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Protein phosphorylation is essential for numerous cellular processes. Large-scale profiling of phosphoproteins continues to enhance the depth and speed at which we understand these processes. The development of effective phosphoprotein and peptide enrichment techniques and improvements to mass spectrometric instrumentation have intensified phosphoproteomic research in recent years, leading to unprecedented achievements. Here, we describe a large-scale phosphorylation analysis of α-factor-arrested yeast. Using a multidimensional separation strategy involving preparative SDS-PAGE for prefractionation, in-gel digestion with trypsin, and immobilized metal affinity chromatography (IMAC) enrichment of phosphopeptides, followed by LC−MS/MS analysis employing a hybrid LTQ-Orbitrap mass spectrometer, we were able to catalog a substantial portion of the phosphoproteins present in yeast whole-cell lysate. This analysis yielded the confident identification of 2288 nonredundant phosphorylation sites from 985 proteins. The ambiguity score (Ascore) algorithm was utilized to determine the certainty of site localization for the entire data set. In addition, the size of the data set permitted extraction of known and novel kinase motifs using the Motif-X algorithm. Finally, a large number of members of the pheromone signaling pathway were found as phosphoproteins and are discussed.

Keywords: α-factor-arrested yeast • IMAC • LC−MS/MS • SDS-PAGE • phosphoproteomics • pheromone signaling pathway

Introduction

Protein phosphorylation is a crucial post-translational modification, forming the foundation of many intracellular signaling networks. Reversible phosphorylation at specific Ser, Thr, or Tyr residues regulates various cellular processes such as cell cycle transitions, differentiation, and regulated proteolysis. Hundreds of protein kinases and phosphatases, as well as thousands of potential substrates, are considered drug targets for a variety of diseases such as cancer and diabetes.

Phosphoproteomics usually refers to a large-scale analysis of protein phosphorylation using mass spectrometry (MS)-based strategies. Recent successes in this area owe much to the development of MS instrumentation such as the linear ion trap and the orbitrap, as well as novel phosphoprotein/peptide enrichment techniques such as immobilized metal affinity chromatography (IMAC), strong cation exchange chromatography (SCX), or the two in combination.

The α-factor signaling pathway, in the budding yeast, Saccharomyces cerevisiae, has served as a model system for how cells respond to secreted pheromones. α-Factor, a short peptide pheromone, is secreted by yeast of the α-mating type to induce α-mating type yeast to differentiate along a mating competency pathway. Signaling through this pathway arrests the cell cycle in a G1-like state, induces a specific transcriptional program, causes rearrangement of the actin cytoskeleton to form a mating projection, and prepares cells for karyogamy and cell fusion. Protein phosphorylation plays a role in the regulation of many of these processes.

Several phosphoproteomic analyses of yeast have been reported in recent years. Ficarro et al. derivatized a tryptic digest of yeast by O-methyl esterification prior to IMAC enrichment and found 383 phosphorylation sites. In addition, Peng and co-workers detected 125 phosphorylation sites during ubiquitination analysis of S. cerevisiae lysate. Recently, Gruhler and colleagues used a combination of SCX and IMAC to enrich phosphopeptides from α-factor-arrested yeast and identified 729 sites. This work presents the single largest exploration of the yeast phosphoproteome described to date. In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to preliminarily separate yeast proteins. After trypsin digestion, IMAC was used to enrich phosphopeptides from α-factor-arrested yeast and identified 729 sites. This work presents the single largest exploration of the yeast phosphoproteome described to date. In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to preliminarily separate yeast proteins.
sites from 985 proteins, more than 3 times the size of any previously reported yeast phosphorylation data set.

Materials and Methods

Treatment of Yeast Cells with α-Factor and Protein Extraction from S. cerevisiae. Strain ADR376 (W303-1a bar1A) was grown to OD_{600} of 0.8 at 30 °C. Cells were arrested in G_1 phase by the addition of 1 μg/mL of α-factor and grown for 150 min to ensure complete arrest. Cells were then harvested and flash-frozen in 0.15 mg aliquots. Yeast protein extract was made by bead beating (multitube bead beater; Biospec) frozen cell pellets in 350 μL of SDS sample buffer (2% SDS, 80 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM EDTA, 0.02% bromophenol blue, 1 mM PMSF, leupeptin, pepstatin, and bestatin all at 1 μg/mL, and 1 mM benzamidine) containing 50 mM glycerol-2-phosphate, pH 8.3, 5 mM NaF, and 1 mM NaVO_4 to inhibit phosphatases. bead beating was performed with an excess of glass beads (Biospec, 0.5 mm) for one pulse of 60 s. The resulting lysate was separated from the glass beads and centrifuged at 14 000 rpm for 5 min to remove insoluble material. Disulfide bonds in proteins were reduced with DTT (3 mM, 60 °C, 30 min), and the nascent free sulfhydryl groups were alkylated with iodoacetamide (~8 mM, 25 °C, 1 h in the dark).

Preparative SDS-PAGE Separation and In-Gel Proteolysis. Yeast extract corresponding to 6 mg of protein was separated by a hand-poured preparative SDS-PAGE (10%) gel (15 × 15 × 0.15 cm). Electrophoresis was stopped when the buffer front had migrated ~5 cm into the gel. The gel was stained with Coomassie blue and then excised into 13 regions (~4 mm × 150 mm). Each band was cut into approximately 1-mm cubes, washed with MilliQ water, and destained using 50% CH_3CN/5% HCOOH, dried completely under vacuum, desalted using stage-tips,23 PEptides were eluted twice by adding 20 μL of elution buffer (50 mM KH_2PO_4/NH_3, pH 10.0) to the beads and incubating at 4 °C for 1 h. The digestion was carried out at 37 °C overnight. Digests were extracted twice using a solution of 50% CH_3CN/5% HCOOH, dried completely under vacuum, and stored at ~8 °C.

Enrichment of Phosphopeptides Using IMAC. Each sample was resuspended in 100 μL of wash/equilibrarate (W/E) buffer (30% CH_3CN and 250 mM CH_3COOH), to which 15 μL of equilibrated Fe(III)-loaded IMAC slurry (liquid/beads = 50:50) (Phos-Select, Sigma) was added. The samples were then vortexed for 90 min at room temperature. IMAC beads were subsequently washed three times with 350 μL of W/E buffer. Peptides were eluted twice by adding 20 μL of elution buffer (50 mM KH_2PO_4/CH_3CN, pH 10.0) to the beads and incubating at room temperature for 5 min. Eluates were acidified with 20 μL of 5% HCOOH, dried under vacuum, desalted using stage-tips,23 and stored at ~8 °C.

LC–MS/MS Analysis. LC–MS/MS experiments were performed on an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, San Jose, CA). Peptide mixtures were loaded onto a 125-μm i.d. fused-silica micropipillary column packed into-house with C_{18} resin (Michrom Bioresources, Inc., Auburn, CA) that were separated using a 40-min gradient from 5% to 28% solvent B (0.15% HCOOH/97.5% CH_3CN). Solvent A was 0.15% HCOOH/2.5% CH_3CN. The LTQ-Orbitrap mass spectrometer was operated in the data-dependent mode using the TOP10 strategy.24 In brief, a scan cycle was initiated with a full scan of high mass accuracy (m/z 350–1700) in the orbitrap, which was followed by MS/MS scans in the linear ion trap on the 10 most abundant precursor ions with dynamic exclusion of previously selected ions. Singly charged ions were excluded from MS/MS analysis.

Neutral loss fragmentation has been considered a major obstacle to confident phosphopeptide identification. In some studies, a third stage MS was employed to improve backbone fragmentation.10,11,25 We did not collect MS^3 scans in this study because we have found them to have reduced usefulness in terms of both identification and site localization using the linear ion trap platform.25

Database Search. Using the Sequest26 search engine, we searched all MS/MS spectra against one protein sequence database consisting of all yeast open reading frames (ORFs) (downloaded March, 2006 from the National Center for Bio technology Information), common contaminant proteins, and these same yeast and contaminant sequences in reversed orientations. Searches were performed with the following parameters: full-trypsin specificity, a mass tolerance of 1.1 Da, static modifications of oxidized Met (+15.9949), carboxymethylated Cys (+57.0215), and dynamic modifications of phosphorylated Ser, Thr, and Tyr (+79.9663). All peptide matches were filtered by XCorr, mass accuracy, and ΔCn (defined as the normalized difference between XCorr values of the top-ranked candidate peptide and the next candidate with a different amino acid sequence). The target/decoy search strategy21,22 permitted the discovery of the following criteria to achieve an estimated false-positive (FP) rate of 0.017%: XCorr >1.8 for +2, +3, and +4 charged precursor ions; ΔCn > 0.14; and a mass accuracy window of 17 ppm (data centered at ± 5 ppm ± 8.5). As a fourth constraint, we required all matches to have a Peptide Score (calculated during site localization with the ambiguity score (Ascore) algorithm25) of at least 45. The number of FP identification in the forward database is estimated to be equal to the number of matches in the reverse database. The FP rate is the percentage of FP to the total identified peptides. The final filtered data set contained 5985 phosphopeptides with only one reverse-sequence match.

Postsearch Data Analysis. Prior to any need for manual validation, we processed our data set using the Ascore algorithm25 for site localization; parameters included a window size of 100 m/z-units and a fragment ion tolerance of 0.6 m/z-unit. Sites with Ascore values of at least 19 (P ≤ 0.01) were considered localized (≥99% certainty), while those with Ascore values less than 19 were considered ambiguous.

Conservative tallying prevented overestimation of total non-redundant sites, which came into play when sequences were detected multiple times with different phosphorylation sites. For instance, if a peptide sequence possessed a phosphorylation site with an Ascore value below 19, and the same sequence was reported again with a different site location of less than 99% certainty, then the existence of a phosphorylation site in the identified sequence was certain, but the location was not. Thus, only a single site was tallied. Even if one of the reported sites was unambiguously localized, together they were still counted as one site, because it is possible that the other ambiguous site actually had the same location as the confirmed one.

Consensus motifs were extracted from the data set of localized sites by the Motif-X program27 (http://motif-x.med.har vard.edu); sequences were centered on each phosphorylation site and extended to 13 amino acids (±6 residues). Only those sites with Ascore values of at least 19 were used. Sites which could not be extended because of N- or C-termini were excluded by the Motif-X algorithm. The significance threshold
was set to $P < 10^{-6}$. The minimum number of motif occurrences was set to 23 for phosphorylated Ser and 5 for phosphorylated Thr.

To assign phosphorylation sites to general sequence categories, the following rules were followed: Acidiphilic, [S/T]#[E/D] or [S/T]#XX[D/E]; Basophilic, RXX[S/T]#; Proline-directed [S/T]#P; other, all remaining.

Results and Discussion

The workflow in this study is outlined in Figure 1. Six milligrams of whole-cell lysate from α-factor-arrested yeast was separated using a preparative polyacrylamide gel. The entire gel was divided into 13 regions and proteolyzed with trypsin. Phosphopeptides were enriched using IMAC before reverse-phase LC-MS/MS analysis. Each sample was then subjected to MS/MS analysis (Figure 1A).

Approximately 70,000 MS/MS spectra were acquired over 13 runs (Figure 1B). All MS/MS spectra were searched against a target/decoy yeast protein database using Sequest. Guided by decoy hits, criteria using mass deviation, XCorr and dCn' values were established to filter these search results, yielding the identification of 2288 nonredundant phosphorylation sites. The exact locations of 66% of these sites were assigned at $P < 0.01$ using the Ascore algorithm.25 Finally, the Motif-X algorithm27 allowed motifs to be extracted from the data set of the precise phosphorylation sites. FP = false positive.

Enrichment of Phosphopeptides. Figure 2A shows the average molecular weights of identified phosphoproteins from each gel region. As expected, molecular masses generally decreased from band 1 (the upper-most band in the gel) to band 13 (the lowest band of the gel). Despite the prominent role of phosphorylation in cell biology, phosphorylated proteins are often rare: proteins regulated by this process may have low stoichiometries, with only a small portion phosphorylated at any given time. Therefore, effective enrichment of phosphopeptides/phosphoproteins is essential for a large-scale phosphorylation study. We evaluated the relative enrichment of phosphopeptides by IMAC by plotting the number of identified phosphopeptides and the specificity of IMAC purification (the fraction of total peptides comprised of phosphopeptides) for each gel region (Figure 2B,C). As we have noted previously,25 more phosphopeptides were identified from higher molecular-weight gel bands than from the lower ones. For all gel bands, a majority (>60%) of the identified phosphopeptides were singly phosphorylated (Figure 2D). Doubly phosphorylated peptides accounted for 20–30% of all phosphopeptides, and less than 6% were triply phosphorylated.

SDS-PAGE separates proteins by molecular weight. SCX separates tryptic peptides based on their solution charge states, and thus, singly phosphorylated peptides are enriched in early fractions.9,10 IMAC, on the other hand, captures phosphopeptides through the interaction between a phosphate group (as an electron donor) and a metal ion bearing at least one free coordination site (as an electron acceptor).28 All pairings of these three techniques have been applied to phosphoproteomic research.
studies: our group has employed SCX to enrich phosphopeptides after SDS-PAGE and in-gel digestion; the combination of SCX and IMAC has also been exploited to study phosphorylation events in yeast and a mammalian central nervous system. In this project, we evaluated the use of SDS-PAGE prior to IMAC. The experimental results suggest that the current approach is a simple and efficient method for phosphopeptide enrichment.

MS Strategy. Previously, we showed that, compared with lower-accuracy mass analysis by the LTQ, accurate mass measurement of precursor ions boosts the rate of confident phosphopeptide identification. To gain a better understanding of the importance of high mass accuracy, we examined the number of identified phosphopeptides at various FP rates with and without filtering the search results using mass accuracy (Figure 3). At a high FP rate (5%), only 13% more phosphopeptides were identified utilizing mass accuracy than those that were identified without utilizing mass accuracy. This value increased to 30% when the results were filtered to a much higher confidence level (FP = 0.5%). These results are more modest than the 2-fold differences that we previously found, likely because of the novel use of dCn′ as a filter in this study. In addition, separate analyses (LTQ only and LTQ FT), and not simply separate filtering with and without mass accuracy, were conducted in that study. Supporting Information Table 2 provides the criteria and thresholds used for filtering the data set. Nevertheless, high-accuracy precursor masses can significantly narrow the range of possible candidates and improve confident phosphopeptide identification. Supporting Information Figure 1 shows the overall distributions for TP and FP with respect to XCorr and dCn′ values in the presence and absence of a mass accuracy filter. The distribution of mass deviations for the entire data set is shown in Supporting Information Figure 2.

CID–MS/MS fragmentation took place in the linear ion trap of an LTQ mass spectrometer. In many cases, rich backbone fragmentation was observed in addition to an observed neutral loss of phosphoric acid from the precursor ion (Figure 4A). Even when neutral loss fragmentation dominated, the large ion capacity of the linear ion trap often allowed measurable numbers of precursor ions to undergo backbone fragmentation (Figure 4B). Finally, the combination of larger ion capacity and high accuracy precursor mass measurement allowed confident peptide identification and site localization. Because of the generally clear detection of b- and y-type fragment ions even for MS/MS with significant phosphate-associated losses (Figure 4B is an example), we did not collect MS3 spectra in this study. For the two examples shown in Figure 4, sites were localized...
with Ascore values of 22.8 (P = 0.0052) and 24.8 (P = 0.0033), which means the chance of incorrect site localization is as low as 0.52% and 0.33%, respectively.

**Analysis of Identified Phosphoproteins.** Most immediate members of the α-factor response pathway were found based on sequenced phosphopeptides (Figure 5). For example, 12

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**Figure 4.** Typical examples of MS/MS spectra with prominent neutral loss fragmentation. (A) Tandem mass spectrum of peptide DSYVSDDVANS#TER from PTR2. Rich backbone fragmentation still occurred in spite of prominent neutral loss fragmentation. (B) Tandem mass spectrum of peptide KVESLGS#PSGATK from CPH1. Confident peptide identification was usually possible even when backbone fragmentation was severely suppressed by neutral loss fragmentation. The identified amino acid sequence is shown, and the phosphorylation site is marked with “#”. The proposed site locations corresponded to Ascore values of 22.8 (P = 0.0052) and 24.8 (P = 0.0033), respectively.
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sites were omitted from this table. A complete list of the identified phospho-peptides/proteins is presented in Table 1 of Supporting Information.

between XCorr values of the most-likely candidate and the second most-likely candidate consisting of a different amino acid sequence.

sites (Ser366, Thr382, Ser385, Thr411, and Thr414, the residues underlined in the first column) were reported previously. 7,11 Phosphopeptides containing redundant sites were also identified in this study.

Table 1.

<table>
<thead>
<tr>
<th>Phosphorylation sites</th>
<th>Phosphopeptide</th>
<th>number of sites</th>
<th>Ascore</th>
<th>m/z</th>
<th>error (ppm)</th>
<th>XCorr</th>
<th>dCn</th>
<th>charge state</th>
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<tr>
<td>Ser310, Thr313</td>
<td>S1T1TIS*DFT#TSTDR</td>
<td>2</td>
<td>32.4, 16.2</td>
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<td>2.942</td>
<td>1.00</td>
<td>2</td>
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<tr>
<td>Thr329, Ser331</td>
<td>FYPGLS*SFT#DSS#NNDK</td>
<td>2</td>
<td>27.1, 26.6</td>
<td>755.6496</td>
<td>8.8</td>
<td>2.646</td>
<td>0.16</td>
<td>3</td>
</tr>
<tr>
<td>Ser366, Thr369, Ser380</td>
<td>ETTSDKH#ERT#FVS#ETADDIEK</td>
<td>3</td>
<td>24.8, 20.3, 7.6</td>
<td>922.3836</td>
<td>7.2</td>
<td>2.501</td>
<td>0.43</td>
<td>3</td>
</tr>
<tr>
<td>Thr382, Ser385, Ser388</td>
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<td>7.8, 28.9, 26.8</td>
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<td>7.1</td>
<td>2.905</td>
<td>1.00</td>
<td>2</td>
</tr>
<tr>
<td>Thr384, Thr387, Thr411, Thr414</td>
<td>EGEVEPDMDY#PTD#AADDIEK</td>
<td>2</td>
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<td>828.6481</td>
<td>4.2</td>
<td>3.600</td>
<td>0.49</td>
<td>3</td>
</tr>
</tbody>
</table>

a Phosphorylation sites were assigned using the Ascore software. 25 The locations of sites with Ascore values of at least 19 were considered significant. In total, 12 phosphorylation sites were identified from 37 redundant phosphopeptides for this protein. Five sites (Ser366, Thr369, Ser380, Thr411, and Thr414, the residues underlined in the first column) were reported previously. 21,22 Phosphopeptides containing redundant sites were omitted from this table. A complete list of the identified phospho-peptides/proteins is presented in Table 1 of Supporting Information. b Phosphorylation sites are indicated by "*". c The Ascore values are numbered corresponding to sites from left to right. d The dCn values listed here are the normalized difference between XCorr values of the most-likely candidate and the second most-likely candidate consisting of a different amino acid sequence.

Some biologically important phosphoproteins, such as the MEK kinase Ste11 and the scaffold protein Ste5 (both are key members of the pheromone signaling pathway (Figure 5)), were not identified in previous studies. 7,11,20 Similarly, Fus1, Fus2, and Fig1, proteins that directly participate in cellular fusion, were also absent. It was proposed by Gruhler et al. that Ste11 and Ste5 were not detected due to extremely low amounts of phosphorylated species, whereas phosphorylation of the latter three proteins (Fus1, Fus2, and Fig1) was speculated to occur only upon contact with cells of the opposite mating type at a later phase of conjugation. 11 All of these proteins were identified with multiple phosphopeptides in the current analysis, demonstrating that phosphorylated Ste11 and Ste5 are indeed present in sufficient amounts, and that phosphorylation of these proteins does occur during α-factor arrest.

To systematically evaluate the sensitivity of our approach, we referenced the codon adaptation index (CAI) values of genes encoding the identified proteins (Figure 6A). This estimate of protein abundance is usually below 0.2 for genes encoding low-abundance proteins. 34,35 A majority of the identified proteins were encoded by genes with CAI values less than 0.2, and the distribution was highly similar to that of the entire genome (Figure 6A inset).

Pheromone signaling initiates numerous cellular processes such as cell cycle arrest and membrane movement, as well as changes in cell polarity, shape, and size. 4,19 Most phosphoproteins we identified could be classified into these categories (Figure 6B). However, whether these phosphorylation events were induced by α-factor or are constitutive remains unclear.

There are no known specific tyrosine kinases in yeast. However, dual-specificity kinases are capable of phosphorylating some tyrosine residues. Two examples of detected tyrosine-phosphorylated peptides are shown in Supporting Information Figure 3.
Motif Analysis of the Precisely Localized Phosphorylation Sites. Amino acid sequences neighboring Ser, Thr, or Tyr mark them as targets for specific kinases. Using the 12 amino acids surrounding the confidently identified phosphorylation sites, we extracted 21 motifs using the Motif-X algorithm, most of which corresponded to kinases of known target sequences (Figure 6C and Supporting Information Table 2). Many known substrates of these kinases were identified from our data set, and most of the identified sites in these proteins were within motifs specific for the kinases. For example, Msn2, Sfl1, and Yak1 have been shown to be phosphorylated by PKA, and Cdc37 and Srp40 are known substrates for CK2. Further study will be necessary to assign the motifs RXXXXS# and S#XXN with specific kinases.

Ser/Thr protein kinases can be divided into 4 general classes based on substrate sequence specificity: acidiphilic, basophilic, proline-directed, and others (see Materials and Methods). The first three categories constituted about 80% of the data set (Figure 6D). More than 40% of the identified phosphorylation events were acidiphilic, whereas basophilic and proline-directed events accounted for 17% and 31%, respectively. We also queried four other data sets (α-factor-arrested yeast, nocodozoole-arrested human HeLa cells, mouse brain, and human HeLa nuclear fraction) with these categories (Supporting Information Figure 4). Finally, the overlap between this study and two other IMAC-based yeast phosphorylation studies is shown in Supporting Information Figure 5.

Conclusion
In conclusion, the addition of SDS-PAGE gel separation prior to IMAC enrichment of trypsinized phosphopeptides allowed the identification of thousands of phosphorylation sites. High mass accuracy precursor ion information provided both increased confidence in identifications and powerful filtering criteria. The target/decoy database searching approach was essential to derive filtering criteria, providing a low estimated FP rate (0.017%). This data set provides an important resource for future studies on phosphorylation in yeast.

Abbreviations: Ascore, ambiguity score; CK2, casein kinase 2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FP: false-positive; IMAC, immobilized metal affinity chromatography; MS, mass spectrometry; NCBI, National Center for Biotechnology Information; OD, optical density; ORF, open reading frame; PKA, cAMP-dependent protein kinase A; PMSF, phenylmethylsulphonylfluoride; SCX, strong cation exchange chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; W/E buffer, wash/equilibrte buffer.

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Supporting Information Available: Complete data set of phosphopeptides identified in this study (SI Table 1). Thresholds used for filtering the data in Figure 3 (SI Table 2). Motifs extracted from our data set using the Motif-X algorithm (SI Table 3). Distribution of TP and FP matches with respect to peptide scoring and mass accuracy (SI Figure 1). Distribution of mass deviations for all phosphopeptides detected in the study (SI Figure 2). Examples of two phosphotyrosine-containing peptides (SI Figure 3). Classification of phosphorylation events into 4 general sequence categories based on kinase specificities (SI Figure 4). Overlap of phosphorylation sites identified in three IMAC-based yeast phosphoproteomic studies (SI Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

References


