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Functional proteomics to identify critical proteins in signal transduction pathways

Review Article

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Summary. Reversible protein phosphorylation plays a crucial role in the regulation of signaling pathways that control various biological responses, such as cell growth, differentiation, invasion, metastasis and apoptosis. Proteomics is a powerful research approach for fully monitoring global molecular responses to the activation of signal transduction pathways. Identification of different phosphoproteins and their phosphorylation sites by functional proteomics provides informational insights into signaling pathways triggered by all kinds of factors. This review summarizes how functional proteomics can be used to answer specific questions related to signal transduction systems of interest. By examining our own example on identifying the novel phosphoproteins in signaling pathways activated by EB virus-encoded latent membrane protein 1 (LMP1), we demonstrated a functional proteomic strategy to elucidate the molecular activity of phosphorylated annexin A2 in LMP1 signaling pathway. Functional profiling of signaling pathways is promising for the identification of novel targets for drug discovery and for the understanding of disease pathogenesis.

Keywords: Proteomics – Phosphoproteomics – Signal transduction – Phosphorylation

Abbreviations: IMAC, immobilized metal affinity chromatography; LMP1, latent membrane protein 1; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMAC, phosphate metal affinity chromatography

1. Introduction

Signal transduction events involve the transmission and amplification of signals from transmembrane receptors to the nucleus. One major conduit of information is mediated via reversible phosphorylation of proteins. Protein phosphorylation and dephosphorylation are regulated by a balanced activity of protein kinases and protein phosphatases. Often, phosphorylation acts as a molecular switch controlling the activities of signaling molecules and downstream target proteins in signal transduction pathways. Phosphorylation can affect the function of a protein in many ways by increasing or decreasing its activity, mediating translocation between subcellular compartments, initiating/disrupting protein–protein interaction, or affecting its stabilization in signaling pathways (Cans et al., 2000). Phosphorylation is thus involved in the control of a wide range of cellular processes such as cell proliferation, differentiation, apoptosis, adhesion and motility and gene transcription. Not surprisingly, disregulated phosphorylation has been regarded as a reason for a variety of human diseases, such as cancer, diabetes, cardiovascular disease, and so on. Especially, the disregulation of protein kinases due to overexpression, constitutive activation and autocrine stimulation is frequently implicated in the initiation of carcinogenesis (Hunter, 2000).

Signal molecules in signaling pathways including protein kinases, receptors, and transcriptional factors are major targets of drug discovery. Researches in this field have resulted in the development of several inhibitors as targeted therapeutics in the past years. For example, the mAb herceptin inhibits the receptor of tyrosine kinase Her2 and is used for the treatment of breast cancer. The small molecule inhibitor, Gleevec, targets BCR-Abl, c-kit, and the platelet-derived growth factor (PDGF) receptor and thus is used for the treatment of chronic myelogenous leukemia (de Graauw et al., 2006). These successful examples demonstrated that identification of components in activated signaling pathways related to the initiation of

diseases may lead to the discovery of novel drug targets or the identification of disease biomarkers.

Identification of phosphorylated proteins is important in order to comprehensively analyze the signaling transduction pathways triggered by various environmental factors. Signal transduction pathways have traditionally been elucidated by identifying the receptor, kinase, and substrate, one by one, and then by individually establishing the connections between them. However, traditional approaches using single readout assays are insufficient to comprehensively describe signaling targets. Thus, systematic strategies are needed in order to globally analyze the network of signaling pathways. Technically, proteomics is a powerful method for monitoring global molecular responses following the activation of signal transduction pathways. However, given the fact that most signaling molecules or downstream target proteins, especially phosphorylated molecules, are usually present in low abundance in cells, basic proteomics may not be sensitive enough for identifying these components in signaling pathways. To address this problem, a more specific, proteome-based analytic approach termed as signalomics (Kenneth, 2002; Hirodshi, 2003) has been proposed. It is a part of functional proteomics, aiming to analyze the global changes of signaling pathways and related signaling molecules. Considering the fact that most components in signaling pathways demonstrate their functions by phosphorylation, investigators in the field also used phosphoproteomics to describe the specific research applying integrated proteomics to explore signal transduction in various biological systems.

To identify the critical proteins in the signal transduction pathways, functional proteomics combined with complementary techniques such as phosphoprotein enrichment may be necessary and important in order to reduce the background and increase the sensitivity of an assay. Phosphoprotein enrichment integrated into proteomics obviously will in many cases be more efficient than other basic systematic approaches in order to study specific cellular signaling networks. In this article, we attempt to give a concise overview of current research strategies in the field of phosphoproteomics. By providing an example in our own research, we demonstrated how phosphoproteomics techniques can be actually used to identify the components in signaling pathways.

2. Strategies for studying signal transduction by functional proteomics

To study signal transduction, experiments are often designed to activate or inhibit specific signaling pathways in order to identify the signaling components and to elucidate the regulation of the signaling pathways. For example, signaling pathways can be activated by treatment of cells with various factors including growth factors, hormones, oncoproteins, and so on. Some specific signaling pathways can be inhibited by drugs, small specific molecules, and RNA interference. Traditionally, proteomic studies in the field of signal transduction centered on the quantitation of differences in protein expression between two or more samples. However, a comparison of protein expression levels only provides one aspect of signal regulation, and it is not likely that most of low abundance signal molecules (mainly phosphoproteins) are detected. Therefore, there is increasing interest in phosphoproteomics as presented by the number of publications describing various strategies to identify phosphoproteins and their phophorylation sites in a global fashion. Here, we summarize recent research strategies for the identification of novel phosphoproteins in signaling pathways.

2.1 ^{32}P radiolabeling

^{32}P labeling is a classical tool for the initial screening of changes in phosphorylation. The technique is highly selective and sensitive, offering a way to easily quantify phosphorylation level of a protein. It is a fine and valuable method for the identification of novel phosphoproteins in signaling pathways. In this approach, cells are labeled in vivo with ^{32}P-orthophosphate, and then cell extracts are prepared. The cell extracts are applied to the analysis of pre-immunoprecipitated and separated signaling complexes for quantification of differentially phosphorylated proteins using SDS-PAGE or 2DE (Immler et al., 1998; Lasern et al., 2001). Alternatively, radiolabeled extracts are fractionated by ion-exchange chromatography, and then each fraction is analyzed by SDS-PAGE or 2DE. Protein phosphorylation is visualized by autoradiography. Proteins that undergo changes in phosphorylation level in response to a biological condition are sequenced and identified by MS and MS/MS. It must be pointed out that metabolically incorporated radiolabeling using ^{32}P or ^{33}P with standard doses and time-course induces DNA fragmentation, elevates p53 tumor suppressor protein expression, alters cell/nuclear morphology and results in cell cycle arrest or apoptosis (Yeargin and Hass, 1995; Hu and Heikka, 2000; Hu et al., 2001). In other words, ^{32}P radiolabeling in vivo may change phosphorylation events in cell. In addition, radiolabeling of phosphoproteins presents challenges to the high-throughput automation of pro-

teomic investigation, due to safety issues associated with handling the materials and the contamination of instrumentation (Conrads et al., 2002).

2.2 Protein kinase profiling

Protein kinase profiling is a way to identify protein kinases and their substrates under certain biological conditions. In this strategy, a cell extract is fractionated by ATP-Sepharose or ion-exchange chromatography and each fraction is incubated with kinase buffer containing γ-^{32}P-ATP to allow labeling of phosphoproteins (Haystead et al., 1993). Individual fractions are resolved by SDS-PAGE or 2DE and proteins that undergo changes in phosphorylation level are selected by autoradiogaphy and then identified by MS. Alternatively, the protein fractions can be applied to γ-^{32}P-ATP-Sepharose to isolate protein kinases (MacDonald et al., 2000, 2001). The most significant disadvantage of the strategy is that the identified phosphoproteins or/and their phosphorylation sites must be subsequently confirmed by in vivo studies, because the phosphorylation is conducted in vitro.

2.3 Phospho-specific immunoprecipitation

A major obstacle of identifying the phosphoproteins in signaling pathways is that phosphoproteins comprise only a small part of the total proteins in a cellular extract. As a result, enrichment of phosphoproteins prior to the proteomic identification is necessary and important, in order to reduce the background and increase the sensitivity of an assay. To enrich phosphoproteins from the total proteins, tyrosine-phosphorylation-specific antibody or serine/threonine-phosphorylation antibody were often applied to immunoprecipitate the tyrosine phosphoproteins or serine/threonine phosphoproteins, respectively. These immunoprecipitated phosphoproteins were gel separated (SDS-PAGE or 2DE), stained with sliver or Coomassie blue, and then analyzed based on their different expressions under different conditions. For example, some researchers used this approach to compare the differences of phosphoproteins in cells treated and untreated with certain factors including EGF, PDGF, IFN, IL-2, and FGFR-1, and the altered protein spots were subsequently identified by MALDI-TOF MS or LC-MS/MS (Lewis et al., 2000; Pandey et al., 2000; Stancato and Petricoin, 2001; Kim et al., 2002; Steen et al., 2002; Hinsby et al., 2003; Yeung and Stanley, 2003). These experiments demonstrated that immunoprecipitation in conjunction with MS analysis can be well utilized to identify the phosphoproteins in signaling pathways. Nevertheless, although high-quality specific antibodies to phosphotyrosine residues are commercially available, antibodies to phosphoserine and phosphothreonine generally do not have sufficient specificity, sensitivity, and affinity for immunoprecipitation. Most studies have therefore focused on the analysis of tyrosine-phosphorylated proteins involved in signaling pathways.

2.4 SH2 profiling

Protein domains such as SH2, SH3, PDZ, PTB, and 14-3-3 mode play critical roles in the propagation of signals in cells, mediating the relocalization and complex formation of proteins in response to changes in tyrosine phosphorylation. As an alternative strategy to enrich phosphorylated proteins, phosphoprotein-binding motifs can also be employed to purify phosphoproteins from cellular extracts (Yaoi et al., 2006). Using a glutathione S-transferase (GST)-Grb2 SH2 domain fusion, Blagoev et al. (2003) identified 228 proteins from EGF-stimulated cells, in which 28 were specifically induced by EGF stimulation. Machida et al. (2003) also developed an SH2 profiling method based on far-Western blotting, in which a battery of SH2 domains was used to probe the global state of tyrosine phosphorylation in biological systems. SH2 profiling is a sensitive and reasonably rapid method for obtaining a fingerprint of the overall pattern of tyrosine phosphorylation, useful in characterizing qualitative and quantitative differences in tyrosine phosphorylation patterns between samples. Further developments of this method will increase sensitivity and allow its application to small amounts of samples or archived clinical samples.

2.5 Phospho-specific immunoblotting with 2DE

Combining phospho-specific antibody immunoblotting with 2DE provides an alternative strategy to detect phosphorylated proteins blotted onto a membrane. This method is sensitive since antibodies can detect as little as a few fmol of phosphorylated proteins. Phosphoproteins can be identified by MS analysis of gel spots excised from a reference gel that was stained by silver or Coomassie blue, which correspond to spots detected by immunoblotting with anti-phosphorylation (tyrosine or serine/threonine) antibodies. Soskic et al. (1999) combined tyrosine phospho-specific antibody and serine phospho-specific antibody immunoblotting with 2DE to elucidate signaling pathways of PDGF β receptor. About 260 and

300 phosphorylated proteins were detected with anti-phosphotyrosine and anti-phosphoserine antibodies, respectively, at least 100 of which showed prominent changes in phosphorylation as a function of time after PDGF stimulation.

Although this approach is sensitive for phosphoprotein discovery, in many cases the amount of a protein in a spot is still not sufficient for the identification of the protein and its phosphorylation sites. Moreover, similar to the situation with ^{32}P-radiolabeling, this method also suffers from the potential identification of false positives. It has recently been shown that tyrosine-phosphorylation antibody can be used to immuno-purify tyrosine-phosphorylated peptides. Therefore, immunoprecipitation could be used as a valuable tool for further enrichment of the tyrosine phosphorylated peptides, and then decreasing the false positive rate in identification. Nevertheless, as for phospho-specific immunoprecipitation, high-quality specific antibodies to phosphoserine and phosphothreonine residues are not commercially available at present.

2.6 Immobilized metal affinity chromatography (IMAC)

In order to decrease sample complexity prior to the analysis by LC-MS/MS, one of the commonly used methods to enrich phosphoryalted proteins or phosphopeptides is immobilized metal affinity chromatography (IMAC) using gallium- or iron-treated affinity columns that can selectively bind to the negatively charged phosphate groups. Recently, IMAC has been combined with LC-MS/MS to identify phosphoproteins and their phosphorylation sites from cell extracts (Posewitz and Tempst, 1999; Cao and Stults, 2000; Xhou et al., 2000; Raska et al., 2002). However, IMAC also binds nonphosphorylated acidic peptides through its carboxylate groups (Mann et al., 2002). To reduce this background binding, Ficarro et al. (2002) modified the carboxyl groups by methylation prior to IMAC, thereby improving the detectability of the phosphopeptides in subsequent MS analysis. Starting with only 500 μg of yeast proteins, more than a thousand phosphopeptides were detected and 383 phosphorylation sites in 216 peptides were identified, showing an excellent sensitivity and high coverage. Besides, Stensballe et al. (2001) also reported that custom-made nanoscale Fe(III)-IMAC columns in combination with 2DE increased the identification rate of phosphorylation sites. The only disadvantage of IMAC technique is that the differences in quantity of phosphoproteins between samples cannot be measured.

As an alternative to IMAC, strong cation exchange can also separate phosphopeptides from non-phosphorylated tryptic peptides on the basis of the charge differences associated with the negatively charged phosphate groups. This enrichment technique either alone or in combination with IMAC has been utilized to fractionate samples before LC-MS/MS analysis (Beausoleil et al., 2004; Gruhler et al., 2005). However, this technique tends to be less specific than IMAC in the enrichment of esterification-modified phosphopeptides.

2.7 Phosphate metal affinity chromatography (PMAC)

Another technique similar to IMAC, Phosphate Metal Affinity Chromatography (PMAC) can be employed to selectively bind a phosphate group on any amino acid including serine, tyrosine, or threonine, so that phosphorylated proteins can be enriched from cellular extracts. It has been demonstrated that phosphorylated proteins and nonphosphorylated proteins were almost completely separated using PMAC. Our experiments combined PMAC with proteomic technique to analyze signaling pathways triggered by latent membrane protein 1 (LMP1), in which 25 signaling molecules and downstream targets of LMP1 were identified and several of them had been implicated in LMP1 signal pathways (Yan et al., 2006a). In comparison, PMAC is usually used to enrich phosphorylated proteins prior to proteomic analysis, while IMAC is mainly used to enrich phosphorylated peptides prior to MS analysis. Similarly, PMAC may also bind non-phosphorylated acidic proteins and thus result in false positives.

2.8 Chemical derivatization and purification

This approach is taking advantage of the unique chemistry of phospho-amino acids in peptides to enrich phosphorylated proteins and peptides. Some specific modifications have been developed for tagging of the phosphate groups. One strategy requires proteolytic digestion of the samples, reduction and alkylation of cysteine residues, N- and C-terminal protections of the peptides, formation of phosphoramidate adducts at phosphorylated residues by carbodimide, condensation with cystamine, capture of the phosphopeptides on glass beads coupled to iodoacetate, elution with trifluoroacetic acid and evaluation by mass spectrometry. Zhou et al. (2001) have demonstrated that the method is equally applicable to enrichment of serine-, threonine- and tyrosine-phosphorylated proteins, and is capable of selectively isolating and identifying phospho-

peptides present in a highly complex peptide mixture. Another strategy involves oxidation of cysteine residues with performic acid, alkaline hydrolysis to induce β-elimination of phosphate groups from phosphoserine- and phosphothreonine-residues, addition of ethanedithiol, and coupling of the resulting free sulfhydryl residues with biotin. These biotin groups can then be used as affinity handles in enrichment by immobilized avidin of the phosphoproteins from the complex mixtures of proteins. The enriched phosphoprotein fractions can be separated by SDS-PAGE or 2DE, and individual phosphoproteins can be identified by MS. Alternatively, the entire protein mixtures were digested by trypsin, and then the biotinylated peptides were enriched with immobilized avidin and identified by LC-MS/MS (Oda et al., 2001).

The main disadvantage of these two methods is that not only phosphate groups but also O-glycans can be removed during β-elimination reaction, resulting in false-negative identification of phosphorylations. Furthermore, phosphotyrosine is not dephosphorylated via β-elimination.

2.9 Phosphoprotein-specific gel staining

Pro-Q Diamond phosphoprotein gel staining is a novel fluorescence-based detection system suitable for specifically and sensitively monitoring the changes in protein phosphorylation directly in SDS-PAGE or 2DE gel. Gels are fixed and stained by a simple incubation in a single solution, and then destained and visualized using a laser-based gel scanner or xenon-arc lamp-based gel scanner. The stain binds noncovalently to phosphoproteins and is thus fully compatible with MS techniques, allowing protein identification after gel electrophoresis. Pro-Q Diamond can easily be combined with total protein staining (e.g. SYPRO Ruby), making quantification of differential phosphorylation easier and allowing protein phosphorylation level and expression level to be monitored in the same gel (Steinberg et al., 2003). The staining technique is also suitable for the detection of phosphorylated proteins and peptides in protein microarrays. However, similar to ^{32}P-radiolabeling technique, the Pro-Q Diamond staining technique does not discriminate tyrosine, serine and threonine, necessitating additional analysis.

3. Phosphoproteomics to identify critical components in LMP1 signaling pathways

Alteration in cellular phosphorylation is a major mechanism for the flow of information from the outside of a cell to the inside. Signal transduction pathways have traditionally been elucidated by identifying the receptor, kinase, and substrate one by one, and then by individually establishing the connections between them. However, the signal transduction pathways comprise a complex network, and the traditional approaches using single readout assays are insufficient to comprehensively describe the entire signaling network. Systematic approaches are thus needed in order to globally analyze the network of signaling pathways. We are interested in establishing methods that would allow to sensitively identify the signaling molecules in a signal transduction system of interest. Our research combined phosphoprotein enrichment with proteomic technique to elucidate signaling pathways triggered by Epstein-Barr virus-encoded LMP1 (Yan et al., 2006a).

The oncogenetic effects of LMP1 can be explained by its ability to constitutively activate nuclear factor kappa B (NFκB), activator protein-1 (AP-1), janus kinase 3 (JAK3), signal transducer and activator of transcription (STAT), p38/MAP, and Ras-mitogen-activated protein kinase (MAPK) (Roberts and Cooper, 1998; Eliopoulos et al., 1999; Gries et al., 1999; Atkinson et al., 2003; Luftig et al., 2003; Saito et al., 2003). However, many signaling molecules and downstream target proteins that could be affected by the oncoprotein LMP1 in epithelial cells, particularly NPC cells, have not been identified thus far. Phosphoproteomics is a powerful strategy to determine the functional components in the signaling cascade activated by LMP1 (Yan et al., 2006a).

In the first attempt, we used phosphatase inhibitors (NaF and Na_3VO_4) combined with PMAC to enrich phosphoproteins. Our results showed that NaF and Na_3VO_4 increased the phosphorylation level of proteins in a dose- and time-dependent manner. But we further found that the phosphorylation level of proteins was not different between nasopharyngeal cancer cells (CNE1) and the cancer cells incorporated with LMP1 (CNE1-LMP1) after treatment with the phosphatase inhibitors, suggesting that the phosphatase inhibitors had a disadvantageous effect on the signaling pathways triggered by LMP1. When PMAC was solely used to enrich phosphorylated proteins from the cellular extract, phosphorylated proteins and nonphosphorylated proteins were almost completely separated. Therefore, we combined PMAC with proteomics to identify phosphoproteins in LMP1 signaling pathways. Our data showed that LMP1 could increase the quantity of total phosphoproteins by 18%, and that 43 proteins exhibited significant changes in the degree of phosphorylation when LMP1 was expressed. Twenty-five signaling mole-

cules or downstream targets of signaling pathways triggered by LMP1 were identified, several of which had been implicated in LMP1 signal pathways. The other proteins, including annexin A2 (Gould et al., 1986; Johnsson et al., 1986), heat shock protein 27 (Satoh and Kim, 1995; Dorion et al., 1999; Paine et al., 2000; Park et al., 2003), stathmin (Sobel, 1991; Drouva et al., 1998), annexin I (Katoh, 2002; Radke et al., 2004), basic transcription factor 3 (Grein and Pyerin, 1999), and porin (Mizuno and Mizushima, 1990), were signaling molecules or targets with no previously known function in LMP1 signal transduction.

Identification of protein phosphorylation without knowing the function has little meaning. To understand the molecular mechanism involved in the phosphorylation of these proteins, we further analyzed the relationship between LMP1 and these phosphoproteins by traditional assay of signal transduction (Yan et al., 2006b). We found that LMP1 increased the serine, but not tyrosine, phosphorylation of annexin A2 by activating another signaling pathway, the protein kinase C (PKC) signaling pathway. LMP1 did not affect the expression level of annexin A2 but induced the nuclear entry of annexin A2 in an energy- and temperature-dependent manner, suggesting that the nuclear entry of annexin A2 is an active process in LMP1-related signal transduction. Treatment of LMP1-expressing cells with the PKC inhibitor myr-ψPKC resulted in annexin A2 being present almost exclusively at cell surface instead of in the nucleus, implicating that the nuclear entry of annexin A2 is associated with serine phosphorylation mediated by PKC.

Based on our research results, the role of annexin A2 involving the LMP1 signaling pathway can be envisaged as follows. Annexin A2 in heterotetramer is phosphorylated by PKC after PKC activation mediated by LMP1; the phosphorylated annexin A2 is then released from the membrane, and annexin A2 becomes dissociated from the heterotetramer. Phosphorylated annexin A2 may then enter the nucleus. The phosphorylated annexin A2 monomer participates in DNA synthesis and mRNA transport. While such a process is speculative at present, this initial discovery that LMP1 can mediate annexin A2 phosphorylation and translocation offers new insights into the LMP1 oncogenic mechanism.

Clearly, the phosphoproteomics method employed here has proven to be suitable for the identification of critical molecules involved in signaling pathways, providing a starting point to construct signaling network concerned for answering key biological questions. We believe that the phosphoproteomic approach could be used to analyze similar signaling pathways.

4. Perspectives

4.1 Combination strategies

Systematic characterization of phosphoproteins obviously requires a combination of selective, specific and sensitive analytical techniques. The research strategies introduced above have been widely used in the field of phosphoproteomics for identifying components in signaling pathways. However, these strategies have their drawbacks respectively. Proteomics combined with a pre-enrichment technology remains a powerful methodology to analyze changes in protein phosphorylation level and their phosphorylation sites. However, more sensitive and specific strategies are needed in the coming years. By integrating specific enrichment techniques into functional proteomics, it is possible to not only identify the differential phosphoproteins but also characterize their phosphorylation sites and determine their time-dependent kinetic profiles.

4.2 Dynamic changes of phosphorylation and phosphorylation sites

It is well known that most proteins have more than one potential phosphorylation site, and phosphorylation/dephosphorylation of different sites in the same protein may lead to different responses. For instant, an activation event may involve a particular protein, but specific phosphorylation sites regulate the cellular activity. Quantitative, time-resolved analysis of phosphorylated molecules will be critical for a more complete understanding of the molecular mechanisms underlying a variety of disease states. So far, most studies in the literature dealt with either the activations of a handful of proteins and phosphorylation sites (Irish et al., 2004; Krutzik et al., 2004) or the global identification of protein phosphorylation sites under static conditions (Salomon et al., 2003; Brill et al., 2004). A dynamic relationship between component proteins in signaling pathways and site- and time-specific phosphorylation events is important in eliciting the desired molecular mechanisms. As a successful example, Zhang et al. (2005) utilized a combination strategy to analyze EGFR signaling pathway, demonstrating a phosphorylation profile for 78 tyrosine phosphorylation sites on 58 proteins at four time points of EGF stimulation in a single analysis, and thus revealing a dynamic module of EGFR signaling pathway.

4.3 Tissue sample and phosphorylation

Phosphorylation studies to date mainly used cell lines to analyze the signaling pathways. The next step toward the

target identification and the understanding of the pathophysiology of diseases would be the profiling of the phosphoproteomes of human tissues. However, the materials are often scarce and not fresh, and the cellular compositions of the diseased tissues can be rather heterogeneous. Even so, some studies have applied phosphoproteomics to clinical specimens to test the feasibility in generating the information needed (Lim et al., 2004). Another way to study phosphorylation changes in diseases is to link experimental data obtained with cell lines or freshly isolated cells to clinical data including disease outcome and/or drug responses. Alternatively, human tissue arrays can be stained with phospho-specific antibodies based on the data obtained from the studies with cell lines, in order to identify disease-specific kinases and/or downstream substrates as novel drug targets.

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