A Multidimensional Chromatography Technology for In-depth Phosphoproteome Analysis*

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Protein phosphorylation is a post-translational modification widely used to regulate cellular responses. Recent studies showed that global phosphorylation analysis could be used to study signaling pathways and to identify targets of protein kinases in cells. A key objective of global phosphorylation analysis is to obtain an in-depth mapping of low abundance protein phosphorylation in cells; this necessitates the use of suitable separation techniques because of the complexity of the phosphoproteome. Here we developed a multidimensional chromatography technology, combining IMAC, hydrophilic interaction chromatography, and reverse phase LC, for phosphopeptide purification and fractionation. Its application to the yeast Saccharomyces cerevisiae after DNA damage led to the identification of 8764 unique phosphopeptides from 2278 phosphoproteins using tandem MS. Analysis of two low abundance proteins, Rad9 and Mrc1, revealed that ~50% of their phosphorylation was identified via this global phosphorylation analysis. Thus, this technology is suited for in-depth phosphoproteome studies. Molecular & Cellular Proteomics 7:1389–1396, 2008.

Cells are highly responsive to their environment. Protein phosphorylation is a widely used post-translational modification that regulates many biological processes in cells. The phosphoproteome, referring to the phosphorylation profile of cells, undergoes many changes in response to various stimuli. Recent advances in MS have made it possible to identify thousands of phosphopeptides (1–5). Furthermore, various stable isotope labeling methods have been used to quantify changes of protein phosphorylation in cells (6–8). These studies illustrate the potential of phosphoproteomics technology to study phosphorylation-mediated signal transduction processes on a large scale.

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The phosphoproteome in cells is highly complex, containing phosphorylation of both high and low abundance proteins on multiple sites. To identify phosphorylation of low abundance proteins in a global phosphorylation analysis, it is necessary to have an in-depth mapping of the phosphoproteome. Toward this goal, several methods were developed to purify phosphopeptides prior to their MS analysis, including the use of titanium dioxide, IMAC, and others (1, 9–11). These methods allowed a high degree enrichment of phosphopeptides for subsequent analysis. To analyze the purified phosphopeptides, the use of reverse phase-based HPLC (RP-HPLC)1 and MS/MS can identify hundreds of phosphopeptides in a single analysis; however, this alone is insufficient for an in-depth mapping of the phosphoproteome.

Strong cation exchange (SCX) chromatography is commonly used to fractionate peptides (12). Recently, an SCX column was used to fractionate phosphopeptides, and as a result, many more phosphopeptides were identified (2, 3, 6, 8). Clearly fractionation of phosphopeptides is necessary for a higher coverage of the phosphoproteome. However, it was found that some phosphopeptides did not bind to the SCX column, and other phosphopeptides were mostly eluted from the SCX column earlier than unphosphorylated peptides (13).2 These observations indicated a relatively weak binding between the SCX resins and phosphopeptides. To analyze phosphopeptides using MS, RP-HPLC is commonly used to further fractionate the phosphopeptides. It was observed that the presence of salt, regardless whether it is volatile or not, would compromise the binding and separation of many phosphopeptides via RP-HPLC. This is likely because many phosphopeptides are hydrophilic and bind less tightly to RP-HPLC columns. Unfortunately, desalting of phosphopeptides is typically performed using reverse phase C18-based columns. Therefore, to minimize loss of phosphopeptides, it is important to have fewer desalting steps. With these considerations, we sought to develop an alternative chromatography method

1 The abbreviations used are: RP-HPLC, reverse phase-based HPLC; HILIC, hydrophilic interaction chromatography; SCX, strong cation exchange; MMS, methyl methanesulfonate; IgG, immunoglobulin G.

2 C. P. Albuquerque, M. B. Smolka, S. H. Payne, V. Bafna, J. Eng, and H. Zhou, unpublished observation.
for phosphopeptide fractionation. Ideally such chromatography does not require the use of salt-containing buffers and provides an orthogonal separation compared with RP-HPLC.

Hydrophilic interaction chromatography (HILIC) is a less commonly used method for peptide fractionation despite that it is often used to fractionate small metabolites (14). Interestingly a recent study of the separation of unphosphorylated peptides using SCX, HILIC, and RP-HPLC indicated that a better orthogonal separation could occur between HILIC and RP-HPLC for unphosphorylated peptides (15, 16). The observed orthogonal separation between HILIC and RP-HPLC likely reflects their different mechanisms of separation. Although RP-HPLC depends on the interaction with the hydrophobic amino acid side chains, HILIC depends on the interaction with those hydrophilic and possibly charged amino acid residues via hydrogen bonding and ionic interactions. Moreover because phosphopeptides are generally hydrophilic and charged, one would expect that phosphopeptides should interact more strongly with HILIC than do unphosphorylated peptides. Thus, it should be possible to separate phosphopeptides using HILIC.

Here we investigated the use of HILIC for phosphopeptide separation and found that it provided a largely orthogonal separation of phosphopeptides with RP-HPLC. A multidimensional chromatography technology combining IMAC, HILIC, and RP-HPLC in a sequential order was thus developed for the purification and separation of phosphopeptides. This technology was designed to have minimal manual steps and found to provide an in-depth and sensitive mapping of the phosphoproteome of the yeast *Saccharomyces cerevisiae* after genotoxic stress.

**EXPERIMENTAL PROCEDURES**

**Preparation of IMAC Resin**—All commonly used chemicals were obtained from Sigma-Aldrich unless noted otherwise. Resins from three silica-nitrilotriacetic acid spin columns (Qiagen, Valencia, CA) were added to 50 ml of buffer containing 5 mM EDTA (pH 8.0) and 1 M NaCl and incubated for 1 h at room temperature under rotation. The resins were then spun down and washed sequentially with 50 ml of water and 50 ml of 0.6% acetic acid and finally incubated with 50 ml of 100 mM FeCl3 in 0.3% acetic acid for 1 h under rotation. The resins were then washed with 50 ml of 0.6% acetic acid; then with 50 ml of a solution containing 25% acetonitrile, 0.1 M NaCl, and 0.1% acetic acid; and then two more times with 50 ml of 0.1% acetic acid. Finally the resins were resuspended in 0.1% acetic acid as a 50% (v/v) slurry and stored at 4 °C (17).

**Purification of Phosphopeptides from Whole Cell Protein Extract**—Yeast cells (RDK2669: MATa, ura3–52, leu2–1, trplΔ63, his3Δ200, lys2Δ8g1, hom3–10, ade2Δ1, ade8) were grown in 2 liters of YPD medium until an A600 of 0.7, then treated with 0.05% MMS for 3 h, and harvested. Protein extract was prepared the same way as above, and 500 mg of proteins were obtained. Protein extract was incubated with 0.1 ml of human immunoglobulin G (IgG)-Sepharose resin (GE Healthcare) for 4 h in the cold room under rotation. The IgG resin was then washed with 10 ml of lysis buffer and then with 5 ml of TBS. The proteins were eluted from the IgG resin using 800 μl of 5% acetic acid and 1 M urea. The eluted proteins were dried under reduced pressure, and then 1 M Tris base was used to neutralize the sample to pH 8. After the sample volume was adjusted to 100 μl, it was reduced by DTT and alkylated by iodoacetamide as described above. The sample was then diluted with TBS to a final concentration of 2 M urea and digested with 2 μg of trypsin at 37 °C overnight. The resulting peptides were desalted using a 50-μg Sep-Pak C18 column (Waters) and dried under vacuum. The dried peptides were resuspended in 100 μl of 1% acetic acid and loaded to a tip column containing 10 μl of IMAC resins. After loading, the IMAC resin was washed twice with 20 μl of wash buffer containing 25% acetonitrile, 100 mM NaCl, and 0.1% acetic acid and then eluted by 100 μl of 1% acetic acid; finally eluted by 100 μl of 1% phosphoric acid; and dried under vacuum.

**Purification of Rad9 and Mrc1 Using a Pulldown Approach**—A TAF tag containing two copies of protein A, tobacco etch virus cleavage site, six histidines, and three copies of the FLAG peptide was inserted at the C terminus of either the Rad9 or Mrc1 protein using a G418 selection marker and RDK2669 yeast background as described previously (18). Rad9-TAF and Mrc1-TAF cells were grown in 2 liters of YPD medium until an A600 of 0.7, then treated with 0.05% MMS for 3 h, and harvested. Protein extract was prepared the same way as above, and 500 mg of proteins were obtained. Protein extract was incubated with 0.1 ml of human immunoglobulin G (IgG)-Sepharose resin (GE Healthcare) for 4 h in the cold room under rotation. The IgG resin was then washed with 10 ml of lysis buffer and then with 5 ml of TBS. The proteins were eluted from the IgG resin using 800 μl of 5% acetic acid and 1 M urea. The eluted proteins were dried under reduced pressure, and then 1 M Tris base was used to neutralize the sample to pH 8. After the sample volume was adjusted to 100 μl, it was reduced by DTT and alkylated by iodoacetamide as described above. The sample was then diluted with TBS to a final concentration of 2 M urea and digested with 2 μg of trypsin at 37 °C overnight. The resulting peptides were desalted using a 50-μg Sep-Pak C18 column (Waters) and dried under vacuum. The dried peptides were resuspended in 100 μl of 1% acetic acid and loaded to a tip column containing 10 μl of IMAC resins. After loading, the IMAC resin was washed twice with 20 μl of wash buffer containing 25% acetonitrile, 100 mM NaCl, and 0.1% acetic acid; then washed with 20 μl of water; finally eluted by 100 μl of 3% ammonium hydroxide; and dried under vacuum.

**The Use of HILIC for Phosphopeptide Separation**—A TSK gel Amide-80 column (2 mm × 15 cm, 5 μm; Tosoh, Grove City, OH) was used for the HILIC experiment. Three buffers were used for the gradient: buffer A, 90% acetonitrile and 0.005% TFA; buffer B, 0.005% TFA; and buffer C, 0.1% phosphoric acid and 0.005% TFA. Phosphopeptides eluted from IMAC were resuspended in 30 μl of 90% acetonitrile with 0.1% formic acid and then injected into the HILIC Amide-80 column via a 100-μl loop with a flow rate of 150 μl/min. One and a half-minute fractions from the HILIC were collected and dried under reduced pressure. The gradient used is shown in Fig. 1B (100% buffer A at time = 0 min, 11% buffer B at 5 min, 29% buffer B at 20 min, 95% buffer C at 45 min, 95% buffer C at 50 min, and finally 100% buffer A at 55 min).

**Mass Spectrometry**—Experiments were performed using the 1100 QuadPump HPLC system (Agilent, Santa Clara, CA), the Ultimate 3000 autosampler (Dionex, Sunnyvale, CA), and the LTQ tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Each HILIC fraction was transferred to a silanized glass insert (National Institute for Standards and Technology, Gaithersburg, MD).
Phosphate Localization.py, is freely available as part of the InsPecT applied to both InsPecT and SEQUEST results. This script, named 3.0 Da, use of such decoy database allows an estimate of the false discovery Database on January 12, 2007) and its reverse protein database. The yeast protein database (downloaded from mass spectra, a composite database was generated using both the relative to the reference mass.

The minimal threshold for the dependent scans was set to 6500 scans according to the ion intensities detected in the full MS scan. To perform one full MS scan followed by six consecutive MS/MS was used for the data acquisition, and the mass spectrometer was set for the use of IMAC-bound, dark blue line) and the unphosphorylated peptides (IMAC flow-through, light blue line) were examined. As shown in Fig. 1B, unphosphorylated peptides generally eluted earlier than the phosphorylated peptides, indicating that phosphopeptides bind more tightly to the HILIC column than do unphosphorylated peptides. The partial overlap of the phosphorylated and unphosphorylated peptides indicated that HILIC is not sufficient for phosphopeptide purification; instead the use of IMAC or other affinity methods for phosphopeptide purification is essential. After IMAC purification, a low level of unphosphorylated peptides was often found in the earlier HILIC fractions, which were no longer considered for later analysis (see Fig. 1B, asterisks). This UV profile of the phosphopeptides on the HILIC was reproducible from multiple experiments (results not shown).

The Use of HILIC to Fractionate Phosphopeptides—To see whether HILIC could provide a high resolution separation of phosphopeptides that is orthogonal to RP-HPLC, the phosphopeptides in HILIC fractions 14–16 were examined. As shown in Fig. 2A, numerous peptide ions are present throughout the RP-HPLC gradient for these fractions, underscoring the need for fractionation of phosphopeptides purified from proteolyzed cell lysate. Interestingly each HILIC fraction has a different elution profile on RP-HPLC, and peptides are present throughout the RP-HPLC gradient, indicating an orthogonal separation of phosphopeptides between HILIC and RP-HPLC. The phosphopeptides in HILIC fractions 14–16 were then identified using SEQUEST and analyzed for potential overlaps between the fractions. As shown in Fig. 2B, there is
an approximate 20% overlap in the identified phosphopeptides between two adjacent HILIC fractions. Interestingly a small overlap (~5%) was found between the non-neighboring HILIC fractions 14 and 16. Similar results were also observed for other HILIC fractions (not shown). These observations showed that HILIC provides a high resolution separation of phosphopeptides that is largely orthogonal to RP-HPLC.

An In-depth Mapping of the Phosphoproteome of the Budding Yeast after DNA Damage—Next SEQUEST and InsPecT were used to identify phosphopeptides present in 20 HILIC fractions (see Fig. 3A) (20, 22). A decoy protein database of the budding yeast S. cerevisiae was included in the database search to evaluate the false discovery rate (19). With a false discovery rate of 1%, a total of 8764 unique phosphopeptides (irrespective of their phosphorylation site assignment) from 2278 proteins were identified using both SEQUEST and InsPecT (see Fig. 3A). Although SEQUEST was able to identify 6419 unique phosphopeptides, InsPecT identified 7681 phosphopeptides from the same data set. Furthermore 5336 unique phosphopeptides were found by both InsPecT and SEQUEST (see Fig. 3B). Thus, SEQUEST and InsPecT are partially complementary for phosphopeptide identification.

This is likely because the scoring mechanisms for SEQUEST and InsPecT are quite different (20).

Of all the top matched phosphorylation sites found, serine phosphorylation was most common, and it constituted ~83% of all phosphorylation found, whereas threonine phosphorylation accounted for the rest. Furthermore the number of doubly phosphorylated peptides was much fewer than singly phosphorylated peptides (756 versus 8008). This is likely because multiply phosphorylated peptides are harder to identify with high confidence using LTQ-MS because of its low mass resolution and the use of collision-induced dissociation. As commonly observed, tandem mass spectra of phosphopeptides obtained via collision-induced dissociation are often of lower quality due to the neutral loss of phosphoric acid, which compromises the assignment of the precise phosphorylation site within a peptide. Although the top matched phosphopeptides are reported in supplemental Tables 1 and 2, only the number of unique phosphopeptides regardless of the phosphorylation site assignment is summarized here (see Fig. 3, A and B). Despite being an underestimation, the large number of unique phosphopeptides identified here showed the poten-
Phosphopeptide Fractionation Using HILIC and RP-HPLC

**Fig. 2.** Comparison of the RP-HPLC profile and phosphopeptide identification from three adjacent HILIC fractions. A, ion abundances of the peptides detected by RP-HPLC-MS for HILIC fractions 14–16. Numerous peptide ions appear throughout RP-HPLC in each fraction that are different from one another. B, Venn diagram indicates the overlaps of the identified phosphopeptides in HILIC fractions 14–16. Although there is an ~20% overlap between adjacent fractions, few overlaps (~5%) were found between fractions 14 and 16.

**Fig. 3.** Summary of the phosphopeptides and phosphoproteins identified from yeast. A, phosphopeptides were purified using 6 mg of total proteins as a starting material. After separation using HILIC, 20 fractions were analyzed by RP-HPLC-MS/MS and searched using SEQUEST and InsPecT. This led to the identification of 8764 unique phosphopeptides from 2278 proteins. Possible redundancy due to charge state, oxidative state, or even possible ambiguity on phosphorylation site assignment was removed to calculate the number of unique phosphopeptides reported here. The false discovery rate is less than 1% as judged by the target-decoy strategy. B, Venn diagram of the phosphopeptides identified using InsPecT and SEQUEST, indicating that these two search tools are partially complementary to each other. C, Venn diagram of the number of phosphopeptides of Rad9 identified using the HILIC, SCX, and pulldown approaches. D, Venn diagram of the number of phosphopeptides of Mrc1 identified using the HILIC, SCX, and pulldown approaches. The amount of starting material used in each case is indicated. In this case, manual examination of each phosphopeptide of Rad9 and Mrc1 was performed.

A key question is how well phosphorylation of low abundance proteins was identified using this approach. To address this question, we chose to further examine phosphorylation of Rad9 and Mrc1, two low abundance proteins with estimated 400 and 721 copies per cell, respectively (23). Rad9 and Mrc1 are two key adaptor proteins in the yeast DNA damage and replication checkpoints, and they are known to be phosphorylated in response to MMS treatment (24, 25). However, their phosphorylation sites have not been mapped previously. To identify the most phosphopeptides of Rad9 and Mrc1 using...
MS, we purified each protein from 500 mg of protein extract derived from MMS-treated Rad9-TAF and Mrc1-TAF cells using a pulldown approach (17). Phosphopeptides of purified Rad9 and Mrc1 were further purified using IMAC and identified (see supplemental Tables 3 and 4). After database search using SEQUEST and InsPecT as described under “Data Analysis Using SEQUEST and InsPecT,” the identified phosphopeptides of Rad9 and Mrc1 were subjected to manual inspection. To this end, all the identified phosphopeptides of Rad9 and Mrc1 were required to be doubly tryptic, and all significant fragment ions in their MS/MS spectra should be correctly assigned. The results are summarized in Fig. 3C and supplemental Tables 3 and 4. Although the pulldown approach identified 38 phosphopeptides from Rad9, 19 of them were also identified by the HILIC method. This corresponds to ~50% coverage of the phosphorylation of Rad9. Next the phosphopeptides of Rad9 identified here were compared with those identified using the SCX-based method (8). Despite that almost 8-fold more starting material (50 mg) was used previously (8), more phosphopeptides of Rad9 were found using the HILIC-based method. Similar results were obtained with Mrc1 (see Fig. 3D). Thus, this IMAC-HILIC-RP-HPLC-based technology is more sensitive, requires fewer manual efforts, and allows a higher coverage of the phosphorylation of low abundance Rad9 and Mrc1 in cells compared with our previous study (8).

**Phosphorylation of SQ/TQ Sites of the Budding Yeast after DNA Damage—**As shown previously, phosphorylation of SQ/TQ sites is a relatively rare event in cells (8). Mec1 and Tel1 are responsible for about 26% of the observed SQ/TQ phosphorylation in cells treated with MMS. In the present study, phosphorylated SQ/TQ sites were found in 355 proteins, which is ~4 times the number of proteins identified by the SCX method (8) (see Fig. 4A and supplemental Table 4). Fig. 4B summarizes the known and potential targets of Mec1/Tel1 that are involved in the DNA damage checkpoint, DNA repair, and DNA replication. Several proteins that were known to undergo DNA damage-induced phosphorylation, including Six4 (26), Rtt107 (27), Mrc1 (25), H2A (28), and others, were identified here (see Fig. 4B, colored in blue), most of them were not described previously (see Fig. 4B, colored in green). Further experiments are in progress to see whether they undergo Mec1- and/or Tel1-dependent phosphorylation in vivo. It should also be noted that additional targets of Mec1 and Tel1 are likely present in the database of SQ/TQ phosphorylated peptides (see supplemental Table 5).

**DISCUSSION**

With the enormously complex phosphoproteome unveiled in several recent studies, a key objective of global phosphorylation analysis is to obtain an in-depth mapping of protein phosphorylation in cells. This is needed to identify and characterize many lower abundance and regulatory phosphorylation events in cells. To this end, a suitable multidimensional chromatography technology should be used to separate phosphopeptides into simpler samples for mass spectrometric analysis. Ideally such technology should be simple, automatic, and allow a sensitive detection of phosphopeptides.

In this study, we described the development of a multidimensional chromatography method based on a combination of IMAC, HILIC, and RP-HPLC to purify and fractionate phosphopeptides. Several features of this technology are demonstrated. First, HILIC was found to be largely orthogonal to RP-HPLC for phosphopeptide separation. Little overlap of phosphopeptides was found between non-neighboring HILIC fractions. As a result, a higher coverage of the phosphoproteome was obtained compared with our previous study (8). During the reviewing of this manuscript, a recent report by McNulty and Annan (29) showed that phosphopeptides bind to HILIC more tightly than do unphosphorylated peptides, and HILIC could be used to fractionate phosphopeptides; this is in agreement with our observation (see Fig. 1B). Second, a salt-free buffer system was developed for the use of HILIC to separate phosphopeptides. As a result, the only sample-han-
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Medical School for helpful suggestions on the automation in sample

loading for MS and SageN for the use of the Sorcerer system for

ogy and the conventional pulldown method showed that

50% of the phosphorylation of Rad9 and Mrc1 was ob-

tained from ~1% of the starting material used in the multi-

dimensional chromatography technology. Compared with

the SCX-based method, this HILIC-based technology ap-

appears to be more sensitive and allows a higher coverage of

phosphoproteome and importantly greater ease of use. Fur-

thermore this study allowed us to generate a more complete database of the SQ/TQ phosphorylation motifs that is expected to contain the potential substrates of Mec1 and Tel1. We suggest that many of these newly identified proteins, with SQ/TQ phosphorylation and known roles in DNA replica-

tion and repair, could be directly phosphorylated by Mec1 and/or Tel1.

Recently it was reported that the use of hybrid instruments,

which combine the high resolution of an FT-ICR detector with the high rate of MS/MS acquisition of a linear ion trap, could lead to a 3-fold increase in the number of identified phos-

phopeptides as compared with the LTQ-MS instrument used here (32). By combining the multidimensional chromatogra-

phy method described here with high mass resolution MS instruments, we anticipate that an even higher coverage of the phosphoproteome could be achieved.

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REFERENCES

1. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002) Phospho-

proteome analysis by mass spectrometry and its application to Saccha-

romycies cerevisiae. Nat. Biotechnol. 20, 301–305

2. Beausoleil, S. A., Jедrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Large-scale characteriz-

ation of HeLa cell nuclear phosphoproteins. Proc. Natl. Acad. Sci. U. S. A. 101, 12130–12135

3. Li, X., Gerber, S. A., Rudner, A. D., Beausoleil, S. A., Haas, W., Villen, J., Elias, J. E., and Gygi, S. P. (2007) Large-scale phosphorylation analysis of

alpha-factor-arrested Saccharomycies cerevisiae. J. Proteome Res. 6, 1190–1197

4. Chi, A., Hutterhoffer, C., Geer, L. Y., Coon, J. J., Syka, J. E., Bai, D. L., Shabanowitz, J., Burke, D. J., Troyanskaya, O. G., and Hunt, D. F. (2007) Analysis of phosphorylation sites on proteins from Saccharomycies cere-

visiae by electron transfer dissociation (ETD) mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. 104, 2193–2198

5. Bodenmiller, B., Malmstrom, J., Gerbits, B., Campbell, D., Lam, H., Schmidt, A., Runnr, O., Mueller, L. N., Shannon, P. T., Pedriol, P. G., Panse, C., Lee, H. K., Schiapbach, R., and Aebersold, R. (2007) PhosphoPep—a phospho-

proteome resource for systems biology research in Drosophila Kc167 cells. Mol. Syst. Biol. 3, 139

6. Gruhler, A., Olsen, J. V., Mohammed, S., Mortensen, P., Faergeman, N. J., Mann, M., and Jensen, O. N. (2005) Quantitative phosphoproteomics applied to the yeast phenome signaling pathway. Mol. Cell. Proteomics 4, 310–327

7. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

8. Smolka, M. B., Albuquerque, C. P., Chen, S. H., and Zhou, H. (2007) Proteome-wide identification of in vivo targets of DNA damage check-

point kinases. Proc. Natl. Acad. Sci. U. S. A. 104, 10364–10369

9. Bodenmiller, B., Mueller, L. N., Mueller, M., Domon, B., and Aebersold, R. (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat. Methods 4, 231–237

10. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jorgensen, T. J. (2005) Highly selective enrichment of phosphorylated pep-

tides from peptide mixtures using titanium dioxide microcolumns. Mol. Cell. Proteomics 4, 873–886

11. Tao, W. A., Wollscheid, B., O’Brien, R., Engel, K. J., Li, X. J., Bodenmiller, B., Wollman, J. D., Hood, L., and Aebersold, R. (2005) Quantitative phospho-

proteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. Nat. Methods 2, 591–598

12. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. 19, 242–247

13. Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., and Gygi, S. P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. 2, 43–50

14. Yoshida, T. (2004) Peptide separation by hydrophilic-interaction chroma-
tography: a review. J. Biochem. Biophys. Methods 60, 265–280

15. Boersema, P. J., Dicehna, N., Heck, A. J., and Mohammed, S. (2007) Evaluation and optimization of ZIC-HILIC-RP as an alternative MudPIT strategy. J. Proteome Res. 6, 937–948

16. Gilur, M., Oillova, P., Daly, A. E., and Gebler, J. C. (2005) Orthogonality of separation in two-dimensional liquid chromatography. Anal. Chem. 77, 6426–6434

17. Smolka, M. B., Albuquerque, C. P., Chen, S. H., Schmidt, K. H., Wei, X. X., Kolodner, R. D., and Zhou, H. (2005) Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine-reactive iso-
tope tag. Mol. Cell. Proteomics 4, 1358–1369

18. Chen, S., Smolka, M. B., and Zhou, H. (2007) Mechanism of Dun1 activation by Rad53 phosphorylation in Saccharomycies cerevisiae. J. Biol. Chem. 282, 986–995

19. Elias, J. E., and Gygi, S. P. (2007) Target-decay search strategy for in-

creased confidence in large-scale protein identifications by mass spec-
20. Tanner, S., Shu, H., Frank, A., Wang, L. C., Zandi, E., Mumby, M., Pavner, P. A., and Bafna, V. (2005) InsPecT: identification of posttranslationally modified peptides from tandem mass spectra. *Anal. Chem.* 77, 4626–4639

21. Beausoleil, S. A., Villen, J., Gerber, S. A., Rush, J., and Gygi, S. P. (2006) A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* 24, 1285–1292

22. Yates, J. R., III, Eng, J. K., McCormack, A. L., and Schieltz, D. (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* 67, 1426–1436

23. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003) Global analysis of protein expression in yeast. *Nature* 425, 737–741

24. Emili, A. (1998) MEC1-dependent phosphorylation of Rad9p in response to DNA damage. *Mol. Cell* 2, 183–189

25. Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bouset, K., Furuya, K., Diffley, J. F., Carr, A. M., and Elledge, S. J. (2001) Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat. Cell Biol.* 3, 958–965

26. Flott, S., and Rouse, J. (2005) Slx4 becomes phosphorylated after DNA damage in a Mec1/Tel1-dependent manner and is required for repair of DNA alkylation damage. *Biochem. J.* 391, 325–333

27. Roberts, T. M., Kobor, M. S., Bastin-Shanower, S. A., II, M., Horte, S. A., Gin, J. W., Emili, A., Rine, J., Brill, S. J., and Brown, G. W. (2006) Slx4 regulates DNA damage checkpoint-dependent phosphorylation of the BRCT domain protein Rtt107/Esc4. *Mol. Biol. Cell* 17, 539–548

28. Downs, J. A., Lowndes, N. F., and Jackson, S. P. (2000) A role for Saccharomyces cerevisiae histone H2A in DNA repair. *Nature* 406, 1001–1004

29. McNulty, D. E., and Annan, R. S. (2008) Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. *Mol. Cell. Proteomics* 7, 971–980

30. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1, 376–386

31. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154–1169

32. Bakalarci, C. E., Haas, W., Dephoure, N. E., and Gygi, S. P. (2007) The effects of mass accuracy, data acquisition speed, and search algorithm choice on peptide identification rates in phosphoproteomics. *Anal. Bioanal. Chem.* 389, 1409–1419