hr/), which identifies redundant terms and chooses representative terms for each group46. The following settings were used with REVIGO: allowed similarity of 0.5: searched against the human Uniprot database; using the SimRel semantic similarity measure45.

**Immunoblot analysis.** Sample preparation for immunoblot analysis of cell states with LPP treatment. First, 1 × 10⁶ HeLa cells were seeded in 10-cm dishes for 24 hours after seeding, medium was replaced with new medium containing thymidine (2 mM). After 18 hours, cells were washed twice with warm DPBS and released into fresh medium. After 9 hours, medium was replaced with medium containing thymidine (2 mM) was reintroduced. At 13 hours later, cells were collected and flash frozen in liquid nitrogen. Mitosis groups were synchronized using a thymidine-nocodazole block and asynchronous groups underwent washing and medium changes at the same time as mitosis groups. After 24 hours, medium was replaced with new medium for asynchronous groups and medium containing thymidine (2 mM) for mitosis groups. After 24 hours, cells were collected and flash frozen in liquid nitrogen.

Cell pellets were resuspended in 250 μl lysis buffer (DPBS with EDTA-free protease inhibitors (Sigma)). Cell pellets were split in half, 10× PhosStop in DPBS (Sigma) was added to a final concentration 1× to all LPP(+) samples and the same volume of DPBS was added to mitosis groups and asynchronous groups, respectively. At 13 hours later, cells were rinsed once with DPBS and released into fresh medium. Then, 30 minutes later, cells were collected and flash frozen in liquid nitrogen.

Sample preparation for immunoblot analysis of ultraviolet treatment in asynchronous vs mitotic cells. Cells were synchronized in mitosis as described in ‘Sample preparation for immunoblot analysis of cell states with LPP treatment’ but directly after release from nocodazole, cells were then ultraviolet irradiated (60 J/m²) and incubated for 30 minutes. Cells were then collected and flash frozen in liquid nitrogen. Cell pellets were resuspended in 250 μl lysis buffer with 1× PhosStop. Samples were processed for immunoblot analysis as described in ‘Sample preparation for immunoblot analysis of cell states with LPP treatment’.

Sample preparation for immunoblot validation of adapted LPP TMT–ABPP protocol. Cells were synchronized, lysed, and labeled with IA-DTBP. Modified proteins were enriched for desphosphorylation as described in ‘Sample preparation for immunoblot analysis of cell states with LPP treatment’ but sample preparation diverged after LPP dephosphorylation. After the second LPP treatment, Samples were washed twice with DPBS and 60 μl of 4X loading dye was added to beads. Samples were briefly boiled and then stored at −20°C.

**Immunoblot analysis.** Proteins were resolved by SDS–PAGE, transferred to 0.45 μM PVDF membranes (GE Healthcare) which were blocked with 5% milk in TBST buffer (20 mM Tris–HCl 7.6, 150 mM NaCl with 0.1% tween 20). Primary antibodies were used at the following concentrations: 1:10,000 anti-GAPDH (Santa Cruz, sc-47724), 1:10,000 anti-ACTB–HPR (Santa Cruz, sc-7761), 1:2,000 anti-phospho-p53 (Cell Signaling, no. 9252), 1:2,000 anti-phospho-p38 (Cell Signaling, no. 4511), 1:2,000 p38 (Cell Signaling, no. 8690), 1:500 CCNB1 (Santa Cruz, sc-245), 1:500 CCNE1 (Santa Cruz, sc-247), 1:1,000 anti-SpTP (Cell Signaling, no. 5243), 1:1,000 anti-MAP2K4 (Cell Signaling, no. 9152), 1:1,000 anti-phospho-MAP2K4 (Cell Signaling, no. 9156), 1:500 anti-MAP2K1 (Cell Signaling, no. 9146), and 1:500 anti-MAP2K1 (Cell Signaling, no. 12671). Blots were incubated with primary antibodies (with the exception of anti-ACTB–HPR) in either 5% BSA in TBST or 5% milk in TBST at 4°C overnight. Membranes were washed with TBST (3 times, 5 minutes), developed with ECL western blotting detection reagent kit (Thermo Scientific) and recorded on CL-XPosure film (Thermo Scientific) and detected with Bio-rad ChemiDoc XRS, or recorded on CL-XPosure film (Thermo Scientific) and detected with Konica Minolta SRX 101 X-ray film processor.

**KEGG cell cycle pathway containing proteins with cysteines quantified by TMT–ABPP.** The KEGG cell cycle pathway (hsa04110) in Fig. 4 was regenerated using gene names used in TMT–ABPP datasets and color coding on the basis of cysteine reactivity47. Members of the KEGG cell cycle pathway that cysteines quantified by TMT–ABPP in two replicates of either the mitosis versus asynchronous dataset or the LPP(−) versus LPP(+) dataset were marked in gray, proteins with cysteine reactivity changes in LPP(−) versus LPP(+) (twofold) were marked in green, in mitosis versus asynchronous (twofold) were marked in blue, and proteins with cysteine reactivity changes in both LPP(−) versus LPP(+) (twofold) and mitosis versus asynchronous (1.6-fold) were marked in light blue. Proteins not quantified were in white.

**Analysis of high occupancy phospho-proteins.** Proteins containing cysteines with cysteine reactivity changes in both LPP(−) vs LPP(+) (twofold) and in mitosis versus asynchronous (>1.6-fold) were cross-referenced to a list of proteins with high occupancy mitotic phosphorylation sites (250%) were taken from Sharma et al.48.

**Analysis of predicted disordered regions.** For Fig. 4d, ‘Cysteines from all proteins’ refers to all cysteines that were quantified in at least two replicates of the LPP(−) versus LPP(+) TMT–ABPP dataset. ‘Phosphorylation-dependent cysteine reactivity changes’ refers to cysteines changing at least 1.6-fold with LPP treatment and changing at least 1.6-fold in mitosis versus asynchronous with those that no longer had phosphorylation-dependent changes with LPP after IA-DTBP labeling removed. Cysteines labeled as ‘Increase’ have twofold more reactivity in LPP(+) and cysteines that are ‘Decrease’ have twofold more reactivity in LPP(−). For Extended Data Fig. 2g, ‘Lower’ and ‘Higher’ refer to cysteines with twofold more or less reactivity in native mitotic proteome relative to mitotic proteome denatured by high molarity urea. ‘Unchanging’ refers to cysteines that change less than twofold upon denaturation.

For all analyses, protein sequences for proteins containing said cysteines were obtained from the same FASTA database used for peptide assignment, namely a reverse concatenated, non-redundant variant of the Human UniProt database (release-2016_07). Protein disorder for the protein sequences was predicted using the tool IUPred2A (download March 2020), which outputs a value from 0 to 0.5 was used to categorize likely disordered regions.

**Analysis of ligandability.** ‘Cysteines from all proteins’, ‘Increase’, and ‘Decrease’ in phosphorylation-dependent cysteine reactivity change are described as ‘Analysis of predicted disordered regions’. Ligandability data were taken from Vinogradova et al., Bar-Peled et al., and Backus et al.49,50,51. Cysteines were considered liganded if they were engaged at least 80% by the small molecule ligands listed in the indicated papers. Cysteines that changed at least twofold with LPP treatment and that changed at least 1.6-fold in mitosis versus asynchronous were grouped and those that no longer had phosphorylation-dependent changes with LPP after IA-DTBP labeling were removed.

**Analysis of (S/T)P phosphorylation.** For Fig. 4i, ‘All liganded cysteines’ are described as ‘Analysis of ligandability’. Of these cysteines, cysteines were defined as ‘Increase’ or ‘Decrease’ in phosphorylation-dependent cysteine reactivity change based on the same criteria as for Fig. 4d in ‘Analysis of predicted disordered regions’.

For Extended Data Fig. 4d, categories are as defined in ‘Analysis of predicted disordered regions’. For Extended Data Fig. 4e, categories are as defined in ‘Adapted LPP TMT–ABPP: categorization of TM–TAPBB cysteine reactivity changes’. In brief, authentic changes were defined as cysteines with at least twofold decrease in LPP(−) versus LPP(+) (+,+), and changing at least 1.6-fold in mitosis versus asynchronous with those that no longer had phosphorylation-dependent changes with LPP after IA-DTBP labeling were removed.
Phosphoproteomic comparison to literature resources. Phosphoproteomic data were taken from Sharma et al. Only phosphoserines and phosphothreonines were considered for analysis, and phosphosites from Sharma et al. were required to have intensities in either mitotic or asynchronous samples. UniProt accession IDs from Sharma et al. were mapped to IDs from a reverse concatenated, non-redundant variant of the Human UniProt database (release-2016_07) used for proteomic analysis in this paper. Sequence windows reported in Sharma et al. were mapped onto the respective protein sequence from the 2016 database. Phosphosites were considered if they were within a sequence of at least 15 consecutive amino acids (of the 31-amino-acid window) that matched the 2016 database sequence. Phosphosite positions from Sharma et al. were remapped to the 2016 database and compared to phosphosites quantified in at least one replicate of either LPP(−)/LPP(+) or Mitosis/Asynch in this study.

Analysis of phospho-proteins with LPP-sensitive phosphosites. For Extended Data Fig. 4b, ‘All proteins with phosphorylation-dependent cysteine reactivity change’ refers to proteins containing cysteines with cysteine reactivity changes in both LPP(−) versus LPP(+) (twofold, at least two replicates) and in mitosis versus asynchronous (>1.6-fold, at least one replicate). Proteins with only artifactual cysteine reactivity changes were removed from analysis (twofold reduced reactivity in LPP(−,−) relative to LPP(+,+)) but changing less than 1.6x-fold in LPP(−,−) versus LPP(+,+)). ‘All proteins’ are any proteins with at least one cysteine quantified in at least two replicates of LPP(−) versus LPP(+). Proteins were considered to have LPP-sensitive phosphosites if a given phosphosite had a twofold change in enrichment between LPP(−) and LPP(+). Proteins with only LPP-insensitive (less than a twofold change) or no quantified phosphosites were grouped and labeled as ‘Proteins with phosphosites not quantified or unchanged’.

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

Data availability
All mass spectrometry data are available via PRIDE with identifier PXD026730. Source data, in addition to Supplementary Dataset 1, is available for all figure panels. Source data are provided with this paper.

Code availability
TMT-based data output from Integrated Proteomics Pipeline (IP2) and isoTOP data output from CIMAGE was further analyzed with custom scripts, available on Zenodo at https://zenodo.org/badge/latestdoi/419072418.

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Acknowledgements
We thank V. Vartabedian from the Teijaro lab for technical assistance and G. Simon (Vividion) and S. Niessen and M. Hayward (Pfizer) for valuable feedback throughout this project. This work was supported by the National Institutes of Health (NIH) (CA231991 to B. F. C), NIH-NCI (CA239556 to E. K. K.), and Vividion Therapeutics.

Author contributions
B. F. C. and E. K. K. conceived of the project, analyzed data, and wrote the manuscript. E. K. K. and M. M. D. developed mass spectrometry methods. E. K. K. and Y. Z. performed experiments.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41592-022-01398-2.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41592-022-01398-2.

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Peer review information
Nature Methods thanks Dustin Maly and the other anonymous, reviewer(s) for their contribution to the peer review of this work. Editor recognition statement Arunima Singh was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Cysteine reactivity profiling of mitotic and asynchronous cells. a, Timeline for mitotic HeLa cell proteome generation. b, Cysteine reactivity values for GAPDH and PARK7 (Mitosis/Asynch) cell proteomes. Horizontal black line for each cysteine marks median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 9 independent experiments (circles). Dotted lines designate boundaries for ≥ twofold changes. c, TMT-ABPP workflow for measuring protein expression (top, blue) and cysteine reactivity (bottom, gray) in the mitotic and asynchronous HeLa cell proteome. d, Venn diagram showing overlap (light blue) in proteins quantified by TMT-ABPP (gray) and unenriched proteomics (dark blue). For inclusion, proteins had at least one quantified cysteine in at least two replicate experiments of TMT-ABPP and/or two unique quantified peptides quantified from at least one replicate of unenriched proteomics. e, GO cellular analysis of proteins with reactivity-based cysteine changes in mitotic vs asynchronous cell proteomes45,46. Proteins with reactivity-based cysteine changes correspond to those defined in Fig. 1d. f, Box plot showing DTYMK cysteine reactivity values (Mitosis/Asynch). Horizontal black line for each cysteine marks median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 5 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ twofold changes. g, Cysteine reactivity values for all quantified DTYMK cysteines following gel filtration of the mitotic cell proteome. Data represent average values +/− standard deviation for n = 3 independent experiments (circles). h, DTYMK C117 reactivity following gel filtration of asynchronous (gray) vs mitotic (blue) cell proteomes. Data represent average values +/− standard deviation for n = 3 (or more) independent experiments (circles). i, X-ray crystal structure of DTYMK in complex with AMP and TMP with C163 and C117 highlighted in yellow (PDB: 1E2D)47. j, Protein expression values for DTWD1 (not detected), NOL8, and RRP15 (Mitosis/Asynch). Horizontal black line for each cysteine marks median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 9 independent experiments (circles). Dotted lines designate boundaries for ≥ twofold changes.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | A proteomic method to map phosphorylation-dependent changes in cysteine reactivity. 

a, Venn diagram of phosphorylated S/T residues quantified by Sharma et al.21 (red) and this study (dark blue) in asynchronous and mitotic cell proteomes. b, Cysteine reactivity ratio values in Native/Denatured mitotic proteome. Light blue and orange data mark cysteine reactivity values that are ≥ twofold higher (boundary marked by dotted lines) in native and denatured cell proteome, respectively. Data are the median value for n = 1 (or more) independent experiments. c, Comparison of cysteine reactivity values from LPP(-)/LPP(+) (y-axis) and Native/Denatured (x-axis) proteomes. Blue and red data mark cysteine reactivity values that are ≥ twofold higher in LPP(-) and LPP(+) cell proteomes, respectively. Data are the median value for n = 1 (or more) Independent experiments. d-f, Cysteine reactivity values across Native/Denatured proteome (orange) and LPP(-)/LPP(+) (green) mitotic proteomes. Dotted lines mark boundaries for cysteines that change ≥ two-fold in reactivity in LPP(-)/LPP(+) and Native/Denatured experiments. Data are the median value for n = 2 (or more) independent LPP(-)/LPP(+) experiments and n = 1 (or more) Native/Denatured experiments. d-f, Cysteine reactivity values for cysteines in d) FLNB, e) NUMA1, and f) BAG3. Horizontal black lines mark median value, boxes mark upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 2 (or more) independent experiments (circles). g, Percentage of cysteines in predicted disordered domains (IUPred ≥ 0.5)48. h, Cysteine reactivity values for MAP2K4 in gel-filtered mitotic cell proteome. Data represent the average values +/- standard deviation for n = 2 (or more) independent experiments (circles). i, MAP2K4 C246 reactivity in gel-filtered asynchronous (gray) vs mitotic (blue) cell proteomes. Data represent the average values +/- standard deviation for n = 4 (or more) independent experiments (circles). j, Left, MAP2K ATP-binding pocket cysteine reactivity. Non-unique peptides are assigned to both MAP2Ks. Horizontal black lines mark median value, boxes mark upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 2 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes. Right, sequence alignment of MAP2K proteins centered on MAP2K4 ATP-binding pocket C246.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Adapted protocol for interpreting proximal phosphorylation-cysteine interactions. 

a, Left, cysteine reactivity values for indicated comparison groups for quantified cysteines from ECD3 (C137, top) and GTF2I (C215, bottom). Horizontal black line for each cysteine marks median value, boxes mark upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 5 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes. Right, tryptic peptides containing EDC3 C137 (asterisks, red, bold; top) and GTF2I C215 (asterisks, red, bold; bottom) and high occupancy phosphorylation sites (black, bold).
b, Left, bar graph showing phosphopeptide enrichment of EDC3 p-S131 (top) and GTF2I p-S210 (bottom). Data were normalized to mitotic proteome without LPP treatment (Mitosis LPP(-)) and represent the median values ± standard deviation for n = 3 independent experiments (circles). Right, tryptic peptides containing phosphorylated (p-, purple, bold) EDC p-S131 (asterisks, purple, bold; top) and GTF2I p-S210 (asterisks, purple, bold; bottom) and cysteines from Extended Data Fig. 3a marked (red, bold).
c, Immunoblot analysis of MAP2K1 with antibody #9146 in mitosis. Data are from a single experiment representative of two independent experiments.
d, FLNA cysteine reactivity values across the indicated comparison groups. Horizontal black line for each cysteine marks median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 5 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes.
e, Left, SLAIN2 C152 reactivity values across the indicated comparison groups. Horizontal black line for each cysteine marks median value, boxes mark the upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 3 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes. Right, tryptic peptide containing SLAIN2 C152 (asterisks, blue, bold) and a potential S/T-P phosphorylation site (black, bold).
f, Left, bar graph showing phosphopeptide enrichment of SLAIN2 p-S147. Data were normalized to mitotic proteome without LPP treatment (Mitosis LPP(-)) and are from n = 1 experiment. Right, tryptic peptide containing phosphorylated (p-, purple, bold) SLAIN2 S147 (asterisks, purple, bold) with cysteine from Extended Data Fig. 3e marked (blue, bold).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Features of proteins with mitotic phosphorylation-dependent changes in cysteine reactivity. a, Proteins with authentic (left) and artifactual (middle) phosphorylation-dependent cysteine reactivity changes are enriched for high stoichiometry mitotic phosphorylation sites21 (blue) compared to all quantified proteins (right). Proteins lacking sufficient data for phosphorylation stoichiometry calculation or exhibiting only low stoichiometry (<50% occupancy) sites were labeled as ‘Low or unquantified stoichiometry’ (orange). b, Proteins with phosphorylation-dependent cysteine reactivity changes (left) are enriched for LPP-sensitive mitotic phosphorylation sites (purple) compared to all quantified proteins (right). Proteins with only artifactual cysteine reactivity changes were removed from analysis. Proteins with LPP-insensitive or no quantified phosphorylation sites were labeled as “Unchanging or unquantified phosphosites” (gray). c, Members of the anaphase-promoting complex (APC/C) of the KEGG cell cycle pathway (hsa04110). Proteins are as described in Fig. 4b. d, Fraction of cysteines showing phosphorylation-dependent reactivity changes within the specified amino acid distances from an S/T-P site. Artifactual phosphorylation-dependent cysteine reactivity changes were omitted from analysis. e, Fraction of cysteines showing authentic (left) versus artifactual (right) phosphorylation-dependent reactivity changes within the specified amino acid distances from an S/T-P site. Authentic and artifactual changes were determined as described in Fig. 3f. f, Sequence alignment of the KLC1 and KLC2 proteins centered on C456 and C441, respectively (asterisks, red, bold). Known (KLC1) and predicted (KLC2) S-P phosphorylation motifs are marked (black, bold). g, KLC1 cysteine reactivity values across the indicated comparison groups. Horizontal black lines mark median value, boxes mark upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 2 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes. h, FXR2 C270 reactivity values for indicated comparison groups. Horizontal black lines mark median value, boxes mark upper and lower quartiles, and whiskers mark 1.5x interquartile range for n = 5 (or more) independent experiments (circles). Dotted lines designate boundaries for ≥ two-fold changes. i, Left, phosphopeptide enrichment of FXR2 p-S450. Data were normalized to Mitosis LPP(-) and represent the median values +/- standard deviation for n = 2 independent experiments (circles). Right, tryptic peptides containing phosphorylated (p-, purple, bold) FXR2 p-S450 (asterisks, purple, bold).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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Only common tests should be described solely by name; describe more complex techniques in the Methods section.

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☐  A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐  For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  Xcalibur v4 was used for mass spectrometry data collection. Konica Minolta SRX 101 x-ray film processor or Bio-Rad ChemiDoc XRS imager used for blotting data collection.

Data analysis  IP2 v6.5.5, RAW Converter v1.1.0.22, ProluCID v1.4 were used to analyze mass spectrometry data. CIMAGE was used for isoTOP-ABPP mass spectrometry data analysis. Data were analyzed and visualized (boxplots, correlation plots, heatmaps, waterfall plots, dot plots, pie charts, venn diagrams) were generated using:

Python (v3.8.1)

 Pandas (v1.1.3)

 Seaborn (v0.11.0)

 Matplotlib (v3.3.2)

 Numpy (1.19.2)

 Matplotlib_venn (v0.11.6)

 Re (v2.2.1)

 Webgestalt (2019)

 REVIGO (2020)

 Code used to process mass spectrometric data are available on Github [in progress]:

https://github.com/cravattlab/Kemper_2021_NM

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. Github). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All mass spectrometry data are available via PRIDE with identifier PX026730. Source data, in addition to Supplementary Dataset 1, is available for all figure panels.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences [x]  Behavioural & social sciences  Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No statistical methods were used to pre-determine sample size. Where possible, for MS-based experiments we performed at least 3 independent replicates and required quantification in at least 2 independent replicates for the main ratio [LPP(+/+)-l(-/-) or Asynch/Mitosis]. For Native/Denatured and phosphoenrichment studies, quantification in one independent replicate was considered sufficient.

Data exclusions
No data were excluded.

Replication
The exact number of experiment replications are noted in the figure legends.

Randomization
Mammalian cells used for this study were grown under identical conditions, so randomization was not used.

Blinding
Mammalian cells used for this study were grown under identical conditions, so blinding was not used.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | Antibodies |
| [x] | Eukaryotic cell lines |
| [x] | Palaeontology and archaeology |
| [x] | Animals and other organisms |
| [x] | Human research participants |
| [ ] | Clinical data |
| [x] | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | ChIP-seq |
| [ ] | Flow cytometry |
| [x] | MRI-based neuroimaging |

Antibodies

Primary antibodies were used at the following concentrations:
1:10000 anti-GAPDH (Santa Cruz, sc-47724)
1:2000 anti-phospho-JNK (Cell Signaling, #4668)
1:2000 anti-JNK (Cell Signaling, #9252)
1:2000 anti-phospho-p38 (Cell Signaling, #4511)
1:2000 p38 (Cell Signaling, #8690)
1:500 CCNB1 (Santa Cruz, sc-245)
1:500 CCNE1 (Santa Cruz, sc-247)
1:1000 anti-Sp1P (Cell Signaling, #5243)
1:1000 anti-MAP2K4 (Cell Signaling, #9152)
1:1000 anti-phospho-MAP2K4 (Cell Signaling, #9156)
1:500 anti-MAP2K1 (Cell Signaling, #9146).
Validation

GAPDH (Santa Cruz, sc-47724): validated for western blotting by manufacturer using extracts from Jurkat, MOLT-4, HeLa, K-562, Bjab and IMR-32 whole cell lysates.

phospho-(Thr183/Tyr185)-JNK (Cell Signaling, #4668): validated for western blot by manufacturer using extracts from 293 cells, untreated or UV-treated, NIH/3T3 cells, untreated or UV-treated and C6 cells, untreated or ansomycin-treated.

JNK (Cell Signaling, #9252): validated for western blotting by manufacturer using extracts from 293 and SK-N-MC cells, untreated or UV-treated [40 μM].

phospho-(Thr180/Tyr182)-p38 (Cell Signaling, #4511): validated for western blotting by manufacturer using extracts from COS and 293 cells, untreated or UV-treated.

p38 (Cell signign, #8690): validated for western blotting by manufacturer using extracts from COS and 293 cells, untreated or UV-treated.

CCNB1 (Santa Cruz, sc-245): validated for western blotting by manufacturer using extracts from K562 cells transfected with cyclin B1 siRNA.

CCNE1 (Santa Cruz, sc-247): validated for western blotting by manufacturer using extracts from HeLa, MEG-01, JAR, MOLT-4, IMR-32 and MCF-7 whole cell lysates.

Phospho-PLK Binding Motif Ser7 (Cell Signaling, #8243): validated for western blotting by manufacturer using extracts from HeLa cells, untreated or nocodazole-treated (100 ng/mL, 16 hrs).

MAP2K4 (Cell Signaling, #9152): validated for western blotting by manufacturer using extracts from HEK293, NIH/3T3, C6, COS and A431 cells.

phospho-(Ser257/Thr261)-MAP2K4 (Cell Signaling, #9156): validated for western blotting by manufacturer using extracts from HeLa and C6 cells, untreated or UV-treated.

MAP2K1 (Cell Signaling, #9146): validated for western blotting by manufacturer using extracts from HeLa, NIH/3T3 and COS cells.

MAP2K1 (Cell Signaling, #12671) validated for western blotting by manufacturer using extracts from MEF wild type and MAP2K1-null cells.

ACTH-HP [ ]: validated for western blotting by manufacturer using extracts from Jurkat, HeLa, MCF-7, and A-431 cells.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
HeLa (ATCC: CCL-2)

Authentication
HeLa cells were authenticated by short tandem repeat loci (STRs) profiling by vendors.

Mycoplasma contamination
HeLa cells were tested negative for mycoplasma.

Commonly misidentified lines (See ITAC register)
No commonly misidentified cell lines were used in this study.