| Protein | Site | Peptide | Stoichiometry (%) | Validation |
|---------|------|---------|-------------------|------------|
|         |      |         | Expt #1 | Expt #2 | Expt #3 | (AQUA) |
| UBP1    | 618  | RIIEHS*DVENENVK  | 59      | 59     | 66     | 69.2   |
| BFR2    | 379  | DSVDDNENS*DDGLDIPK | 97      | 99     | 97     | 99.8   |
| UBP1    | 638  | DNEELQEIDNVS*LDEPK | 75      | 76     | ND     | 61.5   |
| IPP1    | 266  | AASDAIPPAS*PK     | 1       | 1      | 1      | 1.8    |
| NUP145  | 689  | AYEPLDSDFEGIEAS*PK | 68      | 73     | 71     | 78.8   |
| DCP2    | 710  | RGET*FASLANDK     | ND      | 1      | ND     | 2.0    |
| YML093W | 562  | LFES*DEETNGENIQINK | 100     | ND     | 99     | 99.5   |
| MOT2    | 92   | YDDENVRYVTLS*PEELK | 4       | 14     | 13     | 3.5    |
| GNP1    | 45   | QIGS*IEPENEVEYFEK | 1       | 9      | 10     | 8.2    |
| TAT1    | 84   | RQLPPDRNSELES*QEK | 45      | ND     | ND     | 41.4   |

“ND” denotes “not detected” in that analysis.

**ONLINE METHODS**

**Cell culture and lysis, and protein extraction and digestion**

The yeast strain was BY4742 MAT alpha, derived from S288c. Three biological replicate experiments were been carried out in this work. Wild-type cells were grown in YPD media. After ~18 h, cell populations had undergone over ten doublings until they reached log-phase (OD_{600} = 1.0). Yeast cells were harvested, then resuspended at 4°C in a buffer containing 50 mM Tris pH 8.2, 8 M urea, 75 mM NaCl, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and one protease inhibitor cocktail tablet (complete mini, EDTA-free, Roche) per 10 ml. Cells were lysed using the MiniBeadbeater...
(Biospec) for microcentrifuge tubes at maximum speed, four cycles of 60 s each, with 2 min pauses between cycles to avoid overheating of the lysates. After centrifugation, lysates were transferred to new tubes. The protein concentration in the lysate was determined by BCA protein assay (Pierce) and proteins were subjected to disulfide reduction with 5 mM DTT (56°C, 25 min) and alkylation with 15 mM iodoacetamide (RT, 30 min in the dark). Excess iodoacetamide was quenched with 5 mM DTT (room temperature, 15 min in the dark). The denatured protein extract was then digested with 5 ng/μl lys-C (Wako) in 25 mM Tris pH 8.8, 1.5 M urea, at 37°C for 15 h.

**Peptide separation**

Peptide mixtures were acidified by addition of 10% TFA to a final concentration of 0.4%, spun by centrifugation and desalted using a C18 SepPak cartridge (Waters). Purified peptides were separated using hydrophilic interaction chromatography (HILIC) into 20 fractions using a 4.6 × 250mm TSKgel Amide-80 5μm particle column (Tosoh Biosciences, Japan) with a 50-min gradient of 5-28% H2O in acetonitrile (ACN) (0.1% trifluoroacetic acid (TFA)).

**LC-MS/MS analyses**

Dried peptides were dissolved in 5% ACN and 4% formic acid (FA), and 2 μl was loaded onto a microcapillary column packed with C18 beads (Magic C18AQ, 5 μm, 200 Å, 125 μm × 16 cm) using a Famos autosampler (LC Packings). Peptides were separated by reversed-phase chromatography using an Agilent 1100 binary pump with a 70-min gradient of 5-30% ACN (in 0.125% FA) and detected in a hybrid dual-cell quadruple linear ion trap – orbitrap mass spectrometer (LTQ Orbitrap Velos, ThermoFisher) using a data-dependent Top20 method. For each cycle, one full MS scan in the Orbitrap at 3 × 10^6 AGC target was followed by up to 20 MS/MS in the LTQ for the most intense ions. Selected ions were excluded from further analysis for 30 s. Ions with charge 1+ or unassigned were also rejected. Maximum ion accumulation times were 1000 ms for each full MS scan and 150 ms for MS/MS scans.

**Database searches**

Following acquisition of mass spectrometry data, RAW files were converted into mzXML format. Individual precursors selected for MS/MS fragmentation were checked using algorithms that detect and correct errors in monoisotopic peak assignment and refine precursor ion mass.
measurements. All MS/MS spectra were then exported as DTA files and searched using the Sequest algorithm\(^{39}\). Spectra were matched against a database containing sequences of all proteins in the yeast ORFs database (6,607 entries) downloaded from SGD in both forward and reversed orientations. The following parameters were selected to identify peptides for protein expression experiments: 50 ppm precursor mass tolerance; 1.0 Da product ion mass tolerance; up to two missed cleavages; variable modifications: oxidation of methionine (+15.9949); fixed modifications: carbamidomethylation of cysteine (+57.0214). False discovery rates were controlled using the target-decoy strategy to distinguish correct and incorrect identifications\(^{30}\).

**Peptide quantification**

Peptide quantification was performed using the Vista program\(^{40}\). We required a S/N value > 3 for both heavy and light species for quantification. For peptides found exclusively as singlets (only heavy or only light peak present), we reported the peak S/N ratio or its inverse, as a proxy for relative abundance measurement. For such peptides, we required a S/N value > 5 for the observed species. In addition, if the S/N value of one member of a pair was less than three, the partner was required to be greater than five. Finally, to avoid quantifying FPs, any identification from a singlet peak was required to pass a ten-fold more stringent identification threshold (Q-value < 0.001; precision > 99.9%). Raw abundance ratios from each experiment were normalized based on the median distribution ratio.

**Phosphatase reaction**

Desalted peptides from each lys-C digest were split into two tubes equally and then dissolved in the phosphatase reaction buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM DTT, pH 7.9). Calf intestinal phosphatase (1,000 units; CIP; New England BioLabs) was incubated with one tube to dephosphorylate the phosphopeptides. To the other tube, no CIP was added. The reaction proceeded for both tubes at 37 °C for two hours. Reactions were quenched with TFA to final concentration of 0.5%.

To assess phosphatase efficiency, 0.5 mg aliquots from parallel mock- and phosphatase-treated samples were enriched for phosphopeptides using IMAC as described\(^{41}\). LC-MS/MS analysis resulted in the identification of 720 phosphopeptides (no reverse hits) in the mock-treated
sample. No phosphopeptides passed significance testing in the treated sample. It is important to note that reactions occurred on peptides and not proteins. We find no bias toward any sites at the peptide level with CIP.

**Phosphorylation site stoichiometry**

Yeast were seeded, grown, and harvested in three separate experiments. For each replicate, lysate was proteolyzed with endoproteinase lys-C. Two identical 0.5 mg peptide aliquots were subjected to either phosphatase treatment or a mock reaction. Following the reactions, peptides in the phosphatase-treated sample were chemically labeled by reductive dimethylation (ReDi) using deuteroformaldehyde to dimethylate free amines\(^{29}\). The mock reaction sample was chemically labeled with ReDi using formaldehyde. The two aliquots were then mixed, resulting in a 1:1 ratio for nearly all peptides analyzed by LC-MS techniques. However, sequences bearing phosphorylation would result in an increase in their levels directly proportional to the amount tied up by phosphorylation.

Our method requires a data set of phosphorylation sites to identify the ODPs. We chose to use five published data sets\(^{7,10,31-33}\). Each of these presented large-scale analyses of yeast phosphorylation and, importantly, localization information was also presented. We only used localized sites in these experiments. The localization was measured differently in each report. We chose to use the following cutoffs for localization: sites with Phospho (STY) Probabilities\(^{32}\) > 0.9; Ascore\(^{7,10,33}\) > 13, or PLScore\(^{31}\) > 10. Phosphorylation site stoichiometries were directly calculated according to the ratio of H/L \((1-1/\text{Ratio}) \times 100\%\). Values less than 1% were assigned to 1%.

**Validation of site stoichiometries using AQUA**

Ten pairs of heavy phosphopeptides and corresponding non-phosphopeptides were synthesized replacing one residue with an isotopically-enriched version (\(^{13}\)C and \(^{15}\)N) (Cell Signaling Technology). A known amount of heavy phosphopeptides and non-phosphopeptides were spiked into the proteolyzed sample. For the non-phosphopeptide experiment, proteolyzed lysates were separated by HILIC into 20 fractions. In the phosphopeptide experiment, during purification using the C18 SepPak column, the peptides were eluted into five fractions using different
concentrations of ACN and followed by IMAC enrichment of phosphopeptides. Finally, all samples were analyzed by the same LC-MS/MS techniques.

**Secondary structure prediction**
The likely secondary structural environment and level of disorder was predicted for each phosphorylation site using VSL2\textsuperscript{34}, which estimates the probability that each phosphorylation site was located in a region of high disorder. Those sites with disorder probabilities exceeding 0.5 were classified as 'disordered', while those with probabilities below 0.5 were classified as 'ordered'.

**Clustering of phosphoproteins**
Phosphoproteins were grouped into four classes according to their highest stoichiometry site: i.e. 0-10, 10-30, 30-75 and 75-100%. The enrichment analysis for gene oncology (GO) biological process (BP) and cellular compartment (CC) were performed separately for every class using the Database for Annotation, Visualization and Integrated Discovery (DAVID)\textsuperscript{36}, and phosphoproteins identified by mass spectrometric proteomics method were used as background\textsuperscript{7,10,31-33}. Categories without a P-value were assigned a conservative value of 1. The P-values were transformed by the function $x = -\log_{10}(P)$, and then they were z-transformed. Phosphoproteins were then grouped based on their z-scores via hierarchical clustering (Euclidean distance, Centroid linkage) using Gene Cluster 3.0.

**Calculation procedure of evolutionary conservation**
Whole genome DNA sequences for the organisms listed in Supplementary Fig. 3 were retrieved from NCBI and then used in a TBlastN search with the appropriate *S. cerevisiae* protein as the query. This was necessary as many of these organisms do not yet have fully annotated proteomes. These search results were then analyzed using in-house developed software to examine the residue at the corresponding position in the subject’s protein sequence to that of the phosphor-residue from the *S. cerevisiae* protein. For comparison purposes, only the top matching sequence from each organism was selected and when no sequence was found the conservation was designated as “no homologous sequence found”. Because we confined our analysis to just fungal genomes, the alignment was facilitated. A residue was considered conserved if the
corresponding residue position in the subject’s sequence was a perfect match to that of *S. cerevisiae*. After this procedure was applied to each alignment, conservation information was partitioned into 3 occupancy categories (high, ≥ 80%; moderate, < 80%, > 20%; low, ≤ 20%) and then conservation data for each of these categories was clustered using Pearson Correlation and visualized using MultiExperiment Viewer (http://www.tm4.org/mev/).

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