High throughput discovery of functional protein modifications by Hotspot Thermal Profiling

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Mass spectrometry enables global analysis of posttranslationally modified proteoforms from biological samples, yet we still lack methods to systematically predict, or even prioritize, which modification sites may perturb protein function. Here we describe a proteomic method, Hotspot Thermal Profiling, to detect the effects of site-specific protein phosphorylation on the thermal stability of thousands of native proteins in live cells. This massively parallel biophysical assay unveiled shifts in overall protein stability in response to site-specific phosphorylation sites, as well as trends related to protein function and structure. This method can detect intrinsic changes to protein structure as well as extrinsic changes to protein–protein and protein–metabolite interactions resulting from phosphorylation. Finally, we show that functional ‘hotspot’ protein modification sites can be discovered and prioritized for study in a high-throughput and unbiased fashion. This approach is applicable to diverse organisms, cell types and posttranslational modifications.

The quantitative interrogation of endogenous PTMs has been greatly accelerated by methods such as liquid chromatography–mass spectrometry (LC–MS). Subsequent integration of front-end, PTM-specific enrichment protocols with data-dependent and de novo PTM discovery algorithms has resulted in the discovery of tens of thousands of unique PTM sites on proteins from diverse organisms9–11, as well as quantification of PTM dynamics in diverse biological contexts and responses to stimuli12–14. Central questions around how minute changes to chemical structure alter the biophysical properties of a much larger protein, and how these changes lead to signaling and phenotypic consequences, remain only partially explored. This dearth of mechanistic information on PTMs exists because we lack general methods with which to predict, or even prioritize, which modification sites are likely to be functional in the proteome, and, therefore, the one-by-one study of PTMs is intrinsically inefficient and low throughput.

Here we sought to develop a proteomic method that enables direct interrogation of protein stability in response to endogenous PTMs, for thousands of proteins in parallel. This method, Hotspot Thermal Profiling (HTP), couples PTM-specific enrichments and isotopic labeling with global profiling by liquid chromatography–tandem mass spectrometry (LC–MS/MS) to measure the thermal stability of endogenous modified proteoforms, which we refer to as ‘modiforms’, in live cells (Fig. 1). We apply this approach to a ubiquitous yet diminutive PTM (namely, phosphorylation) and demonstrate that HTP can detect both inter- and intramolecular interactions mediated by site-specific PTMs on a wide range of proteins. Moreover, we demonstrate that perturbation of protein stability can be used as a means to discover functionally important and previously uncharacterized ‘hotspot’ modification sites. The resulting dataset represents a rich and generalizable resource to prioritize existing datasets of phosphorylation sites in diverse biological contexts.

Results

HTP detects changes in protein stability in response to site-specific modification. The HTP workflow begins with pulsed exposure of live cell aliquots to temperatures ranging from 37 to 67 °C for 3 min, cooling to room temperature, rapid cell lysis and centrifugation to remove unfolded proteins, in line with reports mapping ligand–protein15–18 and protein–protein19 interactions by thermal proteome profiling (Fig. 1a). The soluble proteome of each aliquot is then tryptolyzed and 5% of each peptide aliquot is labeled with specific tandem mass tag (TMT) isotope channels and reserved for quantification of the ‘unmodified, bulk’ protein pool (Fig. 1a). The remaining 95% of each aliquot is used for phosphopeptide enrichment by TiO2 resin, followed by TMT isotope labeling (Fig. 1b). All aliquots of the unenriched and phosphorylated TMT tryptic peptides are subsequently combined and used for LC–MS/MS detection and quantitation of relative peptide levels across the temperature range.

Peptide-level measurements are analyzed en masse with identification, quantification and automated curve-fitting algorithms to generate peptide-specific melting points (T_m) for tens of thousands of unique peptides per sample (Fig. 1a). Because quantitative proteomic measurements are performed on individual peptides, measurements made on a unique modified peptide (for example, a phosphorylated peptide) serve as a ‘bar code’ to report on any protein that contains a modification at that site to the thermal challenge in live cells. Therefore, this method does not report on single ‘proteoforms’ as recently defined20, but instead reports on an inclusive set of the protein pool that is unambiguously phosphorylated at the
detected site, which we define as the ‘phosphomodiform’. This is a key differentiation from measuring the effect of a modification on the bulk protein pool, such that HTP can detect altered thermal stability even if a modiform is only a minor member of all the modified proteoforms present in the cell (Fig. 1b).

We applied the HTP workflow to detect the $T_m$ values of phosphomodiforms and the bulk, unmodified protein pool in HEK293T cells. We detected >250,000 TMT-labeled peptides from thousands of proteins across a handful of LC–MS/MS runs (Supplementary Fig. 1), and statistical filtering through identification, quantification and curve fitting were combined to generate 4,293 ‘unmodified, bulk’ protein $T_m$ values (Fig. 2a,b). Here we define the unmodified, bulk as all unmodified peptides that map to a given protein of interest, such that this $T_m$ reflects the ‘reference’ population of protein present in cells. Protein and peptide $T_m$ measurements were highly stable across replicates, validating the reproducibility of cellular- and proteome-level manipulations in the HTP workflow, and indicating that even small shifts in thermal stability (for example, ~1.5°C) can be reliably detected with statistical significance (Supplementary Fig. 2a–f).

To measure phosphomodiform-specific $T_m$ values, we integrated metal-dependent phosphopeptide enrichment with subsequent TMT barcoding (Fig. 1). Global phosphorylation was not grossly affected by the short exposure to varied temperature (Supplementary Fig. 3).

Duplicate technical replicates from five biological replicates ($n = 10$, LC–MS/MS runs), as well as grouping phosphopeptides that map to a common site (Supplementary Fig. 4), resulted in identification of 10,800 phosphomodiforms. Automated $T_m$ curve-fitting cutoffs ($R^2 > 0.8$) resulted in a dataset of 9,695 distinct phosphomodiforms. Finally, filtering of only high-confidence $T_m$ values detected in both phospho- and unmodified, bulk proteomes, as well as stringent modification site localization validation, yielded 2,883 high-quality $T_m$ values that directly compare a specific phosphomodiform to its unmodified, bulk protein (Fig. 2a,b and Supplementary Table 1).

The unmodified and phosphorylated proteomes were globally indistinguishable, with mean $T_m$ values of 51.2 and 51.0°C, respectively (Fig. 2b). By contrast, comparison of a given protein to its specific phosphomodiforms yielded a diverse $T_m$ profile that was Gaussian in nature, and highlighted the fact that the most phosphorylated modiforms did not exhibit altered thermal stability relative to their parent proteins (Fig. 2c and Supplementary Table 2). There were a sizable number of phosphosites, however, associated with markedly different thermal stability compared with their unmodified, bulk protein pool in live cells (Fig. 2c). A detailed analysis of specific temperature–response curves verified these trends, with many parent protein–phosphomodiform pairs producing sigmoidal curves that overlay almost exactly (Fig. 2d). In contrast, numerous phosphomodiforms exhibited significantly different temperature–response curves compared with their unmodified, bulk protein counterpart, including pY15 on cyclin-dependent kinase CDK1 and pT926 on kinesin-like protein KIF11 (Fig. 2e,f). Both of these sites have critical roles in regulating the function of their target protein: pY15 has been shown to inactivate CDK1 through structural reorganization and autoinhibition, while pT926 controls intermolecular association of KIF11 with the spindle apparatus during mitosis.

The phosphosite-specific resolution of HTP contrasts with previous reports that have detected shifts in bulk protein $T_m$ values in response to phosphorylation as indirect but related targets of drug action. Instead, the direct interrogation of phosphomodiforms with amino-acid resolution validates the ability to distinguish functional effects at different modification sites, as well as minor phosphomodiforms that might otherwise be masked by more abundant proteoforms. For example, the pS58 phosphomodiform of the enzyme TP11 is associated with significant destabilization, while pT210 has no effect on its stability (Fig. 2g). These data confirmed that modification-dependent changes in protein stability can be detected by thermal profiling of the modified proteome. Most importantly, we hypothesized that ‘hotspot’ modification sites...
Articles not, at least on a global average in this dataset, impart a systematic phosphoamino acid and directly adjacent sequence context does phosphorylation sites in established kinase motifs also did not stability, on average (Fig. 3d). Furthermore, a significant stabiliz- sheet secondary structures demonstrated decreased β helical or major-ity of sites in coil/loop regions, phosphosites within predicted (phospho) independent biological replicates. \( n = 10 \) (phospho) total MS technical replicates from \( n – d \) correspond to mean and s.e.m. from \( n–d \) significant shifts (\( t \)-test for within-protein comparisons) are shown in red (negative shift) and blue (positive shift). Perturbation of protein stability by phosphorylation is site-specific, not systematic. We next asked whether there were any systematic correlations between the types and contexts of protein phosphorylation sites and their effect on protein stability. Comparison of the \( \Delta T_m \) values for phosphorylation on different residues (serine, threonine and tyrosine) across all detected phosphomodiforms did not reveal any general bias for stabilization or destabilization (Fig. 3a). Classes of modiforms harboring multiple phosphorylation sites within the same peptide, and therefore in high proximity on the protein, were also not significantly associ- ated with increased or decreased stability. Global \( \Delta T_m \) values among phosphorylation sites in established kinase motifs also did not reveal significant correlations, indicating that the combination of phosphoamino acid and directly adjacent sequence context does not, at least on a global average in this dataset, impart a systematic stabilizing or destabilizing effect (Fig. 3a,b). As expected, phosphosites detected in our dataset showed enrichment in predicted loop or coil secondary structural elements (Fig. 3c). Relative to the majority of sites in coil/loop regions, phosphosites within predicted helical or β-sheet secondary structures demonstrated decreased stability, on average (Fig. 3d). Furthermore, a significant stabilizing trend was observed for phosphosites located in exposed protein regions, relative to those located in buried regions, as determined by computational predictions of solvent accessibility and local structure organization (Fig. 3e,f). These global trends imply that the effect of protein phosphorylation (as far as can be deduced with this method) is governed by local context within proteins, as opposed to a systemic, biophysical ‘code’ imparted by the phosphoamino acid or linear motif.

HTP detects phosphorylation-mediated protein–protein interactions. We next asked whether significantly perturbed ‘hotspot’ \( \Delta T_m \) values were correlated with altered intra- or intermolecular interactions in cells, or with both. Global correlation of phosphorylation site \( \Delta T_m \) and the number of functional annotations present in the PhosphoSite online database highlighted that most sites (92.5%) detected in this dataset had no annotation, as is true for the vast majority of known posttranslational modification sites in the human proteome. Well-characterized, functional sites in proteins, such as CDK1, STAT3 and STAT1, stood out on this annotation plot (Fig. 4a). Additionally, a cluster of highly perturbed and well-annotated sites in the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) were conspicuous.

The unmodified, bulk 4EBP1 pool exhibited a \( T_m \) of 53.8 ± 0.8 °C by HTP (Fig. 4b), whereas the phosphomodiforms containing phosphorylation sites in the amino (N)-terminal region were signifi-cantly less stable (Fig. 4b). These sites were clustered in a region known to be responsible for direct binding to eukaryotic translation initiation factor 4E (eIF4E), which prevents ribosome binding and translation. A phosphorylation cascade that requires T37, T46 and intervening phosphorylation sites as initiating modifications in the amino (N)-terminal region were sig-nificantly less stable (Fig. 4b). These sites were clustered in a region known to be responsible for direct binding to eukaryotic translation initiation factor 4E (eIF4E), which prevents ribosome binding and translation. A phosphorylation cascade that requires T37, T46 and intervening phosphorylation sites as initiating modifications lead to phosphorylation of Y54, which blocks 4EBP1 binding to eIF4E. The thermal shifts resulting from phosphorylation around

**Fig. 2 | HTP identifies thousands of unmodified, bulk and phosphomodiform \( T_m \) values in HEK293T cells.** a, The total number of unique phosphomodiforms detected in our dataset and the criteria for identifying high-quality \( \Delta T_m \) values. FLS FDR, localization site false discovery rate. b, Global distribution of unmodified, bulk protein level and phosphomodiform \( T_m \) values. c, Volcano plot of high-confidence \( \Delta T_m \) values. Phosphomodiforms with significant shifts (\( P < 0.05 \), two-sided \( t \)-test for within-protein comparisons) are shown in red (negative shift) and blue (positive shift). d–g, Representative \( T_m \) curves from phosphomodiforms that do not affect (d), increase (e) and decrease (f) thermal stability. Distinct phosphomodiforms of the same protein that exhibit unique \( \Delta T_m \) values, as exemplified in TPI1 (g). Detected phosphorylation sites in TPI1 are shown as spheres; PDB 1WYI. T210, T210 Δ\( T_m \) values were correlated with altered intra- or intermolecular interactions in cells, or with both. Global correlation of phosphorylation site \( \Delta T_m \) and the number of functional annotations present in the PhosphoSite online database highlighted that most sites (92.5%) detected in this dataset had no annotation, as is true for the vast majority of known posttranslational modification sites in the human proteome. Well-characterized, functional sites in proteins, such as CDK1, STAT3 and STAT1, stood out on this annotation plot (Fig. 4a). Additionally, a cluster of highly perturbed and well-annotated sites in the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) were conspicuous.
Fig. 3 | Global relationships between local phosphosite environment and altered phosphomodiform stability. **a** Distribution of \(\Delta T_m\) values for tryptic peptides containing indicated phosphoamino acids or coincidental modifications thereof (\(P < 0.0001\), one-way analysis of variance (ANOVA)). **b** Correlation between detected kinase substrate motifs and \(\Delta T_m\) values for identified sites (\(P = 0.3011\), one-way ANOVA). **c** Distribution of all amino acids in the human proteome (left) and detected phosphosites (right) in predicted secondary structural elements. **d–f** Comparisons of \(\Delta T_m\) values and predicted secondary structure elements (d; ***\(P \leq 0.0001\), **\(P \leq 0.001\) two-sided \(t\)-test), ordered structural elements surrounding the phosphosite of interest (e; ***\(P \leq 0.0001\), **\(P \leq 0.001\) two-sided \(t\)-test) and solvent accessibility (f; ***\(P \leq 0.0001\), **\(P \leq 0.001\) two-sided \(t\)-test). Box plots (median, 1–99%) are shown in **a** b **d–f**, with outliers shown as data points. All data were generated from \(n = 12\) (bulk, unmodified) and \(n = 10\) (phospho) total MS technical replicates from \(n = 6\) (bulk, unmodified) and \(n = 5\) (phospho) independent biological replicates.

Fig. 4 | HTP detects the known 4EBP1–EIF4E protein–protein interaction mediated by phosphorylation. **a** Plot of phosphosite \(\Delta T_m\) and number of annotations in the Phosphosite online database. A cluster of 4EBP1 phosphosites are highlighted in red. **b** Representative subset of HTP curves for the unmodified, bulk and N-terminal phosphomodiforms of 4EBP1. **c** X-ray structure of the N-terminal helix-turn motif of 4EBP1 (gray) bound to EIF4E (tan); Y54 in 4EBP1 is shown as spheres. **d** Proposed model correlating observed thermal stability shifts to 4EBP1–EIF4E complexes. **e** HTP curve for C-terminal pS101 phosphomodiform. **f** Schematic showing the location and relative \(\Delta T_m\) values for all detected phosphomodiforms of 4EBP1, with annotation of potentially co-occurring and exclusive modification patterns based on thermal response. Data points shown in **b** e represent the mean and s.e.m. from \(n = 12\), 6, 9, 6, 3, 6, 1, 12 and 2 (in order of \(T_m\) curve appearance) replicate measurements detected in \(n = 6\) (bulk, unmodified) and \(n = 5\) (phospho) independent biological replicates. \(\Delta T_m\) P values were calculated with two-sided \(t\)-tests.
of engaging in new intermolecular interactions. These data provide a proof-of-concept example of the effective prioritization and discovery of uncharacterized, but phenotypically relevant, hotspot modification sites by HTP.

We next interrogated the pS210 phosphomodiform of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was significantly destabilized relative to the unmodified, bulk GAPDH pool, as well as GAPDH protein that is unambiguously unphosphorylated at S210 (Fig. 6a). Other detected sites, such as pT154, were not associated with a significant ΔTm shift (Supplementary Fig. 5a). Published structures show that S210 packs closely to L194 and by extension K195, which is involved in substrate binding (Fig. 6b). To explore the possibility that phosphorylation may affect GAPDH substrate binding and catalysis, and to extend HTP profiling to kinetic/signaling conditions, we performed HTP on cells under the limiting metabolic environment of acute glucose withdrawal, which depleted cellular glyceraldehyde-3-phosphate (GAP) levels fourfold (Supplementary Fig. 5b). Under these conditions the thermal shift between the bulk, unmodified GAPDH pool and the pS210 phosphomodiform disappeared (Fig. 6c). Specifically, the stability of the pS210 phosphomodiform was not affected by the presence of lower GAP levels, which contrasted with the significant destabilization of the bulk, unmodified GAPDH protein pool (Fig. 6d,e). These data suggested that GAP binding contributes to the stability of GAPDH protein that is not phosphorylated at S210, and the negative shift in stability associated with the pS210 phosphomodiform may be capturing the reduced substrate occupancy of these proteins in cells (Fig. 6f).

To specifically test GAP binding and metabolism we tested the enzyme kinetics of wild-type GAPDH relative to mutants that are incapable of phosphorylation (S210A) or that mimic phosphorylation (S210D). Indeed, the reaction kinetics and Michaelis constant (Km) value for GAP was indistinguishable between the nonphosphorylatable S210A mutant and the wild-type enzyme (Fig. 6g,h), whereas the phosphomimetic S210D mutant remained active but exhibited decreased kinetics and a significantly increased Km value for GAP (Fig. 6f). These effects were unique to S210, as the kinetics of nonphosphorylatable and phosphomimetic mutants for a nearby detected site, T182, did not affect conversion rates or Km value for GAP (Supplementary Fig. 5c–e). These data confirmed that altered modiform–ligand interactions can be discovered and characterized by HTP profiling under basal and altered metabolic conditions.

**Discussion**

Here we established a proteomic method enabling the unbiased, global detection of protein stability changes in response to site-specific phosphorylation events on thousands of proteins. In contrast to traditional discovery-mode LC–MS/MS studies, the HTP method provides several novel insights into the functional effects of protein PTMs. First, it provides site-specific, quantitative relationships between the ‘unmodified, bulk’ population of a protein and its distinct phosphomodiforms in the endogenous context of live cells. We have demonstrated that this format promotes detection of both intrinsic alterations in protein structure, as well as context-specific intermolecular events, such as protein–protein and protein–metabolite interactions. We posit that the ability to perform HTP profiling under steady-state and comparative kinetic conditions in response to small molecules or signaling events can be used to discover and characterize PTM-mediated interactions. We demonstrated this attribute of HTP through the identification of previously uncharacterized phosphorylation sites in well-characterized proteins, such as GAPDH, as well as less well-studied structural proteins, such as vinculin.

The information gained through HTP profiling of phosphomodiforms adds another functional layer to that measured by...
quantifying the abundance of modification sites in the proteome, which report on relative ‘writing’ and ‘erasing’ of modifications across the proteome, but provide no information on the dynamic biophysical ‘reading’ of these modifications. HTP profiling alone, or in concert with datasets focused on mapping protein–protein and protein–metabolite interactions, will provide insights into the functional and interconnected roles of site-specific modification in the proteome.

It is important to consider the limitations of HTP-derived ΔTm values in assuming functional impact on a protein of interest. First, this method specifically interrogates the inclusive pool of proteins that unambiguously contain a defining modification site, which we have described here as a ‘modiform’. Thus, this method cannot determine the modification status of other sites that do not fall within this peptide on the same protein molecule, a limitation that is intrinsic to massively parallel detection by LC–MS/MS. We do not view this aspect of the approach as a limitation per se, because the primary goal of the approach is to correlate specific modification sites with putative changes to protein structure and function in cells, regardless of the inherent heterogeneity of the protein pool. As such, HTP may under-sample certain combinations of modifications, or miss them entirely, but it can unequivocally connect changes in apparent stability to the presence of site-specific, ‘hotspot’ modifications. Additionally, the response of individual phosphomodiforms to thermal challenge under a defined cellular condition may be used to provide information about the co-occurrence or mutual exclusivity of specific modification sites on the same molecules in the larger protein pool, as discussed here for TPI1, 4EBP1 and GAPDH. While beyond the scope of the current article, we hope that focused bioinformatic analyses of future datasets could shed light on this aspect of the HTP method. Finally, the method is intended to globally interrogate native, modified proteins in relevant biological contexts,
to identify and prioritize sites for further study and to provide mechanistic hypotheses for functional validation. More than 90% of the modifications detected here have been previously observed in numerous datasets, but have no functional annotation, thus underscoring the low-throughput nature of current PTM annotation. We expect that the phosphorylation dataset presented here, as well as the extension of the HTP method to diverse organisms, cell types and PTMs will greatly expand our current understanding of the role of protein modifications in regulating protein structure, function and signal transduction.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41592-019-0499-3.

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**Methods**

A Supplementary Protocol describing the HTP procedure is available and has also been deposited at ProtocolExchange\(^\text{17}\).

**Cell culture.** Hela and HEK293T cell lines were propagated in RPMI 1640 with 2 mM L-glutamine (HyClone) supplemented with 10% fetal bovine serum (Corning) and 1% penicillin–streptomycin (ThermoFisher Scientific). All cell lines were grown at 37°C in a 5% CO₂, humidified incubator.

HTP proteomic sample preparation. HEK293 cells were grown to 90% confluency in 15 cm cell-culture-treated plates (Denville). Cells were scraped from plates, briefly washed and resuspended in PBS with EDTA-free protease inhibitor tablet (Roche) and phosphatase inhibitor cocktail 3 (Sigma-Aldrich) before being divided into ten equal aliquots. Each aliquot of live cells was exposed to a steady temperature between 37°C to 67°C for 3 min in parallel, incubated at 25°C for another 3 min and lysed by rapid freeze-thawing. Insoluble proteins and cell debris were removed by centrifugation at 17,000g for 10 min. A small amount of supernatant from the 37°C aliquot was set aside to use for protein quantification by Bradford’s assay. The remaining fraction of whole-cell lysates was denatured in 8 M urea, followed by disulfide reduction with dithiothreitol (DTT) (10 mM, 30 min, 65°C), alkylolation (iodoacetamide, 15 mM, 30 min, room temperature, protected from light) and quenching (DTT, 5 mM, 10 min, room temperature). The proteome solution was diluted fourfold with ammonium bicarbonate solution (50 mM, pH 8.0), CaCl₂, added (1 mM) and digested with sequencing grade trypsin (~1:100 enzyme:protein ratio; ThermoFisher) at 37°C while rotating overnight. Peptide digestions were washed in LC–MS/MS buffer A (H₂O with 0.1% formic acid, 0.1% acetic acid, 0.5% CaCl₂) and peptides were then desalted with Sep-Pak C18 cartridges (50 mg, Waters), dried under vacuum, resuspended with LC–MS grade water (Sigma-Aldrich) and then lyophilized. A small fraction (5%) reserved for bulk unmodified proteome thermal profiling was split from each aliquot and lyophilized separately.

**Phosphopeptide enrichment and TMT labeling.** The major fraction of lyophilized peptides (95%) was redissolved in 28.5% lactic acid (Sigma), 0.28% trifluoroacetic acid (Sigma) and applied to TiO₂ spin tips (catalog no. 88303, ThermoFisher) for phosphopeptide enrichment according to the manufacturer’s protocol. Eluted phosphopeptides were dried under vacuum, resuspended with LC–MS/MS buffer A (H₂O with 0.1% formic acid, pH 8.0, 100 µl of 5% hydroxylamine (ThermoFisher). Labeled peptides were combined into a single pool per experiment, acidified with formic acid (pH 2–3), desalted using ZipTip C18 tips (100 µl, Millipore) and lyophilized. The two sets of peptides (phospho-enriched and unenriched) were separately labeled with 10–plex isotopic tandem mass tags (catalog no. 90406, ThermoFisher) according to the manufacturer’s protocol with slight modification. TMT reagents were reconstituted to 8 mg/ml in anhydrous acetonitrile (Sigma) and added to lyophilized peptides dissolved in 100 µl of 200 mM HEPES buffer, pH 8.0 (Δ1 reporter:peptide ratio). The labeling reaction was carried out at room temperature for 1 h with gentle shaking, and quenched with 5 µl of 5% hydroxylamine (ThermoFisher). Labeled peptides were combined into a single pool per experiment, acidified with formic acid (pH 2–3), desalted using ZipTip C18 tips (100 µl, Millipore) and lyophilized. The final processed peptides were dissolved in LC–MS/MS buffer A (Formic acid (0.1% formic acid, LC–MS grade, Sigma–Aldrich) for LC–MS/MS analysis.

**Proteomic LC–MS/MS data analysis.** LC–MS/MS experiments were performed with an Easy-nLC 1000 ultra-high pressure LC system (ThermoFisher) using a reversed-phase PepMap C18 column (75 µm i.d. × 15 cm, 1 µm, 100 Å, ThermoFisher) heated to 40°C coupled to a Q Exactive HF orbitrap and Easy-Spray nanosource (ThermoFisher). TMT-labeled digested peptides in MS/MS buffer A were injected onto the column and separated using the following gradient of buffer B (0.1% formic acid acetonitrile) at 300 nM/min: 0–10% buffer B in 5 min, 10–40% buffer B in 240 min, 40–90% buffer B over 6 min and hold at 90% for 20 min. MS/MS spectra were collected from 0 to 250 min using a data-dependent, top-10 ion selection setting with the following details: full MS scans were acquired at a resolution of 120,000, scan range of 375–1500 m/z, maximum injection time (IT) of 60 ms, automatic gain control target of 1 × 10⁶ and data collection in profile mode. MS/MS scans were performed by higher energy collisional dissociation fragmentation with a data-dependent automatic gain control target of 1 × 10⁶ ions, Δ Δ m of 10 ppm, Δ Δ Δ m(Phosphomodiform) of 50 ppm with modstat and trypstat settings. False discovery rates (FDR) of peptide (spectral false positive rate) were set to 1%. TMT quantification was performed using the isotopic 10-plex labeling algorithm, with a mass tolerance of 5.0 ppm or less in cases where co-eluting peptides interfere. Reporter ions 126.12726, 127.12476, 127.131081, 128.128116, 129.134436, 129.131779, 130.134825, 130.141145 and 131.13838 were used for relative quantification. In general, all quantified peptides have a mass error within ±3 ppm.

**Modification site localization analysis.** Phosphorylation site localization analysis was performed using the LuciPHOR algorithm available on the Integrated Proteomics Pipeline. A global FDR score was calculated for every detected phosphopeptide from each MS run. Phosphopeptides, and therefore phosphophomodiforms, were included in the dataset only if they satisfied a global FDR localization score of <0.01.

**Melting curve analyses.** All detected phosphopeptides that map to the same phosphorylation sites were grouped together and given a new identifier (Gene, p_Site; Supplementary Fig. 4). For example, four different tryptic peptides map to the same GAPHD phosphorylation site: phospho sequence were often detected: R.DGRGALQNIIPAS(79.9963)TGAAKV.GV, R.DGRGALQNIIPAS(79.9963)TGGA.A, R.GALQNIIPAS(79.9963)TGAAKV.GV and R.GALQNIIPAS(79.9963)TGAA.A. These peptides were systematically relabeled with the new custom identifier ‘GAPDH_pS210’. TMT reporter ion intensities of phosphopeptides with the same identifier were combined for each temperature shift fraction from the same MS run (Supplementary Table 1). The change in reporter ion intensity was calculated using the lowest temperature fraction as the reference. Similarly, for the bulk unmodified proteome, TMT reporter ion intensities of all unmodified peptides mapped to the same protein were combined, and fold change values relative to the lowest temperature condition were calculated. To generate unmodified protein and phosphosite-specific melt curves, relative fold changes as a function of temperature was fitted to the equation derived from the chemical denaturation theory using the R package developed by Savitski et al.\(^\text{18}\). Values for Tm were calculated at the point at which the sigmoidal curve crosses the 0.5 fold level change. Only Tm values calculated from melting curves with curve R² > 0.8 were used in subsequent analyses. Shifts in Tm values (for example, ΔTm) induced by phosphorylation were determined by subtracting the Tm of the unmodified protein from the Tm of the phosphomodiform: ΔTm = Tm(Phosphomodiform) – Tm(Unmodified, bulk protein).

Values for ΔTm could only be calculated for phosphopeptides belonging to proteins that were also detected in the unmodified proteome. Values for ΔTm were not determined for phosphopeptides that were detected in fewer than two MS technical replicates. The Tm values of all unmodified proteins and phosphorylation sites, and their ΔTm, values and P values, are summarized in Supplementary Tables 1 and 2.

**Error analyses.** In our workflow, a composite protein-level Tm was calculated by averaging Tm values derived from all detected tryptic peptides of the same protein. To estimate the overall ‘base error’ of our peptide level Tm measurements, that is, on average, how far a peptide Tm value deviates from its composite protein-level Tm, we took four representative datasets and calculated the median peptide ΔTm standard deviation (Supplementary Fig. 2). Every detected tryptic peptide was curve-fitted individually, as well as their composite protein, to calculate Tm values. Each dataset is comprised of 10,000–16,000 peptides and >2,900 protein melt curves. We then subtracted the composite protein-level Tm from each peptide-level Tm to obtain peptide ΔTm values and calculated the standard deviation of each set of peptide ΔTm values. We plotted the distributions of the peptide ΔTm s.d. of the four representative datasets, which resulted in a median peptide ΔTm s.d. average of 1.3 °C, which was used as the vertical cutoff (also known as the smallest high-confidence ΔTm value) in the ΔTm volcano plot (Fig. 2c).

**In-situ measurement of polar metabolites**. For acute glucose withdrawal studies, confluent 15 cm plates of HEK293T cells were switched from RPMI media containing 10 mM glucose into glucose-free RPMI media and grown for 30 min. For control cells the media was replaced with fresh, glucose-containing media. Under both conditions, cells were scraped into ice-cold PBS and isolated by centrifugation at 1,400 g and 4°C. Cell pellets were resuspended in 300 µl of an 80:20 mixture of MeOH:H₂O. Internal deuterated standards, 10 mM d₅-serine, were added to the extraction solution for quantification and sample normalization. LC–MS/MS analysis was then performed as described\(^\text{20}\).

**Gel electrophoresis and western blot.** To determine the effect of the 3-min heat pulses on global phosphorylation level, we exposed cells to the thermal profiling heat pulses as detailed above, but directly homogenized whole proteome by heating to 95°C for 30 min. Western blot analysis using an anti-GAPDH antibody was performed as described above. The GAPDH probe was chosen because it is widely expressed in multiple cell lines and very robustly expressed in our HEK293T cell line. The GAPDH probe was probed at 1 μg/ml and stained with ECL Plus Western Blotting Detection Reagent as described above.
addition of 4X Laemmli buffer containing 50 mM DTT (Supplementary Fig. 3). Samples were prepared for SDS–PAGE by heating to 95 °C for 5 min, cooled to room temperature, resolved on 10% SDS–PAGE gel and transferred onto nitrocellulose membranes by standard western blotting methods. Membranes were blocked in 2% BSA in TBS containing 0.1% Tween-20 (TBST) and probed with primary and secondary antibodies. Antibodies used in this study included: anti-pS/T/Y (1:1,000, ab15556, AbCam). Secondary anti-mouse (Licor) was used at 1:10,000 dilution in 2% BSA-containing TBST and incubated for 1 h before washing and imaging on a Licor infrared scanner. Densitometry measurements were performed with ImageJ software.

**Vinculin expression and focal adhesion confocal microscopy assays.** Full-length Myc-DDK-tagged human vinculin (NM_003573, Origene) was used to generate mutants with PCR primers in Supplementary Table 3 according to the QuikChange II Site-Directed Mutagenesis Kit manufacturer protocol (200523, Agilent). HEK cells were grown to 70% confluency in 6-well plates before transfection of 2 μg plasmid with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, cells were collected by scraping, pelleted by centrifugation, and resuspended in 1× PBS. Following addition of 4X Laemmli buffer containing 50 mM DTT (Supplementary Fig. 3), samples were prepared for SDS–PAGE by heating to 95 °C for 5 min, cooled to room temperature, resolved on 10% SDS–PAGE gel and transferred onto nitrocellulose membranes by standard western blotting methods. Membranes were blocked in 2% BSA in TBS containing 0.1% Tween-20 (TBST) and probed with primary and secondary antibodies. Antibodies used in this study included: anti-pS/T/Y (1:1,000, ab15556, AbCam). Secondary anti-mouse (Licor) was used at 1:10,000 dilution in 2% BSA-containing TBST and incubated for 1 h before washing and imaging on a Licor infrared scanner. Densitometry measurements were performed with ImageJ software.

**References**

36. Huang, J. X., Lee, G. & Moellering, R. E. Discovery and interrogation of functional protein modifications by Hotspot Thermal Profiling. *Protoc. Exch.* https://doi.org/10.21203/rs.2.10602/v1 (2019).

37. Chang, J. W., Lee, G., Coukos, J. S. & Moellering, R. E. Profiling reactive metabolites via chemical trapping and targeted mass spectrometry. *Anal. Chem.* 88, 6658–6661 (2016).
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- [ ] n/a
- [X] Confirmed

- [X] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- [X] An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [X] The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- [X] A description of all covariates tested
- [X] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- [X] A full description of the statistics 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)
- [X] 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.
- [X] 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
- [X] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- [ ] Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

1. Thermo Xcalibur, Qual Browser (Version 4.0.27.19) - Quantitative Proteomics
2. MetaMorph® Microscopy Automation & Image Analysis Software (Version 7.8.8.0) – Fluorescence Microscopy
3. Odyssey Infrared Imaging Software (Version 3.0) – Western Blots Imaging
4. i-control™ software for Tecan Infinite M200 – Absorbance Measurement
5. Agilent MassHunter Workstation Software – Data Acquisition and Analysis for 6400 Series Triple Quadrupole program version B.06.00

Data analysis

1. ImageJ (Fiji version 2.0.0) - Densitometry
2. Integrated Proteomics Pipeline (iP2) (Version 6.0.2), ProLuCID algorithm, LuciPHOR algorithm - Quantitative Proteomics
3. RStudio (Version 1.1.463) – Melting curve analysis
4. TTP: Analyze thermal proteome profiling (TPP) experiments (R package version 3.10.0) – Melting curve analysis
5. RaptorX: raptorx.uchicago.edu - Protein secondary structure prediction
6. Graphs presented were generated in GraphPad Prism 5.0, with analysis algorithms indicated in figure legends
7. All figures were drawn in Adobe Illustrator CC 2018
8. Agilent MassHunter Workstation Software – Data Acquisition and Analysis for 6400 Series Triple Quadrupole program version B.06.00

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/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Primary data for proteomic analyses have been uploaded to MassIVE (dataset DOI: 10.25345/C5CS7H). Raw files can be downloaded from ftp://massive.ucsd.edu/MSV000083786. All other data and materials are available upon reasonable request.

Field-specific reporting

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- Ecological, evolutionary & environmental sciences

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Life sciences study design

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

| Sample size | Sample size for cellular experiments were determined according to the minimal number of independent biological replicates powered to significantly identify an effect. In vitro experiments were performed in replicate independent measurements as indicated in the text. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded. |
| Replication | All experiments were independently replicated, with biological and technical replicates listed in the legends of the corresponding figures. All attempts at replication were successful. |
| Randomization | Randomization was not relevant in this study because no live organisms were used in this study. |
| Blinding | Blinding was not relevant in this study. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|--------------------------------|---------|
| n/a | n/a |
| □ Unique biological materials | □ ChIP-seq |
| ❌ Antibodies | □ Flow cytometry |
| □ Eukaryotic cell lines | □ MRI-based neuroimaging |
| □ Palaeontology | |
| □ Animals and other organisms | |
| □ Human research participants | |

Antibodies

Antibodies used:

| List of commercial antibodies used: |
|-----------------------------------|
| [Antigeg, Company (Cat. No. Lot#), Source, Dilutions] |
| 1. α-pS/T/Y, AbCam (ab15556), Mouse, 1:1000 |
| 2. α-FLAG-M2, Sigma Aldrich (F1804), Mouse, 1:1000 |
| 3. α-FLAG, Cell Signaling (14795, Lot 4), Rabbit, 1:500 |
| 4. α-Paxillin, Millipore (Lot 2798599), Mouse, 1:100 |
| 5. IRDye® 800CW α-Rabbit, Licor (925-32213, C0116-07), Donkey, 1:10000 |
| 6. IRDye® 800CW α-Mouse, Licor (925-32211, CS0129-02), Donkey, 1:10000 |
| 7. Alexafluor488-Conjugated α-Rabbit, Sigma Aldrich (A11034, Lot 1937195), Goat, 1:200 |
| 8. Alexa568-Conjugated Goat-α-Mouse, Sigma Aldrich (A11004, Lot 1698376), Goat, 1:200 |
Validation

Commercial antibodies were validated by relevant citations, validation statements provided by the manufacturer, and data provided in the manuscript.

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | 1. HEK293T (ATCC, CRL-3216)  
2. HeLa (ATCC, CCL-2) |
|---------------------|-------------------------------------------------|
| Authentication      | None of the cell lines were profiled for authentication. |
| Mycoplasma contamination | Cells lines used in this study tested negative for mycoplasma contamination. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used in this study. |