Matthias Trost,[1,2] Gaëlle Bridon,[1,3] Michel Desjardins,[2,4] and Pierre Thibault[1,3,5]***

[1]*Institute for Research in Immunology and Cancer, Université de Montréal, P.O. Box 6128, Station Centre-ville, Montréal, Québec, Canada H3C 3J7*
[2]*Department of Pathology and Cell Biology, Université de Montréal, P.O. Box 6128, Station Centre-ville, Montréal, Québec, Canada H3C 3J7*
[3]*Department of Biochemistry, Université de Montréal, P.O. Box 6128, Station Centre-ville, Montréal, Québec, Canada H3C 3J7*
[4]*Department of Microbiology and Immunology, Université de Montréal, P.O. Box 6128, Station Centre-ville, Montréal, Québec, Canada H3C 3J7*
[5]*Department of Chemistry, Université de Montréal, P.O. Box 6128, Station Centre-ville, Montréal, Québec, Canada H3C 3J7*

Received 18 September 2009; accepted 5 November 2009

Published online 7 October 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mas.20297

Protein phosphorylation represents one of the most extensively studied post-translational modifications, primarily due to the emergence of sensitive methods enabling the detection of this modification both in vitro and in vivo. The availability of enrichment methods combined with sensitive mass spectrometry instrumentation has played a crucial role in uncovering the dynamic changes and the large expanding repertoire of this reversible modification. The structural changes imparted by the phosphorylation of specific residues afford exquisite mechanisms for the regulation of protein functions by modulating new binding sites on scaffold proteins or by abrogating protein–protein interactions. However, the dynamic interplay of protein phosphorylation is not occurring randomly within the cell but is rather finely orchestrated by specific kinases and phosphatases that are unevenly distributed across subcellular compartments. This spatial separation not only regulates protein phosphorylation but can also control the activity of other enzymes and the transfer of other post-translational modifications. While numerous large-scale phosphoproteomics studies highlighted the extent and diversity of phosphoproteins present in total cell lysates, the further understanding of their regulation and biological activities require a spatio-temporal resolution only achievable through subcellular fractionation. This review presents a first account of the emerging field of subcellular phosphoproteomics where cell fractionation approaches are combined with sensitive mass spectrometry methods to facilitate the identification of low abundance proteins and to unravel the intricate regulation of protein phosphorylation.
 Mass Spec Rev 29:962–990, 2010

Keywords: *phosphoproteomics; mass spectrometry; phosphorylation; organelle proteomics; vesicle trafficking; SNARE*

I. INTRODUCTION

Protein phosphorylation is a reversible post-translational modification (PTM) regulating major cellular processes such as cell division, growth, and differentiation through highly dynamic and complex signaling pathways. Approximately one-third of proteins encoded by the human genome are presumed to be phosphorylated during their life cycle accounting for an estimated 100,000 different phosphorylation sites (Zhang et al., 2002a). While a large proportion of total phosphorylation in eukaryotes is found on hydroxylated amino acids such as serine, threonine, and tyrosine (*O*-phosphorylation), previous reports have also documented its occurrence on arginine, lysine, and histidine residues (*N*-phosphorylation) (Matthews, 1995; Klumpp & Krieglstein, 2005; Puttick, Baker, & Delbaere, 2008). Interestingly, histidine phosphorylation can occur to the extent of 6% of the total phosphorylation in eukaryote cells, but its detection remains challenging due to its rapid hydrolysis under acidic conditions (Kleinnijenhuis et al., 2007). In contrast, phosphoester bonds are more stable, and previous estimates suggested that *O*-phosphorylation at serine, threonine, and tyrosine residues can occur at a ratio of 90:10:0.05 (Hunter & Sefton, 1980). However, recent large-scale phosphoproteomics studies reported a distribution of 88:11:1 (Olsen et al., 2006; Villen et al., 2007), though the extent of tyrosine phosphorylation can increase markedly for protein membrane extracts (Trost et al., 2009).

The relevance of phosphorylation is further underscored by its importance during evolution. Until very recently, *O*-phosphorylation was considered to be a major regulatory modification specific to eukaryotic cells. However, increasing genomics and proteomics evidences now suggest that this modification is also present in prokaryotes and more than 100 phosphorylation sites have been uncovered in three different bacteria (Macek et al., 2007, 2008; Soufi et al., 2008). *O*-phosphorylation is regulated by kinases and phosphatases, two classes of enzymes with opposite functions that add or remove a phosphate group from a protein substrate. Still, there are only a few kinases presently known in prokaryotes (Kennelly,

Contract grant sponsors: National Science and Engineering Research Council (NSERC); Canadian Institutes for Health Research (CIHR); Canada Research Chair Program; Canadian Center of Excellence in Commercialization and Research; Canadian Foundation for Innovation; Fonds de la Recherche en Santé du Québec (FRSQ); CIHR Multi-Resource Grant.
**Correspondence to:* Pierre Thibault
E-mail: pierre.thibault@umontreal.ca
***Correspondence to:* Michel Desjardins
E-mail: michel.desjardins@umontreal.ca

2002) while 123 and 251 kinases were reported in *Saccharomyces cerevisiae* (Hunter & Plowman, 1997; Brinkworth, Munn, & Kobe, 2006) and *Drosophila melanogaster* (Morrison, Murakami, & Cleghon, 2000), respectively. In comparison, the human and mouse genomes encode 518 (Manning et al., 2002) and 540 known kinases (Caenepeel et al., 2004). In contrast to kinases, the genomic distribution of phosphatases does not rely solely on the number of genes encoding their catalytic units. Serine/threonine phosphatases are rather multimeric enzymes, assembled from a small number of catalytic and hundreds of non-conserved regulatory subunits (Virshup & Shenolikar, 2009). The combinatorial and regulatory complexity of protein phosphatases provides additional specificity finely adapted to intra- and extracellular cues.

Protein phosphorylation not only imparts structural changes on protein substrates but also affects their outcome via protein–protein interactions, translocation, activation, or inactivation. A well-known example of this is the mitogen-activated protein kinase (MAPK), a multi-level signaling pathway evolutionary conserved from yeast to mammals, where the activated enzymes phosphorylate the kinase at the next level down the cascade (Chang & Karin, 2001). Protein substrates are often phosphorylated at multiple sites providing independent or synergistic scaffold for protein–protein interactions. This is illustrated in Figure 1 for Neph1, a structural protein from kidney cells that interacts with the adaptor protein Grb2 following the phosphorylation of cytoplasmic Tyr 637-638 by the Src tyrosine kinase, Fyn (Harita et al., 2008). Phosphorylated residues can also

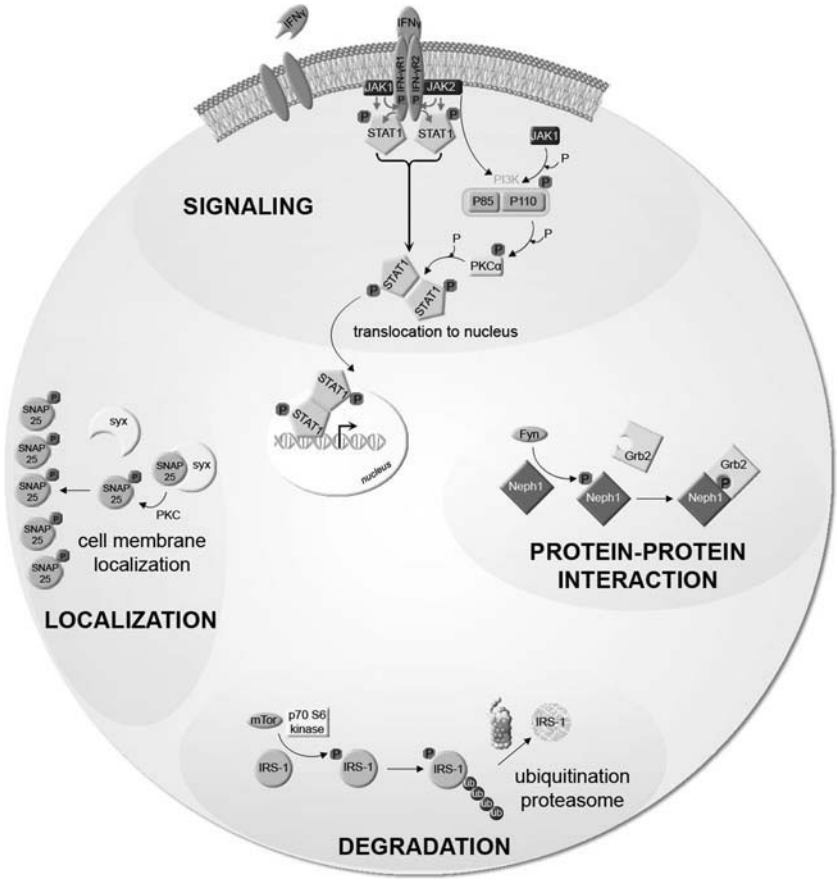

FIGURE 1. Examples for important biological functions regulated by protein phosphorylation. Signaling: Binding of interferon-γ (IFN-γ) to its receptor induces phosphorylation of Tyr440 of the receptor, which promotes the complex formation with the tyrosine-kinases Jak1 and Jak2. This complex phosphorylates Stat1, leading to its dimerization and nuclear translocation, where it regulates gene transcription (Darnell, Kerr, & Stark, 1994; Greenlund et al., 1994; Igarashi et al., 1994; Decker & Kovarik, 2000). Further signaling through MAP-kinases or phosphoinositol-3-kinase (PI3K) can also lead to phosphorylation of Stat1, modulating the primary stimulus. Localization: Phosphorylation of SNAP25 reduces its binding affinity to Syntaxin-1A and induces translocation to the membrane (Shimazaki et al., 1996; Kataoka et al., 2000). Protein–protein interaction: Phosphorylation of Neph1 by the Src-kinase Fyn promotes interaction with the adaptor protein Grb2 (Harita et al., 2008). Degradation: Phosphorylation of the insulin receptor substrate 1 (IRS-1), a critical mediator of insulin signaling, by Ribosomal protein S6 kinase beta-1 induces polyubiquitination of IRS-1 by the E3-ligase CUL7 and its subsequent proteasomal degradation (Xu et al., 2008). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

mediate positive or negative crosstalk with other modifications by favoring the addition/removal of a second PTM or by competing for a site that can also be occupied by another modification (Hunter, 2007). An example of the interplay between phosphorylation and ubiquitin-mediated proteasome degradation is shown in Figure 1 for the insulin receptor substrate 1 (IRS-1), a critical mediator of the insulin/insulin-like growth factor 1 signaling, that is targeted by the CUL7 E3 ligase following its phosphorylation through the activation of the mammalian target of rapamycin (mTOR) and the p70 S6 kinase (Xu et al., 2008).

A hallmark of many signaling pathways is the spatial and temporal regulation of both protein kinases and phosphatases to ensure an appropriate balance of protein phosphorylation (Bauman & Scott, 2002). Indeed, many cellular proteins are phosphorylated and dephosphorylated in separate cell compartments as exemplified for the translocation of the signal transducer and activator of transcription (STAT) following its phosphorylation by Jak tyrosine kinases (Fig. 1). Many cytokines including interferon-γ (IFN-γ) regulate gene expression through a three-protein signaling cascade where activated Jak kinases (Tyk2, Jak1-3) phosphorylate STATs leading to their homo or heterodimerization. The phosphorylated STAT dimers are rapidly translocated to the nucleus where they bind to a specific DNA element in the promoter of the target genes. Additional kinases such as MAPK or PI3K can also phosphorylate STATs on serine residues allowing additional cellular signaling pathways to modulate the primary STAT-activating stimulus (Decker & Kovarik, 2000). The spatial distribution of kinases and phosphatases implies that a gradient of phosphorylated substrates exists across different cellular locations, as suggested previously for the MAPK signaling pathway where both endocytosis and protein scaffolding might be involved in the propagation of intracellular signals (Kholodenko, 2002).

To profile the dynamic changes in protein phosphorylation and its significance in the regulation of different biological activities, current analytical methods must provide an appropriate level of sensitivity and spatio-temporal resolution. To this end, the combination of efficient affinity-enrichment methods with sensitive mass spectrometry instrumentation offers a unique tool for the molecular dissection of complex cell signaling pathways. Several large-scale mass spectrometry-based phosphoproteomics studies have already demonstrated the ability to monitor the temporal dynamics of protein phosphorylation on thousands of phosphorylated sites in response to cell stimulation. While impressive efforts have been deployed to document the extent and diversity of protein phosphorylation from total cell lysates of different species, a more modest number of studies are now providing a refined portrayal of their organelle distribution. Before reviewing the current status of subcellular phosphoproteomics it is appropriate to briefly outline the analytical capabilities of affinity methods and mass spectrometry approaches enabling the identification and profiling of protein phosphorylation. More detailed accounts of affinity methods can be found in excellent recent reviews documenting the use of immunoaffinity ligands, immobilized metal affinity chromatography (IMAC), covalent capture and metal oxide affinity chromatography (MOAC) (Reinders & Sickmann, 2005; Morandell et al., 2006; Schmelzle & White, 2006; D'Ambrosio et al., 2007; Dunn, Reid, & Bruening, 2009).

II. ANALYTICAL TOOLS FOR PHOSPHOPROTEOMICS

Subcellular phosphoproteomics analyses can extend far beyond a cataloguing of phosphorylation sites. Indeed, the further understanding of subcellular protein trafficking and cell signaling also requires the determination of phosphorylation stoichiometry and protein distribution across different experimental conditions and biological replicates. These analyses typically rely on a four-step approach combining cell fractionation, protein and peptide separation, phosphopeptide enrichment, and identification by mass spectrometry (Fig. 2). Each of these steps can be independently adjusted to suit the experimental paradigm under investigation. For example, phosphopeptides bearing a specific phosphorylation motif or a phosphotyrosine residue can be selectively enriched using immunoaffinity methods. Also, protein and peptide separation prior to phosphopeptide enrichment can be used to profile the abundance of putative phosphoproteins from different cell extracts to differentiate changes in phosphorylation from those associated to protein expression. For convenience, these steps will be briefly outlined in the following section.

A. Subcellular Fractionation

1. Practical Considerations

The first electron microscopy observation of subcellular organization in late 1940s led to the development of different cell fractionation techniques (De Duve, 1965; Castle, 2003, 2004). The most frequently used techniques are differential, rate-zonal, and isopycnic gradient centrifugation which separate particles based on their differences in density. For example, continuous density gradient centrifugation in combination with MS, also termed protein correlation profiling, was previously exploited to profile 1,404 proteins from ten subcellular locations of mouse liver cell lysates (Foster et al., 2006). Other techniques such as immunoaffinity capture (Mullock et al., 1987) or free-flow electrophoresis (Canut, Bauer, & Weber, 1999; Zischka et al., 2008) can also provide reliable organelle enrichment.

While several established purification protocols are presently available for most organelles (Castle, 2003, 2004) further optimization is often required to maximize recovery and reproducibility of subcellular protein extracts from different cell types (Dreger, 2003). Certain organelles might be fragilized under different experimental conditions thereby requiring milder isolation protocols. The monitoring of protein translocation can be problematic in situations where the parallel isolation of multiple subcellular structures is required from a single experiment. Careful consideration must also be placed on the selection of detergents and other chemicals required for subcellular fractionation to ensure compatibility with later steps of the proteomics analyses such as proteolytic digestion or mass spectrometry analysis. Protein purification protocols for phosphoproteome analyses also require that phosphatase inhibitors be used at all steps of the organelle purification. Typical phosphatase inhibitor cocktails contain a mixture of some of the inhibitors such as sodium ortho-vanadate (tyrosine phosphatases), imidazole (alkaline phosphatase), sodium tartrate (acid phosphatase), EDTA (alkaline phosphatase, protein phosphatase 2), okadaic acid, β-glycerophosphoric acid, or sodium pyrophosphate (Ser/Thr phosphatases). Sodium fluoride can also be added as a

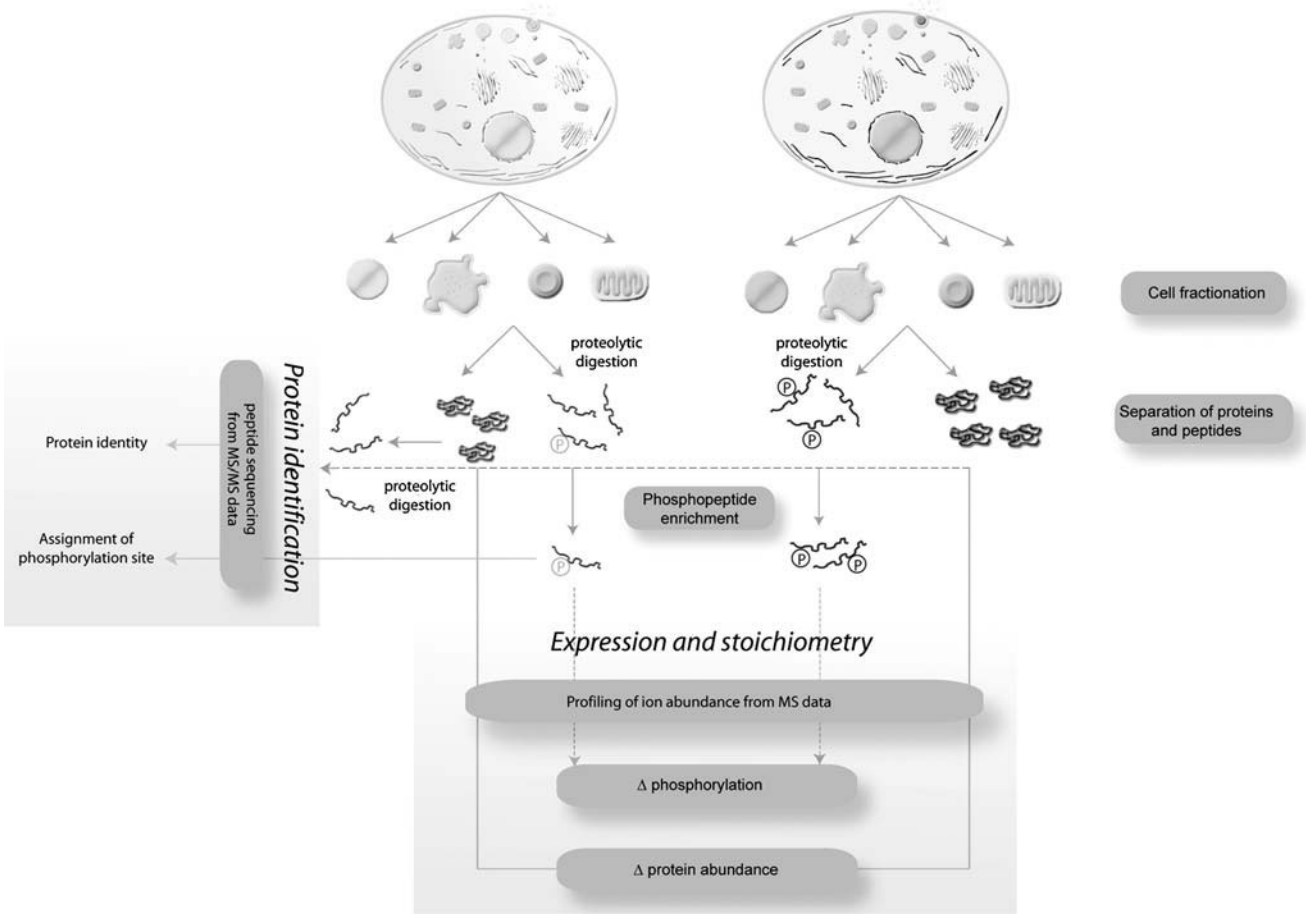

FIGURE 2. Workflow for a subcellular phosphoproteomics experiment. Organelles or subcellular fractions are generated from cells in two different conditions. After digestion of extracted proteins, phosphopeptides are enriched and identified in MS/MS experiments. Using a label-free or stable-isotope approach, the relative and absolute quantities of these peptides can be determined. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

broad-spectrum Ser/Thr-phosphatase inhibitor, but caution must be exercised when working with this toxic salt, especially in acidic solutions.

A significant challenge in subcellular phosphoproteomics relates to the availability of purified protein extract for successful phosphopeptide enrichment. Indeed, the low protein amounts isolated following subcellular fractionation are further compounded with the relatively small proportion of phosphopeptides (typically less than 0.2%) obtainable from the corresponding tryptic digests. The purification of hundreds of nanograms of enriched phosphopeptides for either one- or two-dimensional LC–MS analyses often require 0.5–1.5 mg of purified protein extract, a quantity that is difficult to obtain for many subcellular structures. Cell cultures and sample scale-up must consider the relative proportion of cell volume represented by the target organelle which can be relatively large for the nucleus (~10%) compared to peroxisome (~1%). For example, the recent large-scale phosphoproteomics study on the phagosome, a specialized endocytic vacuole in which pathogens can be killed and digested, required the harvesting of 5×10^{10} mouse macrophage cells (5 g of crude protein extracts) to isolate 1.5 mg of phagosomal proteins of which approximately 1 μg of phosphopeptides was obtained per biological replicate (Trost et al., 2009). Other large-scale studies necessitated purified protein amounts ranging from 8 mg of nuclear extracts from HeLa cell cultures (Beausoleil et al., 2004) to 90 mg of mouse liver tissue (Villen et al., 2007).

2. *Validation of Subcellular Proteomics Data*

The increased sensitivity and dynamic range of modern mass spectrometry instrumentation are placing higher constraints on the purity of isolated organelles to minimize the detection of previously elusive protein contaminants. Therefore, biochemical or immunocytochemistry characterization is normally performed on cell extracts to ensure organelle integrity and purity. Immunoblots with antibodies recognizing specific organelle protein markers can also be used to confirm the purity of the relevant subcellular extracts. However, the quality of the corresponding immunoblots depends on the specificity of the antibody and the dynamic range of detection which is usually less than an order of magnitude in concentration but can be further enhanced using chemiluminescent reagents and a CCD imager.

The dynamic nature of the phosphoproteome and the translocation events characterizing phosphoproteins can be a source of ambiguity when confirming protein location to a specific organelle. For example, changes in protein concentration

and composition or subcellular components can be affected by fusion events with vesicles or the transport of protein cargo through the secretory membrane system. Since purified proteins from subcellular fractionation can contain contaminants from unrelated structures or unknown gene products confirmation by independent methods is required. This can be achieved by confocal microscopy after cloning the target gene products and heterologously expressing the fusion proteins tagged with fluorescent probes (e.g., green fluorescent protein, GFP). The overexpression of fusion proteins must be carefully evaluated to minimize artifactual mislocation or degradation that could ensue from artificial expression levels.

Furthermore, protein phosphorylation can regulate the translocation of a subset of specific proteins to a target organelle while their unphosphorylated counterparts remain largely unaffected. This situation highlights a significant analytical challenge when conducting functional assignment as a relatively small proportion of the cognate protein might be translocated to other subcellular compartments following its phosphorylation. Unambiguous confirmation of protein translocation thus requires the availability of phosphospecific antibodies for immunoblot experiments and/or the production of gene constructs where the modified site has been mutated to a non-phosphorylatable residue (Ala) or a phosphomimetic amino acid (Asp or Glu).

In silico analyses and sequence homology searches can also be used to reveal potential protein function. A comprehensive list of protein subcellular localization prediction tools can be obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/CBBresearch/Lu/subcellular). Among these tools are MITOPROT for the prediction of mitochondrial targeting sequences (Claros & Vincens, 1996) (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), SignalP and TargetP that predict the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms (Emanuelsson et al., 2007) (http://www.cbs.dtu.dk/services/SignalP/; http://www.cbs.dtu.dk/services/TargetP/), TMHMM to predict transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and PredictNLS an automated tool for the analysis and determination of Nuclear Localization Signals (Cokol, Nair, & Rost, 2000) (http://www.rostlab.org/services/predictNLS/). Several tools including Gene Ontology (Ashburner et al., 2000) or the LOCATE database (Fink et al., 2006) are available via the Internet to infer functional assignment and potential subcellular localization.

B. Phosphopeptide Enrichment Methods

The profiling and identification of protein phosphorylation represents a significant analytical challenge in view of the relatively low representation of this modification within cell extracts. Although current estimates suggest that more than 100,000 different phosphorylation sites could be present in human cells, approximately 25,000 sites on 7,000 proteins have been reported thus far as reflected from recent large-scale phosphoproteomics investigations (Lemeer & Heck, 2009). These results underscore several issues also shared with proteomics studies such as the dynamic range of abundance, the uneven distribution across organelles, and the diverse physicochemical properties of phosphoproteins. On the other hand, the characterization of phosphorylation sites does require distinct approaches to enrich the relatively small proportion of peptide segments comprising this modification once the phosphoproteins are proteolytically cleaved. This difficulty is obviously compounded with the variable stoichiometry in the site occupancy characterizing the dynamic nature of protein phosphorylation. Furthermore, not all proteolytic fragments are created equal or identified with comparable ease. While trypsin is a commonly used enzyme for in solution or in gel digestion, the corresponding tryptic peptides may be either too small or hydrophilic to be retained on a C_{18} column or too large to elute or be sequenced efficiently. Therefore, the use of different proteases (e.g., Asp-N, Glu-C, etc.) can be useful to enhance sequence coverage.

To enhance the identification of protein phosphorylation, several affinity methods have been exploited in large-scale phosphoproteomics studies. These include immunoaffinity methods, IMAC with multivalent cations such as Fe^{3+} and Ga^{3+}, precipitation by Ca^{2+}, MOAC with TiO_2, ZrO_2, or Nb_2O_5, and reversible covalent binding via substitution of the phosphate group with a convenient alkylating agent for subsequent fractionation (Fig. 3). The sections below briefly describe current affinity methods for phosphopeptide enrichment.

1. Immunoaffinity Methods

Successful application of immunoaffinity methods for the enrichment of phosphoproteins and phosphopeptides relies on the availability of antibodies recognizing specific epitopes with high affinity. Antibodies can be immobilized on a solid support (e.g., agarose or polymer beads) or as a free form to enrich target proteins from complex cell extracts while eluting non-retained proteins (Fig. 3). A wide range of phosphospecific antibodies are commercially available, but only a few have been described for large-scale phosphoproteomics studies. Amongst these are antibodies against phosphotyrosine that were first introduced more than 25 years ago (Frackelton, Ross, & Eisen, 1983). Numerous large-scale phosphoproteome studies have reported with remarkable success their application for the profiling of phosphotyrosine peptides in cell cultures and in tissue extracts (Oda, Nagasu, & Chait, 2001; Blagoev et al., 2004; Rush et al., 2005; Zhang et al., 2006; Rikova et al., 2007; Guo et al., 2008). For example, quantitative phosphoproteomics analyses enabled the temporal profiling of phosphotyrosine-dependent signaling network in response to epithelial growth factor (EGF) (Blagoev et al., 2004). Rikova et al. (2007) reported the identification of 4,551 sites of tyrosine phosphorylation (2,700 different proteins) from a panel of 41 non-small cell lung cancer (NSCLC) cell lines and over 150 NSCLC tumors.

A more modest number of contributions have used antibodies to phosphoserine and phosphothreonine, possibly due to their lower immunogenicity to the modified residues. Gronborg et al. (2002) previously used a panel of phosphoserine and phosphothreonine antibodies to immunoprecipitate proteins from cells treated with the serine/threonine phosphatase inhibitor calyculin A, and identified several known serine/threonine-phosphorylated proteins including drebrin 1, α-actinin 4, and filamin-1 as well as unknown substrates such as poly(A)-binding protein 2 and Frigg. Phosphopeptide-specific antibodies that recognize a particular phosphorylated sequence motif can also be used to identify putative kinase targets or interacting proteins as previously reported for substrates of ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) (Matsuoka

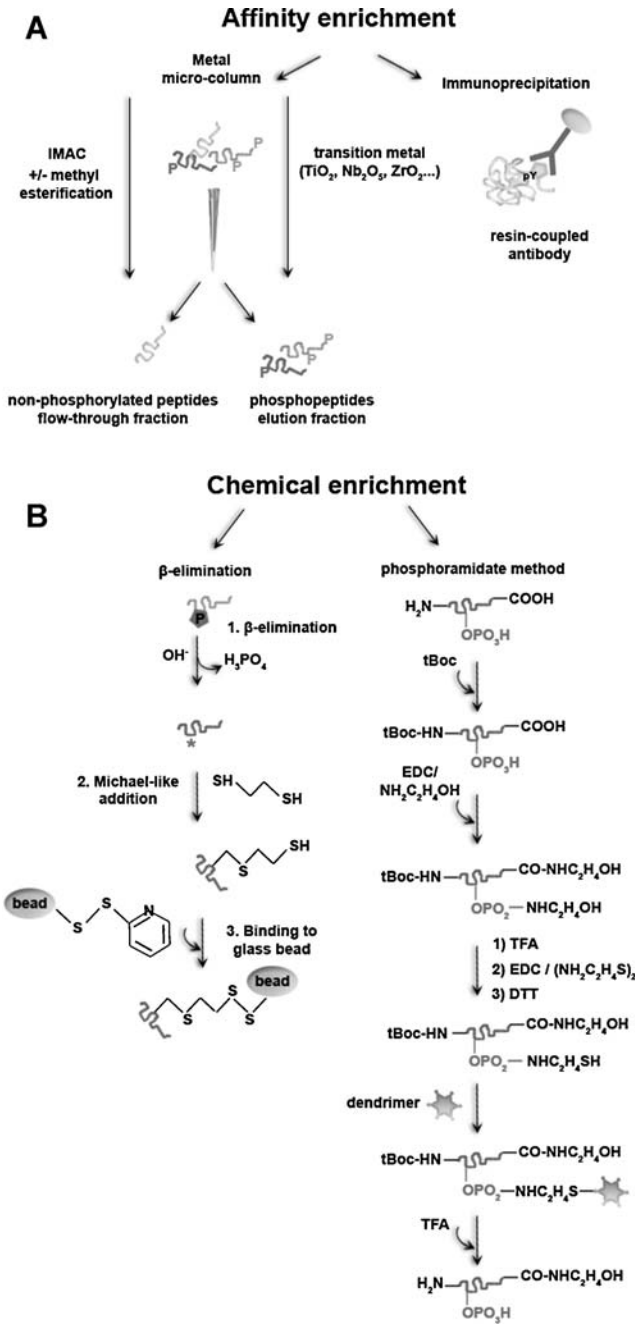

FIGURE 3. Examples for phosphopeptide enrichment methods. **A**: Peptides can be enriched by affinity methods such as IMAC, metal oxide affinity chromatography or by immunoprecipitation using phosphospecific antibodies. **B**: Due to the specific properties of the phosphate group, phosphopeptides can also be enriched by chemical enrichment. In the β-elimination approach, phosphorylated serine or threonine residues are transformed into dehydroalanine or dehydrobutyrine residues respectively. The resulting double-bond reacts easily with thiols in a Michael addition. The modified peptide can then be attached to beads with a second functional group (in this case also a thiol). Using phosphodiamide chemistry, the phosphorus of the phosphate group can undergo a nucleophilic attacked using an amine, provided that primary amines of the peptides are blocked. This phosphodiamide can be further modified and bound to a solid phase such as a dendrimer. Upon acid cleavage the peptides can be retrieved, still bearing the phosphate group at the original amino acid. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

et al., 2007) and 14-3-3 (RSXpSXP and RXY/FXpSXP motifs, where X is any amino acid) (Jin et al., 2004). However, the success of the latter approach depends on the low degeneracy of kinases recognizing a specific consensus sequence and the immunoprecipitation yields which can be relatively low.

2. Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC) is based on the selective binding of metal-ligand with amino acids or ionic functionalities such as phosphate groups to enrich the target molecules and remove impurities (Fig. 3). Selective enrichment of phosphopeptides is typically achieved using Fe(III) immobilized on nitrilotriacetate support although other coordinating metal ions such as Ga(III), Zr(IV), and Al(III) have also been used for this application (Posewitz & Tempst, 1999; Nuhse et al., 2003). To reduce the non-specific binding of peptides containing acidic residues such as aspartic and glutamic acid, the carboxylic groups can be converted to their corresponding methyl esters using methanolic HCl (Ficarro et al., 2002; Moser & White, 2006; Ndassa et al., 2006). While the use of SCX can enhance the selectivity of IMAC, methyl esterification of acidic residues appears to be necessary only when unfractionated samples are used (Villen & Gygi, 2008). Interestingly, IMAC was reported to yield a higher distribution of multiply phosphorylated peptides compared to other affinity media such as MOAC (Jensen and Larsen, 2007). The complementary distribution obtained from separate affinity media was advantageously exploited by Thingholm et al. (2008a) to sequentially separate monophosphorylated peptides and multiply phosphorylated peptides from highly complex biological samples. This approach termed SIMAC (sequential elution from IMAC) provided a two-fold improvement in identified phosphopeptides from lysates of human mesenchymal stem cells. Also, calcium phosphate precipitation combined with Fe(III) IMAC was shown to improve the selectivity of this enrichment method and enabled the identification of 242 phosphopeptides (125 phosphoproteins) from rice embryo (Zhang et al., 2007).

3. Metal Oxide Affinity Chromatography

The use of MOAC for the preconcentration of phosphorylated amino acids was first described by Ikeguchi and Nakamura (1997) using TiO_2. Pinkse et al. (2004) subsequently extended the use of TiO_2 to enrich phosphopeptides and identify eight phosphorylation sites in proteolytic digests of PKG, a cGMP-dependent protein kinase. TiO_2 exhibits high mechanical, chemical, and thermal stability and rapidly gained significant interest as an alternative affinity support for the enrichment of phosphopeptides due to its unique amphoteric ion-exchange properties (Fig. 3). Selective capture of phosphopeptides is performed in acidic solutions (typically 2% trifluoroacetic acid, 80% acetonitrile as loading and washing buffers) containing 100–300 mg/mL of 2,5-dihydroxybenzoic acid (2,5-DHB) in the loading buffer to protonate acidic residues and to minimize adsorption of non-phosphorylated peptides to TiO_2. Phosphopeptide elution is conducted using basic buffer containing 0.5% NH_4OH (pH: 10.5) (Thingholm et al., 2006). Competitive binding using 2,5-DHB, phtalic acid or lactic acid reduces the number of non-phosphorylated peptides enabling the selective capture of phosphopeptides with enrichment levels attaining up to 90% (Larsen et al., 2005; Thingholm et al., 2006;

Mazanek et al., 2007). As mentioned earlier, TiO_2 affinity media preferentially enrich singly and doubly phosphorylated peptides, a trend that mimics the distribution estimated from *in silico* analysis of Phospho.ELM database where these two types of phosphopeptides represent approximately 90% of the overall population (Ndassa et al., 2006). The last 4 years has seen numerous studies reporting the use of TiO_2 in large-scale phosphoproteomics to identify thousands of phosphopeptides from complex biological extracts. For example, Olsen et al. (2006) identified 6,600 phosphorylation sites on 2,244 proteins from HeLa cells and profiled their temporal changes following epidermal growth factor (EGF) stimulation. Recently, phosphopeptide enrichment using TiO_2 was combined with metabolic labeling to monitor more than 10,000 phosphorylation sites in *D. melanogaster* S2 cells following RNAi knockdown of phosphatase Ptp61F, an ortholog of mammalian PTB1B (Hilger et al., 2009). A recent contribution by Daub et al. described the use of multi-columns containing specific kinase inhibitor resins to enrich low cellular abundance kinases that are typically under represented in phosphoproteome studies. This study also enabled the identification of more than 1,000 phosphorylation sites on the corresponding kinases using TiO_2 enrichment of SCX fractionation (Daub et al., 2008). While TiO_2 represents by far the most frequently used MOAC medium, other resin such as $Al(OH)_3$ (Wolschin, Wienkoop, & Weckwerth, 2005; Wang et al., 2007), ZrO_2 (Kweon & Hakansson, 2006; Zhou et al., 2007), and Nb_2O_5 (Ficarro et al., 2008) were also reported. Interestingly, recent phosphoproteomics analyses using Nb_2O_5 indicated that this resin offers complementary selectivity with that of TiO_2 where approximately 30% of non-overlapping phosphopeptide identifications were found in both media.

4. Phosphopeptide Enrichment via Covalent Modification

Different reversible covalent binding approaches have been proposed to convert phosphorylated groups into functionalities amenable to affinity purification. These can be separated into two categories that use either β-elimination or phosphodiamide chemistry (Fig. 3). Phosphoester bonds of modified serine and threonine residues can be cleaved under alkaline conditions via β-elimination with NaOH or $Ba(OH)_2$ to form the corresponding dehydroalanine and dehydrobutyrine amino acids. The nucleophilic addition of an activated thiol compound to the unsaturated bonds (Michael's addition) enables the conversion of phosphate groups to stable thiol-based derivatives for subsequent enrichment steps. It is noteworthy that glycopeptides, sulfopeptides, alkylated cysteine residues and some unmodified serine residues can also undergo β-elimination under the same conditions (Herbert et al., 2003; Taylor, Holst, & Thomas-Oates, 2006; Bernardes et al., 2008). Amongst thiol compounds previously used for Michael's addition are ethanethiol (Jaffe, Veeranna, & Pant, 1998), 1,2-ethanedithiol (Adamczyk, Gebler, & Wu, 2001; Goshe et al., 2001; Oda, Nagasu, & Chait, 2001; McLachlin & Chait, 2003; Qian et al., 2003), 2-dimethylethanediol (Steen & Mann, 2002), 2-aminoethanethiol (Thompson et al., 2003b) and *N*-(2-mercaptoethyl)-6-methylnicotinamide (Tsumoto et al., 2008). The use of bisfunctional thiol substrates enables the coupling to solid-phase thiol–sepharose affinity resins or their conversion to biotinylated derivatives for subsequent purification on streptavidin columns (Oda, Nagasu, & Chait, 2001; McLachlin & Chait, 2003; Qian et al., 2003). An interesting application of the β-elimination and Michael's addition was described by Knight et al. (2003) and involved the substitution of phosphate groups with aminoethylcysteine moieties resulting in isosteric lysine analogs that can be cleaved by trypsin. This reaction affords an unusual *C*-terminus basic amino acid that conveniently identifies the location of the phosphorylated residue.

In contrast to the elimination of the phosphate moiety, a different method involves the activation of the phosphate group to form a reactive phosphoramidate that can be subsequently coupled to glass beads (Zhou, Watts, & Aebersold, 2001) or to a dendrimer (Tao et al., 2005). The former method shown in Figure 3 is accomplished by protecting the amino groups of tryptic peptides with *t*-butyl-dicarbonate (tBoc), and converting the carboxylate and phosphate groups to amides and phosphoramides in a reaction catalyzed by carbodiimide (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, EDC) and imidazole. Acid hydrolysis (10% TFA) is used to regenerate the phosphate group, and cystamine is linked to the phosphate group via an EDC-catalyzed condensation. The cystamine is then converted to its thiol derivative with dithiothreitol (DTT), and this group reacts with iodoacetyl groups immobilized on glass beads. After rinsing the beads with 2 M NaCl, methanol, and water to remove non-phosphorylated peptides, the phosphopeptides are cleaved from the surface and the tBoc protecting groups are removed with 100% TFA.

It is interesting to note that a recent report compared the phosphoramidate method with that of IMAC and TiO_2 affinity media for phosphoproteome analysis of *D. melanogaster* and found that each method reproducibly detected different and partially overlapping phosphopeptide population (Bodenmiller et al., 2007). These results suggest that every phosphopeptide isolation method has its own bias and that comprehensive phosphoproteome analysis must combine different enrichment approaches when sample supply is not limited.

5. Chromatographic Enrichment Methods

Several groups have been taking advantage of the negatively charged phosphate moiety of phosphopeptides to enrich them via ion interaction chromatography methods.

Although strong anion exchange (SAX) would be a natural choice for the enrichment of negatively charged phosphopeptides, relatively few reports have described its application. Recently, different groups described the enrichment of several hundred phosphopeptides using SAX columns (Dai et al., 2007; Nuhse et al., 2007; Han et al., 2008), supporting the relevance of this type of chromatography for phosphoproteomics analyses. In contrast, strong cation exchange (SCX) has been extensively used for the enrichment and fractionation of phosphopeptides. Off-line SCX fractionation was used to collect enriched pools of phosphopeptides in the first salt fractions, an advantage that led to the identification of a large numbers of phosphorylation sites (Beausoleil et al., 2004; Lim & Kassel, 2006; Dai et al., 2007). However, it should be noted that chromatographic fractionation alone yield lower enrichment of phosphopeptides compared to IMAC or TiO_2 methods. Several groups have therefore combined IMAC or TiO_2 enrichment with SCX fractionation to improve recovery yields of phosphopeptides (Gruhler et al., 2005; Villen et al., 2007).

Lately, hydrophilic interaction liquid chromatography (HILIC) previously used for the enrichment of glycoproteins

was shown to be a viable approach for phosphopeptide analysis (Lindner, Sarg, & Helliger, 1997; McNulty & Annan, 2008). HILIC was reported to be truly orthogonal to reversed-phase chromatography (Gilar et al., 2005) and phosphopeptides can be selectively retained due to their high hydrophilicity (McNulty & Annan, 2008). Since high concentration of organic solvent is used, the method is not directly compatible with on-line reverse phase LC. Also, the low enrichment level of HILIC alone requires prefractionation via IMAC or TiO_2 microcolumns (McNulty & Annan, 2008). Electrostatic repulsion hydrophilic interaction chromatography (ERLIC) has been recently described for the enrichment and fractionation of phosphopeptides in a single step using hydrophilic interaction and electrostatic repulsion (Alpert, 2008). At low pH (~2), anionic phosphopeptides can be selectively retained on a weak anion exchange (WAX) column while non-ionized analytes and peptides with protonated amine groups (including their *N*-termini) are electrostatically repulsed by the column. This approach was compared to SCX-IMAC for the analysis of phosphopeptides from human epithelial carcinoma cell line A431 (Gan et al., 2008). Fractionation of phosphopeptides using ERLIC and SCX/IMAC enabled the identification of 926 and 1,315 unique phosphopeptides, respectively. While ERLIC identified a higher number of di- and triphosphopeptides compared to the SCX/IMAC, the relatively low overlap of unique phosphopeptides (12%) between these methods suggested that both approaches provided different subsets of phosphopeptides.

C. Quantitative Phosphoproteomics and Phosphorylation Stoichiometry

1. Quantitation Techniques

The profiling of phosphopeptide abundance across sample sets can be performed with similar techniques to that used for their non-phosphorylated counterparts. Careful considerations must be placed in the experimental design to distinguish changes in phosphorylation from those associated to protein expression. Accordingly, the profiling of ions abundance can be performed separately on the original proteolytic digests and their corresponding enriched phosphopeptide extracts to determine changes in phosphorylation stoichiometry across conditions (Fig. 2). Obviously, this is required when studying cell signaling events over extended stimulation periods (>3 hr) or for extracts originating from separate cell cultures. Another important difference relates to the number of peptide ions defining abundance measurements. While protein expression is determined from different peptide ions of the same cognate protein, phosphorylation stoichiometry relies on a single phosphopeptide for each site under investigation. Appropriate statistical methods must be selected to evaluate errors on measurements from these separate data sets. The following paragraphs highlight different methods that use native peptides (label-free) or stable isotope incorporation for quantitative proteomics. The readers are referred to excellent reviews for more detailed description on these methods (Listgarten & Emili, 2005; Bantscheff et al., 2007; Mueller et al., 2008).

Quantitative proteomics using a label-free approach relies on peptide detection and alignment software enabling the comparison of peptide maps across conditions and sample replicates. Different software and approaches have been reported for the analysis of label-free peptide ions (Kearney & Thibault, 2003; Old et al., 2005; Ono et al., 2006; Bridges et al., 2007; Brusniak et al., 2008; Mueller et al., 2008). Peptide maps contain coordinates (*m*/*z*, charge, elution time, abundance) of all detected peptide ions that are clustered together to identify candidates displaying statistically meaningful changes in abundance across conditions. The correlation of peptide abundance can be performed on all peptide ions whether or not they have been successfully assigned to putative proteins. Unmatched peptide ions showing differential abundance can be targeted for subsequent MS/MS experiments using inclusion lists. Label-free quantitative proteomics is relatively inexpensive to implement and does not impart structural changes or side reactions that can be observed with labeling reagents. However, regular and rigorous instrument performance checks must be performed to ensure reproducible chromatography and mass spectrometry conditions.

A second quantitative proteomics approach involves the use of stable isotope labeling by amino acids in cell cultures (SILAC) (Ong, Kratchmarova, & Mann, 2003; Mann, 2006). Cells are grown in different conditions with ^{15}N as the only nitrogen source or with ^{15}N or ^{13}C labeled amino acids such as arginine or lysine. The use of labeled amino acids reduces dramatically the complexity compared to the ^{15}N-label. The peptides elute simultaneously from the column and appear in LC–MS as peptide pairs separated by a few Daltons. After labeled flies, worms, yeast, and bacteria (Krijgsveld et al., 2003), a whole stable isotope labeled mouse was recently presented, extending SILAC from cell cultures to animal models (Kruger et al., 2008). The inherently high cost of amino acids with stable isotopes is presently one of the disadvantages of SILAC for large-scale phosphoproteomics analyses. For example, the isolation of endosomes or phagosomes enriched extracts requires the culturing of approximately one thousand Petri dishes rendering the cost of SILAC prohibitive for phosphoproteomics studies. Also, the mixing of isotopically labeled peptides for combined LC–MS/MS experiments increases the sample complexity and places higher requirement on instrumentation and software enabling the correlation of MS/MS spectra with co-eluting peptide isotopomers. On the other hand, the use of SILAC can increase reproducibility of abundance measurements since samples are combined at an early stage of sample preparation. Also, the mass spacing of SILAC pairs can be related to specific lysine or arginine labeled amino acids to enhance confidence in protein identification from database searches.

The incorporation of stable isotopes into peptides can also be performed *in vitro* during the proteolytic digestion using either ^{16}O- or ^{18}O-water (Yao et al., 2001; Reynolds, Yao, & Fenselau, 2002). Each heavy peptide weighs 4 Da more than its light counterpart and both peptide pools are mixed and analyzed by LC–MS to determine isotope ratios of co-eluting peptide pairs. This low cost reaction can be performed with a wide range of enzymes (trypsin, chymotrypsin, Lys-C, Glu-C) and provides uniform labeling of peptides from different types of proteins including those post-translationally modified (Fenselau & Yao, 2009). Specific experimental conditions must be maintained to ensure complete $^{18}O_2$-labeling with minimal back exchange.

Another approach enabling the incorporation of ^{13}C or ^{15}N stable isotopes consists of modifying peptides with labeling reagents targeting specific amino acid functionalities. For example, isotope-coded affinity tag (ICAT) reagents target free

thiol groups of cysteine residues and comprise a cleavable biotin moiety for affinity purification (Hansen et al., 2003). Other reagents specifically label free amine groups via isotope-coded protein labeling (ICPL) (Schmidt, Kellermann, & Lottspeich, 2005), isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004; Zhang et al., 2005), tandem mass tags (TMT) (Thompson et al., 2003a) or the amine-reactive isotope tag (*N*-isotag) (Smolka et al., 2005). A recent publication reported the labeling of *N*-terminal amines only by blocking lysines to quantify *N*-terminal peptides (Zappacosta & Annan, 2004). In ICAT, the abundance of the peptide isotopomers are determined from the conventional mass spectrum whereas iTRAQ or TMT reagents permit simultaneous determination of both the identity and relative abundances of co-eluting peptide pairs using a collision-induced dissociation (CID)-based analysis method. However, similarly to metabolic stable isotope labeling, the linear dynamic range of relative quantification is lower than for label-free approaches (Bantscheff et al., 2007). However, chemical labeling can be used for quantitative proteomics of all biological samples including primary tissue and provides an inherent advantage over SILAC. In addition, the possibility of multiplexing sample analysis with up to eight conditions in the same run reduces considerably the instrument time.

2. *Determination of Phosphorylation Stoichiometry*

Quantitative phosphoproteomics combined with phosphopeptide enrichment approaches enable the large-scale profiling of protein phosphorylation to determine changes in phosphopeptides abundance across different sample sets. To provide further insights on the regulatory significance of protein phosphorylation, quantitative proteomics approaches must also determine the extent to which site-specific phosphorylation changes over time or across subcellular location. Changes in phosphorylation stoichiometry can be determined by monitoring the abundance of a phosphopeptide and its unmodified cognate to correlate the signal intensity of both peptide species. This is usually performed for singly phosphorylated species, as stoichiometry analyses of multiply phosphorylated peptides are particularly difficult. Recent development in capillary LC with inductively coupled plasma mass spectrometry enabled the quantitation of phosphoproteins and their degree of phosphorylation (Kruger et al., 2006). The relative abundance of phosphorylated isoforms from gel-separated proteins can be assessed by the relative abundance of phosphorus compared to that of sulfur used to normalized protein amount. The following section outlines other approaches (Fig. 4) based on electrospray mass spectrometry to determine changes in phosphorylation stoichiometry.

Changes in the degree of phosphorylation for specific sites can be determined in a targeted fashion using immunoaffinity enrichment (Fig. 4A). In this approach, proteins from stimulated and control cells are isolated by immunoprecipitation and then separated on SDS–PAGE prior to in-gel digestion. The abundance of non-modifiable tryptic peptides (unchanged peptides) can be use to normalize protein amounts across sample sets. Combined ion profiling and MS/MS peptide sequencing analyses are performed to correlate abundance changes of identified phosphopeptides and their non-phosphorylated counterparts across the different experimental paradigms (Fig. 4A).

This approach was previously used by Steen et al. (2005), who isolated the yeast transcription factor Pho4 after *in vitro* phosphorylation by the Pho80/85 kinase complex. Phosphorylation stoichiometry measurements were obtained by following the temporal changes of ion currents of the phosphopeptides and their unphosphorylated cognates and correcting for differences in their respective ionization efficiencies. Similarly, Carriere et al. (2008) used immunoaffinity enrichment and quantitative proteomics to profile changes in phosphorylation of Raptor in HEK293 cells stimulated with phorbol ester and in presence of kinase inhibitors UO126 and BI-D1870 kinase inhibitors. They determined that RSK specifically phosphorylates Raptor on Ser719 within an evolutionarily conserved region with no previously known function.

A second approach to profile changes in phosphorylation stoichiometry makes use of an unspecific phosphatase to compare ion abundance following the dephosphorylation of modified residues. In this approach, samples are splitted in two and one portion is incubated with alkaline phosphatase or λ-phosphatase. The degree of phosphorylation can be obtained by comparing the signal relative abundance of the phosphopeptides and their dephosphorylated counterparts in the two samples, assuming that the changes in ion intensities are solely due to phosphatase activity (Fig. 4B) (Zhang et al., 2002b; Hegeman et al., 2004). This simple approach requires a near-100% dephosphorylation activity of the enzyme, but avoids the problem of different ionization efficiencies of the phosphorylated and non-phosphorylated species (Marcantonio et al., 2008).

Phosphopeptide quantitation can also be achieved by absolute quantification (AQUA), a technique that uses peptide standards labeled with stable isotopes to determine the concentration of peptides in complex extracts (Barr et al., 1996; Gerber et al., 2003). For the determination of the degree of phosphorylation, both the phosphorylated and non-phosphorylated version of the peptide need to be synthesized and spiked into the sample at different concentrations. By comparing the areas of the ion chromatogram of the labeled and unlabeled peptide ions, a very precise absolute quantification can be obtained (Fig. 4C).

D. Phosphopeptide Identification by Mass Spectrometry

The identification of phosphorylation sites by tandem mass spectrometry has been extensively reviewed and the readers are referred to recent contributions for more details (Kalume, Molina, & Pandey, 2003; Reinders & Sickmann, 2005; Savitski & Falth, 2007; Smith & Figeys, 2008).

The detection of phosphopeptides can be achieved using either negative or positive ion modes. Several groups have successfully reported the use of negative ion ionization of phosphopeptides (Dass & Mahalakshmi, 1995; Annan et al., 2001) and their identification from complex cell extracts (Kocher, Allmaier, & Wilm, 2003; Old et al., 2009). However, the unpredictable nature of negative ion fragmentation and the limited sequence information obtainable from the dissociation of peptide anions make large-scale analyses very challenging. In view of these limitations, positive ion fragmentation is typically used for the sequencing and determination of modification sites in phosphopeptides. Nevertheless, negative ion mode can correctly identify the phosphorylation site in phosphopeptides comprising several modified serine/threonine residues (Edelson-Averbukh, Pipkorn, & Lehmann, 2007).

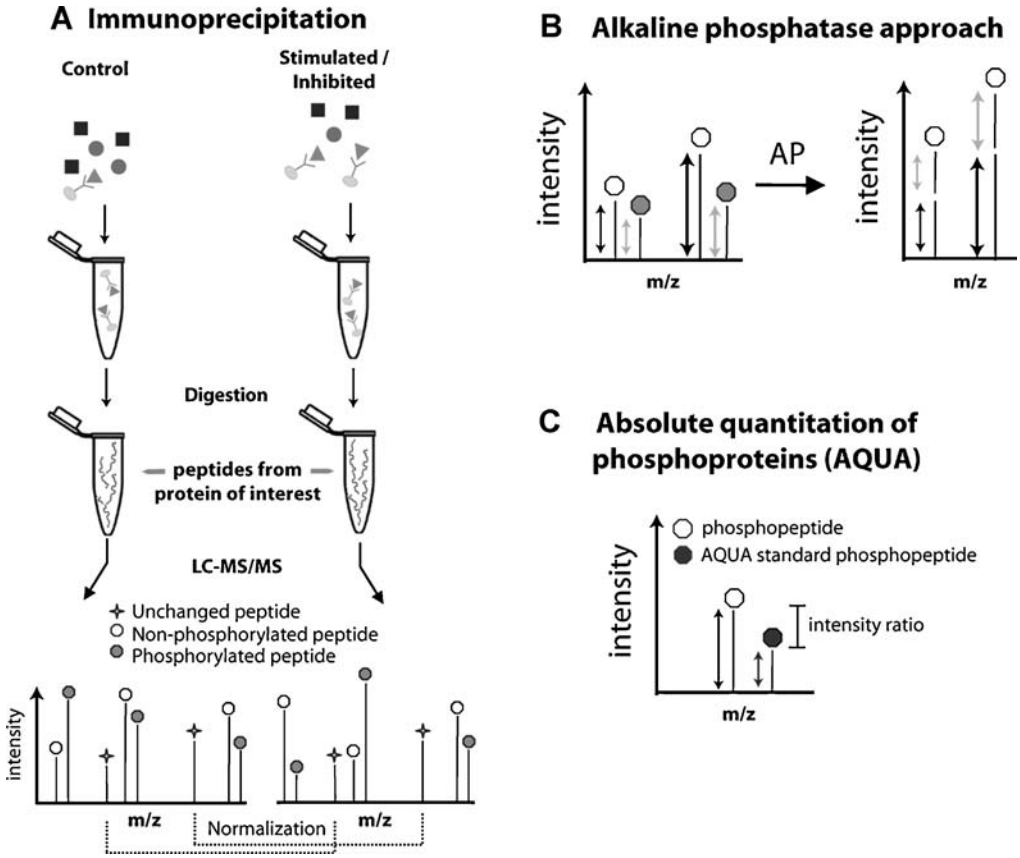

FIGURE 4. Proteomics approaches for the identification of phosphorylation stoichiometry. **A**: A protein from stimulated and control cells is isolated by immunoprecipitation to identify phosphopeptides and their corresponding dephosphorylated counterparts. The intensity of non-phosphorylated peptide ions is used to normalize ion intensity to account for variation of protein abundance across samples. **B**: In the phosphatase approach, samples are split in two and treatment with alkaline phosphatase is applied to only one sample to remove all phosphate groups. The comparison of peptide maps enables the correlation of intensities from phospho- and dephosphopeptides. **C**: The absolute quantification (AQUA) technique uses a known spike concentration of an isotopically labeled version of an expected phosphopeptide. For the determination of the degree of phosphorylation, both the phosphorylated and non-phosphorylated version of the peptide can be synthesized and spiked into the sample at different concentrations. By comparing the areas of the ion chromatogram of the peptide of interest and the AQUA peptide, a very precise absolute quantification can be obtained. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

1. Collision-Induced Dissociation (CID)

Collision-induced dissociation (CID) is the most commonly used fragmentation technique for peptide sequencing (Sleno & Volmer, 2004; Wells & McLuckey, 2005). Multiply protonated peptide ions are accelerated under low (<300 eV) or high (keV) kinetic energy regime undergoing collisional activation with neutral gas molecules such as helium, nitrogen or argon. A portion of the kinetic energy is converted into internal energy during the collision, resulting in the dissociation of the peptide ions into smaller fragments via intramolecular vibrational energy redistribution. In CID, the most common peptide fragments arise from the cleavage of the backbone amide bond, generating b-type ions and y-type ions (Steen & Mann, 2004; Medzihradszky, 2005).

Collision-induced dissociation (CID) fragmentation of phosphopeptides often produces MS/MS spectra dominated by the loss of H_3PO_4 (−97.9769 Da) (DeGnore & Qin, 1998), originating from a ''charge-directed'' mechanism and the formation of a cyclic intermediate (Palumbo, Tepe, & Reid, 2008). While this mechanism is prevalent in ion trap instruments, this problem is less commonly observed in MS/MS spectra from Q-TOF instruments that also benefit from a higher mass resolution to facilitate the assignment of phosphorylation site (Trinidad et al., 2006). The specific loss of H_3PO_4 can on the other hand be used in neutral loss experiments to identify all precursor and fragment ion pairs separated by 98 Da (Hunter & Games, 1994). However, this scanning mode is subject to errors arising from false-positive identification due to conflicting fragments showing similar nominal mass losses (Lehmann et al., 2007). This is further complicated by the occurrence of gas phase rearrangement of phosphoester bonds with other hydroxyl-containing amino acids rendering the identification of phosphorylation sites more difficult (Palumbo & Reid, 2008). The loss of HPO_3 (−79.9663 Da) from *O*-phosphorylated residues including tyrosine has also been reported although its occurrence is less frequent than the loss of H_3PO_4 (Annan & Carr, 1996; Annan et al., 2001; Salek et al., 2003). It is noteworthy that

the loss of 80 Da might also be attributed to the loss of SO_3 (−79.9568 Da) from sulfated amino acids whether they are present as a genuine biological modification (Monigatti, Hekking, & Steen, 2006) or introduced as an artifact through silver-staining protocols (Gharib et al., 2009). The availability of mass spectrometers providing high accuracy mass measurements (<5 ppm) combined with sensitive multi-stage fragment ion spectra is critical to obtain unambiguous assignment of phosphorylation sites.

Several reports described alternate approaches to enhance the identification rates and fragmentation efficiencies of phosphopeptides. First, MS^3 or multi-stage activation can be performed to sequence phosphopeptides showing a prominent loss of 98 Da (Olsen & Mann, 2004; Ulintz et al., 2008). The overall gain in identification appears to be counterbalanced by the increased duty cycle required to acquire the additional MS/MS spectra (Bakalarski et al., 2007; Villen, Beausoleil, & Gygi, 2008). Also, the lower fragmentation yield and ionization efficiency typically observed for phosphopeptides can be circumvented using alkaline phosphatase although the exact location of the phosphorylation site can be lost as part of the enzymatic digestion (Ishihama et al., 2007; Marcantonio et al., 2008). A major cause for unfavorable fragmentation of phosphopeptides is insufficient proton ''mobility'' due to charge localization at the guanidino functionality of arginine. The conversion of the guanidino group to a malondialdehyde can reduce the basicity of arginine residues leading to increase peptide fragmentation and reduction of neutral loss of phosphoric acid from phosphopeptides (Leitner, Foettinger, & Lindner, 2007).

2. Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD)

Selective fragmentation of peptide ions leading to complementary c-type and z-type fragment ions can be achieved using ECD where precursor ions are exposed to near-thermal energy electron (~6 eV) leading to an exothermic reaction that causes cleavage of the peptide backbone in a non-ergodic process (i.e., no intramolecular vibrational energy redistribution) (Zubarev, Kelleher, & McLafferty, 1998; Creese & Cooper, 2008). Fragmentation of peptide ions is specific to c- and z-type fragment ions and preserves the actual moiety attached to the amino acid thereby facilitating the identification of the modification site. ECD is mainly used in Penning traps of Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers and can be implemented on a millisecond time scale to yield product ion conversion efficiencies approaching 30% (Haselmann et al., 2001; Tsybin et al., 2001).

More recently, conditions leading to the introduction of thermal electrons into RF fields of quadrupole ion trap were successfully achieved using electron transfer dissociation (ETD) with anthracene and fluoranthene anions (Syka et al., 2004). In ETD, reactive anions generated in a chemical ionization source transfer an electron to multiply protonated peptides and induce peptide backbone cleavages similar to those observed in ECD. ETD is generally well suited to the sequencing of large multiply charged peptide cations, and many groups have used the endopeptidase Lys-C (Chi et al., 2007), Lys-N (Taouatas et al., 2008) or incomplete tryptic digestion (Swaney, McAlister, & Coon, 2008) to generate peptides of suitable size for ETD fragmentation. The use of Lys-C or Lys-N has been favored for quantitative proteomics to avoid difficulties associated with incomplete proteolytic digestion. Increased fragmentation of small doubly charged peptides under ETD has been facilitated using supplemental low energy collisional activation of $[M + 2H]^{2+*}$ species (ETcaD) (Swaney et al., 2007).

Electron transfer dissociation (ETD) has been shown to yield complementary results to that of CID, and a recent report described the combination of both fragmentation modes in a data-dependent decision tree fashion to enhance the identification rates in proteomics experiments (Swaney, McAlister, & Coon, 2008). The ability to fragment peptide ions while simultaneously preserving the labile phosphoester bonds provides ETD with an inherent advantage in large-scale phosphoproteome analyses (Chi et al., 2007; Molina et al., 2007; Swaney, McAlister, & Coon, 2008).

III. BIOINFORMATIC TOOLS FOR PHOSPHOPROTEOMICS

A. Identification of Phosphorylation Sites

The precise identification of phosphorylated residues within peptides containing multiple serine and threonine residues can be challenging in view of the prompt loss of H_3PO_4 and the low abundance of dehydroalanine or dehydrobutyrine fragment ions in CID. The situation is less ambiguous for phosphotyrosine residues due to the relative stability of the corresponding phosphoester bond (DeGnore & Qin, 1998). Also, MS/MS spectra of phosphopeptides obtained using ETD can facilitate the localization of the modification site for highly charged precursor ions as the non-ergodic nature of the fragmentation typically yield c' and z-type fragment ions without loss of the phosphate group. While large-scale phosphoproteomics can combine both CID and ETD MS/MS spectra, search engines must also consider the ambiguity in the localization of modified sites and attribute confidence scores or ranking for the different assignments. A number of different approaches have been reported to calculate confidence scores for phosphopeptides identification. For example, the A-Score, identifies specific fragments ions as ''site-determining ions'' and calculates a probability score based on the likelihood of identifying site-determining ions compared to random chance (Beausoleil et al., 2006). This approach was further extended by the open-source PhosphoScore algorithm which also considers MS^n data (Ruttenberg et al., 2008). Both approaches are currently limited to data output from Sequest search engine. For MS/MS spectra processed using Mascot, the MSQuant software enables the calculation of a confidence score for phosphosites based on a previous MS^3 scoring algorithm (Olsen & Mann, 2004) that correlates the number of matches between the observed and theoretical b- and y-fragment ions for all possible combination of phosphorylation sites in a peptide sequence (Olsen et al., 2006) (http://msquant.sourceforge.net/).

Other approaches were developed to identify phosphopeptides from MS/MS spectra that remained unassigned by Sequest and other database search engines. A scoring function modeled from validated phosphopeptides spectra was developed as part of the software Inspect and enhanced the number of identifications by up to 43% compared to Sequest or X!Tandem search engines (Payne et al., 2008). A phosphosite scoring scheme was elaborated in the software Phosm to provide in-depth analysis

of small data sets represented by a single or a few phosphoproteins (Schlosser, Vanselow, & Kramer, 2007). It is noteworthy that a number of algorithms rely on the observation of a neutral loss of 98 Da ($-H_3PO_4$) for confident assignment of phosphorylation. This feature could result in false-positive identification for situations where abundant conflicting fragment ions are detected ''close-to-98/z'' neutral loss fragmentations (Lehmann et al., 2007). For more detailed information on software and databases for phosphoproteome analyses the reader is referred to a recent review (Hjerrild & Gammeltoft, 2006).

B. *In Silico* Characterization of Phosphoproteins

The identification of phosphorylation sites from large-scale phosphoproteomics studies becomes an obvious starting point for subsequent experiments designed to understand the biological and functional significance of selected phosphoprotein substrates. However, these subsequent analyses require reliable assignment of phosphorylation sites which in some cases can be problematic given the uncertainty associated with their precise location as indicated in the previous section. In this context, different bioinformatics tools are available to provide further insights on the conservation of phosphorylation sites, kinase consensus motifs, interaction domains, Gene Ontology annotation and protein–protein interaction networks (Fig. 5).

Motif-x is a software tool enabling the extraction of over-represented patterns from any sequence data set including large-scale phosphoproteome data sets (Schwartz & Gygi, 2005) (http://motif-x.med.harvard.edu/). This software is similar to Web logo (Crooks et al., 2004) and provides a graphical representation of amino acid sequence alignment where the overall height of the stack indicates the sequence conservation, while the height of individual residues shows the relative frequency of each amino or nucleic acid at that position. Motif-x can be used not only to describe the motif composition of large-scale phosphoproteome data sets, but also to probe the overall changes in phosphorylation motifs associated to known or unknown kinases in response to cellular stimuli.

Linear phosphorylation motifs can be relatively short within an unstructured region of the protein substrate, but can never-

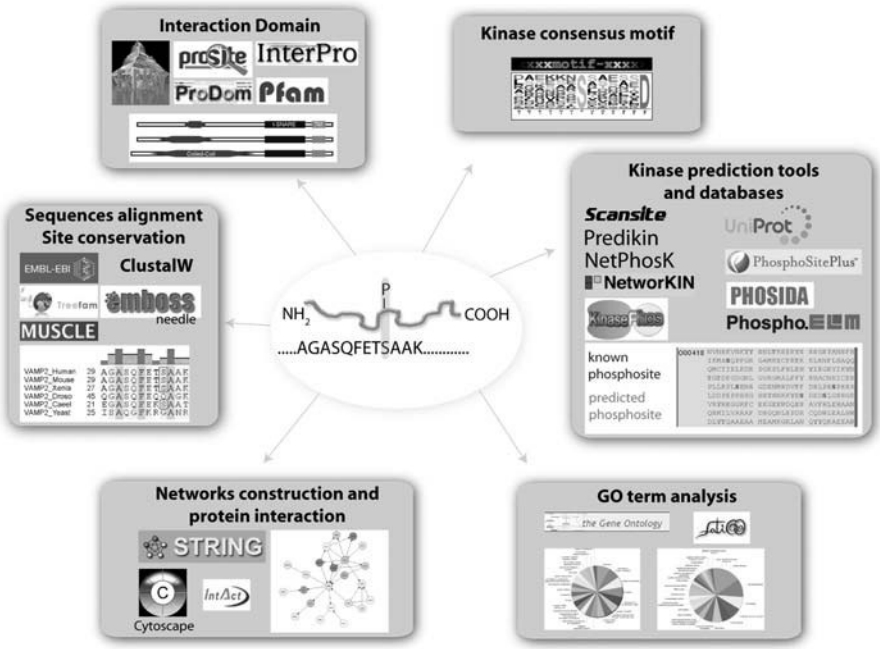

FIGURE 5. Bioinformatic tools for phosphoproteomics. Several tools have shown to provide considerable help in analyzing large-scale phosphoproteome data. Among these, kinase prediction tools such as Scansite (http://scansite.mit.edu/), Predikin (http://predikin.biosci.uq.edu.au/), NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/), NetworKin (http://networkin.info), and KinasePhos (http://kinasephos.mbc.nctu.edu.tw/). Phosphorylation databases including UniProt (http://www.uniprot.org/), Phosida (http://www.phosida.com/), PhosphoELM (http://phospho.elm.eu.org/), and Phosphosite Plus (http://www.phosphosite.org/). Furthermore, functional analyses can be performed by Gene Ontology (GO) (http://www.geneontology.org/) and FatiGO (http://babelomics.bioinfo.cipf.es/). Tools for network and protein–protein interaction analysis include the Intact Database (http://www.ebi.ac.uk/intact/site/index.jsf), the String database (http://string.embl.de/) and the visualization software Cytoscape (http://www.cytoscape.org/). Sequence alignment can be performed using EBI's ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), Emboss (http://www.ebi.ac.uk/Tools/emboss/) or Muscle (http://www.drive5.com/muscle/). The database Treefam contains phylogeny of many proteins in the Ensembl database (http://www.treefam.org/). Protein domain analysis can be performed using Prosite (http://ca.expasy.org/prosite/), PFAM (http://pfam.sanger.ac.uk/), InterPro (http://www.ebi.ac.uk/interpro/), or ProDom (http://prodom.prabi.fr/). Sequence motifs of phosphorylated peptides can be generated by Motif-X (http://motif-x.med.harvard.edu/), a software of the group of Steven Gygi, based on weblogo (http://weblogo.berkeley.edu/). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

theless be recognized by specific kinases. Numerous methods are available for the prediction of kinase specificity based on known consensus motifs or advanced machine-learning algorithms. Amongst those using consensus motifs from data sets of mined literature are phospho.ELM (Puntervoll et al., 2003) (http://phospho.elm.eu.org/), Prosite (Sigrist et al., 2002) (http://ca.expasy.org/prosite/), and Phosphomotif finder (Amanchy et al., 2007) (http://www.hprd.org/PhosphoMotif_finder) as well as NetworKin, a database that integrates consensus substrate motifs with an improved prediction of cellular kinase–substrate relations (Linding et al., 2008) (http://networkin.info). Other methods using position-specific scoring matrices (Predikin (Saunders et al., 2008) (http://predikin.biosci.uq.edu.au/)) and Scansite (Obenauer, Cantley, & Yaffe, 2003) (http://scansite.mit.edu/)) hidden markov model (KinasePhos (Huang et al., 2005) (http://kinasephos.mbc.nctu.edu.tw/)) or artificial neural network (NetphosK (Blom et al., 2004) (http://www.cbs.dtu.dk/services/NetPhosK/)) have also been reported. A recent review by Miller and Blom (2009) provides a more comprehensive list of kinase-specific prediction methods.

Phosphorylation sites are often conserved within protein families, pointing to specific regulatory mechanisms common across related species. The conservation of specific sites can be used to prioritize subsequent mutagenesis experiments aimed at understanding their functional significance. Alignment of sequences can be obtained in a pairwise fashion using Emboss (http://www.ebi.ac.uk/Tools/emboss/align/index.html), or with MUSCLE (Edgar, 2004) (http://www.drive5.com/muscle/) or ClustalW (Larkin et al., 2007) (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to create multiple protein sequence alignments. Comparison of genes across species can be achieved using Treefam, a curated resource of animal gene families (>170,000 genes from nine fully sequenced animal genomes and other UniProt proteins) with ortholog and paralog assignments (Li et al., 2006) (http://www.treefam.org/). Other tools such as ProDom (http://prodom.prabi.fr/prodom/current/html/home.php), Pfam (http://pfam.sanger.ac.uk/), or Interpro (http://www.ebi.ac.uk/interpro/) provide additional information relating to interaction domains comprising the phosphorylation site.

Network analyses are also used to visualize static phosphoprotein interactions or dynamic changes resulting from different environmental stimuli. This can be achieved using BLAST against protein sequences from STRING (http://string.embl.de/) (von Mering et al., 2007). Protein interaction data can also be analyzed using the open source database system IntAct (http://www.ebi.ac.uk/intact/site/index.jsf). These interactions can be visualized using Cytoscape (http://www.cytoscape.org/) (Shannon et al., 2003) to obtain an overall constellation of nodes and edges. For convenience in data reporting the identified phosphoproteins can be regrouped under Gene Ontology terms (http://www.geneontology.org/) representing cellular component, molecular functions, or biological processes.

IV. THE DYNAMIC REPERTOIRE OF ORGANELLE PHOSPHOPROTEOME

Phosphoproteomics has moved far beyond a simple catalogue of phosphorylation sites and is contributing to important cell biology discoveries by unveiling the dynamic changes in protein phosphorylation regulating numerous cellular functions. The combination of cell fractionation techniques enabling the purification of organelles is now expanding the contribution of phosphoproteomics to the further understanding of intricate molecular machines not yet accessible by conventional approaches. Phosphoproteomics of subcellular structures still represents a major analytical challenge as only a few studies were reported over the past 5 years although efficient phosphopeptide enrichment methods were available since. This is in stark contrast to the large number of subcellular proteomics studies that have been reported over the same time period (reviewed in Brunet et al., 2003; Dreger, 2003; Yates et al., 2005). While a description of organelle proteomics is beyond the scope of the present contribution, selected examples of subcellular phosphoproteomics were chosen to illustrate how this emerging field already uncovered important biological paradigms (Fig. 6).

A. Nucleus

The nucleus is a membrane-enclosed organelle found in all eukaryotes, containing most the cell's genetic material. It is divided from the cytoplasm by the nuclear envelope and the nuclear lamina and trafficking can only occur through the well-studied nuclear pores. The nucleus itself has several substructures such as the Nucleolus, Cajal bodies, PML bodies or the splicing speckles to name a few. Several of these nuclear substructures have been characterized by large-scale proteomics experiments (Neubauer et al., 1998; Rout et al., 2000; Andersen et al., 2002; Cronshaw et al., 2002; Rappsilber et al., 2002; Schirmer et al., 2003; Andersen et al., 2005).

In one of the first large-scale phosphoproteome analyses, Beausoleil et al. (2004) reported 2,002 phosphorylation sites on 967 proteins from purified HeLa nuclear extracts. This was achieved following the separation of nuclear proteins (8 mg) into 10 bands using preparative gel electrophoresis with subsequent fractionation by off-line SCX chromatography. Although no affinity chromatography step was used, fractions of low salt concentration enhanced the proportion of phosphopeptides for subsequent LC–MS/MS analyses on an LCQ mass spectrometer. Sequest searches identified Ser and Thr phosphopeptides from this large collection of tandem mass spectra (525,000 MS^2 and 12,000 MS^3 spectra acquired). The analysis of amino acid sequences flanking the sites of phosphorylation revealed that Pro-directed and acidophilic sites accounted for 77% of all detected phosphorylation motifs. Scansite (Obenauer, Cantley, & Yaffe, 2003) also matched several phosphorylation sites to potential kinases including Pro-directed kinases (e.g., glycogen synthase kinase 3 and CDC2), acidophilic kinases (e.g., casein kinase 2), and basophilic kinases (AKT, PKA, and Clk2).

Nuclear phosphoproteome analyses have also been reported for *Arabidopsis thaliana*, a popular model organism for understanding plant traits including flower development and light sensing. An early phosphoproteomics study by de la Fuente van Bentem et al. (2006) revealed *in vivo* phosphorylation of Arabidopsis proteins involved in RNA metabolism. By using IMAC, the authors identified a total of 79 unique phosphorylation sites in 22 phosphoproteins including 12 Ser/Arg-rich (SR) splicing factors. The prevalence of phosphorylation in the SR domains of splicing factors led to the proposal that the mRNA splicing machinery and other proteins involved in RNA metabolism might be targeted by SR protein-specific kinases (SRPK). This proposal was further validated by *in vitro* kinase assay with SRPK4 using the protein RSp31 as a substrate. Several

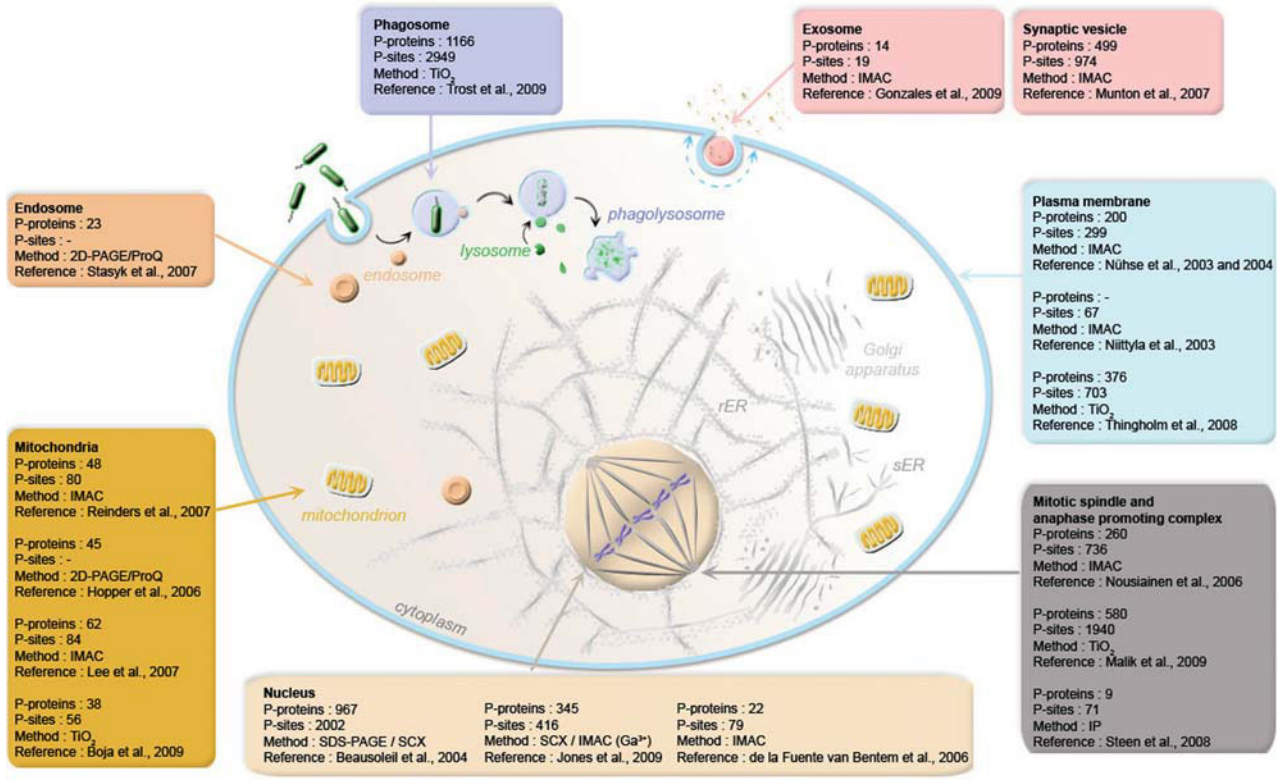

FIGURE 6. Examples of subcellular phosphoproteomics analyses. Phosphoproteomics experiments were performed on following subcellular structures: plasma membrane, mitotic spindle, nucleus, mitochondria, endosomes, phagosomes, and exosomes. See text for more details.

plant proteins containing SR domains display distinct splice variants often differing in their *C*-terminal SR domains. Alternative splice forms lacking one or more phosphorylation sites may be impaired in binding to other components of the splicing machinery. A recent study using IMAC (Ga^{3+}) enriched nuclei fractions from *A. thaliana* identified 416 phosphopeptides from 345 phosphoproteins including proteins in nuclear transport (e.g., Ran-associated proteins), transcriptions factors, chromatin remodeling proteins, and spliceosome components (Jones et al., 2009). Interestingly, the unexpected identification of Golgi proteins involved in vesicle trafficking suggested their possible participation in cell plate formation during cytokinesis.

B. Mitotic Spindle and Anaphase Promoting Complex

During cell cycle, chromosomes have to be replicated and separated, a process highly regulated by phosphorylation (Daub et al., 2008; Dephoure et al., 2008). The mitotic spindle is a part of the microtubule network that segregates chromosomes to the opposite poles of the cell during transition from interphase to mitosis (Karsenti & Vernos, 2001). Failure of correct spindle assembly might lead to aneuploidy and may be involved in cancer and aging (Baker, Chen, & van Deursen, 2005). Due to the importance of the mitotic spindle, most of its proteins are tightly regulated by phosphorylation and many of them in a cell-cycle-dependent manner (Cassimeris & Spittle, 2001).

The isolation of mitotic spindles can be achieved using protocols based on the stabilization of microtubules by taxol that offer suitable protein amounts for mass spectrometric analysis (Sillje & Nigg, 2006). Using this isolation method, Nousiainen et al. extended an earlier proteomics study on mitotic spindle proteins (Sauer et al., 2005) to identify phosphorylation sites on proteins from this subcellular structure (Nousiainen et al., 2006). The purification of mitotic spindle proteins from HeLa cells with subsequent separation by SDS–PAGE and IMAC enrichment (Fe^{3+}) enabled the mass spectrometry identification of 736 phosphorylation sites in 260 proteins of which more than 300 sites were found in known spindle proteins. The most extensively phosphorylated protein was INCENP with 33 phosphorylation sites, of which 3 (Thr892, Ser893, and Ser894), are located in the *C*-terminal IN-box, a region previously shown to be important for Aurora-B kinase activation (Honda, Korner, & Nigg, 2003). The analysis of consensus recognition sites revealed that Cdk1 (or other Pro-directed kinases such as the MAPK family) and Polo-Like Kinase1, Plk1 (D/E-X-pS/pT) were the most frequently observed phosphorylation sites. A large proportion (e.g., 46%) of the sites identified was also evolutionary conserved in at least three species suggesting that many of the detected phosphorylation sites may be functionally relevant during mitosis.

Recently, the same group identified 1,940 unique phosphorylation sites on isolated mitotic spindles and quantified differences during mitotic stages (Malik et al., 2009). Their results showed that during late mitosis the spindle phosphoproteome is drastically altered and that phosphothreonines are preferably dephosphorylated during this stage.

Another important subcellular structure important for mitosis is the anaphase promoting complex (APC). The APC controls the degradation of proteins during exit from mitosis and entry into S-phase and its activity is regulated by phosphorylation. Steen et al. (2008) characterized changes in phosphorylation of the APC upon arrest of the cell cycle by different drugs. They identified 71 phosphorylation sites on nine subunits of the APC and showed differences in the relative stoichiometries of the phosphorylation, indicating that mitotic arrest induced by these drugs was not a static condition.

C. Mitochondria

Mitochondria consist of inner and outer membrane-bound compartments having distinct protein composition and functions. Integral proteins from the outer membrane control the access of low molecular weight metabolites necessary for ATP synthesis while the more permeable inner membrane comprises proteins required for diverse functions including oxidative phosphorylation, ATP production, import machinery and metabolite transport within the mitochondria matrix. Mitochondria play a central role in many cellular functions, including bioenergetics, apoptosis, and the metabolism of amino acids, lipids, and iron (Newmeyer & Ferguson-Miller, 2003). Many diseases have been attributed to mitochondrial defects (Schon, 2000). Hence, mitochondria of several organisms, including yeast (Sickmann et al., 2003; Prokisch et al., 2004), mouse (Mootha et al., 2003; Pagliarini et al., 2008), rat (Forner et al., 2006), and pig (Boja et al., 2009), have been well studied by large-scale proteomics analyses. Due to their high density, mitochondria can be isolated in high purity by density ultracentrifugation.

Several recent reports have described the phosphoproteome analyses of mitochondria. However, the number of phosphorylation sites (~200 sites) identified to date on more than 1,000 proteins characterizing this organelle remains relatively low in spite of the preliminary estimates obtained from 2D-gel and ^{32}P-labeling experiments (Bykova, Egsgaard, & Moller, 2003; Hopper et al., 2006). The use of ProQ phosphospecific stain (Steinberg et al., 2003) in 2D gel electrophoresis experiments, enabled the identification of 45 phosphorylated proteins in porcine heart mitochondria including members from mitochondrial respiratory chain complexes and enzymes involved in intermediary metabolism, such as pyruvate dehydrogenase (PDH), citrate synthase, and acyl-CoA dehydrogenases (Hopper et al., 2006). Exposure to extramitochondrial Ca^{2+} was used to induce cytochrome *c* release and simulate the initial stages of mitochondria-induced apoptosis. Ca^{2+}-stimulation led to dynamic changes of phosphorylation in mitochondrial matrix proteins, most notably the dephosphorylation of PDH, manganese superoxide dismutase and the F_0F_1-ATPase.

Reinders et al. (2007) used a combination of analytical approaches ranging from SDS–PAGE followed by IMAC (Fe^{3+}, Ga^{3+}, Zr^{4+}) to SCX enrichment of phosphopeptides and two steps IMAC to enhance the coverage of the yeast mitochondrial phosphoproteome. This study enabled the identification of 80 unique phosphorylation sites on 48 different proteins involved in mitochondrial energy metabolism, transport and redox processes, protein folding, and genome maintenance. Interestingly, seven subunits of the F_0F_1-ATPase were found to be phosphorylated, and site mutagenesis together with native gel assay showed that phosphorylation of S62 from Atp20 (subunit g) was found to impair ATP synthase dimerization, a critical step for the functionality of ATP synthase (Bornhovd et al., 2006). Accordingly, the phosphorylation of Atp20 could regulate the bioenergetic state of mitochondria as shown in Figure 7A.

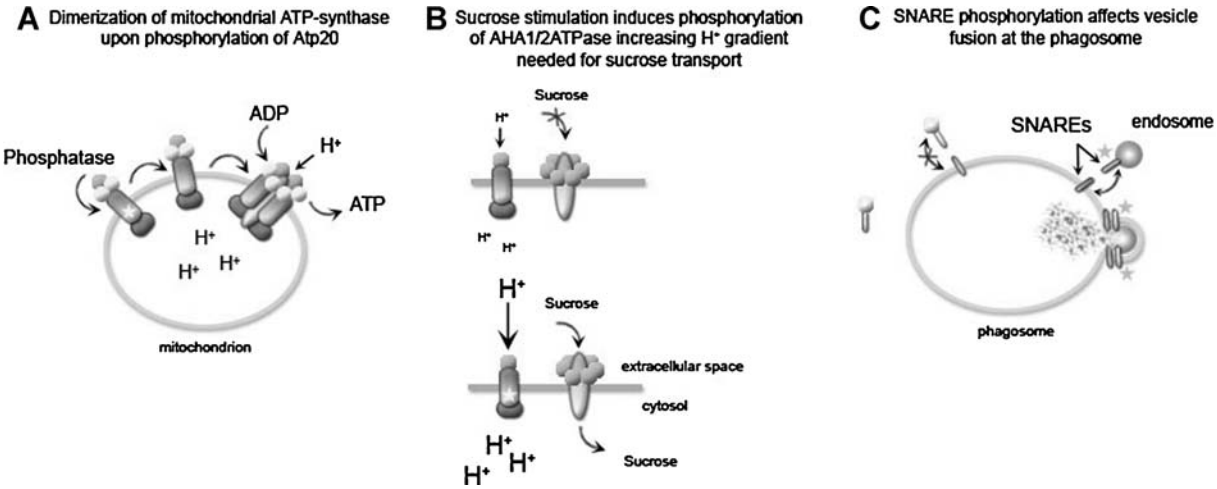

FIGURE 7. Biological functions regulated by phosphorylation identified in subcellular phosphoproteomics experiments. **A**: Phosphorylation of residue Ser62 of the Atp20 subunit of the mitochondrial ATP-synthase abolishes dimerization of the protein required for its functionality (Reinders et al., 2007). **B**: Stimulation of Arabidopsis with sucrose induces a phosphorylation of the AHA1/2 ATPase enhancing its proton transport activity. The resulting proton motive force drives the sucrose transport into cells (Niittyla et al., 2007). **C**: Activation of macrophages by interferon-γ induces differential phosphorylation of SNARE proteins possibly modulating vesicle-trafficking proteins of the phagosome. SNARE phosphorylation is likely associated with phagosome–endosome and phagosome–lysosome fusion events in activated macrophages (Trost et al., 2009). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Recently, Boja et al. isolated mitochondria from porcine heart and enriched phosphopeptides by TiO_2 chromatography. They quantified changes upon three different stimuli such as Ca^{2+}, de-energization or the experimental drug dichloroacetate using iTRAQ and identified 56 phosphorylation sites on 38 proteins (Boja et al., 2009). The use of an optimized IMAC protocol (Fe^{3+}) with complete conversion of peptides to their corresponding methyl esters led to the identification of 84 phosphorylation sites on 64 proteins from purified mitochondria extracts of mouse liver (Lee et al., 2007). Identification of phosphopeptides was also facilitated using the observation of H_3PO_4 loss in first-generation product ion spectrum to trigger the acquisition of MS^3 scan on a LTQ ion trap mass spectrometer (Olsen & Mann, 2004).

D. Synaptic Vesicles

Synaptosomes are isolated synapses of neurons and are comprised of phospholipids and synaptic proteins such as receptors. They contain the molecular machinery necessary for the uptake, storage, and release of neurotransmitters (Bai & Witzmann, 2007). Synaptic vesicles have been implicated in many neurological diseases (Yao, 2004) and have been studied extensively by proteomics (Li & Jimenez, 2008). Phosphorylation of pre- and post-synaptic proteins also plays an important role in modulating the activity of kinases and receptors involved in neurotransmission and synaptic plasticity.

Munton et al. (2007) reported the subcellular fractionation of mouse cortices to isolate synaptic membranes, post-synaptic density (PSD), and synaptic vesicles through sequential sucrose density centrifugation. Tryptic or Glu-C peptides (0.8–2 mg) from synaptic membranes, PSD, or synaptic vesicles were separated on a SCX column and phosphopeptides from the resulting fractions were enriched using IMAC (Fe^{3+}) prior to their LC–MS/MS analyses using either ESI-MS/MS or MALDI-MS/MS. Altogether, they identified 974 unique phosphorylation sites on 499 proteins including novel sites on glutamate receptor NMDA (NR2), disks large-associated protein (DAP), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). The use of isobaric peptide tags (iTRAQ) enabled the relative quantitation of proteins and highlighted the higher proportion of vesicle proteins, proton pumps, and proteins involved in lipid metabolism in synaptic vesicles compared to PSD or synaptic membrane preparations. Absolute quantitation measurements using synthetic peptides enabled the profiling of activity-dependent changes in phosphorylation in KCl-stimulated synaptosome extracts. For example, increase phosphorylation of T286 from CaMKIIα and T287 from CaMKIIβ was observed in KCl-stimulated synaptosome extracts with no significant change in protein abundance levels. The use of quantitative phosphoproteomics also highlighted the variable stoichiometry of basal phosphorylation in different proteins. The modulation of phosphorylation sites with activity provides meaningful insights for subsequent biological studies to underpin their functional regulation.

E. Plasma Membrane

The plasma membrane (PM) provides an interface between the cell environment and the intracellular components. It is composed of a lipid bilayer and a diversity of embedded hydrophobic proteins involved in selective transport of ions and nutrients, transmembrane signaling to cytoplasmic proteins or enzymatic reactions catalyzing reactions related to the PM. Many transmembrane receptors mediate cell signaling through protein phosphorylation.

The isolation of PM proteins requires more elaborate fractionation procedures typically involving immunoaffinity enrichment, detergents and/or multi-step ultracentrifugation. Nuhse et al. (2003) purified PM proteins from *A. thaliana* via sucrose, dextran/polyethylene glycol ultracentrifugation and detergent solubilization. Tryptic digests of PM-enriched protein extracts (500 μg) were first fractionated by strong anion exchange chromatography (SAX). Salt fractions were desalted and individually incubated with IMAC (Fe^{3+}) resins prior to mass spectrometry analysis on MALDI-TOF and LC–MS/MS. This study reported a total of 299 phosphopeptides including several novel phosphosites, of which two were identified on different H^+-ATPase isoforms. In plant and fungi, these H^+-ATPase generate proton gradients providing active transport of nutrients by H^+-symport (Palmgren, 2001). Interestingly, phosphorylated residues T881, S899, and T947 were identified close to the postulated *C*-terminus regulatory R1 region of ATPase 2 (At4g30190). The phosphorylation of the penultimate T947 residue promotes binding of 14-3-3 proteins necessary for the activation of the H^+-ATPase.

In a separate study using iTRAQ, the same group reported differential regulation of phosphorylation sites of H^+-ATPases following 3–15 min exposure to the bacterial elicitor peptide flagellin-22 (flg22) (Nuhse et al., 2007). Residues T881 and T947 showed more than threefold decrease in phosphorylation whereas the opposite trend was observed for residue S899. The similarity of phosphorylation profiles observed at each of these sites in different paralogs suggested a coordinated regulation of the overall proton pumping activity in response to microbial elicitors whereby dephosphorylation of T947 reduced proton pumping (Palmgren, 2001).

Similarly, Niittyla et al. (2007) analyzed PM protein extracts from *A. thaliana* and performed label-free quantitative phosphoproteomics analyses following sucrose resupply after depletion. Altogether they profiled the abundance of 67 phosphopeptides from 50 to 150 μg of PM protein digest using IMAC (Fe^{3+}) enrichment and LC–MS/MS analyses on a LTQ mass spectrometer. The phosphorylation of residue T947 from H^+-ATPases 1 and 2 was also correlated with increased proton pumping activity upon sucrose resupply, enhancing the proton gradient necessary for sucrose up-take (Fig. 7B). In addition to H^+-ATPase, this study also enabled the profiling of phosphorylation sites in other important classes of PM proteins such as transporters (e.g., PIP2e, SUC1, ACA8) and kinases (e.g., At5g41260, LRR-RLK At3g02880).

Quantitative phosphoproteomics using phosphotyrosine immunoprecipitation and metabolic labeling in a continuous quench-flow system was investigated for the rapid profiling of cell signaling events in receptor tyrosine kinases following EGF stimulation of HeLa S3 cells (Dengjel et al., 2007). This study enabled the identification of eight phosphorylation sites on EGF receptor, of which three (Y1092, Y1172, and Y1197) displayed more than twofold increase in phosphorylation within 5 sec exposure to EGF. Increase in tyrosine phosphorylation was also observed for down stream substrates such as Src homologous

and collagen-like protein, Shc (Y427) and phospholipase C γ1, PLCγ1 (Y771 and Y1253).

In a recent phosphoproteomics study Thingholm et al. (2008b) used sucrose centrifugation in combination with sodium carbonate extraction to obtain microgram amounts of PM proteins from $\sim 10^7$ human mesenchymal stem cells. Phosphopeptide enrichment using TiO_2 affinity medium followed by LC–MS/MS analyses enabled the assignment of 703 unique phosphorylation sites in 376 phosphoproteins, of which 12 sites were identified in Ephrin Type-A Receptor 2. Treatment of cells with different phosphatase inhibitors such as calyculin A and sodium pervanadate afforded the isolation of distinct populations of phosphopeptides.

F. Endosomes

Endocytosis represents a route of entry into the cell for biomolecules internalized through the PM leading to their encapsulation into membrane-bound cargoes (Pollard, Earnshaw, & Lippincott-Schwartz, 2007). Different mechanisms of endocytosis are being used by the cell depending on the mode of uptake and fate of internalized cargo. These include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-dependent uptake, and non-clathrin/non-caveolae endocytosis. Four classes of endosomes typically characterize clathrin-dependent endocytosis, and these can be distinguished based on their morphological appearance and the presence of specific markers such as GTPases and phosphoinositides (Behnia & Munro, 2005). Newly internalized proteins are uptaken by *early endosomes* composed of a network of tubules and vacuoles. Receptors together with specific PM proteins can be recycled to the cell surface via *recycling endosomes*. Early endosomes can fuse with other vacuolar compartment to progressively acquire internal membrane vesicles leading to the formation of *multi-vesicular bodies* that can subsequently mature into *late endosomes*. The acquisition of vacuolar ATPase during this maturation process lowers the luminal pH from 6.5 in early endosomes to approximately pH 5.0 in late endosomes. The fusion of late endosome with *lysosomes* leads to the acquisition of other lytic enzymes with a further reduction of pH to approximately 3.5 facilitating the degradation of the internalized cargo. It is noteworthy that endosomes are involved in many signal transduction pathways (Miaczynska, Pelkmans, & Zerial, 2004) and diseases (Kahn, Fu, & Roy, 2002; Harjes & Wanker, 2003) and the elucidation of the dynamic regulation of their protein composition represents a topic of significant interest.

The isolation of endosomes represents a challenging task in view of their rapid composition and morphological changes. Their isolation can be achieved by density gradient centrifugation (de Araujo, Huber, & Stasyk, 2008). A relatively limited number of contributions highlighted the significance of protein phosphorylation in endosomes. In a recent article, Stasyk et al. (2007) profiled the changes in endosomal proteins from EpH4 mouse cells in response to EGF and in the presence of the EGF receptor tyrosine kinase inhibitor gefitinib using 2D-gel and mass spectrometry. Phosphoproteins were stained with ProQ-diamond and subsequently identified on a MALDI TOF/TOF mass spectrometer. A total of 23 EGF-regulated proteins were found to be involved in endosomal trafficking and cytoskeleton rearrangement including Alix, myosin-9, myosin regulatory light chain, Trap1, moesin, cytokeratin 8, septins 2 and 11, and CapZβ. Interestingly they found that R-Ras was associated with late endosomes in an EGF-dependent manner and this association was inhibited by gefitinib suggesting a new signaling function for this protein.

G. Phagosomes

Phagocytosis is a specialized form of endocytosis associated with the internalization of large particles ($>0.2\,\mu m$) such as dust, apoptotic cells, or microbes. Professional phagocytic cells including dendritic cells or macrophages internalize particles through a series of regulated steps involving cell surface receptors, phospholipids, and Rho-GTPase mediated signaling cascades. The nascent phagosome undergoes progressive maturation by successive fusion events with endosomes and lysosomes resulting in the acidification and the recruitment of hydrolytic enzymes to the phagolysosome. Phagosomes also harbor MHC class I and II presentation machineries and consequently play an important role in innate and adaptive immunity (Jutras & Desjardins, 2005). Moreover, several pathogens including *Mycobacterium tuberculosis* have developed strategies to survive in the hostile environment of professional phagocytic cells and the further understanding of these persistence mechanisms may provide new therapeutic avenues for the treatment of bacterial infection (Mueller & Pieters, 2006).

The availability of efficient fractionation procedures relying on latex-bead internalization and density gradient centrifugation have significantly facilitated the isolation of this subcellular compartment with high purity (Desjardins et al., 1994; Desjardins & Griffiths, 2003). Proteomics analyses of phagosome-enriched protein extracts uncovered new cell biology paradigms such as the involvement of the ER-mediated phagocytosis (Garin et al., 2001; Gagnon et al., 2002), and the identification of the MHC class I machinery enabling antigen cross-presentation (Guermonprez et al., 2003; Houde et al., 2003).

A recent large-scale quantitative proteomics study profiled the changes in protein composition and phosphorylation of phagosomal proteins from mouse macrophage following stimulation with IFN-γ (Trost et al., 2009). The latex-bead/density gradient centrifugation approach was used to enrich phagosome proteins prior to their fractionation by gel electrophoresis and LC–MS/MS analysis on a LTQ-Orbitrap mass spectrometer. They reported the identification of 2,415 proteins (average sequence coverage 24%) including several low abundance proteins. TiO_2 enrichment led to the identification of 2,949 unique phosphosites on more than 1,100 phagosomal proteins of which 459 phosphoproteins (861 phosphorylation sites) were regulated by IFN-γ. A total of 2,131 novel phosphorylation sites were identified, on proteins such as Rab proteins, Src-family kinases, PKC, and several members of the JNK and MAP kinase-pathways. The analysis of protein interaction network from the combined proteome and phosphoproteome data sets uncovered specific molecular machineries involved in phagocytosis such as proteins involved in the cytoskeleton rearrangement (e.g., Ena-VASP-like, Zyxin, Arp2/3, CDC42, kinesins, myosins), antigen cross-presentation (e.g., tapasin, TAP1/2, immunoproteasome activator 28a) or vesicle trafficking (e.g., Rabs, VAMPs, syntaxins). Interestingly, the observation on the phagosome of several phosphoproteins involved in vesicle trafficking including

soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNAREs) provided additional insights on the regulation of vesicle fusion in response to cytokines. Phosphorylation of membrane trafficking proteins could play an important role in the fusion of specific vesicles as shown in Figure 7C and further detailed in the following section.

V. PHOSPHORYLATION AND VESICLE TRAFFICKING

The previous section highlighted the importance of phosphorylation in a number of subcellular compartments including those involved in the release of neurotransmitters and the internalization of biomolecules or pathogens through the PM. Intracellular trafficking is an important process involved in almost all cellular functions and is often regulated by membrane fusion. The fusion process requires the rearrangement of the membrane of adjoining vesicles to favor the mixing of their luminal contents. Specific fusion of vesicles and membranes is mediated by interaction of compartmentally specific protein complexes formed from SNAREs, small GTPases of the Rab family and tethering factors (Snyder, Kelly, & Woodbury, 2006; Pfeffer, 2007; Malsam, Kreye, & Sollner, 2008; Martens & McMahon, 2008; Wickner & Schekman, 2008). The spatial specificity of fusion events is regulated by multiple parameters. First, protein complexes can be formed by many different members, resulting in a large number of combinatorial protein–protein interactions. For example, there are approximately 36 SNARE proteins (Hong, 2005) which could in theory form hundreds of combinations. However, only a dozen or so combinations of SNAREs lead to actual membrane–membrane fusion events *in vitro* (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000). Additionally, there are several known SNARE interactors (Gallwitz & Jahn, 2003; Ungar & Hughson, 2003; Jahn & Scheller, 2006), at least 66 Rab GTPases (Colicelli, 2004), a long list of Rab effector proteins (Grosshans, Ortiz, & Novick, 2006) and numerous tethering proteins in mammalians (Lupashin & Sztul, 2005; Sztul & Lupashin, 2006; Pfeffer, 2007). Second, Ca^{2+} concentration appears to play also an important role in the regulation of membrane fusion (Rizo, Chen, & Arac, 2006; Martens & McMahon, 2008). And lastly, phosphorylation is rapidly emerging as a key regulator of vesicle trafficking (Snyder, Kelly, & Woodbury, 2006).

Most SNAREs are transmembrane proteins that comprise a cytoplasmic *N*-terminal domain and a *C*-terminal domain anchored to the lipid bilayer. The SNARE motif contains heptad repeats of 60–70 amino acids that can assemble together into a SNARE complex consisting of a bundle of α-helices. SNAREs are distributed into vesicle (v-) and complementary target membrane (t-) SNAREs. The formation of a SNARE complex involves the pairing of cognate v- and t-SNAREs. For example, the t-SNAREs Syntaxins can assemble with the SNARE motifs of a v-SNARE such as that of SNAP-23. v-SNARE from vesicle-associate membrane proteins (VAMPs) can form a loose *trans*-complex with the t-SNAREs, supported by SNARE-binding proteins such as proteins of the Sec1/Munc18 (SM) family (Sudhof & Rothman, 2009). These complexes proceed by ''zippering-up'' to a tight *trans*-complex by Ca^{2+}-binding, followed by the opening of the fusion pore. The SNAREs then form a *cis*-complex which is finally disassembled by the *N*-ethylmaleimide sensitive factor (NSF) and other co-factors (Fig. 8A).

Previous phosphoproteome analyses of phagosomal proteins identified 37 phosphorylation sites on 22 different SNARE proteins, of which only 10 were known before (Trost et al., 2009). This study also highlights the sensitivity of subcellular phosphoproteomics as most of these sites have not been identified in previous large-scale studies. A list of phosphorylation sites identified is presented in Table I and their location within the SNARE proteins is shown in Figure 8 B. Interestingly, many of the identified phosphorylation sites are located within the SNARE domains and are likely to be involved in the regulation of membrane fusion. Although SNAREs are widely studied, little is known about the functional significance of SNARE phosphorylation (Table II). For example, VAMP-4 which is involved in endosome to *trans*-Golgi network trafficking (Steegmaier et al., 1999; Mallard et al., 2002; Tran, Zeng, & Hong, 2007), was differentially phosphorylated on five different residues in phagosome extracts from IFN-γ activated macrophages (Trost et al., 2009). Only one of these phosphorylation sites (Ser30) has been more extensively studied. Previous reports indicated that phosphorylation of Ser30 by casein kinase II (CK-II), increased VAMP-4 binding to the adaptor protein AP-1 and mutation of this residue resulted in mislocalization of VAMP4 (Hinners et al., 2003). As VAMP-4 binds to a number of proteins including SNAP23, Syntaxin-6 (Cocucci et al., 2008), PACS-1 (Hinners et al., 2003), Syntaxin-13, VTI1A (Zwilling et al., 2007), and SNAP-29 (Feldmann et al., 2009), it is likely that other phosphorylation sites also play an important role in SNARE complex formation and in regulating fusion of VAMP-4 containing endosomes to fuse with the phagosome (Trost et al., 2009). It is noteworthy that identified phosphorylation sites are not necessarily conserved amongst family members but in some cases (e.g., VAMP-2) display a remarkable conservation across species (Fig. 8C). The different distribution of phosphorylation sites amongst family members could confer a convenient mechanism for the association of specific SNAREs. The identification of multiple phosphorylation sites on SNARE proteins now available from large scale subcellular phosphoproteomics studies leave an open question concerning their potential role in the regulation of SNARE complex formation and vesicle fusion. Obviously, this observation will require detailed functional characterization to fully understand its biological significance.

VI. OVERVIEW AND FUTURE PERSPECTIVES

The past decade has seen significant technological advances in the development of affinity chromatography techniques enabling the enrichment of phosphopeptides from complex cell extracts. In particular, the availability of IMAC and MOAC affinity media combined with high-performance mass spectrometers have greatly facilitated the identification of low abundance phosphopeptides representing typically less than 1% of proteolytic digests. However, multiple enrichment techniques are often necessary to obtain a comprehensive portrayal of the cellular phosphoproteome. Recent large-scale phosphoproteomics investigations have provided a detailed account of the expanding repertoire of the human phosphorylome currently populated by approximately 25,000 unique phosphorylation sites on 7,000 proteins. While these numbers appear relatively modest

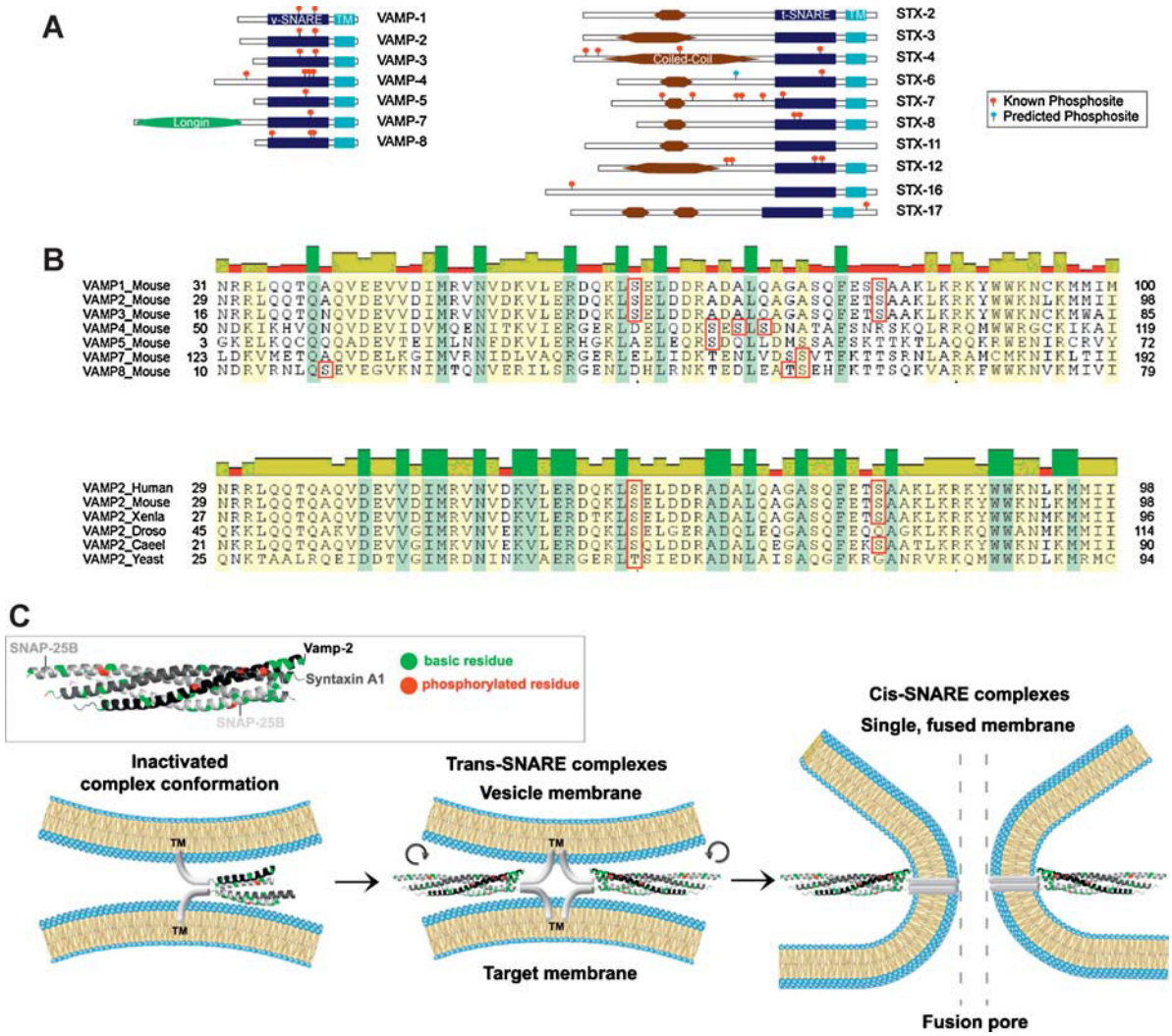

FIGURE 8. The phosphorylation of SNARE proteins. **A**: Phosphorylation sites identified on VAMP and Syntaxin proteins from phagosomal extracts (Trost et al., 2009). Many of the sites are within the SNARE domain. **B**: Alignment of the SNARE domains of seven VAMP proteins in mouse (upper panel). The identified phosphorylation sites are highlighted by red boxes. Phosphorylation sites of VAMP2 are highly conserved across species (lower panel). **C**: Phosphorylation in the SNARE domain possibly affects the formation of SNARE complex as shown for the SNAP25–VAMP2–Syntaxin-1A complex (Stein et al., 2009). The formation of salt bridges between basic and phosphorylated residues could provide a mechanism for modulating trans-SNARE complex assembly thereby affecting vesicle fusion.

compared to the estimated 100,000 different phosphorylation sites on human proteins, they do highlight some important aspects relating to the analytical capabilities of current phosphoproteomics approaches. As for any mass spectrometry-based proteomics approaches the successful identification of phosphorylation sites is also influenced by protein abundance, the dynamic range of detection, the variable stoichiometry of the modification, and the physicochemical properties of phosphoproteins. In this context, subcellular fractionation enables the enrichment of proteins and phosphoproteins that are unevenly distributed across organelles and may not be easily detectable from total cell lysates.

The protein composition of organelles is not static, but undergoes dynamic changes following interactions with other organelles or in response to various intra and extracellular cues. Some of these changes can be mediated by protein phosphorylation, as exemplified by the nuclear translocation of STAT1 or the membrane localization of SNAP25 (Fig. 1). The combined variation in phosphorylation and protein distribution associated with the remodeling of organelles underscore the necessity of phosphoproteomics approaches to provide a higher level of resolution and comprehensiveness to fully understand the nature of subcellular interactions. To this end, efforts must be placed on the integration of efficient cell fractionation techniques and quantitation strategies using label-free or isotopically labeled reagents to determine changes in both protein abundance and phosphorylation stoichiometry.

While subcellular phosphoproteomics has the potential to uncover new regulatory pathways, the validation of these findings and the further understanding of their biological significance often require independent methods. Confocal microscopy and molecular cloning experiments are typically used to confirm

TABLE I. SNARE phosphorylation identified by mass spectrometry (Villen et al., 2007; Trost et al., 2009, http://www.phosida.de)

Name	IPI	Protein ratio [IFN-g/Ctrl]	Phosphopeptide Sequence	Site1	Site2	Ratio [IFN-g/Ctrl]	RSD	p-value
ABI1	IPI00454179	n/a	TNPPTQKPPpSPPVSGR	183		n/a		
			LGpSQHSPGR	222		n/a		
			LGSQHpSPGR	225		n/a		
			HNpSTTSSTSSGGYR	296		n/a		
ABI2	IPI00380956	n/a	HTPPTIGGpSLPYR	301		n/a		
BET1	IPI00132685	n/a	pSLSIEIGHEVK	48		n/a		
			SLpSIEIGHEVK	50		5.8	0.12	0.0071
BET1L	IPI00315437	n/a	AQSpSGAVEDILDR	9		n/a		
SEC22b	IPI00114368	1.2	NLGpSINTELQDVQR	136		1.0	0.24	0.8308
SNAP-23	IPI00622270	1.8	AHQVTDEpSLESTRR	20		2.3	0.38	0.2992
			AHQVTDEpSLEpSTRR	*20*	*23*	1.1	0.26	0.7372
			ILGLAIEpSQDAGIK	34		n/a		
			ATWGDGGDNpSPSNVVSK	110		1.0	0.25	0.9853
			QPpSRITNGQPQQTTGAASGGYIK	*120*		5.1	0.14	0.0075
SNAP-29	IPI00321581	1.7				n/a		
Stx-2	IPI00653302	1.5				n/a		
Stx-3	IPI00137866	1.8				n/a		
Stx-4a	IPI00109335	1.7	QGDNIpSDDEDEVR	*15*		2.2	0.28	0.0210
			VALVVHpSGAAR	29		n/a		
			AIEPQKEEADENYNpSVNTR	117		1.6	0.25	0.1234
			NILSpSADYVER	248		0.7	0.18	0.1388
Stx-6	IPI00109506	1.2	IGGELEEQAVMLDDFpSHELESTQSR	205				
Stx-7	IPI00118217	1.0	TLNQLGTPQDpSPELR	44		n/a		
			EFGSLPTpTPSEQR	78		0.7	0.41	0.3870
			ASpSRVpSGGFPEDSSK	125	128	0.6	0.22	0.1637
			VpSGGFPEDSSK	128		0.6	0.25	0.1819
			NLVpSWESQTQPQVQVQDEEITEDDLR	143		0.8	0.30	0.4347
			EpSSIRQLEADIMDINEIFK	172		n/a		
Stx-8	IPI00136653	0.9	IIQEQDAGLDALSpSIISR	160		6.9	0.10	0.0018
			IIQEQDAGLDALSpSIIpSR	160	163	0.17	0.12	0.0033
Stx-11	IPI00654086	11.3						
Stx-12	IPI00111416	1.1	AGpSRLpSAEDR	139	142	1.8	0.38	0.1058
			AGSRLpSAEDR	142		1.1	0.31	0.6577
			DLAMMIHDQGDLIDpSIEANVEpSSEVHVER	218	225			
Stx-16	IPI00621076	2.0	QLLAEQVSSHTTSpSPLHSR	35		n/a		
Stx-17	IPI00316431	0.8	LTSpSCPDLPSQSDK	287		n/a		
Uaca	IPI00229465	6.9	NLpSHTQDEGSVK	280		n/a		
Vti-1a	IPI00131540	1.0				n/a		
Vti-1b	IPI00130115	1.0	ALLLQGTEpSLNR	138		8.9	0.31	0.0658
VAMP-1	IPI00125990	n/a	ADALQAGASQFETpSAAK *			0.17	0.01	0.0000
VAMP-2	IPI00229703	1.3	ADALQAGASQFETpSAAK *	80		0.17	0.01	0.0000
VAMP-3	IPI00132276	0.7	DQKLpSELDDR	48		0.8	0.22	0.8308
			ADALQAGASQFETpSAAK *	67		0.17	0.01	0.0000
VAMP-4	IPI00118372	0.2	HLNDDDVTGpSVK	17		1.70	0.65	0.4488
			NLLEDDpSDEEEDFFLR	30		0.23	0.23	0.0309
			LDELQDKpSESLSDNATAFSNR	88	0.07	0.14	0.0042	
			LDELQDKpSEpSLSDNATAFSNR	88	90	0.02	0.21	0.0147
			LDELQDKpSESLpSDNATAFSNR	88	92	0.12	0.34	0.0901
			LDELQDKSEpSLSDNATAFSNR	90	0.06	0.26	0.0365	
			LDELQDKpSESLpSDNATAFSNR	90	92	0.2	0.50	0.3059
VAMP-5			LAELEQRpSDQLLDMSSAFSK	41				
VAMP-7	IPI00137647	0.7	TENLVDSpSVTFK	168		0.1	0.35	0.1022
VAMP-8	IPI00453589	0.7	NLQpSEVEGVK	18		0.12	0.17	0.0110
			NKTEDLEApTSEHFK	54		0.6	0.46	0.3597
			TEDLEATpSEHFK	55		0.23	0.14	0.0125

*Sequence shared by VAMP-1, VAMP-2, and VAMP-3.
Sites known before Trost et al. (2009) are given in italics.

TABLE II. Known functions of SNARE phosphorylations (adapted from Snyder, Kelly, & Woodbury, 2006)

Protein	Kinase	Site(s) (Mouse)	Function	Reference(s)
Syntaxin-1/4	CKI/CKII	14/15	Increased Interaction with Synaptotagmin; Directs localization	(Risinger & Bennett, 1999; Foletti et al., 2000; Dubois et al., 2002)
Syntaxin-1a	DAPK	188	Decreased Interaction with Munc18	(Tian, Das & Sheng, 2003)
Syntaxin-7	PKC/Akt	124, 125, 128	Increased binding to Vti1b,Stx-8,VAMP-8	(Achuthan et al., 2008)
SNAP-25	PKC	28, 29, 187	Decreased Interaction with Stx; Localization to Membrane; Inhibits Voltage-gated Calcium Channels; Enhances Exocytosis	(Shimazaki et al., 1996; Genoud et al., 1999; Kataoka et al., 2000; Nagy et al., 2002; Finley, Scheller & Madison, 2003; Takahashi, Itakura & Kataoka, 2003; Pozzi et al., 2008; Shu et al., 2008)
	PKA/PKC	138		(Risinger & Bennett, 1999; Nagy et al., 2004)
SNAP-23	PKC	23, 24	Decreased Interaction with Stx-4	(Polgar et al., 2003)
	IKK2	95, 110, 161	Increased Interaction with Stx-4 and VAMP-2; Degranulation of Mast Cells	(Hepp et al., 2005; Suzuki & Verma, 2008)
VAMP-2	CKII	75		(Nielander et al., 1995)
	CaMKII	35, 61		(Nielander et al., 1995)
VAMP-4	CKII	30	Increased interaction with AP-1 mu	(Hinners et al., 2003)

the presence of proteins on specific organelles. In the case of differential phosphorylation, functional assignment studies rely on the availability of phosphospecific antibodies for immunoblot experiments. This strategy is further limited by the fact that many of these antibodies are of poor specificity and provide semiquantitative results at best. In view of the large number of phosphorylation sites that can now be identified from phosphoproteomics studies, their individual validation becomes an impractical task. There is thus an opportunity for phosphoproteomics to provide multiplex quantitative measurements that would otherwise be impossible to obtain with traditional immunoblot experiments one phosphoprotein at a time. Contemporary biological research is experiencing important paradigm shifts that are shaped by the accessibility of new OMICS technologies. At this juncture, subcellular phosphoproteomics is poised to become an enabling technology contributing to the emergence of high definition models of intracellular signaling.

VII. ABBREVIATIONS

CID	collision-induced dissociation
ECD	electron capture dissociation
ESI	electrospray ionization
ETD	electron-transfer dissociation
FA	formic acid
HILIC	hydrophilic interaction liquid chromatography
IMAC	immobilized metal affinity chromatography
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MS/MS	tandem mass spectrometry
MOAC	metal oxide affinity chromatography
pS	phosphoserine
pT	phosphothreonine
pY	phosphotyrosine
RP	reversed-phase
SAX	strong anion exchange
SCX	strong cation exchange
SDS–PAGE	sodium dodecyl-polyacrylamide gel electrophoresis
SNARE	*N*-ethylmaleimide sensitive factor attachment protein receptor
TFA	trifluoroacetic acid
TOF	time-of-flight
VAMP	vesicle-associated membrane protein

ACKNOWLEDGMENTS

M.T. thank for a fellowship of the Deutsche Forschungsgemeinschaft (DFG). This work was carried out with the financial support of operating grants from the National Science and

Engineering Research Council (NSERC), the Canadian Institutes for Health Research (CIHR) and the Canada Research Chair program. IRIC is supported in part by the Canadian Center of Excellence in Commercialization and Research, the Canadian Foundation for Innovation and the Fonds de la Recherche en Santé du Québec (FRSQ) and a CIHR multi-resource grant.

REFERENCES

Achuthan A, Masendycz P, Lopez JA, Nguyen T, James DE, Sweet MJ, Hamilton JA, Scholz GM. 2008. Regulation of the endosomal SNARE protein syntaxin 7 by colony-stimulating factor 1 in macrophages. Mol Cell Biol 28:6149–6159.

Adamczyk M, Gebler JC, Wu J. 2001. Selective analysis of phosphopeptides within a protein mixture by chemical modification, reversible biotinylation and mass spectrometry. Rapid Commun Mass Spectrom 15:1481–1488.

Alpert AJ. 2008. Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. Anal Chem 80:62–76.

Amanchy R, Periaswamy B, Mathivanan S, Reddy R, Tattikota SG, Pandey A. 2007. A curated compendium of phosphorylation motifs. Nat Biotechnol 25:285–286.

Andersen JS, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, Mann M, Lamond AI. 2002. Directed proteomic analysis of the human nucleolus. Curr Biol 12:1–11.

Andersen JS, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI, Mann M. 2005. Nucleolar proteome dynamics. Nature 433:77–83.

Annan RS, Carr SA. 1996. Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. Anal Chem 68:3413–3421.

Annan RS, Huddleston MJ, Verma R, Deshaies RJ, Carr SA. 2001. A multidimensional electrospray MS-based approach to phosphopeptide mapping. Anal Chem 73:393–404.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. 2000. Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–29.

Bai F, Witzmann FA. 2007. Synaptosome proteomics. Subcell Biochem 43:77–98.

Bakalarski CE, Haas W, Dephoure NE, Gygi SP. 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.

Baker DJ, Chen J, van Deursen JM. 2005. The mitotic checkpoint in cancer and aging: What have mice taught us? Curr Opin Cell Biol 17:583–589.

Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. 2007. Quantitative mass spectrometry in proteomics: A critical review. Anal Bioanal Chem 389:1017–1031.

Barr JR, Maggio VL, Patterson DG, Jr., Cooper GR, Henderson LO, Turner WE, Smith SJ, Hannon WH, Needham LL, Sampson EJ. 1996. Isotope dilution–mass spectrometric quantification of specific proteins: Model application with apolipoprotein A-I. Clin Chem 42:1676–1682.

Bauman AL, Scott JD. 2002. Kinase- and phosphatase-anchoring proteins: Harnessing the dynamic duo. Nat Cell Biol 4:E203–E206.

Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. 2004. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci USA 101:12130–12135.

Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. 2006. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat Biotechnol 24:1285–1292.

Behnia R, Munro S. 2005. Organelle identity and the signposts for membrane traffic. Nature 438:597–604.

Bernardes GJ, Chalker JM, Errey JC, Davis BG. 2008. Facile conversion of cysteine and alkyl cysteines to dehydroalanine on protein surfaces: Versatile and switchable access to functionalized proteins. J Am Chem Soc 130:5052–5053.

Blagoev B, Ong SE, Kratchmarova I, Mann M. 2004. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat Biotechnol 22:1139–1145.

Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S. 2004. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4:1633–1649.

Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R. 2007. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat Methods 4:231–237.

Boja ES, Phillips D, French SA, Harris RA, Balaban RS. 2009. Quantitative mitochondrial phosphoproteomics using iTRAQ on an LTQ-orbitrap with high energy collision dissociation. J Proteome Res. 8:4665–4675.

Bornhovd C, Vogel F, Neupert W, Reichert AS. 2006. Mitochondrial membrane potential is dependent on the oligomeric state of F1F0-ATP synthase supracomplexes. J Biol Chem 281:13990–13998.

Bridges SM, Magee GB, Wang N, Williams WP, Burgess SC, Nanduri B. 2007. ProtQuant: A tool for the label-free quantification of MudPIT proteomics data. BMC Bioinformatics 8(Suppl 7):S24.

Brinkworth RI, Munn AL, Kobe B. 2006. Protein kinases associated with the yeast phosphoproteome. BMC Bioinformatics 7:47.

Brunet S, Thibault P, Gagnon E, Kearney P, Bergeron JJ, Desjardins M. 2003. Organelle proteomics: Looking at less to see more. Trends Cell Biol 13:629–638.

Brusniak MY, Bodenmiller B, Campbell D, Cooke K, Eddes J, Garbutt A, Lau H, Letarte S, Mueller LN, Sharma V, Vitek O, Zhang N, Aebersold R, Watts JD. 2008. Corra: Computational framework and tools for LC-MS discovery and targeted mass spectrometry-based proteomics. BMC Bioinformatics 9:542.

Bykova NV, Egsgaard H, Moller IM. 2003. Identification of 14 new phosphoproteins involved in important plant mitochondrial processes. FEBS Lett 540:141–146.

Caenepeel S, Charydczak G, Sudarsanam S, Hunter T, Manning G. 2004. The mouse kinome: Discovery and comparative genomics of all mouse protein kinases. Proc Natl Acad Sci USA 101:11707–11712.

Canut H, Bauer J, Weber G. 1999. Separation of plant membranes by electromigration techniques. J Chromatogr B Biomed Sci Appl 722:121–139.

Carriere A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, Roux PP. 2008. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol 18:1269–1277.

Cassimeris L, Spittle C. 2001. Regulation of microtubule-associated proteins. Int Rev Cytol 210:163–226.

Castle JD. 2003. Purification of organelles from mammalian cells. Curr Protoc Immunol Chapter 8: Unit 8 1B.

Castle JD. 2004. Overview of cell fractionation. Curr Protoc Protein Sci Chapter 4: Unit 4 1.

Chang L, Karin M. 2001. Mammalian MAP kinase signalling cascades. Nature 410:37–40.

Chi A, Huttenhower C, Geer LY, Coon JJ, Syka JE, Bai DL, Shabanowitz J, Burke DJ, Troyanskaya OG, Hunt DF. 2007. Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proc Natl Acad Sci USA 104:2193–2198.

Claros MG, Vincens P. 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241:779–786.

Cocucci E, Racchetti G, Rupnik M, Meldolesi J. 2008. The regulated exocytosis of enlargeosomes is mediated by a SNARE machinery that includes VAMP4. J Cell Sci 121:2983–2991.

Cokol M, Nair R, Rost B. 2000. Finding nuclear localization signals. EMBO Rep 1:411–415.

Colicelli J. 2004. Human RAS superfamily proteins and related GTPases. Sci STKE 2004 250:13.

Creese AJ, Cooper HJ. 2008. The effect of phosphorylation on the electron capture dissociation of peptide ions. J Am Soc Mass Spectrom 19:1263–1274.

Cronshaw JM, Krutchinsky AN, Zhang W, Chait BT, Matunis MJ. 2002. Proteomic analysis of the mammalian nuclear pore complex. J Cell Biol 158:915–927.

Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: A sequence logo generator. Genome Res 14:1188–1190.

D'Ambrosio C, Salzano AM, Arena S, Renzone G, Scaloni A. 2007. Analytical methodologies for the detection and structural characterization of phosphorylated proteins. J Chromatogr B Anal Technol Biomed Life Sci 849:163–180.

Dai J, Jin WH, Sheng QH, Shieh CH, Wu JR, Zeng R. 2007. Protein phosphorylation and expression profiling by Yin-yang multidimensional liquid chromatography (Yin-yang MDLC) mass spectrometry. J Proteome Res 6:250–262.

Darnell JE, Jr., Kerr IM, Stark GR. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421.

Dass C, Mahalakshmi P. 1995. Amino acid sequence determination of phosphoenkephalins using liquid secondary ionization mass spectrometry. Rapid Commun Mass Spectrom 9:1148–1154.

Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O, Mann M. 2008. Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 31:438–448.

de Araujo ME, Huber LA, Stasyk T. 2008. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol Biol 424:317–331.

De Duve C. 1965. The separation and characterization of subcellular particles. Harvey Lect 59:49–87.

de la Fuente van Bentem S, Anrather D, Roitinger E, Djamei A, Hufnagl T, Barta A, Csaszar E, Dohnal I, Lecourieux D, Hirt H. 2006. Phosphoproteomics reveals extensive in vivo phosphorylation of Arabidopsis proteins involved in RNA metabolism. Nucleic Acids Res 34:3267–3278.

Decker T, Kovarik P. 2000. Serine phosphorylation of STATs. Oncogene 19:2628–2637.

DeGnore JP, Qin J. 1998. Fragmentation of phosphopeptides in an ion trap mass spectrometer. J Am Soc Mass Spectrom 9:1175–1188.

Dengjel J, Akimov V, Olsen JV, Bunkenborg J, Mann M, Blagoev B, Andersen JS. 2007. Quantitative proteomic assessment of very early cellular signaling events. Nat Biotechnol 25:566–568.

Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP. 2008. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci USA 105:10762–10767.

Desjardins M, Celis JE, van Meer G, Dieplinger H, Jahraus A, Griffiths G, Huber LA. 1994. Molecular characterization of phagosomes. J Biol Chem 269:32194–32200.

Desjardins M, Griffiths G. 2003. Phagocytosis: Latex leads the way. Curr Opin Cell Biol 15:498–503.

Dreger M. 2003. Subcellular proteomics. Mass Spectrom Rev 22:27–56.

Dubois T, Kerai P, Learmonth M, Cronshaw A, Aitken A. 2002. Identification of syntaxin-1A sites of phosphorylation by casein kinase I and casein kinase II. Eur J Biochem 269:909–914.

Dunn JD, Reid GE, Bruening ML. 2009. Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. Mass Spectrom Rev 29:29–51.

Edelson-Averbukh M, Pipkorn R, Lehmann WD. 2007. Analysis of protein phosphorylation in the regions of consecutive serine/threonine residues by negative ion electrospray collision-induced dissociation. Approach to pinpointing of phosphorylation sites. Anal Chem 79:3476–3486.

Edgar RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2:953–971.

Feldmann A, Winterstein C, White R, Trotter J, Kramer-Albers EM. 2009. Comprehensive analysis of expression, subcellular localization, and cognate pairing of SNARE proteins in oligodendrocytes. J Neurosci Res 87:1760–1772.

Fenselau C, Yao X. 2009. 18O2-labeling in quantitative proteomic strategies: A status report. J Proteome Res 8:2140–2143.

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. 2002. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat Biotechnol 20:301–305.

Ficarro SB, Parikh JR, Blank NC, Marto JA. 2008. Niobium(V) oxide (Nb2O5): Application to phosphoproteomics. Anal Chem 80:4606–4613.

Fink JL, Aturaliya RN, Davis MJ, Zhang F, Hanson K, Teasdale MS, Kai C, Kawai J, Carninci P, Hayashizaki Y, Teasdale RD. 2006. LOCATE: A mouse protein subcellular localization database. Nucleic Acids Res 34:D213–D217.

Finley MF, Scheller RH, Madison DV. 2003. SNAP-25 Ser187 does not mediate phorbol ester enhancement of hippocampal synaptic transmission. Neuropharmacology 45:857–862.

Foletti DL, Lin R, Finley MA, Scheller RH. 2000. Phosphorylated syntaxin 1 is localized to discrete domains along a subset of axons. J Neurosci 20:4535–4544.

Forner F, Foster LJ, Campanaro S, Valle G, Mann M. 2006. Quantitative proteomic comparison of rat mitochondria from muscle, heart, and liver. Mol Cell Proteomics 5:608–619.

Foster LJ, de Hoog CL, Zhang Y, Zhang Y, Xie X, Mootha VK, Mann M. 2006. A mammalian organelle map by protein correlation profiling. Cell 125:187–199.

Frackelton AR, Jr., Ross AH, Eisen HN. 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. Mol Cell Biol 3:1343–1352.

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, Sollner TH. 2000. Functional architecture of an intracellular membrane t-SNARE. Nature 407:198–202.

Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, Paiement J, Bergeron JJ, Desjardins M. 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110:119–131.

Gallwitz D, Jahn R. 2003. The riddle of the Sec1/Munc-18 proteins—New twists added to their interactions with SNAREs. Trends Biochem Sci 28:113–116.

Gan CS, Guo T, Zhang H, Lim SK, Sze SK. 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.

Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, Desjardins M. 2001. The phagosome proteome: Insight into phagosome functions. J Cell Biol 152:165–180.

Genoud S, Pralong W, Riederer BM, Eder L, Catsicas S, Muller D. 1999. Activity-dependent phosphorylation of SNAP-25 in hippocampal organotypic cultures. J Neurochem 72:1699–1706.

Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. 2003. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci USA 100:6940–6945.

Gharib M, Marcantonio M, Lehmann SG, Courcelles M, Meloche S, Verreault A, Thibault P. 2009. Artifactual sulfation of silver-stained proteins: Implications for the assignment of phosphorylation and sulfation sites. Mol Cell Proteomics 8:506–518.

Gilar M, Olivova P, Daly AE, Gebler JC. 2005. Orthogonality of separation in two-dimensional liquid chromatography. Anal Chem 77:6426–6434.

Goshe MB, Conrads TP, Panisko EA, Angell NH, Veenstra TD, Smith RD. 2001. Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. Anal Chem 73:2578–2586.

Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J 13:1591–1600.

Gronborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, Jensen ON, Pandey A. 2002. A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: Identification of a novel protein, Frigg, as a protein kinase A substrate. Mol Cell Proteomics 1:517–527.

Grosshans BL, Ortiz D, Novick P. 2006. Rabs and their effectors: Achieving specificity in membrane traffic. Proc Natl Acad Sci USA 103:11821–11827.

Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON. 2005. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. Mol Cell Proteomics 4:310–327.

Guermonprez P, Saveanu L, Kleijmeer M, Davoust J, Van Endert P, Amigorena S. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425:397–402.

Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, Wang Y, MacNeill J, Mitchell J, Gygi SP, Rush J, Polakiewicz RD, Comb MJ. 2008. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci USA 105:692–697.

Han G, Ye M, Zhou H, Jiang X, Feng S, Jiang X, Tian R, Wan D, Zou H, Gu J. 2008. Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography. Proteomics 8:1346–1361.

Hansen KC, Schmitt-Ulms G, Chalkley RJ, Hirsch J, Baldwin MA, Burlingame AL. 2003. Mass spectrometric analysis of protein mixtures at low levels using cleavable 13C-isotope-coded affinity tag and multidimensional chromatography. Mol Cell Proteomics 2:299–314.

Harita Y, Kurihara H, Kosako H, Tezuka T, Sekine T, Igarashi T, Hattori S. 2008. Neph1, a component of the kidney slit diaphragm, is tyrosine-phosphorylated by the Src family tyrosine kinase and modulates intracellular signaling by binding to Grb2. J Biol Chem 283:9177–9186.

Harjes P, Wanker EE. 2003. The hunt for huntingtin function: Interaction partners tell many different stories. Trends Biochem Sci 28:425–433.

Haselmann KF, Budnik BA, Olsen JV, Nielsen ML, Reis CA, Clausen H, Johnsen AH, Zubarev RA. 2001. Advantages of external accumulation for electron capture dissociation in Fourier transform mass spectrometry. Anal Chem 73:2998–3005.

Hegeman AD, Harms AC, Sussman MR, Bunner AE, Harper JF. 2004. An isotope labeling strategy for quantifying the degree of phosphorylation at multiple sites in proteins. J Am Soc Mass Spectrom 15:647–653.

Hepp R, Puri N, Hohenstein AC, Crawford GL, Whiteheart SW, Roche PA. 2005. Phosphorylation of SNAP-23 regulates exocytosis from mast cells. J Biol Chem 280:6610–6620.

Herbert B, Hopwood F, Oxley D, McCarthy J, Laver M, Grinyer J, Goodall A, Williams K, Castagna A, Righetti PG. 2003. Beta-elimination: An unexpected artefact in proteome analysis. Proteomics 3:826–831.

Hilger M, Bonaldi T, Gnad F, Mann M. 2009. Systems-wide analysis of a phosphatase knock down by quantitative proteomics and phosphoproteomics. Mol Cell Proteomics 8:1908–1920.

Hinners I, Wendler F, Fei H, Thomas L, Thomas G, Tooze SA. 2003. AP-1 recruitment to VAMP4 is modulated by phosphorylation-dependent binding of PACS-1. EMBO Rep 4:1182–1189.

Hjerrild M, Gammeltoft S. 2006. Phosphoproteomics toolbox: Computational biology, protein chemistry and mass spectrometry. FEBS Lett 580:4764–4770.

Honda R, Korner R, Nigg EA. 2003. Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. Mol Biol Cell 14:3325–3341.

Hong W. 2005. SNAREs and traffic. Biochim Biophys Acta 1744:493–517.

Hopper RK, Carroll S, Aponte AM, Johnson DT, French S, Shen RF, Witzmann FA, Harris RA, Balaban RS. 2006. Mitochondrial matrix phosphoproteome: Effect of extra mitochondrial calcium. Biochemistry 45:2524–2536.

Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, Princiotta MF, Thibault P, Sacks D, Desjardins M. 2003. Phagosomes are competent organelles for antigen cross-presentation. Nature 425:402–406.

Huang HD, Lee TY, Tzeng SW, Horng JT. 2005. KinasePhos: A web tool for identifying protein kinase-specific phosphorylation sites. Nucleic Acids Res 33:W226–W229.

Hunter AP, Games DE. 1994. Chromatographic and mass spectrometric methods for the identification of phosphorylation sites in phosphoproteins. Rapid Commun Mass Spectrom 8:559–570.

Hunter T, Sefton BM. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA 77:1311–1315.

Hunter T, Plowman GD. 1997. The protein kinases of budding yeast: Six score and more. Trends Biochem Sci 22:18–22.

Hunter T. 2007. The age of crosstalk: Phosphorylation, ubiquitination, and beyond. Mol Cell 28:730–738.

Igarashi K, Garotta G, Ozmen L, Ziemiecki A, Wilks AF, Harpur AG, Larner AC, Finbloom DS. 1994. Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. J Biol Chem 269:14333–14336.

Ikeguchi Y, Nakamura H. 1997. Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on Titania. Anal. Sc 13:479–483.

Ishihama Y, Wei FY, Aoshima K, Sato T, Kuromitsu J, Oda Y. 2007. Enhancement of the efficiency of phosphoproteomic identification by removing phosphates after phosphopeptide enrichment. J Proteome Res 6:1139–1144.

Jaffe H, Veeranna, Pant HC. 1998. Characterization of serine and threonine phosphorylation sites in beta-elimination/ethanethiol addition-modified proteins by electrospray tandem mass spectrometry and database searching. Biochemistry 37:16211–16224.

Jahn R, Scheller RH. 2006. SNAREs—Engines for membrane fusion. Nat Rev Mol Cell Biol 7:631–643.

Jensen SS, Larsen MR. 2007. Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques. Rapid Commun Mass Spectrom 21:3635–3645.

Jin J, Smith FD, Stark C, Wells CD, Fawcett JP, Kulkarni S, Metalnikov P, O'Donnell P, Taylor P, Taylor L, Zougman A, Woodgett JR, Langeberg LK, Scott JD, 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.

Jones AM, MacLean D, Studholme DJ, Serna-Sanz A, Andreasson E, Rathjen JP, Peck SC. 2009. Phosphoproteomic analysis of nuclei-enriched fractions from *Arabidopsis thaliana*. J Proteomics 72:439–451.

Jutras I, Desjardins M. 2005. Phagocytosis: At the crossroads of innate and adaptive immunity. Annu Rev Cell Dev Biol 21:511–527.

Kahn RA, Fu H, Roy CR. 2002. Cellular hijacking: A common strategy for microbial infection. Trends Biochem Sci 27:308–314.

Kalume DE, Molina H, Pandey A. 2003. Tackling the phosphoproteome: Tools and strategies. Curr Opin Chem Biol 7:64–69.

Karsenti E, Vernos I. 2001. The mitotic spindle: A self-made machine. Science 294:543–547.

Kataoka M, Kuwahara R, Iwasaki S, Shoji-Kasai Y, Takahashi M. 2000. Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells: A possible involvement in the regulation of SNAP-25 localization. J Neurochem 74:2058–2066.

Kearney P, Thibault P. 2003. Bioinformatics meets proteomics—Bridging the gap between mass spectrometry data analysis and cell biology. J Bioinform Comput Biol 1:183–200.

Kennelly PJ. 2002. Protein kinases and protein phosphatases in prokaryotes: A genomic perspective. FEMS Microbiol Lett 206:1–8.

Kholodenko BN. 2002. MAP kinase cascade signaling and endocytic trafficking: A marriage of convenience? Trends Cell Biol 12:173–177.

Kleinnijenhuis AJ, Kjeldsen F, Kallipolitis B, Haselmann KF, Jensen ON. 2007. Analysis of histidine phosphorylation using tandem MS and ion-electron reactions. Anal Chem 79:7450–7456.

Klumpp S, Krieglstein J. 2005. Reversible phosphorylation of histidine residues in vertebrate proteins. Biochim Biophys Acta 1754:291–295.

Knight ZA, Schilling B, Row RH, Kenski DM, Gibson BW, Shokat KM. 2003. Phosphospecific proteolysis for mapping sites of protein phosphorylation. Nat Biotechnol 21:1047–1054.

Kocher T, Allmaier G, Wilm M. 2003. Nanoelectrospray-based detection and sequencing of substoichiometric amounts of phosphopeptides in complex mixtures. J Mass Spectrom 38:131–137.

Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH, Heck AJ. 2003. Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. Nat Biotechnol 21:927–931.

Kruger M, Moser M, Ussar S, Thievessen I, Luber CA, Forner F, Schmidt S, Zanivan S, Fassler R, Mann M. 2008. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. Cell 134:353–364.

Kruger R, Kubler D, Pallisse R, Burkovski A, Lehmann WD. 2006. Protein and proteome phosphorylation stoichiometry analysis by element mass spectrometry. Anal Chem 78:1987–1994.

Kweon HK, Hakansson K. 2006. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. Anal Chem 78:1743–1749.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. 2005. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics 4:873–886.

Lee J, Xu Y, Chen Y, Sprung R, Kim SC, Xie S, Zhao Y. 2007. Mitochondrial phosphoproteome revealed by an improved IMAC method and MS/MS/MS. Mol Cell Proteomics 6:669–676.

Lehmann WD, Kruger R, Salek M, Hung CW, Wolschin F, Weckwerth W. 2007. Neutral loss-based phosphopeptide recognition: A collection of caveats. J Proteome Res 6:2866–2873.

Leitner A, Foettinger A, Lindner W. 2007. Improving fragmentation of poorly fragmenting peptides and phosphopeptides during collision-induced dissociation by malondialdehyde modification of arginine residues. J Mass Spectrom 42:950–959.

Lemeer S, Heck AJ. 2009. The phosphoproteomics data explosion. Curr Opin Chem Biol 13:414–420.

Li H, Coghlan A, Ruan J, Coin LJ, Heriche JK, Osmotherly L, Li R, Liu T, Zhang Z, Bolund L, Wong GK, Zheng W, Dehal P, Wang J, Durbin R. 2006. TreeFam: A curated database of phylogenetic trees of animal gene families. Nucleic Acids Res 34:D572–D580.

Li KW, Jimenez CR. 2008. Synapse proteomics: Current status and quantitative applications. Expert Rev Proteomics 5:353–360.

Lim KB, Kassel DB. 2006. Phosphopeptides enrichment using on-line two-dimensional strong cation exchange followed by reversed-phase liquid chromatography/mass spectrometry. Anal Biochem 354:213–219.

Linding R, Jensen LJ, Pasculescu A, Olhovsky M, Colwill K, Bork P, Yaffe MB, Pawson T. 2008. NetworKIN: A resource for exploring cellular phosphorylation networks. Nucleic Acids Res 36:D695–D699.

Lindner H, Sarg B, Helliger W. 1997. Application of hydrophilic-interaction liquid chromatography to the separation of phosphorylated H1 histones. J Chromatogr A 782:55–62.

Listgarten J, Emili A. 2005. Statistical and computational methods for comparative proteomic profiling using liquid chromatography-tandem mass spectrometry. Mol Cell Proteomics 4:419–434.

Lupashin V, Sztul E. 2005. Golgi tethering factors. Biochim Biophys Acta 1744:325–339.

Macek B, Mijakovic I, Olsen JV, Gnad F, Kumar C, Jensen PR, Mann M. 2007. The serine/threonine/tyrosine phosphoproteome of the model bacterium Bacillus subtilis. Mol Cell Proteomics 6:697–707.

Macek B, Gnad F, Soufi B, Kumar C, Olsen JV, Mijakovic I, Mann M. 2008. Phosphoproteome analysis of E. coli reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. Mol Cell Proteomics 7:299–307.

Malik R, Lenobel R, Santamaria A, Ries A, Nigg EA, Korner R. 2009. Quantitative analysis of the human spindle phosphoproteome at distinct mitotic stages. J Proteome Res 8:4553–-4563.

Mallard F, Tang BL, Galli T, Tenza D, Saint-Pol A, Yue X, Antony C, Hong W, Goud B, Johannes L. 2002. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J Cell Biol 156:653–664.

Malsam J, Kreye S, Sollner TH. 2008. Membrane fusion: SNAREs and regulation. Cell Mol Life Sci 65:2814–2832.

Mann M. 2006. Functional and quantitative proteomics using SILAC. Nat Rev Mol Cell Biol 7:952–958.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. 2002. The protein kinase complement of the human genome. Science 298:1912–1934.

Marcantonio M, Trost M, Courcelles M, Desjardins M, Thibault P. 2008. Combined enzymatic and data mining approaches for comprehensive phosphoproteome analyses: Application to cell signaling events of interferon-gamma-stimulated macrophages. Mol Cell Proteomics 7:645–660.

Martens S, McMahon HT. 2008. Mechanisms of membrane fusion: Disparate players and common principles. Nat Rev Mol Cell Biol 9:543–556.

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316:1160–1166.

Matthews HR. 1995. Protein kinases and phosphatases that act on histidine, lysine, or arginine residues in eukaryotic proteins: A possible regulator of the mitogen-activated protein kinase cascade. Pharmacol Ther 67:323–350.

Mazanek M, Mituloviae G, Herzog F, Stingl C, Hutchins JR, Peters JM, Mechtler K. 2007. Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis. Nat Protoc 2:1059–1069.

McLachlin DT, Chait BT. 2003. Improved beta-elimination-based affinity purification strategy for enrichment of phosphopeptides. Anal Chem 75:6826–6836.

McNew JA, Parlati F, Fukuda R, Johnston RJ, Paz K, Paumet F, Sollner TH, Rothman JE. 2000. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407:153–159.

McNulty DE, Annan RS. 2008. Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. Mol Cell Proteomics 7:971–980.

Medzihradszky KF. 2005. Peptide sequence analysis. Methods Enzymol 402:209–244.

Miaczynska M, Pelkmans L, Zerial M. 2004. Not just a sink: Endosomes in control of signal transduction. Curr Opin Cell Biol 16:400–406.

Miller ML, Blom N. 2009. Kinase-specific prediction of protein phosphorylation sites. Methods Mol Biol 527:299–310.

Molina H, Horn DM, Tang N, Mathivanan S, Pandey A. 2007. Global proteomic profiling of phosphopeptides using electron transfer

dissociation tandem mass spectrometry. Proc Natl Acad Sci USA 104:2199–2204.

Monigatti F, Hekking B, Steen H. 2006. Protein sulfation analysis—A primer. Biochim Biophys Acta 1764:1904–1913.

Mootha VK, Bunkenborg J, Olsen JV, Hjerrild M, Wisniewski JR, Stahl E, Bolouri MS, Ray HN, Sihag S, Kamal M, Patterson N, Lander ES, Mann M. 2003. Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell 115:629–640.

Morandell S, Stasyk T, Grosstessner-Hain K, Roitinger E, Mechtler K, Bonn GK, Huber LA. 2006. Phosphoproteomics strategies for the functional analysis of signal transduction. Proteomics 6:4047–4056.

Morrison DK, Murakami MS, Cleghon V. 2000. Protein kinases and phosphatases in the Drosophila genome. J Cell Biol 150:F57–F62.

Moser K, White FM. 2006. Phosphoproteomic analysis of rat liver by high capacity IMAC and LC-MS/MS. J Proteome Res 5:98–104.

Mueller LN, Brusniak MY, Mani DR, Aebersold R. 2008. An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. J Proteome Res 7:51–61.

Mueller P, Pieters J. 2006. Modulation of macrophage antimicrobial mechanisms by pathogenic mycobacteria. Immunobiology 211:549–556.

Mullock BM, Hinton RH, Peppard JV, Slot JW, Luzio JP. 1987. The preparative isolation of endosome fractions: A review. Cell Biochem Funct 5:235–243.

Munton RP, Tweedie-Cullen R, Livingstone-Zatchej M, Weinandy F, Waidelich M, Longo D, Gehrig P, Potthast F, Rutishauser D, Gerrits B, Panse C, Schlapbach R, Mansuy IM. 2007. Qualitative and quantitative analyses of protein phosphorylation in naive and stimulated mouse synaptosomal preparations. Mol Cell Proteomics 6:283–293.

Nagy G, Matti U, Nehring RB, Binz T, Rettig J, Neher E, Sorensen JB. 2002. Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. J Neurosci 22:9278–9286.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sorensen JB. 2004. Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. Neuron 41:417–429.

Ndassa YM, Orsi C, Marto JA, Chen S, Ross MM. 2006. Improved immobilized metal affinity chromatography for large-scale phosphoproteomics applications. J Proteome Res 5:2789–2799.

Neubauer G, King A, Rappsilber J, Calvio C, Watson M, Ajuh P, Sleeman J, Lamond A, Mann M. 1998. Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex. Nat Genet 20:46–50.

Newmeyer DD, Ferguson-Miller S. 2003. Mitochondria: Releasing power for life and unleashing the machineries of death. Cell 112:481–490.

Nielander HB, Onofri F, Valtorta F, Schiavo G, Montecucco C, Greengard P, Benfenati F. 1995. Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous protein kinases. J Neurochem 65:1712–1720.

Niittyla T, Fuglsang AT, Palmgren MG, Frommer WB, Schulze WX. 2007. Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of Arabidopsis. Mol Cell Proteomics 6:1711–1726.

Nousiainen M, Sillje HH, Sauer G, Nigg EA, Korner R. 2006. Phosphoproteome analysis of the human mitotic spindle. Proc Natl Acad Sci USA 103:5391–5396.

Nuhse TS, Stensballe A, Jensen ON, Peck SC. 2003. Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. Mol Cell Proteomics 2:1234–1243.

Nuhse TS, Bottrill AR, Jones AM, Peck SC. 2007. Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. Plant J 51:931–940.

Obenauer JC, Cantley LC, Yaffe MB. 2003. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res 31:3635–3641.

Oda Y, Nagasu T, Chait BT. 2001. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. Nat Biotechnol 19:379–382.

Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, Ahn NG. 2005. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics 4:1487–1502.

Old WM, Shabb JB, Houel S, Wang H, Couts KL, Yen CY, Litman ES, Croy CH, Meyer-Arendt K, Miranda JG, Brown RA, Witze ES, Schweppe RE, Resing KA, Ahn NG. 2009. Functional proteomics identifies targets of phosphorylation by B-Raf signaling in melanoma. Mol Cell 34:115–131.

Olsen JV, Mann M. 2004. Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. Proc Natl Acad Sci USA 101:13417–13422.

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

Ong SE, Kratchmarova I, Mann M. 2003. Properties of 13C-substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). J Proteome Res 2:173–181.

Ono M, Shitashige M, Honda K, Isobe T, Kuwabara H, Matsuzuki H, Hirohashi S, Yamada T. 2006. Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. Mol Cell Proteomics 5:1338–1347.

Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong SE, Walford GA, Sugiana C, Boneh A, Chen WK, Hill DE, Vidal M, Evans JG, Thorburn DR, Carr SA, Mootha VK. 2008. A mitochondrial protein compendium elucidates complex I disease biology. Cell 134:112–123.

Palmgren MG. 2001. Plant plasma membrane H+-ATPases: Powerhouses for nutrient uptake. Annu Rev Plant Physiol Plant Mol Biol 52:817–845.

Palumbo AM, Reid GE. 2008. Evaluation of Gas-phase rearrangement and competing fragmentation reactions on protein phosphorylation site assignment using collision induced dissociation-MS/MS and MS(3). Anal Chem 80:9735–9747.

Palumbo AM, Tepe JJ, Reid GE. 2008. Mechanistic insights into the multistage gas-phase fragmentation behavior of phosphoserine- and phosphothreonine-containing peptides. J Proteome Res 7:771–779.

Parlati F, McNew JA, Fukuda R, Miller R, Sollner TH, Rothman JE. 2000. Topological restriction of SNARE-dependent membrane fusion. Nature 407:194–198.

Payne SH, Yau M, Smolka MB, Tanner S, Zhou H, Bafna V. 2008. Phosphorylation-specific MS/MS scoring for rapid and accurate phosphoproteome analysis. J Proteome Res 7:3373–3381.

Pfeffer SR. 2007. Unsolved mysteries in membrane traffic. Annu Rev Biochem 76:629–645.

Pinkse MW, Uitto PM, Hilhorst MJ, Ooms B, Heck AJ. 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.

Polgar J, Lane WS, Chung SH, Houng AK, Reed GL. 2003. Phosphorylation of SNAP-23 in activated human platelets. J Biol Chem 278:44369–44376.

Pollard TD, Earnshaw WC, Lippincott-Schwartz J. 2007. Cell biology, 2nd edition. Elsevier.

Posewitz MC, Tempst P. 1999. Immobilized gallium(III) affinity chromatography of phosphopeptides. Anal Chem 71:2883–2892.

Pozzi D, Condliffe S, Bozzi Y, Chikhladze M, Grumelli C, Proux-Gillardeaux V, Takahashi M, Franceschetti S, Verderio C, Matteoli M. 2008. Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. Proc Natl Acad Sci USA 105:323–328.

Prokisch H, Scharfe C, Camp DG II, Xiao W, David L, Andreoli C, Monroe ME, Moore RJ, Gritsenko MA, Kozany C, Hixson KK, Mottaz HM, Zischka H, Ueffing M, Herman ZS, Davis RW, Meitinger T, Oefner PJ,

Smith RD, Steinmetz LM. 2004. Integrative analysis of the mitochondrial proteome in yeast. PLoS Biol 2:e160.

Puntervoll P, Linding R, Gemund C, Chabanis-Davidson S, Mattingsdal M, Cameron S, Martin DM, Ausiello G, Brannetti B, Costantini A, Ferre F, Maselli V, Via A, Cesareni G, Diella F, Superti-Furga G, Wyrwicz L, Ramu C, McGuigan C, Gudavalli R, Letunic I, Bork P, Rychlewski L, Kuster B, Helmer-Citterich M, Hunter WN, Aasland R, Gibson TJ. 2003. ELM server: A new resource for investigating short functional sites in modular eukaryotic proteins. Nucleic Acids Res 31:3625–3630.

Puttick J, Baker EN, Delbaere LT. 2008. Histidine phosphorylation in biological systems. Biochim Biophys Acta 1784:100–105.

Qian WJ, Goshe MB, Camp DG II, Yu LR, Tang K, Smith RD. 2003. Phosphoprotein isotope-coded solid-phase tag approach for enrichment and quantitative analysis of phosphopeptides from complex mixtures. Anal Chem 75:5441–5450.

Rappsilber J, Ryder U, Lamond AI, Mann M. 2002. Large-scale proteomic analysis of the human spliceosome. Genome Res 12:1231–1245.

Reinders J, Sickmann A. 2005. State-of-the-art in phosphoproteomics. Proteomics 5:4052–4061.

Reinders J, Wagner K, Zahedi RP, Stojanovski D, Eyrich B, van der Laan M, Rehling P, Sickmann A, Pfanner N, Meisinger C. 2007. Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase. Mol Cell Proteomics 6:1896–1906.

Reynolds KJ, Yao X, Fenselau C. 2002. Proteolytic 18O labeling for comparative proteomics: Evaluation of endoprotease Glu-C as the catalytic agent. J Proteome Res 1:27–33.

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 JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ. 2007. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131:1190–1203.

Risinger C, Bennett MK. 1999. Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. J Neurochem 72:614–624.

Rizo J, Chen X, Arac D. 2006. Unraveling the mechanisms of synaptotagmin and SNARE function in neurotransmitter release. Trends Cell Biol 16:339–350.

Ross PL, Huang YN, Marchese JN, 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, Pappin DJ. 2004. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–1169.

Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, Chait BT. 2000. The yeast nuclear pore complex: Composition, architecture, and transport mechanism. J Cell Biol 148:635–651.

Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ. 2005. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 23:94–101.

Ruttenberg BE, Pisitkun T, Knepper MA, Hoffert JD. 2008. PhosphoScore: An open-source phosphorylation site assignment tool for MS(n) data. J Proteome Res 7:3054–3059.

Salek M, Alonso A, Pipkorn R, Lehmann WD. 2003. Analysis of protein tyrosine phosphorylation by nanoelectrospray ionization high-resolution tandem mass spectrometry and tyrosine-targeted product ion scanning. Anal Chem 75:2724–2729.

Sauer G, Korner R, Hanisch A, Ries A, Nigg EA, Sillje HH. 2005. Proteome analysis of the human mitotic spindle. Mol Cell Proteomics 4:35–43.

Saunders NF, Brinkworth RI, Huber T, Kemp BE, 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.

Savitski MM, Falth M. 2007. Peptide fragmentation and phospho-site detection. Expert Rev Proteomics 4:445–446.

Schirmer EC, Florens L, Guan T, Yates JR III, Gerace L. 2003. Nuclear membrane proteins with potential disease links found by subtractive proteomics. Science 301:1380–1382.

Schlosser A, Vanselow JT, Kramer A. 2007. Comprehensive phosphorylation site analysis of individual phosphoproteins applying scoring schemes for MS/MS data. Anal Chem 79:7439–7449.

Schmelzle K, White FM. 2006. Phosphoproteomic approaches to elucidate cellular signaling networks. Curr Opin Biotechnol 17:406–414.

Schmidt A, Kellermann J, Lottspeich F. 2005. A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5:4–15.

Schon EA. 2000. Mitochondrial genetics and disease. Trends Biochem Sci 25:555–560.

Schwartz D, Gygi SP. 2005. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat Biotechnol 23:1391–1398.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res 13:2498–2504.

Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M. 1996. Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. J Biol Chem 271:14548–14553.

Shu Y, Liu X, Yang Y, Takahashi M, Gillis KD. 2008. Phosphorylation of SNAP-25 at Ser187 mediates enhancement of exocytosis by a phorbol ester in INS-1 cells. J Neurosci 28:21–30.

Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schonfisch B, Perschil I, Chacinska A, Guiard B, Rehling P, Pfanner N, Meisinger C. 2003. The proteome of Saccharomyces cerevisiae mitochondria. Proc Natl Acad Sci USA 100:13207–13212.

Sigrist CJ, Cerutti L, Hulo N, Gattiker A, Falquet L, Pagni M, Bairoch A, Bucher P. 2002. PROSITE: A documented database using patterns and profiles as motif descriptors. Brief Bioinform 3:265–274.

Sillje HH, Nigg EA. 2006. Purification of mitotic spindles from cultured human cells. Methods 38:25–28.

Sleno L, Volmer DA. 2004. Ion activation methods for tandem mass spectrometry. J Mass Spectrom 39:1091–1112.

Smith JC, Figeys D. 2008. Recent developments in mass spectrometry-based quantitative phosphoproteomics. Biochem Cell Biol 86:137–148.

Smolka MB, Albuquerque CP, Chen SH, Schmidt KH, Wei XX, Kolodner RD, Zhou H. 2005. Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine-reactive isotope tag. Mol Cell Proteomics 4:1358–1369.

Snyder DA, Kelly ML, Woodbury DJ. 2006. SNARE complex regulation by phosphorylation. Cell Biochem Biophys 45:111–123.

Soufi B, Gnad F, Jensen PR, Petranovic D, Mann M, Mijakovic I, Macek B. 2008. The Ser/Thr/Tyr phosphoproteome of Lactococcus lactis IL1403 reveals multiply phosphorylated proteins. Proteomics 8:3486–3493.

Stasyk T, Schiefermeier N, Skvortsov S, Zwierzina H, Peranen J, Bonn GK, Huber LA. 2007. Identification of endosomal epidermal growth factor receptor signaling targets by functional organelle proteomics. Mol Cell Proteomics 6:908–922.

Steegmaier M, Klumperman J, Foletti DL, Yoo JS, Scheller RH. 1999. Vesicle-associated membrane protein 4 is implicated in trans-Golgi network vesicle trafficking. Mol Biol Cell 10:1957–1972.

Steen H, Mann M. 2002. A new derivatization strategy for the analysis of phosphopeptides by precursor ion scanning in positive ion mode. J Am Soc Mass Spectrom 13:996–1003.

Steen H, Mann M. 2004. The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol 5:699–711.

Steen H, Jebanathirajah JA, Springer M, Kirschner MW. 2005. Stable isotope-free relative and absolute quantitation of protein phosphorylation stoichiometry by MS. Proc Natl Acad Sci USA 102:3948–3953.

Steen JA, Steen H, Georgi A, Parker K, Springer M, Kirchner M, Hamprecht F, Kirschner MW. 2008. Different phosphorylation states of the anaphase promoting complex in response to antimitotic drugs: A quantitative proteomic analysis. Proc Natl Acad Sci USA 105:6069–6074.

Stein A, Weber G, Wahl MC, Jahn R. 2009. Helical extension of the neuronal SNARE complex into the membrane. Nature 460:525–528.

Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, Schulenberg B, Hendrickson J, Beechem JM, Haugland RP, Patton WF. 2003. Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. Proteomics 3:1128–1144.

Sudhof TC, Rothman JE. 2009. Membrane fusion: Grappling with SNARE and SM proteins. Science 323:474–477.

Suzuki K, Verma IM. 2008. Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation. Cell 134:485–495.

Swaney DL, McAlister GC, Wirtala M, Schwartz JC, Syka JE, Coon JJ. 2007. Supplemental activation method for high-efficiency electron-transfer dissociation of doubly protonated peptide precursors. Anal Chem 79:477–485.

Swaney DL, McAlister GC, Coon JJ. 2008. Decision tree-driven tandem mass spectrometry for shotgun proteomics. Nat Methods 5:959–964.

Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. 2004. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci USA 101:9528–9533.

Sztul E, Lupashin V. 2006. Role of tethering factors in secretory membrane traffic. Am J Physiol Cell Physiol 290:C11–C26.

Takahashi M, Itakura M, Kataoka M. 2003. New aspects of neurotransmitter release and exocytosis: Regulation of neurotransmitter release by phosphorylation. J Pharmacol Sci 93:41–45.

Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, Watts JD, Hood L, Aebersold R. 2005. Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. Nat Methods 2:591–598.

Taouatas N, Drugan MM, Heck AJ, Mohammed S. 2008. Straightforward ladder sequencing of peptides using a Lys-N metalloendopeptidase. Nat Methods 5:405–407.

Taylor AM, Holst O, Thomas-Oates J. 2006. Mass spectrometric profiling of O-linked glycans released directly from glycoproteins in gels using in-gel reductive beta-elimination. Proteomics 6:2936–2946.

Thingholm TE, Jorgensen TJ, Jensen ON, Larsen MR. 2006. Highly selective enrichment of phosphorylated peptides using titanium dioxide. Nat Protoc 1:1929–1935.

Thingholm TE, Jensen ON, Robinson PJ, Larsen MR. 2008a. SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. Mol Cell Proteomics 7:661–671.

Thingholm TE, Larsen MR, Ingrell CR, Kassem M, Jensen ON. 2008b. TiO2-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. J Proteome Res 7:3304–3313.

Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. 2003a. Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75:1895–1904.

Thompson AJ, Hart SR, Franz C, Barnouin K, Ridley A, Cramer R. 2003b. Characterization of protein phosphorylation by mass spectrometry using immobilized metal ion affinity chromatography with on-resin beta-elimination and Michael addition. Anal Chem 75:3232–3243.

Tian JH, Das S, Sheng ZH. 2003. Ca2+-dependent phosphorylation of syntaxin-1A by the death-associated protein (DAP) kinase regulates its interaction with Munc18. J Biol Chem 278:26265–26274.

Tran TH, Zeng Q, Hong W. 2007. VAMP4 cycles from the cell surface to the trans-Golgi network via sorting and recycling endosomes. J Cell Sci 120:1028–1041.

Trinidad JC, Specht CG, Thalhammer A, Schoepfer R, Burlingame AL. 2006. Comprehensive identification of phosphorylation sites in postsynaptic density preparations. Mol Cell Proteomics 5:914–922.

Trost M, English L, Lemieux S, Courcelles M, Desjardins M, Thibault P. 2009. The phagosomal proteome in interferon-gamma-activated macrophages. Immunity 30:143–154.

Tsumoto H, Ra M, Samejima K, Taguchi R, Kohda K. 2008. Chemical derivatization of peptides containing phosphorylated serine/threonine for efficient ionization and quantification in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 22:965–972.

Tsybin YO, Hakansson P, Budnik BA, Haselmann KF, Kjeldsen F, Gorshkov M, Zubarev RA. 2001. Improved low-energy electron injection systems for high rate electron capture dissociation in Fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun Mass Spectrom 15:1849–1854.

Ulintz PJ, Yocum AK, Bodenmiller B, Aebersold R, Andrews PC, Nesvizhskii AI. 2008. Comparison of MS(2)-only, MSA, and MS(2)/MS(3) methodologies for phosphopeptide identification. J Proteome Res 8:887–899.

Ungar D, Hughson FM. 2003. SNARE protein structure and function. Annu Rev Cell Dev Biol 19:493–517.

Villen J, Beausoleil SA, Gerber SA, Gygi SP. 2007. Large-scale phosphorylation analysis of mouse liver. Proc Natl Acad Sci USA 104:1488–1493.

Villen J, Beausoleil SA, Gygi SP. 2008. Evaluation of the utility of neutral-loss-dependent MS3 strategies in large-scale phosphorylation analysis. Proteomics 8:4444–4452.

Villen J, Gygi SP. 2008. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. Nat Protoc 3:1630–1638.

Virshup DM, Shenolikar S. 2009. From promiscuity to precision: Protein phosphatases get a makeover. Mol Cell 33:537–545.

von Mering C, Hugenholtz P, Raes J, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P. 2007. Quantitative phylogenetic assessment of microbial communities in diverse environments. Science 315:1126–1130.

Wang Y, Chen W, Wu J, Guo Y, Xia X. 2007. Highly efficient and selective enrichment of phosphopeptides using porous anodic alumina membrane for MALDI-TOF MS analysis. J Am Soc Mass Spectrom 18:1387–1395.

Wells JM, McLuckey SA. 2005. Collision-induced dissociation (CID) of peptides and proteins. Methods Enzymol 402:148–185.

Wickner W, Schekman R. 2008. Membrane fusion. Nat Struct Mol Biol 15:658–664.

Wolschin F, Wienkoop S, Weckwerth W. 2005. Enrichment of phosphorylated proteins and peptides from complex mixtures using metal oxide/hydroxide affinity chromatography (MOAC). Proteomics 5:4389–4397.

Xu X, Sarikas A, Dias-Santagata DC, Dolios G, Lafontant PJ, Tsai SC, Zhu W, Nakajima H, Nakajima HO, Field LJ, Wang R, Pan ZQ. 2008. The CUL7 E3 ubiquitin ligase targets insulin receptor substrate 1 for ubiquitin-dependent degradation. Mol Cell 30:403–414.

Yao PJ. 2004. Synaptic frailty and clathrin-mediated synaptic vesicle trafficking in Alzheimer's disease. Trends Neurosci 27:24–29.

Yao X, Freas A, Ramirez J, Demirev PA, Fenselau C. 2001. Proteolytic 18O labeling for comparative proteomics: Model studies with two serotypes of adenovirus. Anal Chem 73:2836–2842.

Yates JR III, Gilchrist A, Howell KE, Bergeron JJ. 2005. Proteomics of organelles and large cellular structures. Nat Rev Mol Cell Biol 6:702–714.

Zappacosta F, Annan RS. 2004. N-terminal isotope tagging strategy for quantitative proteomics: Results-driven analysis of protein abundance changes. Anal Chem 76:6618–6627.

Zhang G, Spellman DS, Skolnik EY, Neubert TA. 2006. Quantitative phosphotyrosine proteomics of EphB2 signaling by stable isotope

labeling with amino acids in cell culture (SILAC). J Proteome Res 5:581–588.

Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR, Polakiewicz RD, Comb MJ. 2002a. Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. J Biol Chem 277:39379–39387.

Zhang X, Jin QK, Carr SA, Annan RS. 2002b. N-Terminal peptide labeling strategy for incorporation of isotopic tags: A method for the determination of site-specific absolute phosphorylation stoichiometry. Rapid Commun Mass Spectrom 16:2325–2332.

Zhang X, Ye J, Jensen ON, Roepstorff P. 2007. Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. Mol Cell Proteomics 6:2032–2042.

Zhang Y, Wolf-Yadlin A, Ross PL, Pappin DJ, Rush J, Lauffenburger DA, White FM. 2005. Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. Mol Cell Proteomics 4:1240–1250.

Zhou H, Watts JD, Aebersold R. 2001. A systematic approach to the analysis of protein phosphorylation. Nat Biotechnol 19:375–378.

Zhou H, Tian R, Ye M, Xu S, Feng S, Pan C, Jiang X, Li X, Zou H. 2007. Highly specific enrichment of phosphopeptides by zirconium dioxide nanoparticles for phosphoproteome analysis. Electrophoresis 28:2201–2215.

Zischka H, Lichtmannegger J, Jagemann N, Jennen L, Hamoller D, Huber E, Walch A, Summer KH, Gottlicher M. 2008. Isolation of highly pure rat liver mitochondria with the aid of zone-electrophoresis in a free flow device (ZE-FFE). Methods Mol Biol 424:333–348.

Zubarev RA, Kelleher NL, McLafferty FW. 1998. Electron capture dissociation of multiply charged protein cations. J Am Chem Soc 120:3265–3266.

Zwilling D, Cypionka A, Pohl WH, Fasshauer D, Walla PJ, Wahl MC, Jahn R. 2007. Early endosomal SNAREs form a structurally conserved SNARE complex and fuse liposomes with multiple topologies. EMBO J 26: 9–18.

Matthias Trost received his Ph.D. degree in Cellular Microbiology and Proteomics from the Helmholtz Centre for Infection Research in Braunschweig, Germany, in 2004 under the supervision of Prof. Jürgen Wehland where he studied the functional genome analysis of secretory proteins and host–pathogen interactions of the human pathogen *Listeria monocytogenes*. He is currently a post-doctoral fellow with Pierre Thibault and Michel Desjardins at the Institute for Research in Immunology and Cancer of the Université de Montréal. His research interests include proteome and phosphoproteome analyses of organelles in macrophages and leukemic stem cells.

Gaëlle Bridon obtained her M.Sc. degree in biochemistry and proteomics from the Université des Sciences et Technologies de Lille, France working with Prof. Michel Salzet. She is presently conducting her Ph.D. degree at the biochemistry department and the Institute for Research in Immunology and Cancer of the Université de Montréal. Her thesis is centered on the further understanding of signaling pathways involved in innate immunity using phosphoproteomics.

Michel Desjardins obtained his Ph.D. degree in anatomy cell biology in 1991 from the Université de Montréal and his post-doctoral training in cell biology at the European Molecular Biology Laboratories in Heidelberg, Germany. He is a professor at the department of pathology and cell biology of the Université de Montréal since 1994. His laboratory pioneered the use of proteomics to unravel the molecular machines associated with phagosomes and decipher the fine details of the mechanisms governing the functions of this cellular organelle. His research program provides further understanding of the molecular machineries regulating phagolysosome biogenesis to facilitate the development of novel therapeutic approaches against infectious diseases.

Pierre Thibault received his Ph.D. degree in analytical chemistry from the Université de Montréal in 1988 and conducted his post-doctoral fellowship at the Institute for Marine Biosciences of the National Research Council in Halifax, Canada. He is a bioanalytical chemist specializing in mass spectrometry and proteomics with more than 20 years experience as a principal investigator in academic, government and industry laboratories. Since 2004, he is a professor at the department of Chemistry, and a principal investigator at the Institute for Research in Immunology and Cancer of the Université de Montréal where he also directs the Proteomics Core Facility. His research program focuses on the development and implementation of new technologies in proteomics and cell biology to provide a deeper understanding of molecular mechanisms and post-translational modifications regulating the function and translocation of proteins involved in immunity and in signaling of cancer cells.