Identification of phosphosites that alter protein thermal stability

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Proteomic methods have enabled the discovery of hundreds of thousands of protein phosphorylation sites; however, we lack methods to systematically annotate their function. Phosphorylation has numerous biological functions that biochemically involve changes in protein structure and interactions. These biochemical changes can be detected by measuring the difference in thermal stability between the protein and the phosphoprotein. Recently, Huang et al. developed the Hotspot Thermal Profiling (HTP) method to identify phosphosites that alter protein thermal stability, reporting 719 of 2,883 (25%) measured human phosphoisoforms from HEK293T cells with significant effects.

Huang et al’s reported melting temperatures ($T_m$) for phosphoisoforms and their corresponding proteins showed low correlation ($R^2 = 0.18$) (Fig. 1a), implying that phosphorylation largely functions by structurally reshaping the proteome. However, upon careful examination of their data, we found poor reproducibility for $T_m$ values between replicates (mean pairwise $R^2 = 0.42$ for proteins and 0.21 for phosphoisoforms, Fig. 1b). This suggests that the large effects observed may be due to technical variation instead of biological variation, given that critical steps of the method are conducted separately for phosphopeptides and proteins and for the different temperature sample points (Supplementary Discussion).

The HTP workflow described by Huang et al. consists of phosphopeptide enrichment followed by separate isotopic labeling and mass spectrometric analysis to derive $T_m$ values for phosphoisoforms and proteins, respectively. To assess technical variation between the two samples, we analyzed unmodified peptides identified in phosphopeptide samples, which are expected to yield the same $T_m$ values as the parent proteins (Supplementary Discussion). Our reanalysis revealed significant stability effects on 1,223 of 3,074 (40%) of the phosphoisoforms (average $R^2 = 0.92$) for proteins (average $R^2 = 0.77$) and phosphoisoforms (average $R^2 = 0.66$). The stability of unmodified peptides detected in phosphopeptide-enriched samples was also correlated with that of their proteins ($R^2 = 0.92$ for mean $R_s$ comparisons), indicating that $R_s$ measurements in phosphopeptide samples and protein samples can be reliably compared (Extended Data Fig. 1b). Finally, our analysis yielded 68 yeast phosphopeptide isoforms of 1,978 (3%) with significantly different thermal stability than that of the unmodified protein (Fig. 1g and Supplementary Data 3). Most of these peptides (six of eight) were cases in which the protein is known to be posttranslationally processed via cleavage (for example, ribosomal protein RPS31 (ref. 3)) or splicing (for example, the ATPase VMA1 (ref. 3)), resulting in proteins and/or proteoforms of different thermal stability, as our method reliably measured (Extended Data Fig. 3).
Among phosphosites that decreased protein thermal stability, we identified four sites located at protein interfaces (Ser56 on PUP2, Ser59 on ARO8, Ser79 on TPI1 and Ser201 on GAPDH) (Fig. 2a and Extended Data Fig. 4) that may act by disrupting protein–protein interactions. For example, PUP2 is the α5 subunit of the 20S proteasome, and Ser56 is a known substrate of kinase CDC28 (ref. 8) located at the protein interaction interface with PRE6, the 20S proteasome α4 subunit (Fig. 2a). The stability measured for the phosphopeptide spanning Ser56 is significantly lower than the stability of PUP2, which is similar to other proteins in the 20S proteasome, suggesting that Ser56 phosphorylation may dissociate PUP2 from the 20S proteasome. We identified stabilizing phosphosites that may...
play a role in the protein translation process. For example, we found that phosphorylation at Ser38 on ribosomal protein RPL12 (uL11) significantly increased protein stability (Fig. 2b). This phosphosite is an evolutionarily conserved CDC28 substrate that is regulated during the cell cycle and was depleted in polysomes and influenced mitotic translation. Considering RPL12 location at the ribosome during the cell cycle and was depleted in polysomes and influenced may stabilize RPL12. We also identified a stabilizing phosphorylation on the translation termination and ribosome biogenesis factor NEW1 at Thr1191 (ΔR = 1.23) (Fig. 2c). A NEW1 T1191A mutant has growth defects, suggesting that phosphorylation is important for NEW1 function (Supplementary Discussion). Additionally, we identified phosphosites that may modulate the kinetics of key glycolytic enzymes (Thr331 on PGK1 destabilization, Ser149 on GAPDH stabilization) (Extended Data Fig. 5 and Supplementary Discussion). Further experimental characterization of these phosphosites will help establish their precise function.

Dali enables robust comparison of thermal stability between proteins and phosphorylated proteoforms. One potential limitation of our method is that the sensitivity to detect changes in thermal stability is lower for proteins with extreme melting temperatures, which can be circumvented by performing the experiment using different temperature gradients. Our method can be extended to other model organisms and cell culture systems, as well as to other posttranslational modifications, expanding the proteomic toolkit to functionally annotate dynamic protein modifications at scale.

Online content
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References
1. Hornbeck, P. V. et al. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res. 43, D512–D520 (2015).
2. Huang, J. X. et al. High throughput discovery of functional protein modifications by Hotspot Thermal Profiling. Nat. Methods 16, 894–901 (2019).
3. Potel, C. M. et al. Impact of phosphorylation on thermal stability of proteins. Nat. Methods https://doi.org/10.1038/s41592-021-01177-5 (2021).
4. Gaetani, M. et al. Proteome Integral Solubility Alteration: a high-throughput proteomics assay for target deconvolution. J. Proteome Res. 18, 4027–4037 (2019).
5. Savitski, M. M. et al. Tracking cancer drugs in living cells by thermal profiling of the proteome. Science 346, 1255784 (2014).
6. Finley, D., Bartel, B. & Varshavsky, A. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature 338, 394–401 (1989).
7. Kane, P. M. et al. Protein splicing converts the yeast TFP1 gene product to the 69-kD subunit of the vacuolar H+/adenosine triphosphatase. Science 250, 651–657 (1990).
8. Holt, L. J. et al. Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science 325, 1682–1686 (2009).
9. Dephoure, N. et al. A quantitative atlas of mitotic phosphorylation. Proc. Natl Acad. Sci. USA 105, 10762–10767 (2008).
10. Imami, K. et al. Phosphorylation of the ribosomal protein RPL12/uL11 affects translation during mitosis. Mol. Cell 72, 84–98 (2018).
11. Vieitez, C. et al. Towards a systematic map of the functional role of protein phosphorylation. Preprint at bioRxiv https://doi.org/10.1101/872770 (2019).

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Fig. 2 | Examples of phosphosites that alter protein thermal stability. a, Box plot of R values and distributions for the phosphopeptide containing PUP2 Ser56, PUP2 protein and all the proteins in the 20S proteasome. Shown at the right is the structure of the 20S proteasome with PUP2 in blue, Ser56 in red and PRE6 in gray. b, R values for RPL12 and the RPL12 Ser38 phosphopeptide isoform. c, R values for NEW1 and the phosphopeptide isoform containing NEW1 T1191. Box plots show results from six biological replicates, the line represents the median, the box designates the IQR, and the whiskers define 1.5×IQR from the box ends.
Matters arising

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S. cerevisiae growth, stable isotope labeling and cell collection. Two overnight yeast cultures were grown at 30 °C in synthetic complete medium containing 6.7 g−1 yeast nitrogen base, 2.1 g−1 synthetic complete mix minus lysine and 2% glucose, supplemented with either regular lysine (light culture) or H4-lysine (heavy culture) at a final concentration of 0.872 mM. These cultures were used to seed three 50-ml cultures each of light and heavy cultures at an OD405 of 0.15, which were grown at 30 °C, and 45 ml was collected at OD405 = 1 by centrifugation at 7,000 × g for 10 min. Yeast pellets were washed by resuspension in 1.5 ml ice-cold sterile water and centrifugation in 2 ml screw cap tubes at 21,000 × g for 10 min and then snap frozen in liquid nitrogen and stored at −80 °C.

Cell lysis and protein extract temperature treatment. Frozen yeast cell pellets were resuspended in 700 μl non-denaturing lysis buffer (50 mM HEPES, pH 7.0, 75 mM NaCl) containing 0.5X protease inhibitors (Pierce) and phosphatase inhibitors (50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium orthovanadate) on ice. Cells were lysed by bead beating with 0.5-mm zirconia-silica beads for four cycles of mechanical agitation for 60 s, followed by a 90-s rest on ice. Lysates were clarified by sequential centrifugation, first at 1,200 × g for 1 min to remove beads and then at 21,000 × g for 10 min at 4 °C to remove cell debris. To bring all protein extracts to the same concentration, extract volumes were adjusted to 1 OD405 unit from a 45-ml culture in 1 ml.

Each cell extract was divided into two steps of eight right PCR tubes each (1 × 8 for the temperature gradient and 1 × 8 for the 30 °C control), dispensing 50 μl protein extract per tube. All samples were initially equilibrated to 30 °C for 5 min. Temperature-gradient samples were subjected to temperatures of 45.6 °C, 46.8 °C, 48.3 °C, 50 °C, 52 °C, 53.6 °C, 54.9 °C and 57 °C (one tube for each temperature) for 5 min. In parallel, controls were subjected to an additional treatment at 30 °C for 5 min. All samples were cooled down to room temperature for 10 min. For each replicate, temperature-gradient samples were all pooled into one tube and 30 °C controls were pooled into a separate tube before centrifugation at 21,000 × g for 30 min at 4 °C. The soluble protein fractions for the temperature gradient and 30 °C controls were combined at a ratio of 2:1; three replicates with the temperature gradient labeled with the heavy isotope, the 30 °C controls labeled with the light isotope and three additional replicates with the labels swapped. We generated additional controls in which heavy- and light-isotope-labeled 30 °C controls were combined to assess potential differences in protein expression due to different labeling. Protein concentration was measured by the BCA assay.

Protein reduction, alkylation, LysC digestion and desalting. Samples were diluted twofold with a buffer containing 8 M urea, 500 mM HEPES, pH 8.9, 75 mM NaCl, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate and 50 mM NaF. Protein samples were subjected to reduction with 5.75 mM dithiothreitol for 30 min at 55 °C and alkylation with 22.5 mM iodoacetamide for 30 min at room temperature in the dark with agitation. The alkylation reaction was quenched with an additional 7.5 mM dithiothreitol for 30 min at 37 °C and 1 ml 0.1% TFA. Peptides were then diluted twofold with a buffer containing 8 M urea, 500 mM HEPES, pH 8.9, 75 mM NaF, 10 mM sodium pyrophosphate, 0.25% acetic acid, 1 ml 40% acetonitrile, 1 ml 0.1% TFA and 1 ml 0.5% acetic acid. Peptides were eluted with 60 μl 40% acetonitrile, 0.5% acetic acid and 400 μl 70% acetonitrile, 0.25% acetic acid and aliquoted as follows: 40 μl for high-pH reversed-phase fractionation, 200 μg for Fe3+-NTA IMAC phosphopeptide enrichment and 10 μg for preliminary LC–MS/MS analysis to assess sample quality. All samples were dried by vacuum centrifugation and stored at −80 °C.

High-pH reversed-phase fractionation. Peptides were fractionated by high-pH reversed-phase fractionation on a 200-μl pipette tip packed with four layers of SDB-XC material (Empore, 3M). The material was washed with 50 μl methanol, 30 μl 80% acetonitrile, 20 mM ammonium formate and 3 × 50 μl 20 mM ammonium formate. Peptides (40 μg) were solubilized in 40 μl 5% acetonitrile, 20 mM ammonium formate and loaded onto the SDB-XC tip, and the flow through was collected in an MS vial (fraction 1). Peptide fractions 2–5 were obtained by stepwise elution with 40 μl 20 mM ammonium formate in 10%, 15%, 20% and 80% acetonitrile and collection in MS vials. Peptide fractions were dried by vacuum centrifugation, solubilized in 3% acetonitrile, 4% formic acid, and ~1 μg of each fraction was analyzed by LC–MS/MS.

Fe3+-NTA IMAC phosphopeptide enrichment. Phosphopeptide enrichment was conducted by immobilized iron cation affinity chromatography in batch mode and automated in a 96-well format on a KingFisher magnetic particle processor as we described previously. For each sample, ~200 μg peptides were solubilized in 70 μl 0.1% TFA, 80% acetonitrile and incubated with 80 μl of a 5% slurry of magnetic Fe3+-NTA beads (Cobe Biotech) for 30 min at room temperature. The beads were washed three times with 150 μl 0.1% TFA, 80% acetonitrile, and phosphopeptides were eluted with 50 μl 50% acetonitrile, 0.37 M ammonium hydroxide. Eluates were acidified with 30 μl 10% formic acid, 75% acetonitrile and filtered over two-layer C18 extraction disks (Empore, 3M) packed in 200-μl pipette tip, which had been previously conditioned with 50 μl 100% methanol, 50 μl 100% acetonitrile and 50 μl 70% acetonitrile, 0.25% acetic acid. Filtered peptides were collected in an MS vial, and the peptides in the extraction disk were further eluted with 50 μl 70% acetonitrile, 0.25% acetic acid and collected into the same MS vial. Phosphopeptide-enriched samples were dried by vacuum centrifugation and solubilized in 3% acetonitrile, 4% formic acid, and one-third of each sample was analyzed by LC–MS/MS.

Liquid chromatography coupled to tandem mass spectrometry. Peptide samples were analyzed by nLC–MS/MS on a nanoAcquity UPLC (Waters) coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher) with Xcalibur (4.1.50) and Thermo Foundation 3.1 SP4 (3.1.1990). Samples were loaded on a 100-μm × 3-μm trap column packed with 3-μm C18 beads (Dr. Maisch), separated on a 100-μm × 30-cm capillary analytical column, packed with 1.9-μm C18 beads (Dr. Maisch) and set at 50 °C, using a 90-min reverse-phase gradient of acetonitrile in 0.125% formic acid and analyzed online by MS using data-dependent acquisition. Each cycle consisted of 3 s data-dependent MS scan was acquired on the Orbitrap at a resolution of 120,000 from 300 to 1,575 m/z using an automatic gain control (AGC) of 7 × 105 and a maximum injection time of 50 ms, followed by MS/MS-dependent scans on the most intense precursor m/z ions (only considering z = 2–5) until the 3-s cycle time was exhausted, using a 1.6-m/z isolation window and HCD fragmentation at a normalized collision energy (NCE) of 35%, acquired at a resolution of 15,000 on the Orbitrap with 0–1.9 × 105 (peptide samples) and an AGC of 1 × 106 (phosphopeptide samples) with a maximum injection time of 22 ms. Dynamic exclusion was enabled to exclude fragmented precursors from repeated MS/MS selection for 30 s. To increase coverage, phosphopeptide samples were injected twice, and data from two technical replicates were combined.

Database searching, peptide quantification, phosphosite localization and Rn calculation. MS data files for proteome samples were analyzed with MaxQuant (version 1.6.7.0) to obtain peptide identifications and quantifications using the following parameters: the protein sequence database for S. cerevisiae downloaded from SGD in July 2014, LysC enzyme specificity (cleavage C terminal to K), a maximum of two missed cleavages, a mass tolerance of 20 ppm for MS and 20 ppm for MS/MS, fixed modification of carbamidomethyl on cysteines and variable oxidation modifications on methionine residues and acetylation on protein N termini. Lysine residues were only allowed to be all light isotopes or all H4-lysines within the same peptide. Data from phosphoproteome samples were processed in MaxQuant in a similar way, with additional phosphoserine modification on serine, threonine and tyrosine residues. All searches were combined for MaxQuant filtering set to 1% FDR at the level of peptide-spectral matches and protein.

Quantification values for heavy and light peptide features were extracted from the evidence.txt file. Quantification values for features corresponding to the same peptide sequence (for example, the same peptide identified at multiple charge states or fractions) were summed. Phosphopeptide quantification features were aggregated to the phosphopeptide isoform level by summing features corresponding to the same peptide sequence (for example, the same peptide identified at multiple charge states or replicate injections) as well as overlapping peptide sequences sharing the same combination of modifications. For each phosphopeptide isoform, we required the maximum localization probability to be greater than 75% for at least one site. Rn values were calculated as log, ratios of the quantification value for samples treated with a gradient of temperatures divided by the respective quantification for those samples treated with a gradient of temperatures with the respective quantification values for those of their unmodified protein counterpart, we performed a t-test comparing phosphopeptide isoform Rn values (n = 6) to protein Rn values (n = 6), assuming unequal variance. Phosphopeptide isoforms and protein counterparts were required to be observed in at least three replicates. P values were corrected for multiple-hypothesis testing using the Benjamini–Hochberg method. All data analysis was conducted using R (version 3.6.1) and Rstudio (version 1.2.1335), and data figures were generated in R and Adobe Illustrator CS3 (version 15.0.0).
Structure visualization and bioinformatics. Protein complex annotations were extracted from the CYC2008 resource 1. Protein structure coordinates were downloaded from the PDB and visualized and manipulated with PyMOL 2. For PPI2-interface analysis, we extracted 20S proteasome protein structure from PDB 1RPP 3. Protein interface structures for ARO8, TPI1 and GAPDH were extracted from the PDB (4HE5 (ref. 4), INEY 5 and 3PYM, respectively). To assess the stabilizing effect of Ser149 phosphorylation at the catalytic site of GAPDH, we aligned crystal structures of GAPDH with bound G3P (1NQO 6) and inorganic phosphates (1GP 7) to an NAD-bound yeast GAPDH structure (3PYM) 8. Data on sequence conservation, protein interfaces and predicted stability effects of mutations (\(\Delta \Delta G_{\text{pred}}\)) were obtained from the mutfunc resource 9.

Reanalysis of the Huang et al. data. Supplementary data from Huang et al.2 was used to calculate the correlation between \(T_P\) values for phosphopeptides and proteins and to learn about their statistical parameters. For data reanalysis, all MS files from the study were downloaded from the MassIVE data repository (dataset identifier, MSV000083786), converted to open-format mXML files with ReAdW (2015.1.0) and database searched with Comet 23 (version 2015.02, revision 2) to obtain peptide and phosphopeptide identifications, with the exception of the ‘Bulk_6_2’ file, which failed to convert. Database search parameters were the human protein sequence database from UniProt (UP000005640), a mass tolerance of 50 ppm for precursor m/z and 0.2 Da for fragment ions, trypsin enzyme specificity (cleavage Ct to K, R, except for KP, RP, a maximum of two missed cleavages, fixed modification of carbamidomethyl on cysteines and TMT10 (+229.1629 Da) on lysine residues and peptide N termini and variable oxidation modifications on methionine residues and acetylation on protein N termini. Phosphorylation samples included variable phosphorylation modification at serine, threonine and tyrosine. Search results were filtered to <1% FDR at the PSM level with Percolator 10. Phosphosite localization was conducted with an in-house C++ implementation of Ascore 11, and sites with Ascore > 13 were considered confidently localized (P < 0.05). TMT10 reporter ion intensities were extracted from MS/MS scans using in-house TMT quantification software (IsobaricQuant 0.5).

We attempted to replicate the analysis conducted by Huang et al.2 by following the method description provided in their study. Biological and technical replicates were treated equally. For each replicate, TMT reporter ion intensities for all peptide-spectral matches from proteome files were summed to the protein level, and TMT reporter ion intensities for phosphopeptide-spectral matches were summed to the phosphopeptide isofrom level. In addition, we used the same strategy to aggregate TMT signals to the peptide level for PSMs mapping to the same unmodified peptide observed in the phosphopeptide-enriched samples. We implemented the TPP package in R to fit melting curve data for proteins, phosphopeptide isoforms, and unmodified peptides in the phosphorylation-enriched sample. To recapitulate the reported results, we had to conduct the fitting for all samples together (Supplementary Discussion). Melting curves were filtered for fitting R>0.8. t-tests were conducted by comparing \(T_P\) values for phosphopeptide isoforms or unmodified peptides observed in phosphopeptide-enriched samples to unmodified protein \(T_P\) values, assuming equal variances, without multiple-hypothesis correction (as implemented by Huang et al.2). Of note, our reanalysis revealed that one of the phosphoproteome technical injections for biological replicate 5 was instead a repeated MS analysis of biological replicate 4.

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

Data availability
The MS proteomic data generated for this study were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016790.

Code availability
All code to reproduce analysis and data figures is available at https://gitlab.com/public_villenlab/dali_phospho_thermalstability.

References
12. Leutert, M., Rodriguez-Mias, R. A., Fukuda, N. K. & Villén, J. R2–P2 rapid-robotic phosphoproteomics enables multidimensional cell signaling analysis. Mol. Syst. Biol. 15, e9021 (2019).
13. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).
14. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B (Methodol.) 57, 289–300 (1995).
15. Pu, S., Wong, J., Turner, B., Cho, E. & Wodak, S. I. Up-to-date catalogues of yeast protein complexes. Nucleic Acids Res. 37, 825–831 (2009).
16. The PyMOL Molecular Graphics System v. 1.2.3pre (Schrodinger, LLC.).
17. Groll, M. et al. Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386, 463–471 (1997).
18. Bulfer, S. L., Brunzelle, J. S. & Trievel, R. C. Crystal structure of Saccharomyces cerevisiae Aro8, a putative α-aminoacidate aminotransferase. Protein Sci. 22, 1417–1424 (2013).
19. Jogl, G., Rozovsky, S., McDermott, A. E. & Tong, L. Optimal alignment for enzymatic proton transfer: structure of the Michaelis complex of triosephosphate isomerase at 1.2 Å resolution. Proc. Natl Acad. Sci. USA 100, 50–55 (2003).
20. Didierjean, c. et al. Crystal structure of two ternary complexes of phosphorylating glyceraldehyde-3-phosphate dehydrogenase from Bacillus steaothermophilus with NAD and d-glyceraldehyde-3-phosphate. J. Biol. Chem. 278, 12968–12976 (2003).
21. Kim, H., Fei, i. K., Verlinde, C. L. M. J., Petra, P. H. & Hol, W. G. J. Crystal structure of glycosylated glyceraldehyde-3-phosphate dehydrogenase from Leishmania mexicana: implications for structure-based drug design and a new position for the inorganic phosphate binding site. Biochemistry 34, 14975–14986 (1995).
22. Wagith, O. et al. A resource of variant effect predictions of single nucleotide variants in model organisms. Mol. Syst. Biol. 14, e8430 (2018).
23. Eng, J. K., Jahn, T. A. & Hoopmann, M. R. Comet: an open-source MS/MS sequence database search tool. Proteomics 13, 22–24 (2013).
24. Käll, L., Canterbury, J. D., Weston, J., Noble, W. S. & MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat. Methods 4, 923–925 (2007).
25. Beausoleil, S. A., Villén, J., Gerber, S. A., Rush, J. & Gygi, S. P. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat. Biotechnol. 24, 1285–1292 (2006).

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Author contributions
I.R.S., K.N.H., R.A.R.-M. and I.V. conceived the study and designed experiments. I.R.S. conducted experiments with advice from K.N.H., R.A.R.-M. and J.V. and assistance from I.R.S., K.N.H., R.A.R.-M. and J.V. I.R.S., K.N.H., R.A.R.-M. and J.V. supervised the conducted experiments with advice from K.N.H., R.A.R.-M. and J.V. and assistance from I.R.S., K.N.H., R.A.R.-M. and J.V. I.R.S. and J.V. wrote the paper, and all authors edited it.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Reproducibility and robustness of Dali compared to HTP. **a**, Scatter plot and Pearson correlation between the mean $T_m$ for unmodified peptides observed in the phosphopeptide enriched samples ($n=10$) and the mean $T_m$ for their corresponding proteins ($n=11$). Results from the Huang et al. data reanalysis conducted by us. **b**, Scatter plot and Pearson correlation as in **a** with $R_s$ values obtained from the Dali method ($n=6$).
Extended Data Fig. 2 | Phosphosites that significantly alter protein thermal stability using two different statistical settings. Volcano plots showing ΔT_m for mean phosphopeptide isoform to mean protein counterpart in the x axis, and the two-sided Student’s t-test probability in the y axis. a, Huang et al. implementation shows a P value because multiple hypothesis correction was not applied. Significant phosphopeptide isoforms (blue) are defined by P value < 0.05. b, Our proposed analysis consolidates data from MS reanalysis prior to statistical testing using a two-sided Welch’s t-test, which is performed assuming unequal variances between phosphopeptide isoform and proteins. Benjamini-Hochberg adjustment was used to correct P values for multiple hypothesis testing. Significant phosphopeptide isoforms (blue) are defined by q value < 0.05.
Extended Data Fig. 3 | Examples of significant hits on proteins that undergo posttranslational splicing or cleavage. a, \( R_s \) values for observed VMA1 unmodified peptides identified in phosphopeptide-enriched samples and proteome samples displayed across the length of VMA1. Spliced products from amino acid 2–283 and 738–1031 are joined to generate the V-type proton ATPase catalytic subunit A proteoform, extinguishing the 284–737 segment. Peptides derived from the proteome samples are colored in gray and significant unmodified peptides found in the phosphopeptide-enriched sample are in red. b, Similar plot to a for RPS31, which is cleaved to generate ubiquitin (1–76 amino acid segment) and 40S ribosomal protein S31 (77–152 amino acid segment) proteins.
Extended Data Fig. 4 | Examples of phosphosites that alter protein thermal stability and are located at protein interfaces. R, boxplots for a, ARO8 S59, b, TPI1 S79, and c, GAPDH S201 phosphopeptide isoforms and their protein counterparts. All boxplots show results from n = 6 biological replicates, and the line represents the median, the box designates the interquartile range (IQR), and the whiskers define 1.5*IQR from the box ends. ARO8 S59, TPI1 S79, and GAPDH S201 reside at dimerization interfaces as shown in the structures to the right (PDB accession: 4JE5, 1NEY, and 3PYM, respectively). Phosphomimetic mutations ARO8 S59E and TPI1 S79E are predicted to disrupt protein interfaces (ΔΔG<sub>pred</sub> = 3.78 and ΔΔG<sub>pred</sub> = 8.04 respectively). Additionally, TPI1 S79E mutation is predicted to alter protein conformational stability (ΔΔG<sub>pred</sub> = 2.39). ΔΔG<sub>pred</sub> > 2 is predicted to be destabilizing.
Extended Data Fig. 5 | Examples of phosphosites that alter protein thermal stability on glycolytic enzymes. a, $R_s$ values for PGK1 and all measured PGK1 phosphopeptide isoforms, with significantly destabilizing phosphosite S331 shown in red. $\Delta \Delta G_{\text{pred}}$ for all glutamic acid phosphomimetic substitutions were obtained from mutfunc, with $\Delta \Delta G_{\text{pred}} > 2$ considered likely destabilizing. b, GAPDH S149 phosphopeptide is shared across all GAPDH paralogs (TDH1, TDH2, and TDH3). Boxplot shows $R_s$ values and distributions for peptides unique to one isoform (TDH1, TDH2, TDH3), peptides shared among all GAPDH isoforms (all), all peptides for TDH3, and the S149 phosphopeptide isoform. Bottom panel shows localization of S149 on the GAPDH structure near the binding site of the enzyme substrate. Boxplots show results from 6 biological replicates, the line represents the median, the box designates the interquartile range (IQR), and the whiskers define 1.5*IQR from the box ends.
Statsitics

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Software and code

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Data collection

1) Thermo Foundation 3.1 SP4 [3.1.190.0] and Xcalibur [4.1.50]: MS data acquisition

Data analysis

1) RStudio [1.2.1335] and R [3.6.1]: data analysis and figure generation

2) MaxQuant [1.6.7.0]: MS database search for Dalil data for peptide identification and quantification

3) ReAdW [2015.1.0]: MS raw file converter to.mzXML

4) Comet (2015.02 rev. 2): MS database search used here for Huang et al. re-analysis

5) Percolator (3.0.1): Data filtering and FDR calculation of peptide spectral matches of Huang et al. data re-analysis

6) IsobaricQuant (0.5): TMT quantification, used here to extract TMT-reporter ion intensities from Huang et al. MS/MS spectra

7) In-house implementation of Ascore algorithm: site localization for phosphopeptides [algorithm described in Beausoleil et al. Nat. Biotech. 2005]

8) Adobe Illustrator CSS [15.0.0]: figure generation

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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

The mass spectrometry proteomics data generated for this manuscript have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016750. Huang et al. (Nat. Methods 2019) mass spectrometry datasets are publicly available at the MassIVE repository.
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.

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Life sciences study design

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

Sample size
We chose the sample size (number of replicates N = 6) according to previous phosphoproteomic studies in the lab (Lawerence et al. Nat. Methods 2016; Searle et al. Nat. Methods 2019). This sample size provides a high diversity of phosphopeptides (that are observed in at least N=3, half of the replicates) for biological analysis and evaluation of the Dali method, while factoring in time of analysis.

Data exclusions
All data is included.

Replication
We used Pearson’s correlation to assess reproducibility between biological replicates for protein and phosphopeptide measurements (Extended Data Figure 1a). We further show that protein and phosphopeptide samples can be confidently compared by showing the Pearson’s correlation between Rs for the unmodified peptides (measured in the phosphopeptide samples) and the protein. For internal assessment, we have also conducted an independent experiment using a different labeling scheme, which fully replicates our results and conclusions. For simplicity, this second experiment was not included in the manuscript.

Randomization
Each sample was internally controlled by mixing with a heavy lysine labeled sample that was incubated at 30°C. Digestion and enrichment of phosphopeptides were performed after mixing, to minimize technical variability. Protein fractions and their matched phosphopeptide fractions were then analyzed in blocks of three samples at a time. First phosphopeptide fractions 1-3, then protein fractions 1-3, then phosphopeptide fractions 4-6, and finally protein fractions 4-6. Phosphopeptide fractions 1-6 were subsequently measured with a second injection. Wash steps were performed when switching fraction type. This blocking structure was chosen to minimize time between analyzing phosphopeptide fractions and their matched protein fractions while also minimizing carry over of peptides between different fraction types.

Blinding
Blinding was not relevant in this study because knowledge of the sample identity would not impact the results.

Reporting for specific materials, systems and methods

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### Materials & experimental systems

| Material/Experiment | Relevant |
|---------------------|----------|
| n/a                 |          |
| Antibodies          | x        |
| Eukaryotic cell lines | x      |
| Palaeontology       | x        |
| Animals and other organisms | x |
| Human research participants | x |
| Clinical data       | x        |

### Methods

| Method               | Relevant |
|----------------------|----------|
| n/a                  |          |
| ChiP-seq             | x        |
| Flow cytometry       | x        |
| MRI-based neuroimaging | x  |