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The Coming of Age of Phosphoproteomics—from Large Data Sets to Inference of Protein Functions*

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Protein phosphorylation is one of the most common post-translational modifications used in signal transduction to control cell growth, proliferation, and survival in response to both intracellular and extracellular stimuli. This modification is finely coordinated by a network of kinases and phosphatases that recognize unique sequence motifs and/or mediate their functions through scaffold and adaptor proteins. Detailed information on the nature of kinase substrates and site-specific phosphoregulation is required in order for one to better understand their pathophysiological roles. Recent advances in affinity chromatography and mass spectrometry (MS) sensitivity have enabled the large-scale identification and profiling of protein phosphorylation, but appropriate follow-up experiments are required in order to ascertain the functional significance of identified phosphorylation sites. In this review, we present meaningful technical details for MS-based phosphoproteomic analyses and describe important considerations for the selection of model systems and the functional characterization of identified phosphorylation sites. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.R113.032862, 3453–3464, 2013.***

Protein phosphorylation affects protein activity and controls a wide range of important cellular processes, including cell signaling and metabolism, as well as cell growth, differentiation, and proliferation. This post-translational modification (PTM)[1] also modulates protein–protein interactions and provides a framework through which signaling networks integrate and relay signals (1). The reciprocal actions of protein kinases and phosphatases that add or remove phosphate groups from specific substrates afford an exquisite mechanism to ensure coordinated and selective responses to internal and external cell stimuli (2). Although phosphorylation-specific protein interactions are crucial for transducing intracellular signals, mutations or overexpression of kinases and phosphatases can perturb the dynamic regulation of protein phosphorylation, a situation encountered in many diseases, including cancer (3). This is best exemplified by gain-of-function mutations in components of the Ras/mitogen-activated protein kinase (MAPK) pathway that lead to various developmental disorders, as well as cancer (4).

Unraveling the connectivity between kinase activity and effects on downstream substrates is a major endeavor that requires complementary approaches to determine direct interactions, functional significance, and relationships between genotype and phenotype. Contemporary phosphoproteomic approaches that rely on affinity purification of phosphopeptides and mass spectrometry (MS) instrumentation are playing a key role in the large-scale identification of modified residues, the determination of phosphorylation stoichiometry, and the correlation of kinase activity. The level of detail now offered by phosphoproteomics complements more traditional biochemical approaches with which kinases and their activities are studied on an individual basis. Quantitative phosphoproteomics allows researchers to investigate aberrantly activated signaling pathways in different cellular model systems of disease to identify patterns of phosphorylation that vary in terms of stoichiometry and duration according to substrates and experimental paradigms. Here, we describe key technical points for successful MS-based phosphoproteomic analyses and discuss important considerations in the selection of model systems and the characterization of phosphorylation sites to determine their functional significance.

The Basics of MS-based Phosphoproteomic Analyses—Our understanding of cell signaling has been greatly facilitated by the availability of efficient phosphopeptide enrichment techniques and sensitive MS instrumentation enabling large-scale phosphoproteomic analyses. Far beyond a simple catalogu-

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[1] The abbreviations used are: GlcNAc, *N*-acetylglucosamine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PTM, post-translational modification; SILAC, stable isotope labeling with amino acids in cell culture; SUMO, small ubiquitin-related modifier.

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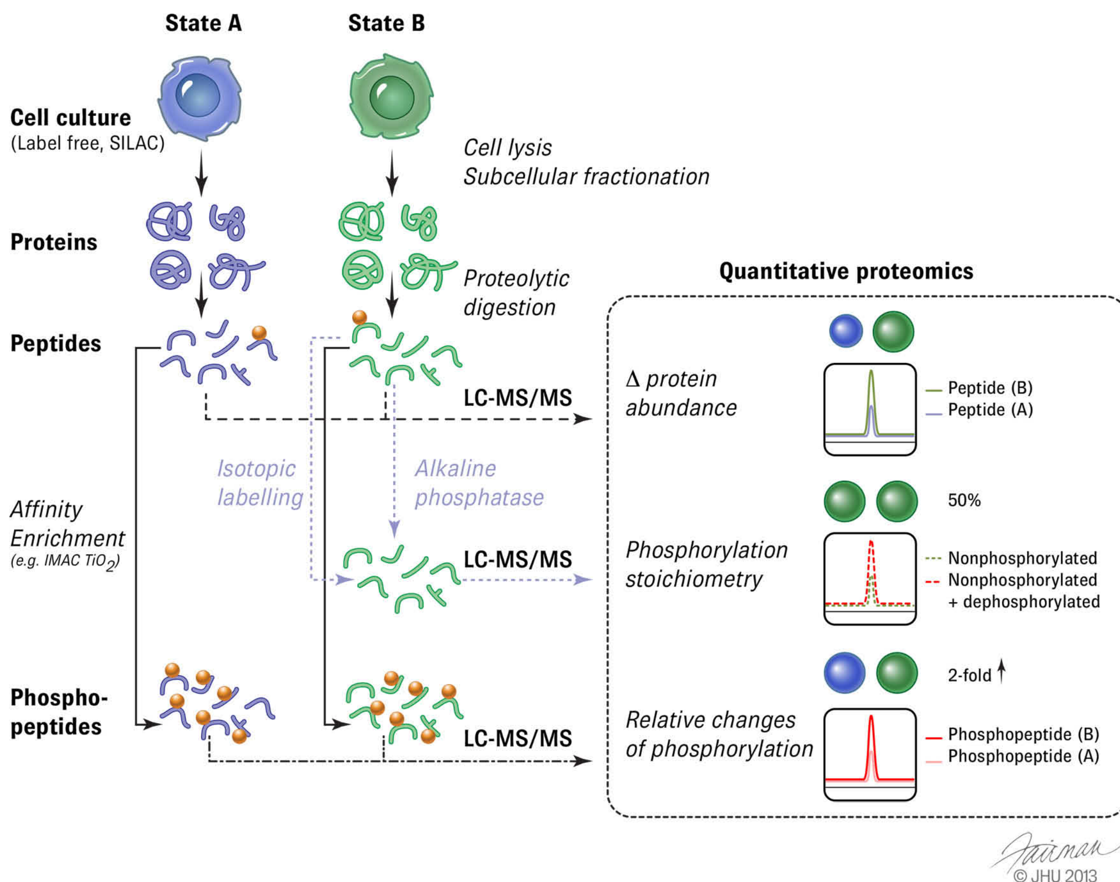

FIG. 1. **Schematic overview of MS-based phosphoproteomic approaches.** Outline of cell fractionation, affinity purification, and quantitative MS analyses. Information derived from quantitative proteomics such as changes in protein and phosphopeptide abundances and variation in phosphorylation stoichiometry are outlined by a rectangle (lower right). Blue and green circles depict protein abundances from different cell extracts, and shaded red circles indicate phosphorylation stoichiometry at a single site. When profiling phosphorylation events over extended stimulation periods (>1 h), phosphoproteomic results should be normalized to account for relative changes in protein abundances.

ing of modified residues, current phosphoproteomic approaches provide quantitative measurements that profile phosphorylation stoichiometry and protein abundance across different cellular paradigms. Current strategies for phosphoproteomic analyses typically comprise four important steps: cell fractionation, protein digestion, enrichment of phosphopeptides, and analysis via liquid chromatography (LC) coupled with tandem MS (Fig. 1). These steps can be customized depending on the type of quantitative information required or, for example, to specifically enrich phosphopeptides with a specific consensus motif (*e.g.* 14-3-3-binding or Akt consensus motifs) (5–7) or modified residues present at low proportions in cell extracts (*e.g.* phosphotyrosine) (8–10). The following sections outline key aspects of the experimental design that should be considered when planning quantitative phosphoproteomic analyses.

Sample Preparation—The amount of protein extract needed for quantitative phosphoproteomics depends on the type of information required. Whereas protein expression measurements can be performed with a few micrograms of protein digests, phosphoproteomic analyses typically require 50 to 100 times more material to enrich low-abundance phosphopeptides (<5%) present in an overwhelming population of unmodified peptides. For example, large-scale phosphoproteomic experiments performed on mammalian cells often require 3 to 5 mg of protein extracts in order to identify more than 10,000 distinct phosphorylation sites. However, the identification of low-abundance peptides with phosphotyrosine residues typically requires larger amounts of protein (>20 mg) for successful immunoaffinity-based phosphopeptide enrichment. Also, experiments involving cell stimulation that extend over several hours must measure both protein and phosphopeptide abundances on the same extracts in order to accurately determine changes in phosphorylation stoichiometry (Fig. 1) (11).

Appropriate protease and phosphatase inhibitors must be used during all steps of cell lysis and protein extraction to preserve the integrity of protein and phosphorylation status, as different phosphatase inhibitors can produce distinct phosphopeptide populations (12). Proper attention must be

placed on protein isolation procedures to minimize contaminants from cell cultures (*e.g.* serum proteins) or changes in phosphorylation associated with variations in osmolarity or temperature. Accordingly, cell harvesting and lysis must be performed rapidly at a low temperature (*e.g.* −80 °C ethanol), or, alternatively, cell pellets can be snap-frozen in liquid nitrogen and kept at −80 °C prior to protein extraction and digestion. Although trypsin is commonly used in proteomic experiments to proteolyse protein extracts, phosphopeptides present in these digests may be too small or too large to be recovered and identified efficiently. Alternate enzymes (*e.g.* lysyl endopeptidase, Asp-N, glutamyl endopeptidase, chymotrypsin) used alone or in combination with trypsin can improve sequence coverage and phosphorylation site mapping, especially for modified residues that lie within basic protein domains (13, 14).

Enrichment of Phosphopeptides via Affinity Purification—Phosphopeptides are typically present in low proportions in protein digests and can exhibit variable site occupancy on a wide dynamic range of expressed proteins. Successful phosphoproteomic experiments thus rely on the use of selective enrichment techniques that enhance the relative abundance of phosphopeptides to levels detectable via MS. Different analytical strategies that combine LC fractionation (*e.g.* ion exchange and hydrophilic interaction chromatography) before or after phosphopeptide enrichment have been proposed to enhance selectivity (15–18). The most popular sorbent methodologies for phosphopeptide enrichment are immobilized metal affinity chromatography with Fe^{3+} (19–21) and metal oxide affinity chromatography with TiO_2 beads (22, 23). However, several alternate and complementary approaches have also been described, including phosphotyrosine immunoaffinity purification (8, 9), immobilized metal affinity chromatography with Ga^{3+} (24), metal oxide affinity chromatography with ZrO_2 or Nb_2O_5 (25, 26), and a combination of immobilized metal affinity chromatography and metal oxide affinity chromatography (27). More details on phosphopeptide affinity media can be found in recent reviews on this topic (28–31).

Localization of Phosphorylation Sites—Another important consideration relates to the confidence in the localization of phosphorylation sites identified from MS/MS data. The localization of phosphorylation sites is typically based on the observation of sequence-specific fragment ions comprising the intact phosphorylated moiety or resulting from the cleavage of the phosphoester bond. The prompt loss of the phosphate moiety from the precursor or fragment ions can give rise to ambiguous site localization, a situation that is partly alleviated using electron transfer dissociation (32). Large-scale phosphoproteomics experiments using electron transfer dissociation activation were first described for the analysis of *E. coli* and human embryonic kidney 293T cells (33, 34). Despite the quality of the acquired MS/MS spectra, the confidence in site localization can be compromised by the occurrence of phosphopeptide isomers that can represent up to 3% to 6% of all identifications (35). To address the difficulty of site localization, different algorithms have been developed to provide a probabilistic measure of phosphorylation site assignment based on results from database search engines. These include PTM score (36), Ascore (37), PhosphoScore (38), PhosphoScan (39), and PhosCalc (40) for MS/MS spectra acquired using collision-induced dissociation and SLoMo (41), Phosphinator (42), and PhosphoRS (43) for spectra acquired using both collision-induced dissociation and electron transfer dissociation fragmentation modes.

Quantitative Phosphoproteomics—The emergence of MS instruments with high resolution and sensitivity combined with reproducible sample preparation strategies and data mining algorithms has provided robust analytical platforms for quantitative proteomics. Changes in phosphopeptide abundance with different conditions and replicates can be determined using quantitative approaches similar to those used for their nonphosphorylated counterparts. Essentially, we distinguish two types of quantitative analyses in which MS signals can be used to profile relative changes in phosphoprotein abundance or phosphorylation stoichiometry (Fig. 1). In each case, the comparison of ion abundances can be accomplished using native peptides (label-free) (44) and stable isotopes incorporated either as labeled amino acids in cell culture (SILAC) (45) or as chemical tags using functionalized reagents after protein digestion (46, 47). Alternative approaches for enhancing sample multiplexing have recently been proposed using both SILAC and isobaric chemical tags (48). Quantitation of specific peptides can also be performed using absolute quantitation (AQUA) (49) by spiking known amounts of isotopically labeled peptides in protein digests. More detailed descriptions of these methods can be found in recently published reviews on quantitative proteomics (50–53).

The profiling of phosphoprotein abundance must distinguish changes in phosphorylation from those associated with protein expression. For experimental paradigms involving rapid cell stimulation ($<$1 h) in which protein expression remains relatively constant, changes in protein phosphorylation between a given condition and its reference sample serve as a proxy for relative changes in phosphorylation stoichiometry. This approach was used in combination with phosphotyrosine immunoaffinity enrichment and SILAC to profile rapid ($<$1 min) and site-specific changes in the phosphorylation of the epithelial growth factor receptor following stimulation with its ligand (54). Quantitative phosphoproteomics has also been used to identify downstream signaling intermediates of other receptors, including EphB (55), Her2/Neu (56), and the T-cell receptor (57). Recently, the use of SILAC combined with TiO_2 and anti-phosphotyrosine antibodies was described for quantitative phosphoproteomics analysis of signaling pathways activated by a low-abundance thymic stromal lymphopoietin cytokine (58).

The accurate measurement of phosphorylation stoichiometry is significantly more challenging, but is often necessary

TABLE I
Model systems and paradigms used in large-scale phosphoproteomic studies

Model systems or paradigms	Examples	Advantages	Limitations
Growth factors and other agonists	Insulin, EGF, serum, DNA damaging agents, metformin	Allows for acute stimulations as well as temporal studies	Specificity will vary. Needs to be combined with more specific approaches (i.e., inhibitors or RNAi).
Constitutively activated protein kinase	Oncogenes (e.g., activated B-Raf or PI3K)	Specific activation of a particular pathway. Often, clinically relevant mutations can be used.	May lead to the identification of indirect mechanisms
Loss-of-function of pathway inhibitors	Tumor suppressors (e.g., TSC2, PTEN)	Specific activation of a particular pathway. Often, clinically relevant mutation can be used.	May lead to the identification of indirect mechanisms
Pharmacological inhibitors	Various (e.g., rapamycin, PI-103, PD184352)	Allows for acute inhibition and temporal studies. May be very specific or target several related proteins.	Specificity could be an issue
RNA interference	shRNA or siRNA	Can be highly specific. Possibility of use *in vivo* and with an inducible system.	May lead to the identification of indirect mechanisms

for assessment of the functional significance of identified sites. In large-scale phosphoproteomic experiments, these measurements require the normalization of protein expression for proper interpretation of the phosphorylation dynamics (11). Recent quantitative analyses of the proteome and phosphoproteome of the human cell cycle enabled the determination of phosphorylation stoichiometry on more than 5000 sites and revealed the site-specific up-regulation of cyclin-dependent kinase 1 or 2 substrates during mitosis (59). Isotopic labeling and enzymatic dephosphorylation can also be used to determine phosphorylation stoichiometry by measuring the abundance of the nonphosphorylated peptides with and without phosphatase treatment (Fig. 1). This was recently demonstrated in *Saccharomyces cerevisiae* at mid-log phase, where low phosphorylation stoichiometry was observed for abundant cytosol, ribosome, and mitochondria phosphoproteins relative to nuclear and mitotic bud phosphoproteins (60). However, correct relationships between phosphorylation stoichiometry, protein abundance, and site conservation must also consider the background conservation rate of residues, structural protein regions, and proteins (61).

Model Systems and Paradigms for Improved Large-scale Studies—One often underappreciated factor contributing to the success of large-scale studies is the choice and optimization of the biological system. For most protein kinases, many different types of gain- and loss-of-function paradigms amenable to quantitative phosphoproteomics are available (Table I). These include the use of extracellular agonists combined with specific pharmacological inhibitors, as recently demonstrated for the characterization of the Ras/MAPK-dependent phosphoproteome (62). In these studies, the accumulation of phosphoproteins is monitored in response to an acute stimulus (*i.e.* serum or EGF) and compared with cells in which a pathway inhibitor was added to prevent MAPK activation. Another common approach involves the use of cells expressing a constitutively activated or inactive allele of a pathway component (*e.g.* expression of an oncogene or inhibition of a tumor suppressor), leading to constitutive downstream signaling. Combined with pharmacological inhibitors, this approach can be highly effective but relies on substrate dephosphorylation during inhibitor treatment, which may vary greatly among phosphoproteins. This approach was recently used to characterize phosphorylation events occurring downstream of mTOR (63, 64) and the phosphoproteome of melanoma cells harboring an activating mutation in the kinase B-Raf (65).

Pharmacological inhibitors are indispensable tools for acutely inhibiting the activity of a particular protein kinase; however, these tools have varying specificities and potencies (66). A viable approach is to validate phosphoproteomic data using a second inhibitor with a different chemical structure, on the premise that nonspecific kinase substrates will be excluded from the overlap between data sets. If the availability of pharmacological inhibitors is an issue, a similar comparison can be made using inhibitors acting at two different levels of a signaling cascade. Alternatively, RNA interference or other gene-targeted strategies can be considered to more specifically inactivate a protein kinase or a pathway of interest. Such an approach was recently used to determine the phosphoproteome of TANK-binding kinase 1 in lung cancer cells (67) and to characterize mTOR-dependent signaling events in mouse fibroblasts (68). Although these techniques appear to be more specific than pharmacological inhibitors, one needs to keep in mind that they also involve long-term down-regulation, which increases the possible rate of indirect events. Other important considerations when designing large-scale studies include the spatial and temporal regulation of substrate phosphorylation. Indeed, many cellular proteins are

phosphorylated or dephosphorylated in specific cell compartments, and subcellular fractionation may be beneficial to increase the identification of relevant phosphorylation sites. The ability to profile temporal changes in the regulation of protein phosphorylation also increases the quality of data sets that often rely on a single time point for the assessment of dynamic changes. For example, a recent application of quantitative phosphoproteomics to profile the temporal regulation of EGF networks showed that mammalian Shc1 responds through multiple waves of distinct phosphorylation events and protein interactions (69). Overall, an effort toward the careful optimization of biological systems is usually time well spent early in assay design, as more reliable data sets should, in principle, help in the characterization of the identified phosphorylation sites.

Characterization of Identified Phosphorylation Sites—The substantial number of phosphorylation sites identified by large-scale phosphoproteomic studies becomes an obvious starting point for subsequent experiments aimed at understanding both the regulation and functional consequences of protein phosphorylation. However, even under the best possible conditions, phosphorylation sites can be missed by MS because of several different factors affecting peptide purification and/or detection. Thus, one should keep in mind that phosphoproteomic screens are not currently saturating and that the failure to identify a phosphorylation site is often insufficient to rule out its nonexistence. When possible, a viable alternative is to perform targeted phosphorylation site mapping using individually purified proteins to increase sequence coverage and phosphorylation site identification.

As most proteins appear to be phosphorylated at more than one residue, and often by several different protein kinases, phosphorylation mapping data can rapidly become overwhelming. The phosphorylation status of a particular protein may also depend on the cellular context and its environment, underscoring the need for *in silico* approaches to better characterize individual phosphorylation sites. In this context, different bioinformatics tools can provide insights into the conservation of phosphorylation sites (*e.g.* MUSCLE, ClustalW) (70, 71), protein kinase consensus motifs (*e.g.* Phospho.ELM, Predikin, NetworKin, Scansite) (72–75), potential interaction domains (*e.g.* ProDom, Pfam, Interpro) (76), and protein–protein interaction networks (*e.g.* STRING, BioGRID, IntAct) (77–79). With these tools, several predictions can be made about the regulation and function of site-specific protein phosphorylation, which can then be tested using standard biochemical and cell biological approaches.

Confirmation of in Vitro Phosphorylation—A protein kinase may be required for the phosphorylation of a protein substrate in cells, but it may also contribute to the activation of another kinase that directly acts on the substrate (*i.e.* protein kinases within the same signaling cascade). For this reason, the demonstration of direct *in vitro* phosphorylation is a necessary step to define a kinase–substrate relationship. Even when specific phosphorylation sites are analyzed, it is preferable to demonstrate phosphorylation using a full-length protein substrate, rather than synthetic peptides or protein fragments. This will help one determine the relevant phosphorylation sites and whether the putative protein kinase has any preference, at least *in vitro*. This is usually done by monitoring radioactive phosphate incorporation, but it can also be achieved using phospho-specific or phospho-motif antibodies if there is prior knowledge about the exact phospho-acceptor sites or targeted consensus phosphorylation sequences, respectively. This method can also be used for the *de novo* identification of phosphorylation sites using MS-based approaches, but caution must be exerted during the interpretation of the results, as many protein kinases are promiscuous *in vitro* if given enough time and protein substrate. Thus, *in vitro* phosphorylation is not sufficient to define a new phosphorylation site for a given protein kinase, and stoichiometry measurements could disambiguate specific *versus* promiscuous phosphorylation events (60).

Analysis of Phosphorylation Events in Cells—An important and often limiting step in characterizing phosphorylation events is the optimization of a reliable assay to monitor *in vivo* phosphorylation. This can occasionally be detected as an electrophoretic mobility shift, in which case the detection is rapid and simple and allows the determination of phosphorylation stoichiometry. An obvious limitation of this approach is that not all phosphorylation events cause mobility shifts, but several methods have been reported to facilitate their detection, such as altering the ratio of acrylamide to bisacrylamide (80) or the incorporation of a phosphate-binding molecule prior to SDS-PAGE (*e.g.* Phos-Tag) (81). *In vivo* [^{32}P]-orthophosphate labeling is also used to map and characterize site-specific phosphorylation, but because of its time-consuming and low-throughput nature, this approach is somewhat less popular for experimental readouts. Generally preferred are phospho-specific antibodies, but not all phosphorylation sites and/or residues can be detected with this approach. Antibodies recognizing phosphotyrosine have been used successfully for decades, but phosphoserine and phosphothreonine are much less immunogenic, and no comparable antibodies are currently available. As mentioned above, phospho-motif antibodies can be used to monitor phosphorylation by specific classes of protein kinases (82), but caution must be exerted when using these tools, as the recognition of phosphorylation sites can also depend on surrounding sequences. A better approach is to use a combination of phospho-motif antibodies targeted against similar motifs to reduce undesired nonspecific binding (7). This can be combined with site-directed mutagenesis to characterize the phosphorylation sites responsible for the immunoreactivity (83, 84). Once specific phosphorylation sites have been identified, phospho-specific antibodies can be generated and used to monitor exact phosphorylation sites. Although these tools are both expensive and imperfect, they are presently

viewed as the gold standard for monitoring site-specific phosphorylation in cells.

Characterization of the Protein Kinase(s) Involved—As briefly mentioned above, the characterization of the protein kinase(s) starts with the analysis of consensus recognition sequences. Many computational tools exist for protein kinase predictions, which can be used to match identified phosphorylation sites with putative protein kinases. Once established, both pharmacological inhibitors and loss-of-function approaches can be used to determine the requirement for the protein kinase using the preferred phosphorylation site readout. The pharmacological approach has the advantage of allowing for acute kinase inhibition, thus decreasing the possibility of indirect mechanisms. Of course, one must also be aware of potential redundancies between protein kinases of the same family, which often complicate the analysis of substrate phosphorylation. Complementary experiments can be performed with exogenously expressed protein kinase mutants, which in some cases have been shown to behave as dominant-negative alleles. Alternatively, cell lines in which the protein kinase of interest has been depleted or deleted can be used to verify its involvement in substrate phosphorylation. Genetic epistasis allows the ordering of genes within a pathway based on the expression of their phenotypes. This can be useful for positioning new candidate substrates of a particular protein kinase, but more rigorous approaches are needed to demonstrate direct regulation through phosphorylation. Although kinase domains do not generally show significant affinity for their substrates, many protein kinases associate with their substrates using specialized docking motifs, such as the D-domain found in some MAPK substrates (85). Also, the identification of physical interaction between a protein kinase and its substrate increases confidence that the regulation is direct.

Functional Significance of Protein Phosphorylation—The evaluation of the biological significance of regulated phosphorylation sites identified from large-scale phosphoproteomic studies represents a significant undertaking. Although there are different bioinformatics tools for determining the conservation of phosphorylation sites and their possible location within functional domains, large-scale functional analyses remain very limited. For a large subset of proteins, phosphorylation is tightly associated with protein activity and is a key point of protein function regulation. Protein phosphorylation commonly induces structural changes that modulate the proteins' intrinsic functional properties (Fig. 2). These changes generally result from the disruption or creation of hydrogen bonds between the phosphate groups and neighboring amino acids, and they typically involve the positively charged guanidinium side chain of arginine residues and backbone nitrogens of α-helices. Assessment of the impact of protein phosphorylation usually involves mutation of the sites (*i.e.* Ser/Thr to Ala, or Tyr to Phe) so that their biological significance can be ascertained. When the mutation of phosphorylation sites causes a loss of function, it is usually important to ensure that the mutation did not result in nonspecific changes to the protein. So-called phosphomimetic mutations can also be generated by substituting the phosphorylation site with an aspartic or glutamic acid residue. Although these mutations can participate in the creation of new hydrogen bonds, they usually do not mimic binding sites for phospho-dependent adaptor proteins. Indeed, another common outcome of protein phosphorylation is the creation of phospho-dependent binding sites for proteins such as 14-3-3 and Forkhead-associated (FHA) domain-containing proteins (Fig. 2), which show specificity for phosphorylated serine or threonine residues (86, 87). Additional examples include Src homology 2 (SH2) and phosphotyrosine-binding (PTP) domain-containing proteins, which selectively bind phosphotyrosine in a sequence-dependent context (88, 89). Because phosphomimetic mutations often fail to reproduce the changes to a protein caused by phosphorylation, the behavior of these mutants can sometimes be difficult to interpret.

As mentioned above, protein phosphorylation leads to changes in the structural properties of substrates, but it can also affect protein–protein interaction networks. These changes can have diverse biological outcomes, such as effects on protein subcellular localization (*e.g.* nuclear translocation or cytoplasmic retention), degradation, and stability, as well as variations in intrinsic catalytic activity (*i.e.* activation or inactivation) (Fig. 2). For example, PDCD4 phosphorylation at Ser457, located near its C-terminal NLS, was shown to promote its nuclear translocation in response to Akt activation by growth factors (90). Protein kinases are themselves very good examples of the latter, as the majority are activated via phosphorylation of residues within their activation loop (*i.e.* T-loop). Protein phosphorylation can also occur within sequences that are recognized by ubiquitin ligase complexes, such as $SCF^{\beta TrCP}$, which recognizes the consensus DpSGXX(X)pS where the serine residues are phosphorylated by specific protein kinases (91). Protein phosphorylation can also reveal or obstruct sequences that regulate the subcellular localization of substrates, such as nuclear exclusion and localization signals (*i.e.* NES and NLS). Alternatively, phosphorylation of residues located within adapter binding sites can induce substrate translocation to the plasma membrane, such as with Grb2 and the p85 subunit of PI3K, which contain SH2 domains that interact with phosphotyrosines within specific motifs (89). The complication is that protein substrates are often phosphorylated at multiple sites that provide independent or synergistic effects on protein function. Because many proteins can be phosphorylated by multiple protein kinases, special care must be taken to ensure that hypotheses originate from rigorous and high-confidence phosphoproteomic data.

Crosstalk and Interplay with Other Post-translational Modifications—Phosphorylation can positively or negatively regulate other types of PTMs. Positive crosstalk involves a situation in which phosphorylation initiates the addition or removal

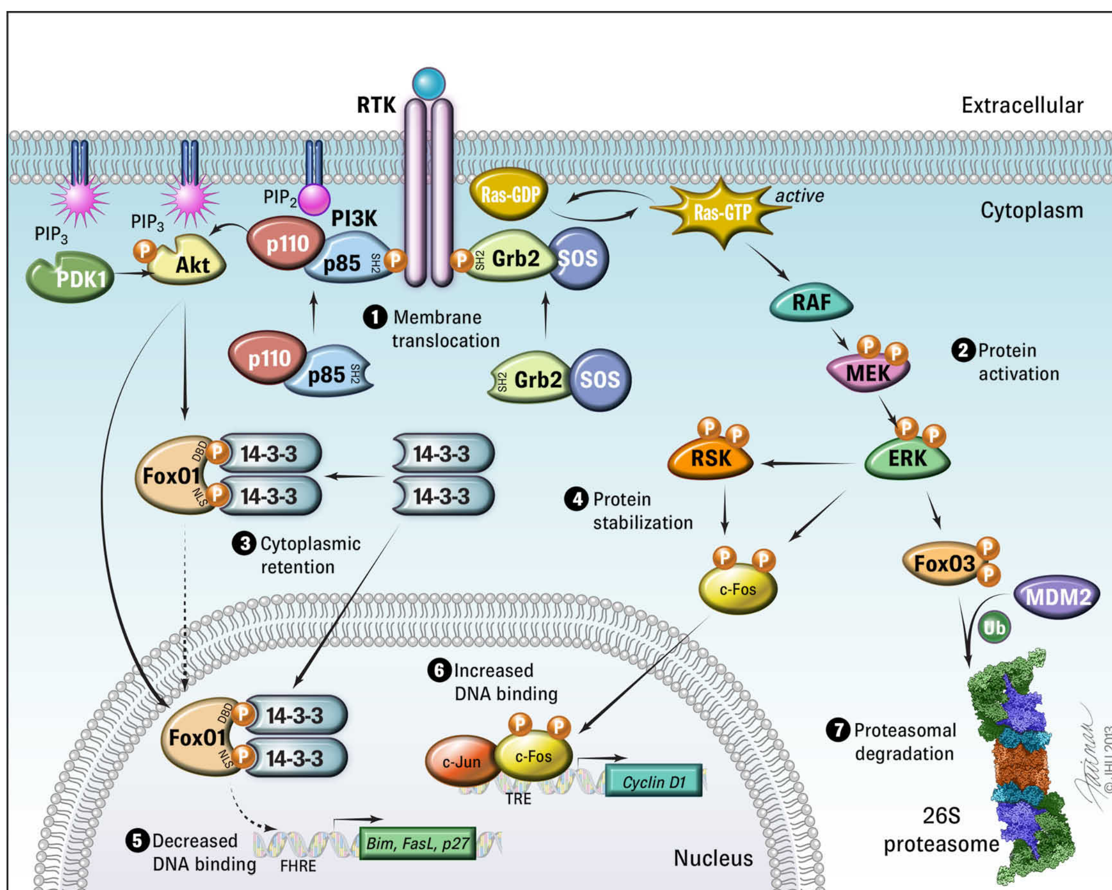

FIG. 2. **Functional significance of protein phosphorylation.** Schematic model illustrating the different roles of protein phosphorylation in mediating diverse biological outcomes. (1) Phosphorylation-dependent membrane translocation can occur when SH2 domain-containing proteins, such as Grb2 and p85, bind to tyrosine-phosphorylated receptors at the plasma membrane. (2) Phosphorylation of protein kinases within their activation loop (T-loop), such as with MEK, ERK, and RSK, is a common activation mechanism that relies on structural rearrangements within the kinase domain. (3) Phosphorylation-dependent binding of scaffold proteins such as 14-3-3 can act as a cytoplasmic anchor for certain kinase substrates such as FoxO1 when phosphorylated by Akt. (4) In some instances, protein phosphorylation can promote protein stability, such as is the case for the immediate-early gene product c-Fos. (5), (6) Phosphorylation of certain transcription factors has been shown to promote or impede DNA binding, as was demonstrated for FoxO1 and c-Fos. (7) Phosphorylation of certain proteins within sequences termed “phosphodegron” can promote their recognition by specific ubiquitin ligases and subsequent proteosomal degradation, as was shown for the transcription factor FoxO3 and the E3 ligase MDM2.

of another modification, whereas negative crosstalk arises from a competition with a modification at the same site or through the inhibition of a second modification (Fig. 3). Processive and inhibitory actions of protein phosphorylation can also take place on multiple sites that are modified by different kinases or phosphatases. Well-known examples include the sequential phosphorylation of MAPK at the TxY motif (where x is any amino acid) (92), processive phosphorylation on the N-terminal (*e.g.* glycogen synthase kinase 3) or C-terminal (*e.g.* CKI) side of the priming phosphate, and the inhibition of Src tyrosine kinase activity through the formation of an inactive folded state following the phosphorylation of the conserved C-terminal tyrosine by the kinase Csk (93).

Crosstalk between phosphorylation and ubiquitylation probably represents one of the most studied intersections between protein modifications (reviewed in Ref. 94). Phosphorylation can regulate the activity of E3 ligases by affecting target protein binding (*e.g.* homologous to the E6 AP carboxyl terminus (HECT) domain) (95) or by causing allosteric activation/inhibition (96, 97). Substrate phosphorylation can also create a recognition signal (phosphodegron) on protein substrates that favors the binding of E3 ligases and subsequent proteasomal degradation (98). For example, several phosphodegrons are recognized by two subfamilies of F-box proteins (*e.g.* WD40 repeat and leucine-rich repeat) contained within Skp1/cullin/F-box E3 ligases (94). In addition to ubiquitylating enzymes, phosphorylation can also regulate the activity of deubiquitinases as evidenced for USP44, for which it enhances activity and prevents premature activation of the anaphase-promoting complex (99). Interestingly, two recent large-scale proteomic studies described different approaches for the serial enrichment of phosphorylated and ubiquitylated

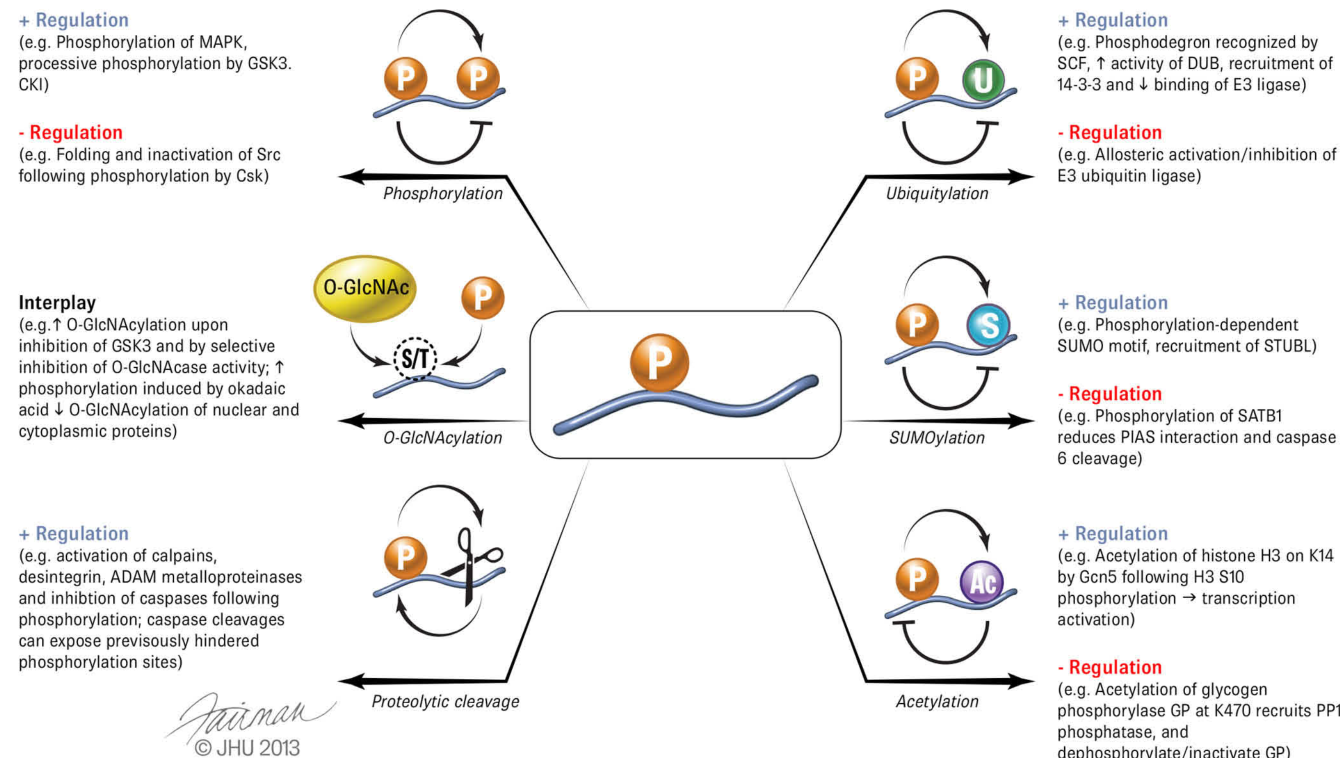

FIG. 3. **Crosstalk between protein phosphorylation and other modifications.** Examples of positive and negative regulation of protein phosphorylation on different modifications are highlighted in rounded rectangles. Interplay between phosphorylation and *O*-GlcNAcylation on specific Ser/Thr residues is also shown.

substrates (100, 101) and revealed novel regulatory mechanisms of kinases and 14-3-3 scaffold proteins via proteasome-independent ubiquitylation (101).

Phosphorylation can also affect neighboring lysine residues modified by SUMOylation, acetylation, or methylation. For example, a phosphorylation-dependent SUMO motif ψKxExxSP (where ψ is a hydrophobic amino acid and x is any amino acid) was identified in several proteins that are substrates for both SUMO conjugation and proline-directed kinases (102). Positive regulation of SUMOylation by phosphorylation was previously shown for several substrates, including FEN1 (103), where the sequential modification recruits SUMO-targeted ubiquitin ligases that ubiquitylate substrates, leading to their degradation via the proteasome pathway (104). In contrast, phosphorylation can antagonize the binding of E3 SUMO ligases as for the Special AT-rich sequence-binding protein 1, where its phosphorylation by protein kinase C impedes interaction with protein inhibitor of activated STAT and prevents its cleavage by caspase 6 (105).

Also, proteases such as calpains, desintegrin, and A disintegrin and metalloproteases (ADAM) can be activated by phosphorylation, whereas the opposite effect is observed for several caspases and separases (106, 107). Previous studies that examined functional effects of phosphorylation on caspase cleavage indicated that phosphorylation mainly prevents caspase cleavages (106, 108, 109). However, a recent large-scale proteomic study performed under apoptotic conditions revealed that caspase cleavages can expose phosphorylation sites that would otherwise be inaccessible, and that phosphorylation at the +3 position of cleavage sites can promote substrate proteolysis by caspase 8 (110).

A complex interplay was also described for phosphorylation and the attachment of *O*-linked *N*-acetylglucosamine (GlcNAc) to specific Ser/Thr residues, where they can either compete for the same site or occupy different sites on a substrate (111). Phosphorylation can affect the activity of *O*-GlcNAc-modifying enzymes, and *O*-GlcNAcylation can modulate kinase activity. Recent proteomic investigations highlighted the drastic changes in *O*-GlcNAcylation upon the inhibition of glycogen synthase kinase 3 and via the selective inhibition of *O*-GlcNAcase activity (112). Proteins showing actively cycling phosphorylated sites upon elevated *O*-GlcNAcylation included cytoskeletal proteins, metabolic enzymes, kinases, transcription factors, and RNA-processing factors. The interplay between *O*-GlcNAc and phosphorylation can in principle be used as a logic gate in signaling networks.

Conclusions and Perspectives—Although the first protein kinase was discovered over 50 years ago, the past decade has seen an exponential increase in the number of identified phosphorylation sites. This is mostly due to recent advances in LC-MS/MS and phosphopeptide enrichment strategies that

now allow the rapid identification of phosphorylation sites with precision and sensitivity. Despite these accomplishments, comparative studies have revealed that further improvements in MS instrumentation and other analytical tools will be required in order for saturation to be reached. This is best exemplified by recent phosphoproteomics studies on MAPK signaling that have used complementary technological platforms and identified different subsets of phosphorylated substrates (62, 65, 113). Although it appears that these limitations can be reduced through the use of different strategies for quantitative phosphoproteomics, absolute quantification of phosphopeptides and the determination of phosphorylation stoichiometry will require further development.

Despite the vast amount of quantitative phosphoproteomics data currently available, validation remains quite limited, and only a very small proportion of phosphorylation sites have been associated to a particular biological function. A major goal in the future will be to move from the large compendium of phosphorylation sites to the challenging task of teasing out biologically relevant phosphorylation events. Further advances in bioinformatics and computational biology that integrate different -omics fields should provide useful information on kinase-regulated phosphorylation events. Phosphoproteomics data can also be used to understand crosstalk between signaling pathways and the spatiotemporal regulation of phosphorylation necessary to decipher the complex circuitry at a systems biology level. Another important research area is the characterization of crosstalk between different types of PTMs. Whereas the crosstalk between ubiquitylation and phosphorylation is reasonably well understood, much remains to be known regarding potential crosstalk between phosphorylation and other types of PTMs, such as SUMOylation, glycosylation, methylation, and acetylation. Obviously, detailed biochemical and cell biological analyses of selected phosphorylation sites and their kinases are important for the elucidation of their regulatory functions. Major advances could thus be made toward finding better tools with which to validate and functionally characterize phosphorylation sites arising from phosphoproteomic studies.

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REFERENCES

1. Pawson, T. (2004) Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* **116,** 191–203
2. Ubersax, J. A., and Ferrell, J. E., Jr. (2007) Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol.* **8,** 530–541
3. Brognard, J., and Hunter, T. (2011) Protein kinase signaling networks in cancer. *Curr. Opin. Genet. Dev.* **21,** 4–11
4. Tidyman, W. E., and Rauen, K. A. (2009) The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. *Curr. Opin. Genet. Dev.* **19,** 230–236
5. Jin, J., Smith, F. D., Stark, C., Wells, C. D., Fawcett, J. P., Kulkarni, S., Metalnikov, P., O'Donnell, P., Taylor, P., Taylor, L., Zougman, A., Woodgett, J. R., Langeberg, L. K., Scott, J. D., and Pawson, T. (2004) Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization. *Curr. Biol.* **14,** 1436–1450
6. Atrih, A., Turnock, D., Sellar, G., Thompson, A., Feuerstein, G., Ferguson, M. A., and Huang, J. T. (2010) Stoichiometric quantification of Akt phosphorylation using LC-MS/MS. *J. Proteome Res.* **9,** 743–751
7. Moritz, A., Li, Y., Guo, A., Villen, J., Wang, Y., MacNeill, J., Kornhauser, J., Sprott, K., Zhou, J., Possemato, A., Ren, J. M., Hornbeck, P., Cantley, L. C., Gygi, S. P., Rush, J., and Comb, M. J. (2010) Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci. Signal.* **3,** ra64
8. Jedrychowski, M. P., Huttlin, E. L., Haas, W., Sowa, M. E., Rad, R., and Gygi, S. P. (2011) Evaluation of HCD- and CID-type fragmentation within their respective detection platforms for murine phosphoproteomics. *Mol. Cell. Proteomics* **10,** M111.009910
9. Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., Nardone, J., Lee, K., Reeves, C., Li, Y., Hu, Y., Tan, Z., Stokes, M., Sullivan, L., Mitchell, J., Wetzel, R., Macneill, J., Ren, J. M., Yuan, J., Bakalarski, C. E., Villen, J., Kornhauser, J. M., Smith, B., Li, D., Zhou, X., Gygi, S. P., Gu, T. L., Polakiewicz, R. D., Rush, J., and Comb, M. J. (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* **131,** 1190–1203
10. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **23,** 94–101
11. Wu, R., Dephoure, N., Haas, W., Huttlin, E. L., Zhai, B., Sowa, M. E., and Gygi, S. P. (2011) Correct interpretation of comprehensive phosphorylation dynamics requires normalization by protein expression changes. *Mol. Cell. Proteomics* **10,** M111.009654
12. Thingholm, T. E., Larsen, M. R., Ingrell, C. R., Kassem, M., and Jensen, O. N. (2008) TiO(2)-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *J. Proteome Res.* **7,** 3304–3313
13. Wisniewski, J. R., and Mann, M. (2012) Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal. Chem.* **84,** 2631–2637
14. Bian, Y., Ye, M., Song, C., Cheng, K., Wang, C., Wei, X., Zhu, J., Chen, R., Wang, F., and Zou, H. (2012) Improve the coverage for the analysis of phosphoproteome of HeLa cells by a tandem digestion approach. *J. Proteome Res.* **11,** 2828–2837
15. Ficarro, S. B., Zhang, Y., Carrasco-Alfonso, M. J., Garg, B., Adelmant, G., Webber, J. T., Luckey, C. J., and Marto, J. A. (2011) Online nanoflow multidimensional fractionation for high efficiency phosphopeptide analysis. *Mol. Cell. Proteomics* **10,** O111.011064
16. Villen, J., and Gygi, S. P. (2008) The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* **3,** 1630–1638
17. 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
18. Gan, C. S., Guo, T., Zhang, H., Lim, S. K., and Sze, S. K. (2008) A comparative study of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) versus SCX-IMAC-based methods for phosphopeptide isolation/enrichment. *J. Proteome Res.* **7,** 4869–4877
19. Andersson, L., and Porath, J. (1986) Isolation of phosphoproteins by

immobilized metal ($Fe3+$) affinity chromatography. *Anal. Biochem.* **154,** 250–254
20. Nuhse, T., Yu, K., and Salomon, A. (2007) Isolation of phosphopeptides by immobilized metal ion affinity chromatography. *Curr. Protoc. Mol. Biol.* Chapter 18, Unit 18.13
21. Thingholm, T. E., and Jensen, O. N. (2009) Enrichment and characterization of phosphopeptides by immobilized metal affinity chromatography (IMAC) and mass spectrometry. *Methods Mol. Biol.* **527,** 47–56, xi
22. Ikeguchi, Y., and Nakamura, H. (1997) Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on Titania. *Anal. Sci.* **13,** 479–483
23. Pinkse, M. W., Uitto, P. M., Hilhorst, M. J., Ooms, B., and Heck, A. J. (2004) Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem.* **76,** 3935–3943
24. Posewitz, M. C., and Tempst, P. (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.* **71,** 2883–2892
25. Ficarro, S. B., Parikh, J. R., Blank, N. C., and Marto, J. A. (2008) Niobium(V) oxide (Nb2O5): application to phosphoproteomics. *Anal. Chem.* **80,** 4606–4613
26. Kweon, H. K., and Hakansson, K. (2006) Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal. Chem.* **78,** 1743–1749
27. Thingholm, T. E., Jensen, O. N., Robinson, P. J., and Larsen, M. R. (2008) SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol. Cell. Proteomics* **7,** 661–671
28. Beltran, L., and Cutillas, P. R. (2012) Advances in phosphopeptide enrichment techniques for phosphoproteomics. *Amino Acids* **43,** 1009–1024
29. Dunn, J. D., Reid, G. E., and Bruening, M. L. (2009) Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. *Mass Spectrom. Rev.* **29,** 29–54
30. Engholm-Keller, K., and Larsen, M. R. (2013) Technologies and challenges in large-scale phosphoproteomics. *Proteomics* **13,** 910–931
31. Kanshin, E., Michnick, S., and Thibault, P. (2012) Sample preparation and analytical strategies for large-scale phosphoproteomics experiments. *Semin. Cell Dev. Biol.* **23,** 843–853
32. Mikesh, L. M., Ueberheide, B., Chi, A., Coon, J. J., Syka, J. E., Shabanowitz, J., and Hunt, D. F. (2006) The utility of ETD mass spectrometry in proteomic analysis. *Biochim. Biophys. Acta* **1764,** 1811–1822
33. Chi, A., Huttenhower, 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 *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 2193–2198
34. Molina, H., Horn, D. M., Tang, N., Mathivanan, S., and Pandey, A. (2007) Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 2199–2204
35. Courcelles, M., Bridon, G., Lemieux, S., and Thibault, P. (2012) Occurrence and detection of phosphopeptide isomers in large-scale phosphoproteomics experiments. *J. Proteome Res.* **11,** 3753–3765
36. 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
37. 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
38. Ruttenberg, B. E., Pisitkun, T., Knepper, M. A., and Hoffert, J. D. (2008) PhosphoScore: an open-source phosphorylation site assignment tool for MSn data. *J. Proteome Res.* **7,** 3054–3059
39. Wan, Y., Cripps, D., Thomas, S., Campbell, P., Ambulos, N., Chen, T., and Yang, A. (2008) PhosphoScan: a probability-based method for phosphorylation site prediction using MS2/MS3 pair information. *J. Proteome Res.* **7,** 2803–2811
40. MacLean, D., Burrell, M. A., Studholme, D. J., and Jones, A. M. (2008) PhosCalc: a tool for evaluating the sites of peptide phosphorylation from mass spectrometer data. *BMC Res. Notes* **1,** 30
41. Bailey, C. M., Sweet, S. M., Cunningham, D. L., Zeller, M., Heath, J. K., and Cooper, H. J. (2009) SLoMo: automated site localization of modifications from ETD/ECD mass spectra. *J. Proteome Res.* **8,** 1965–1971
42. Swaney, D. L., Wenger, C. D., Thomson, J. A., and Coon, J. J. (2009) Human embryonic stem cell phosphoproteome revealed by electron transfer dissociation tandem mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 995–1000
43. Taus, T., Kocher, T., Pichler, P., Paschke, C., Schmidt, A., Henrich, C., and Mechtler, K. (2011) Universal and confident phosphorylation site localization using phosphoRS. *J. Proteome Res.* **10,** 5354–5362
44. Kearney, P., and Thibault, P. (2003) Bioinformatics meets proteomics—bridging the gap between mass spectrometry data analysis and cell biology. *J. Bioinform. Comput. Biol.* **1,** 183–200
45. 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
46. 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
47. Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A. K., and Hamon, C. (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* **75,** 1895–1904
48. McAlister, G. C., Huttlin, E. L., Haas, W., Ting, L., Jedrychowski, M. P., Rogers, J. C., Kuhn, K., Pike, I., Grothe, R. A., Blethrow, J. D., and Gygi, S. P. (2012) Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. *Anal. Chem.* **84,** 7469–7478
49. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 6940–6945
50. Nilsson, C. L. (2012) Advances in quantitative phosphoproteomics. *Anal. Chem.* **84,** 735–746
51. Pimienta, G., Chaerkady, R., and Pandey, A. (2009) SILAC for global phosphoproteomic analysis. *Methods Mol. Biol.* **527,** 107–116, x
52. Ong, S. E., Foster, L. J., and Mann, M. (2003) Mass spectrometric-based approaches in quantitative proteomics. *Methods* **29,** 124–130
53. Neilson, K. A., Ali, N. A., Muralidharan, S., Mirzaei, M., Mariani, M., Assadourian, G., Lee, A., van Sluyter, S. C., and Haynes, P. A. (2011) Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics* **11,** 535–553
54. Dengjel, J., Akimov, V., Olsen, J. V., Bunkenborg, J., Mann, M., Blagoev, B., and Andersen, J. S. (2007) Quantitative proteomic assessment of very early cellular signaling events. *Nat. Biotechnol.* **25,** 566–568
55. Jorgensen, C., Sherman, A., Chen, G. I., Pasculescu, A., Poliakov, A., Hsiung, M., Larsen, B., Wilkinson, D. G., Linding, R., and Pawson, T. (2009) Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. *Science* **326,** 1502–1509
56. Bose, R., Molina, H., Patterson, A. S., Bitok, J. K., Periaswamy, B., Bader, J. S., Pandey, A., and Cole, P. A. (2006) Phosphoproteomic analysis of Her2/neu signaling and inhibition. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 9773–9778
57. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T cell receptor activation. *Mol. Cell. Proteomics* **8,** 2418–2431
58. Zhong, J., Kim, M. S., Chaerkady, R., Wu, X., Huang, T. C., Getnet, D., Mitchell, C. J., Palapetta, S. M., Sharma, J., O'Meally, R. N., Cole, R. N., Yoda, A., Moritz, A., Loriaux, M. M., Rush, J., Weinstock, D. M., Tyner, J. W., and Pandey, A. (2012) TSLP signaling network revealed by SILAC-based phosphoproteomics. *Mol. Cell. Proteomics* **11,** M112.017764
59. Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnad, F., Cox, J., Jensen, T. S., Nigg, E. A., Brunak, S., and Mann, M. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal.* **3,** ra3

60. Wu, R., Haas, W., Dephoure, N., Huttlin, E. L., Zhai, B., Sowa, M. E., and Gygi, S. P. (2011) A large-scale method to measure absolute protein phosphorylation stoichiometries. *Nat. Methods* **8,** 677–683
61. Tan, C. S., and Bader, G. D. (2012) Phosphorylation sites of higher stoichiometry are more conserved. *Nat. Methods* **9,** 317
62. Courcelles, M., Fremin, C., Voisin, L., Lemieux, S., Meloche, S., and Thibault, P. (2013) Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions. *Mol. Syst. Biol.* **9,** 669
63. Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* **332,** 1317–1322
64. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villen, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* **332,** 1322–1326
65. Old, W. M., Shabb, J. B., Houel, S., Wang, H., Couts, K. L., Yen, C. Y., Litman, E. S., Croy, C. H., Meyer-Arendt, K., Miranda, J. G., Brown, R. A., Witze, E. S., Schweppe, R. E., Resing, K. A., and Ahn, N. G. (2009) Functional proteomics identifies targets of phosphorylation by B-Raf signaling in melanoma. *Mol. Cell* **34,** 115–131
66. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. *Biochem. J.* **371,** 199–204
67. Kim, J. Y., Welsh, E. A., Oguz, U., Fang, B., Bai, Y., Kinose, F., Bronk, C., Remsing Rix, L. L., Beg, A. A., Rix, U., Eschrich, S. A., Koomen, J. M., and Haura, E. B. (2013) Dissection of TBK1 signaling via phosphoproteomics in lung cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 12414–12419
68. Robitaille, A. M., Christen, S., Shimobayashi, M., Cornu, M., Fava, L. L., Moes, S., Prescianotto-Baschong, C., Sauer, U., Jenoe, P., and Hall, M. N. (2013) Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science* **339,** 1320–1323
69. Zheng, Y., Zhang, C., Croucher, D. R., Soliman, M. A., St-Denis, N., Pasculescu, A., Taylor, L., Tate, S. A., Hardy, W. R., Colwill, K., Dai, A. Y., Bagshaw, R., Dennis, J. W., Gingras, A. C., Daly, R. J., and Pawson, T. (2013) Temporal regulation of EGF signalling networks by the scaffold protein Shc1. *Nature* **499,** 166–171
70. Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32,** 1792–1797
71. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23,** 2947–2948
72. Gould, C. M., Diella, F., Via, A., Puntervoll, P., Gemund, C., Chabanis-Davidson, S., Michael, S., Sayadi, A., Bryne, J. C., Chica, C., Seiler, M., Davey, N. E., Haslam, N., Weatheritt, R. J., Budd, A., Hughes, T., Pas, J., Rychlewski, L., Trave, G., Aasland, R., Helmer-Citterich, M., Linding, R., and Gibson, T. J. (2010) ELM: the status of the 2010 eukaryotic linear motif resource. *Nucleic Acids Res.* **38,** D167–D180
73. Saunders, N. F., Brinkworth, R. I., Huber, T., Kemp, B. E., and Kobe, B. (2008) Predikin and PredikinDB: a computational framework for the prediction of protein kinase peptide specificity and an associated database of phosphorylation sites. *BMC Bioinformatics* **9,** 245
74. Linding, R., Jensen, L. J., Pasculescu, A., Olhovsky, M., Colwill, K., Bork, P., Yaffe, M. B., and Pawson, T. (2008) NetworKIN: a resource for exploring cellular phosphorylation networks. *Nucleic Acids Res.* **36,** D695–D699
75. Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* **31,** 3635–3641
76. Hunter, S., Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duquenne, L., Finn, R. D., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Laugraud, A., Letunic, I., Lonsdale, D., Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., Mistry, J., Mitchell, A., Mulder, N., Natale, D., Orengo, C., Quinn, A. F., Selengut, J. D., Sigrist, C. J., Thimma, M., Thomas, P. D., Valentin, F., Wilson, D., Wu, C. H., and Yeats, C. (2009) InterPro: the integrative protein signature database. *Nucleic Acids Res.* **37,** D211–D215
77. Chatr-Aryamontri, A., Breitkreutz, B. J., Heinicke, S., Boucher, L., Winter, A., Stark, C., Nixon, J., Ramage, L., Kolas, N., O'Donnell, L., Reguly, T., Breitkreutz, A., Sellam, A., Chen, D., Chang, C., Rust, J., Livstone, M., Oughtred, R., Dolinski, K., and Tyers, M. (2013) The BioGRID interaction database: 2013 update. *Nucleic Acids Res.* **41,** D816–D823
78. Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., von Mering, C., and Jensen, L. J. (2013) STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* **41,** D808–D815
79. Kerrien, S., Aranda, B., Breuza, L., Bridge, A., Broackes-Carter, F., Chen, C., Duesbury, M., Dumousseau, M., Feuermann, M., Hinz, U., Jandrasits, C., Jimenez, R. C., Khadake, J., Mahadevan, U., Masson, P., Pedruzzi, I., Pfeiffenberger, E., Porras, P., Raghunath, A., Roechert, B., Orchard, S., and Hermjakob, H. (2012) The IntAct molecular interaction database in 2012. *Nucleic Acids Res.* **40,** D841–D846
80. Nishiwaki, T., Satomi, Y., Kitayama, Y., Terauchi, K., Kiyohara, R., Takao, T., and Kondo, T. (2007) A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J.* **26,** 4029–4037
81. Kinoshita, E., Kinoshita-Kikuta, E., and Koike, T. (2012) Phos-tag SDS-PAGE systems for phosphorylation profiling of proteins with a wide range of molecular masses under neutral pH conditions. *Proteomics* **12,** 192–202
82. Zhang, H., Zha, X., Tan, Y., Hornbeck, P. V., Mastrangelo, A. J., Alessi, D. R., Polakiewicz, R. D., and Comb, M. J. (2002) Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J. Biol. Chem.* **277,** 39379–39387
83. Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P., and Blenis, J. (2004) Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 13489–13494
84. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell* **10,** 151–162
85. Cargnello, M., and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75,** 50–83
86. Durocher, D., Henckel, J., Fersht, A. R., and Jackson, S. P. (1999) The FHA domain is a modular phosphopeptide recognition motif. *Mol. Cell* **4,** 387–394
87. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91,** 961–971
88. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268,** 1177–1179
89. Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S., and Pawson, T. (1990) Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 8622–8626
90. Palamarchuk, A., Efanov, A., Maximov, V., Aqeilan, R. I., Croce, C. M., and Pekarsky, Y. (2005) Akt phosphorylates and regulates Pdcd4 tumor suppressor protein. *Cancer Res.* **65,** 11282–11286
91. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91,** 209–219
92. Aoki, K., Yamada, M., Kunida, K., Yasuda, S., and Matsuda, M. (2011) Processive phosphorylation of ERK MAP kinase in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 12675–12680
93. Cole, P. A., Shen, K., Qiao, Y., and Wang, D. (2003) Protein tyrosine kinases Src and Csk: a tail's tale. *Curr. Opin. Chem. Biol.* **7,** 580–585
94. Hunter, T. (2007) The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Mol. Cell* **28,** 730–738
95. Ichimura, T., Yamamura, H., Sasamoto, K., Tominaga, Y., Taoka, M., Kakiuchi, K., Shinkawa, T., Takahashi, N., Shimada, S., and Isobe, T. (2005) 14-3-3 proteins modulate the expression of epithelial Na^+ channels by phosphorylation-dependent interaction with Nedd4–2 ubiquitin ligase. *J. Biol. Chem.* **280,** 13187–13194
96. Gallagher, E., Gao, M., Liu, Y. C., and Karin, M. (2006) Activation of the E3

ubiquitin ligase Itch through a phosphorylation-induced conformational change. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 1717–1722
97. Yang, C., Zhou, W., Jeon, M. S., Demydenko, D., Harada, Y., Zhou, H., and Liu, Y. C. (2006) Negative regulation of the E3 ubiquitin ligase itch via Fyn-mediated tyrosine phosphorylation. *Mol. Cell* **21,** 135–141
98. Cardozo, T., and Pagano, M. (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell Biol.* **5,** 739–751
99. Stegmeier, F., Rape, M., Draviam, V. M., Nalepa, G., Sowa, M. E., Ang, X. L., McDonald, E. R., 3rd, Li, M. Z., Hannon, G. J., Sorger, P. K., Kirschner, M. W., Harper, J. W., and Elledge, S. J. (2007) Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* **446,** 876–881
100. Mertins, P., Qiao, J. W., Patel, J., Udeshi, N. D., Clauser, K. R., Mani, D. R., Burgess, M. W., Gillette, M. A., Jaffe, J. D., and Carr, S. A. (2013) Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nat. Methods* **10,** 634–637
101. Swaney, D. L., Beltrao, P., Starita, L., Guo, A., Rush, J., Fields, S., Krogan, N. J., and Villen, J. (2013) Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nat. Methods* **10,** 676–682
102. Yang, X. J., and Gregoire, S. (2006) A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol. Cell* **23,** 779–786
103. Guo, Z., Kanjanapangka, J., Liu, N., Liu, S., Liu, C., Wu, Z., Wang, Y., Loh, T., Kowolik, C., Jamsen, J., Zhou, M., Truong, K., Chen, Y., Zheng, L., and Shen, B. (2012) Sequential posttranslational modifications program FEN1 degradation during cell-cycle progression. *Mol. Cell* **47,** 444–456
104. Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J., Tainer, J. A., McGowan, C. H., and Boddy, M. N. (2007) SUMO-targeted ubiquitin ligases in genome stability. *EMBO J.* **26,** 4089–4101
105. Tan, J. A., Song, J., Chen, Y., and Durrin, L. K. (2010) Phosphorylation-dependent interaction of SATB1 and PIAS1 directs SUMO-regulated caspase cleavage of SATB1. *Mol. Cell. Biol.* **30,** 2823–2836
106. Kurokawa, M., and Kornbluth, S. (2009) Caspases and kinases in a death grip. *Cell* **138,** 838–854
107. Lopez-Otin, C., and Hunter, T. (2010) The regulatory crosstalk between kinases and proteases in cancer. *Nat. Rev. Cancer* **10,** 278–292
108. Duncan, J. S., Turowec, J. P., Vilk, G., Li, S. S., Gloor, G. B., and Litchfield, D. W. (2010) Regulation of cell proliferation and survival: convergence of protein kinases and caspases. *Biochim. Biophys. Acta* **1804,** 505–510
109. Tozser, J., Bagossi, P., Zahuczky, G., Specht, S. I., Majerova, E., and Copeland, T. D. (2003) Effect of caspase cleavage-site phosphorylation on proteolysis. *Biochem. J.* **372,** 137–143
110. Dix, M. M., Simon, G. M., Wang, C., Okerberg, E., Patricelli, M. P., and Cravatt, B. F. (2012) Functional interplay between caspase cleavage and phosphorylation sculpts the apoptotic proteome. *Cell* **150,** 426–440
111. Hart, G. W., Housley, M. P., and Slawson, C. (2007) Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* **446,** 1017–1022
112. Wang, Z., Pandey, A., and Hart, G. W. (2007) Dynamic interplay between O-linked N-acetylglucosaminylation and glycogen synthase kinase-3-dependent phosphorylation. *Mol. Cell. Proteomics* **6,** 1365–1379
113. Pan, C., Olsen, J. V., Daub, H., and Mann, M. (2009) Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Mol. Cell. Proteomics* **8,** 2796–2808