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Clinical Applications of Phosphoproteomics: Illuminating Cancer Signaling and Enabling Rational Therapeutic Strategies

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ABSTRACT

Protein phosphorylation is a central post-translational modification regulating cellular signaling, frequently dysregulated in cancer. Mass spectrometry (MS)-based phosphoproteomics has emerged as a powerful approach to systematically profile phosphorylation events, thereby revealing aberrant kinase activity and therapeutic vulnerabilities that are not captured by genomic or transcriptomic analyses. Recent advances across the workflow—including optimized sample preparation and phosphopeptide enrichment, isotope- or label-free quantitative strategies, high-resolution mass spectrometry platforms, specialized algorithms for site identification and quantification, and integrative informatics analyses—have enabled the detection of tens of thousands of phosphorylation sites even from small clinical specimens. These developments have facilitated the characterization of signaling pathways across diverse cancer types, leading to the identification of targetable kinases and informing therapeutic strategies. In this review, we highlight studies that employed phosphoproteomic analyses of clinical specimens or patient-derived cancer cells to delineate signaling characteristics and to propose and validate therapeutic targets. Collectively, MS-based phosphoproteomics is poised to become a cornerstone of precision oncology. By enabling comprehensive and quantitative mapping of phosphorylation events, this technology allows mechanistic dissection of cancer signaling pathways and uncovers therapeutic vulnerabilities that may be exploited with targeted agents.

Abbreviations: ABL, abelson murine leukemia viral oncogene homolog; AML, acute myeloid leukemia; AXL, anexelekto receptor tyrosine kinase; CML, chronic myeloid leukemia; CPTAC, clinical proteomic tumor analysis consortium; CRC, colorectal cancer; DDA, data-dependent acquisition; DIA, data-independent acquisition; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; ETD, electron transfer dissociation; EThcD, electron transfer/higher energy collision dissociation; FDA, U.S. Food and Drug Administration; FT-ICR, fourier transform ion cyclotron resonance; HCD, higher-energy collisional dissociation; IMAC, immobilized metal ion affinity chromatography; IMS, ion mobility separation; iTRAQ, isobaric tags for relative and absolute quantification; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LFQ, label-free quantification; MOAC, metal oxide affinity chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; mTOR, mechanistic target of rapamycin; NAT, normal adjacent tissue; PAK1, p21-activated kinase 1; PASEF, parallel accumulation–serial fragmentation; PDAC, pancreatic ductal adenocarcinoma; PDX, patient-derived xenograft; PI3K, phosphoinositide 3-kinase; PRM, parallel reaction monitoring; Rb, retinoblastoma protein.

1 | Introduction

Protein phosphorylation is one of the most common and best-studied post-translational modifications involved in regulating key biological processes in normal and cancer cells. Understanding these dynamic changes is crucial across a wide range of fields, from fundamental life sciences to disease research and drug discovery. Dysregulation of kinase signaling pathways is commonly associated with various cancers [1–3]. Thus, research on the cancer phosphoproteome provides unique biological information regarding the activity of signaling pathways and the circuitry of kinase networks that are not captured by genetic or transcriptomic technologies. These studies have not only advanced cancer biology but also contributed to the development of cancer therapies. Abnormalities in signal transduction within cancer cells serve as targets for cancer treatment. Pioneering examples of precision medicine in cancer treatment based on aberrant signaling include the use of imatinib for chronic myeloid leukemia (CML) [4, 5] and erlotinib for non-small cell lung cancer [6, 7]. These drugs inhibit BCR-ABL1 and EGFR tyrosine kinase, respectively. It is reported that the number of small molecule protein kinase inhibitors currently approved by the FDA has reached 85 drugs which primarily target 38 protein kinases [8], while humans have over 500 protein kinases [9]. It is reported that over 300 kinases have crystal structures, and more than 400 have at least one assay, while only 24% of kinases have high-quality chemical probes [10]. These findings indicate that for small-molecule inhibitors, the lack of tools limits biological exploration and target validation. However, development of inhibitors based on novel modalities such as PROTACs (Proteolysis targeting chimeras), ADCs (Antibody-drug conjugates), and nucleic acid therapeutics is advancing at a rapid pace, and the druggability of kinases is expected to expand significantly in the near future [11–13].

This situation strongly supports a strategy of identifying targetable kinases for specific cancer patient groups through phosphoproteome analysis of clinical cancer specimens and applying existing or developing kinase inhibitors.

This review provides an overview of MS-based phosphoproteomics and discusses its technological advances. Next, we review examples of phosphoproteomics applications for therapeutic target discovery, including analyses using cultured cancer cells, experimental animals such as PDX mice, and human cancer clinical specimens.

Finally, we review phosphoproteomic studies that illustrate how this technique is contributing, and can contribute in the future, to our understanding of cancer cell signaling and biology and the development of cancer treatments.

2 | Technological Advances in MS-Based Phosphoproteomics

MS-based phosphoproteomics, supported by recent advances in analytical chemistry, informatics, and computational science, has evolved to enable the analysis of over 20,000 phosphorylation sites in a single experiment. This section outlines the technological development of MS-based phosphoproteomics from six perspectives: (i) sample preparation and phosphopeptide enrichment, (ii) quantitative methods for large-scale sample analysis, (iii) mass spectrometry measurements, (iv) identification and quantification, (v) informatics analysis, and (vi) limitation of MS-based phosphoproteomics. It also discusses future prospects.

2.1 | Sample Preparation and Phosphopeptide Enrichment

In phosphoproteome analysis, pretreatment steps prior to mass spectrometry (MS) are critical for the highly sensitive detection of low-stoichiometry and unstable phosphorylation modifications. Particularly when analyzing clinical samples, it has been reported that the process from sample collection to storage can significantly impact the phosphoproteome. Regarding the impact of time until freezing, a study measured time-dependent changes in the phosphoproteome via mass spectrometry for tumors collected from ovarian cancer tissue and breast cancer xenograft mouse models, left at room temperature for 0 to 60 min [14]. It reported detectable changes in phosphosites as early as 5 min, with up to 24.3% of phosphorylation sites showing significant variation by 60 min. Furthermore, a recent study evaluated the effects of time from collection to storage at the transcriptome, proteome, and phosphoproteome levels using over 600 colorectal cancer surgical specimens [15]. Molecules showing differences between tumors and non-cancerous tissue frozen within 10 min (T1) were evaluated based on whether these differences were maintained under conditions where storage time was varied: 10–12 min (T2), 13–15 min (T3), 16–18 min (T4), and 19–20 min (T5). The results showed that in the phosphoproteome, 25% and 80% of phospho-sites at T2 and T5, respectively, failed to maintain their differences. This indicates that a mere 10-min difference in time to freezing causes significant fluctuations in the phosphorylation state of cancer tissue. Furthermore, for the transcriptome and proteome at T5, 25% and 50% of phospho-sites failed to maintain the differences observed at T1, respectively. This demonstrates that the phosphoproteome is most susceptible to the time interval between sample collection and storage. To address this issue, we achieved preservation levels equivalent to fresh cultured cells by freezing endoscopic biopsy specimens with liquid nitrogen within 20 s of collection [16–18]. This method avoids the effects of ischemia in surgical specimens, making it highly suitable for phosphoproteome analysis. Furthermore, compared to needle biopsy, endoscopic biopsy specimens offer the advantage of a higher probability of obtaining tissue from the cancerous area. Furthermore, a major advantage of using endoscopic biopsy specimens is the ability to analyze consecutive biopsy specimens before and after treatment. In gastric cancer, an inhibitory signature of the ErbB signaling was observed in the post-treatment HER2-positive group compared with the pre-treatment HER2-positive group [17]. Furthermore, subsequent serial biopsy analysis collected pre-treatment and on-treatment of paclitaxel and ramucirumab has revealed the dynamic mesenchymal transitions within gastric cancer cells, along with the concomitant rewiring of the kinome network [16].

The method for phosphorylated peptide enrichment is also a critical factor for cancer phosphoproteomics. In the early 2000s,

selective enrichment methods were developed using derivatization, such as introducing tags via β-elimination and Michael addition of phosphate groups of phosphorylated serine and threonine residues [19, 20]. Subsequently, the development and refinement of enrichment techniques using immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) with metal oxides such as titanium dioxide (TiO_2) enabled large-scale identification of phosphorylated peptides [21–27]. These methods remain widely used today. In IMAC, metal ions such as Fe^{3+}, Al^{3+}, Ga^{3+}, and Zr^{4+} are immobilized on a stationary phase (e.g., silica or agarose beads) via ligands like nitrilotriacetic acid (NTA) to capture phosphopeptides. In MOAC, metal oxides (e.g., titanium dioxide, TiO_2) act as anchors, forming multivalent bonds to capture phosphopeptides. When using phosphopeptide enrichment methods like IMAC or MOAC, phosphorylated serine and threonine are primarily identified, while phosphorylated tyrosine is often only identified at 1%–5% of the total. Therefore, for high-sensitivity analysis of phosphorylated tyrosine, immunoprecipitation using anti-phosphotyrosine antibodies [28–30] or enrichment methods using phosphotyrosine-binding domains (e.g., SH2/PTB) [31] are frequently employed. Furthermore, combining peptide separation using high-pH reverse-phase chromatography prior to mass spectrometry can dramatically improve the number of identified phosphopeptides [32]. This approach is particularly employed to enhance sensitivity in the analysis of multiplexed minute samples, such as cancer biopsy specimens.

2.2 | Quantitative Methods for Large-Scale Sample Analysis

Quantitative analysis using MS-based phosphoproteomics is essential for elucidating dynamic changes in signaling networks. Phosphorylation, in particular, undergoes stimulus-dependent and reversible changes, necessitating quantitative comparisons beyond mere identification. Quantitative methods are classified into targeted and non-targeted approaches, each offering distinct advantages and limitations depending on the biological question and experimental constraints. Non-targeted proteomics aims to achieve a comprehensive, unbiased survey of the phosphoproteome and enables the simultaneous identification and quantification of tens of thousands of phosphorylated peptides without prior knowledge of the analytes. Thus, non-targeted phosphoproteomics is often used for exploratory research for hypothesis generation and systems-level phosphoproteome profiling.

Non-targeted analysis can be classified into isotope-labeled quantification and label-free quantification. Isotope-labeled quantification is further divided into metabolic labeling using stable isotopes and isobaric chemical labeling. A representative metabolic labeling method is metabolic labeling using stable isotopes (SILAC: stable isotope labeling by amino acids in cell culture [33]). SILAC enables accurate relative protein quantification in MS–based experiments. In SILAC, cells are cultured in media supplemented with either "light" (natural) or "heavy" (stable isotope–labeled) essential amino acids, most commonly arginine and lysine. During several cell divisions, the labeled amino acids are incorporated into newly synthesized proteins, leading to complete metabolic labeling of the proteome. After labeling, proteins from differentially treated cell populations (e.g., control vs. stimulated) are mixed, digested into peptides, and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Since "light" and "heavy" peptides are chemically identical but differ in mass due to isotopic substitution (e.g., 13C, 15N), their ionization efficiencies are equivalent, allowing highly accurate relative quantification based on peak intensities. However, due to difficulties in applying SILAC to clinical samples and its low throughput, the SILAC method is mainly used in cultured cell-based experiments. For example, SILAC is an indispensable labeling method for analyzing protein turnover, particularly leveraging the characteristics of metabolic labels. Among isobaric chemical labeling methods, iTRAQ [34] and TMT [35] are widely used. Each reagent has the same overall mass but is designed with a balance of isotopes in the reporter and balance groups. During MS/MS fragmentation, the tags release reporter ions of distinct m/z values, enabling relative quantification of peptides from different samples while maintaining identical chromatographic and MS1 properties. TMT labeling, in particular, allows for multiplexing of 32 samples, reducing the sample quantity required per analysis. Isobaric labeling (e.g., TMT) offers high throughput, scalability, and sensitivity when combined with fractionation. Accordingly, many Clinical Proteomic Tumor Analysis Consortium (CPTAC) projects have employed TMT-based phosphoproteome quantification.

Label-free quantification (LFQ), meanwhile, is frequently used in both basic and clinical research due to its simplicity in sample preparation and broad applicability. Recently, LFQ has been combined with the DIA method described later. Comparing TMT and LFQ, TMT can show lower quantitative accuracy than LFQ due to ratio compression caused by reporter ion interference. However, within the same batch, TMT tends to show higher quantitative precision than LFQ [36]. For both chemical labeling and label-free quantification, experimental design to minimize batch effects is crucial when analyzing multiple clinical samples. Quantification in proteome analysis can be performed using multiple peptides derived from each protein. However, in phosphoproteome analysis, quantification occurs at the peptide level, making it prone to missing values. Particularly in multi-sample analysis, the proportion of missing values increases significantly, making experimental design crucial for minimizing missing values. As shown in Figure 1, when we analyzed endoscopic biopsy specimens from gastric cancer, we adopted TMT labeling and placed a boost sample [37] (a mixture of gastric cancer cultured cells) in one channel of each batch. By designing the boost sample to constitute two-thirds of the total sample amount for the entire batch, we suppressed batch effects, enhanced signal intensity and increased the number of valid quantitative values, enabling quantification of 21,103 phosphorylation sites per sample.

Targeted proteomics focuses on the precise and sensitive quantification of predefined peptides or proteins of interest. The most established techniques—selected reaction monitoring (SRM) and parallel reaction monitoring (PRM)—achieve high specificity by monitoring predetermined precursor/fragment ion pairs [38, 39]. These assays offer superior quantitative accuracy, linearity, and reproducibility. Classically, the AQUA (absolute quantification) strategy [40] has been used for absolute quantitation, where synthetic stable isotope-labeled phosphorylated peptides are added as internal standards to precisely quantify

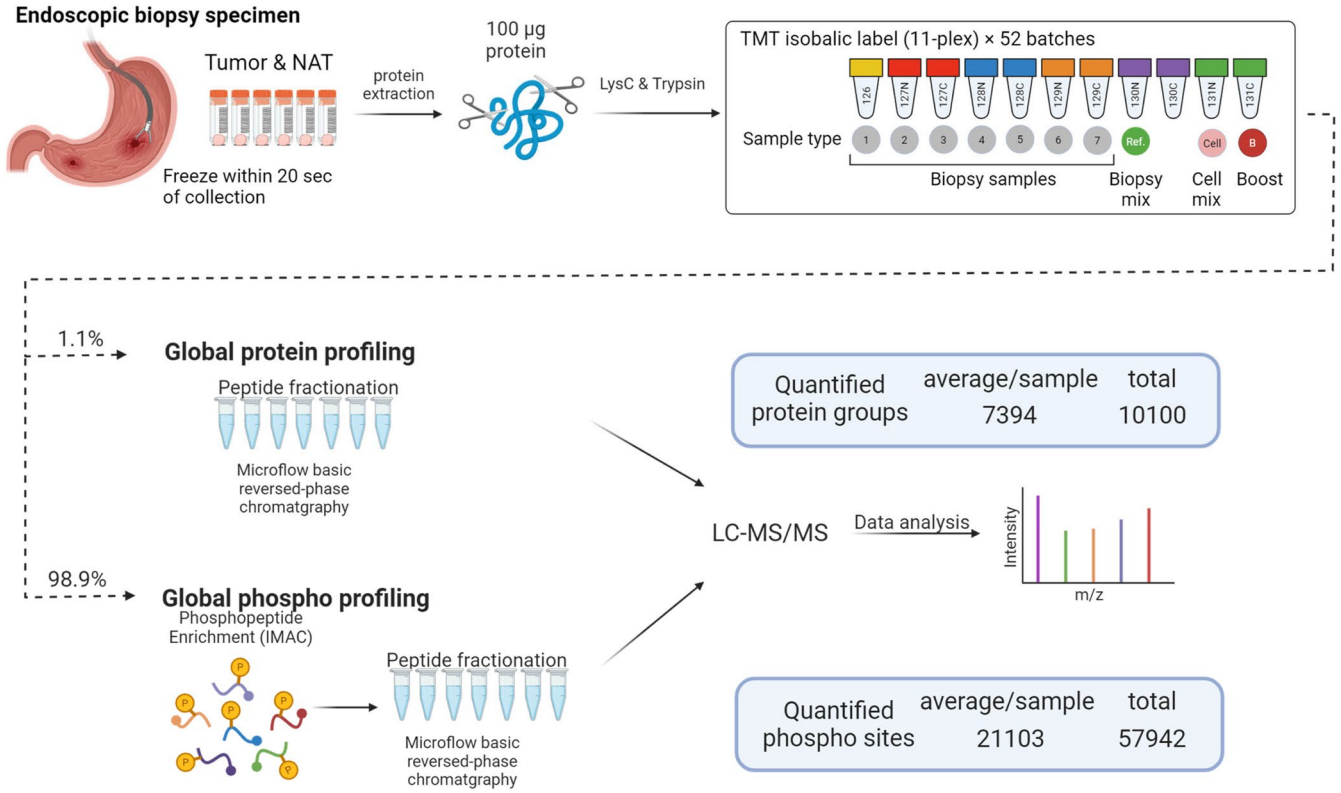

FIGURE 1 | Experimental design and workflow for quantitative phosphoproteomics using TMT reagent for gastric cancer endoscopic biopsy specimens [16].

the target peptide amount. Absolute quantification is useful for directly determining the stoichiometry of phosphorylation sites or the modification frequency per molecule. In recent years, the development of targeted quantification technologies such as SureQuant [41] has enabled highly sensitive and accurate monitoring of specific phosphorylation sites. These absolute quantitative approaches are particularly useful for validating candidate biomarkers in clinical samples.

Targeted affinity-based proteomics platforms such as Olink [42] and SomaScan [43] enable high-throughput, multiplexed quantification of predefined protein panels using specific binding reagents. Olink employs proximity extension assays (PEA), in which paired antibodies linked to oligonucleotides generate a DNA reporter only when bound to the target protein, providing exceptional specificity, low sample input, and broad dynamic range. SomaScan, in contrast, utilizes modified DNA aptamers with enhanced binding affinities, enabling simultaneous measurement of thousands of proteins in a single assay. Compared with mass spectrometry–based discovery proteomics, these platforms offer higher sensitivity but are restricted to predefined targets and dependent on the availability and performance of affinity reagents. They are therefore particularly well suited for blood biomarker studies. However, quantification of phosphorylated proteins is not supported.

2.3 | Mass Spectrometry Measurements

The advancement of MS-based phosphoproteomics has relied heavily not only on improvements in sample preparation and quantification methods but also on the evolution of mass spectrometers themselves. Identifying phosphorylated peptides demands high sensitivity, high resolution, and fast scanning simultaneously. Responding to these demands, mass spectrometry technology has made tremendous strides over the past 20 years, achieving orders of magnitude improvements in analytical depth.

Early phosphorylation analyses utilized triple quadrupole (QqQ) and ion trap (IT) instruments, but these had limitations in detection sensitivity and resolution. Subsequent time-of-flight (TOF) and FT-ICR instruments significantly improved resolution, though challenges remained in mass accuracy and measurement speed, respectively. The turning point came with the introduction of the Orbitrap mass spectrometer [44]. The Orbitrap, with its high resolution and excellent mass accuracy, proved ideal for the precise identification and quantification of phosphorylated peptides. It subsequently became the standard instrument widely adopted for proteomics research.

Advances in fragmentation techniques have also contributed to expanding analytical depth. Conventional CID (collision-induced dissociation) frequently caused neutral loss of phosphate groups, making it difficult to identify phosphorylation sites. In contrast, the introduction of ETD (electron transfer dissociation) and EThcD enabled the accurate mapping of modified sites while preserving the peptide backbone [45].

Furthermore, improvements in higher-energy collisional dissociation (HCD) have enhanced the efficiency of phosphosite identification and quantitative accuracy. Combining this with

ion mobility separation (IMS) further expands the depth of analysis for complex phosphoproteomes. Specifically, the combination of trapped ion mobility spectrometry (TIMS) and parallel accumulation–serial fragmentation (PASEF) achieves both high measurement speed and sensitivity, making phosphorylation analysis at the single-cell level increasingly feasible [46, 47]. Furthermore, the recently developed Astral analyzer enables quantification of over 30,000 phosphorylation sites in a single run at a measurement speed reaching 200 Hz [48, 49].

Regarding data acquisition methods, alongside DDA (Data-Dependent Acquisition)—which selects the strongest precursor ion from the MS1 scan for sequential MS/MS identification—DIA (Data-Independent Acquisition) methods have been developed [50, 51]. These divide the MS1 precursor ions across a broad window, independent of intensity, and comprehensively measure all via MS/MS. This enables the analysis of low-intensity peptides and improves reproducibility. This allows comprehensive analysis of low-intensity peptides, improving reproducibility. While interpreting complex spectra was an early challenge for DIA, the emergence of algorithms using machine learning and neural networks (e.g., DIA-NN [52]) has enabled results surpassing conventional DDA in both depth and accuracy.

2.4 | Identification and Quantification

In MS-based phosphoproteomics analysis, the evolution of mass spectrometers has led to the generation of massive amounts of data, making the development of software for identification and quantification indispensable. Particularly in phosphorylation analysis, the accurate identification and quantification of modified sites determine the reliability of research results, driving the successive development of specialized algorithms and integrated analysis environments.

Initially, database search engines like SEQUEST [53] and Mascot [54] were mainstream, identifying peptides by matching MS/MS spectra against known sequence databases. However, the identification of modified sites proved difficult due to phosphorylation-specific neutral losses and spectral complexity caused by multiple modifications. To overcome this, methods like Ascore [55] and Localization Probability [56] were developed. These techniques, which statistically calculate the localization probability of phosphorylation sites, are now widely used.

Subsequently, alongside improvements in identification accuracy, MaxQuant emerged and became standardized as an integrated platform supporting diverse quantification methods such as label-free quantification, SILAC, and TMT [57]. Notably, the Andromeda search engine incorporated into MaxQuant enables analysis considering phosphorylation modifications, such as calculating localization probability, and accelerating large-scale phosphorylation analysis studies.

Recent developments include DIA-specific analysis software, exemplified by Spectronaut [58] and DIA-NN [52]. These leverage machine learning algorithms to enable bulk quantification of phosphorylated peptides. Furthermore, Skyline, specialized for target-based analysis like PRM and SRM, is widely adopted as the gold standard software for targeted proteomics [59].

2.5 | Informatics Analysis

Data obtained from MS-based phosphoproteomics is vast and complex, requiring advanced informatics analysis for biological interpretation. Phosphorylation, in particular, is a core modification in signaling pathways. Understanding it demands more than mere site identification; it requires system-level comprehension through kinase activity estimation, pathway reconstruction, and network analysis.

Computational inference of upstream kinase activity from phosphoproteomic data relies on three broad classes of information sources. Sequence motif–based approaches exploit the preference of kinases for specific amino acid patterns surrounding phosphorylated residues, scoring phosphosites using position-specific scoring matrices or consensus motifs to estimate kinase compatibility. Curated kinase–substrate database–based methods aggregate phosphorylation changes across experimentally validated substrate sets derived from resources such as PhosphoSitePlus, enabling enrichment or activity scoring that reflects coordinated regulation of known targets. Furthermore, learned models, including machine-learning and deep-learning approaches, have been developed to infer kinase–substrate relationships and kinase activity directly from data by integrating sequence context with additional features such as structural information, evolutionary conservation, or network connectivity [60–62]. Together, these approaches differ in their assumptions and coverage but share the common goal of translating phosphosite-level measurements into interpretable estimates of kinase signaling activity [63]. However, kinase activity estimation often favors well-studied kinases and may complicate biological interpretation. Therefore, developing methods to systematically and large-scale acquire kinase-substrate information is critically important. We have successfully identified multiple ALK substrates simultaneously by combining phosphoproteome and interactome analysis using a doxycycline-inducible kinase expression system. Extending this approach to other kinases is expected to expand kinase-substrate information. Additionally, a novel substrate and signaling pathway identification approach focusing on co-phosphorylation changes in large-scale clinical sample phosphoproteome data has been reported [64]. While reducing the false positive rate remains a challenge, the exponential growth of phosphoproteome data from clinical samples is anticipated, making it possible to obtain more accurate kinase-substrate information.

2.6 | Limitation of MS-Based Phosphoproteomics

Mass-spectrometry–based phosphoproteomics has become an indispensable tool for mapping signaling networks at scale, offering unparalleled breadth and the ability to uncover previously uncharacterized phosphorylation events. Nonetheless, several practical considerations are important for appropriately interpreting MS-derived phosphoproteomic data and understanding how these methods complement targeted approaches.

One central consideration is that MS does not inherently guarantee detection of specific phosphosites of interest, particularly when they are present at very low abundance. This is not a limitation of MS per se, but rather a reflection of the extraordinary

TABLE 1 | Representative examples of phosphoproteome analysis in cancer clinical specimens or patient-derived cells and therapeutic target identification.

Study	Cancer type	Clinical samples	Sample type	Target patient subgroup	Quantitation methods/reagents for phosphoproteomics/ data acquisition method	Kinase activity prediction	Target kinase/ pathway	Representative findings	Drug associations	Validation experiments	References
1 (CPTAC)	Colon adenocarcinoma	110 tumor–NAT pairs	Surgical resection	High-Rb phosphorylation tumors	Isobaric chemical labeling, TMT10plex/DDA	in-house predeiction	CDK–Rb axis	RB1 hyperphosphorylation	CDK inhibitors	—	[67]
2 (CPTAC)	Clear cell renal cell carcinoma	110 tumors, 83 NATs	Surgical resection	Tumor vs. NAT	Isobaric chemical labeling, TMT10plex/DDA	in-house predeiction	PI3K–AKT–mTOR/RTK-MEK–ERK	AKT1S1 T246, MAPK3 Y204, CDK substrates	VEGFR, EGFR, MEK, ERK, AKT, mTOR, CDK inhibitors	—	[68]
3 (CPTAC)	Breast cancer	122 tumors	Surgical resection	Rb-high TNBC subtype	Isobaric chemical labeling, TMT10plex/DDA	PTM-SEA	CDK–Rb axis	Rb-high expresion	CDK4/6 inhibitors	—	[69]
4	Acute myelogenous leukemia	44 primary AML patient samples	Bone marrow aspirates/ peripheral blood	Selinexor-resistant AML with AKT-high signature	Isobaric chemical labeling, TMT11plex/DDA	KSEA	AKT–FOXO3 axis	AKT S473; FOXO3 S253; 4EBP1 T37/46; S6K T389	Selinexor + AKT inhibitor	Ex vivo drug response; cell-line	[70]
5	Colorectal cancer	30 metastatic CRC PDX models	PDX (surgical grafts)	Cetuximab-resistant CRC	Label-free quantification/–/DDA	INKA	EGFR, ephrin kinases	EGFR signaling activation	Dasatinib	Organoid; PDX model	[71]
6	Pancreatic ductal adenocarcinoma	9 PDAC cells	Organoids/PDX (from surgical tumors)	—	Label-free quantification/–/DDA	INKA	RTK/MAPK, PI3K–AKT pathways	pERK, pAKT	Low-dose combination of multiTKIs	Organoid; PDX model	[72]
7	Colorectal liver metastases	24 paired primary and recurrent CRC liver metastases	Surgical resections (hepatectomy)	Recurrent CRC liver mets, PAK1-high	Isobaric chemical labeling, TMT11plex/DDA	PTM-SEA	PI3K–PAK1 axis	PAK1 sites	PI3K inhibitor (copanlisib)	Cell-line; mouse xenograft model	[73]
8	Gastric cancer	127 baseline endoscopic biopsies + longitudinal biopsies in 9 patients	Endoscopic biopsy	Gastric cancers shifting to EMT during chemo	Isobaric chemical labeling, TMT11plex/DDA	PTM-SEA	AXL	AXL activated in EMT subtype	AXL inhibitors (gilteritinib/ONO-7475) + paclitaxel	Cell-line; mouse xenograft model	[16]

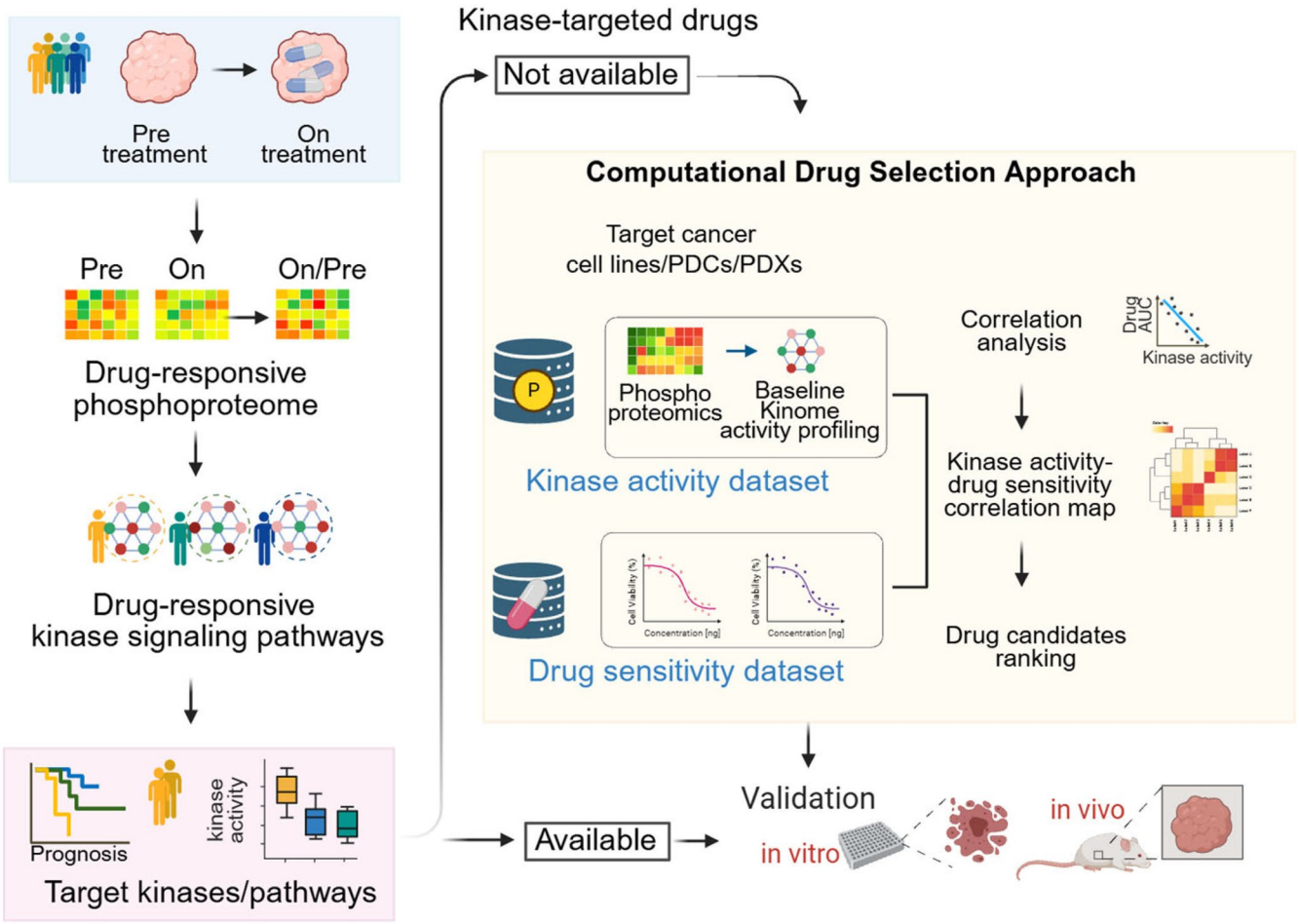

FIGURE 2 | Conceptual workflow for therapeutic target discovery using phosphoproteomics. This workflow outlines a method for identifying patient populations, kinases, and phosphoproteomic signaling pathways for therapeutic development by integrating prognostic information through differential analysis of phosphoproteome and kinase activity before and after drug administration. Furthermore, it illustrates drug ranking based on correlation analysis using kinase activity and drug sensitivity datasets, as well as drug validation.

dynamic range and biochemical complexity of the phosphoproteome. Although enrichment strategies and instrument sensitivity continue to improve rapidly, low-stoichiometry sites may still fall below detection thresholds, especially in highly complex samples. Additionally, ionization efficiencies differ among peptides, leading to preferential detection of certain phosphopeptides. Advances in chromatography, ion mobility, and acquisition strategies continually mitigate these issues, but sampling depth remains finite due to inherent constraints in instrument duty cycles and chromatographic peak capacity. Further improvements in instrument sensitivity and data analysis are expected to alleviate these limitations in the future.

Furthermore, kinase activity in vivo is modulated by protein abundance, subcellular localization, activation loop phosphorylation, and allosteric regulation, as well as docking interactions, priming phosphorylation, and counter-regulation by phosphatases. Additionally, many phosphosites are targeted by multiple kinases, further confounding straightforward attribution. Unraveling these complexities is challenging, but combining high-resolution spatial proteomics technologies, chemical biology methods that directly quantify kinase activity, and computational protein structure analysis may hold the key to solving them.

Formalin-fixed, paraffin-embedded (FFPE) tissues represent one of the most valuable resources for translational research, offering access to well-annotated clinical specimens. Recent methodological innovations have significantly enhanced the depth of phosphoproteomic profiling from FFPE samples [65, 66]. Recent studies analyzing fresh frozen tissue and FFPE sections from rats have reported high correlation between the phosphoproteomes of fresh frozen tissue and FFPE sections when rapidly pretreated at 4°C. Therefore, as mentioned in Section 2.1, minimizing the time until fixation is crucial for the quality of FFPE phosphoproteome analysis. Furthermore, the biological interpretability of FFPE-derived phosphoproteome datasets remains unclear, as there are still few reported cases and insufficient information regarding the conservation quality at individual phosphorylation sites.

3 | Applications of Phosphoproteomics in Cancer Signaling Characteristics and Identification of Therapeutic Targets

Phosphoproteomics is a powerful approach that systematically analyzes phosphorylation events—central to cellular signaling—and visualizes disease-specific signaling abnormalities. Table 1

shows examples of phosphoproteome analysis in cancer clinical specimens or patient-derived cells, along with therapeutic target development research. In Phase 2 of CPTAC, initiated in 2011, proteome analysis of clinical specimens from various cancer types was conducted [74]. In most cases, surgical specimens were used, and proteogenomic analysis was employed to elucidate cancer characteristics. Notably, in colon adenocarcinoma and breast cancer, activation of the CDK-Rb axis was identified in patients with phosphorylated Rb protein or high Rb expression, leading to the proposal of CDK inhibitor-based therapy [67, 69]. Furthermore, in clear cell renal cell carcinoma, compared to normal tissue, normal adjacent tissue (NAT), the PI3K–AKT–mTOR pathway and RTK-MEK-ERK pathways are activated in tumors. Utilization of inhibitors targeting upstream and downstream molecules of these pathways—VEGFR, EGFR, MEK, ERK, AKT, mTOR, and CDK—has been proposed [68]. Since 2020, studies have been reported that not only identify target patient populations, target kinases, target pathways, and therapeutic candidates using phosphoproteomics, but also validate therapeutic agents in vitro and/or in vivo models (Table 1, studies 4–8, [16, 70–73]). As shown in Table 1, previous studies mainly used bulk tissue or biopsy samples. Thus, findings regarding the intratumoral heterogeneity were limited. In the future, combining high-depth proteome data from bulk samples with single-cell-level spatial proteomics data will enable us to elucidate tumor heterogeneity. This will allow for more precise target selection.

Figure 2 illustrates the workflow for therapeutic target discovery using phosphoproteomics. We present an example comparing the phosphoproteome before and during drug treatment. By comparing the phosphoproteome before and during drug treatment in each patient, it becomes possible to capture the drug's effects while minimizing individual variability. Next, by functionally annotating phosphorylation sites and estimating kinase activity using substrate motif analysis and kinase-substrate databases, the drug-responsive kinase signaling pathway in each patient becomes apparent. For example, Figure 3 shows kinase activity profiles for the proliferation subtype, mesenchymal subtype, and oxidative phosphorylation subtype, as defined by unsupervised clustering of the phosphoproteome data of untreated gastric cancer patients [16]. Subsequently, within patient cohorts stratified based on prognostic information or omics data, we focus on the patient group eligible for treatment and identify the kinases and signaling pathways activated in that cohort. If no drugs targeting the identified kinases or signaling pathways have been developed or are unapproved, we adopt a computational approach for drug selection. We select as many cultured cells, patient-derived cells, or PDX mouse models as possible for the target cancer type. For cultured cells, we typically select 30 to 50 cell lines. We then perform phosphoproteome analysis to obtain kinase activity profiles and simultaneously acquire public or in-house drug-sensitivity profiles. Correlation analysis of both datasets creates a kinase-activity-drug-sensitivity correlation map. Drugs exhibiting high sensitivity to the pre-selected therapeutic target kinase activity are selected. Subsequently, the anti-tumor efficacy of these drugs is validated in vitro and in vivo.

Regarding cancer diagnosis using phosphoproteomics, a clinical proteomics and phosphoproteomic workflow for integration

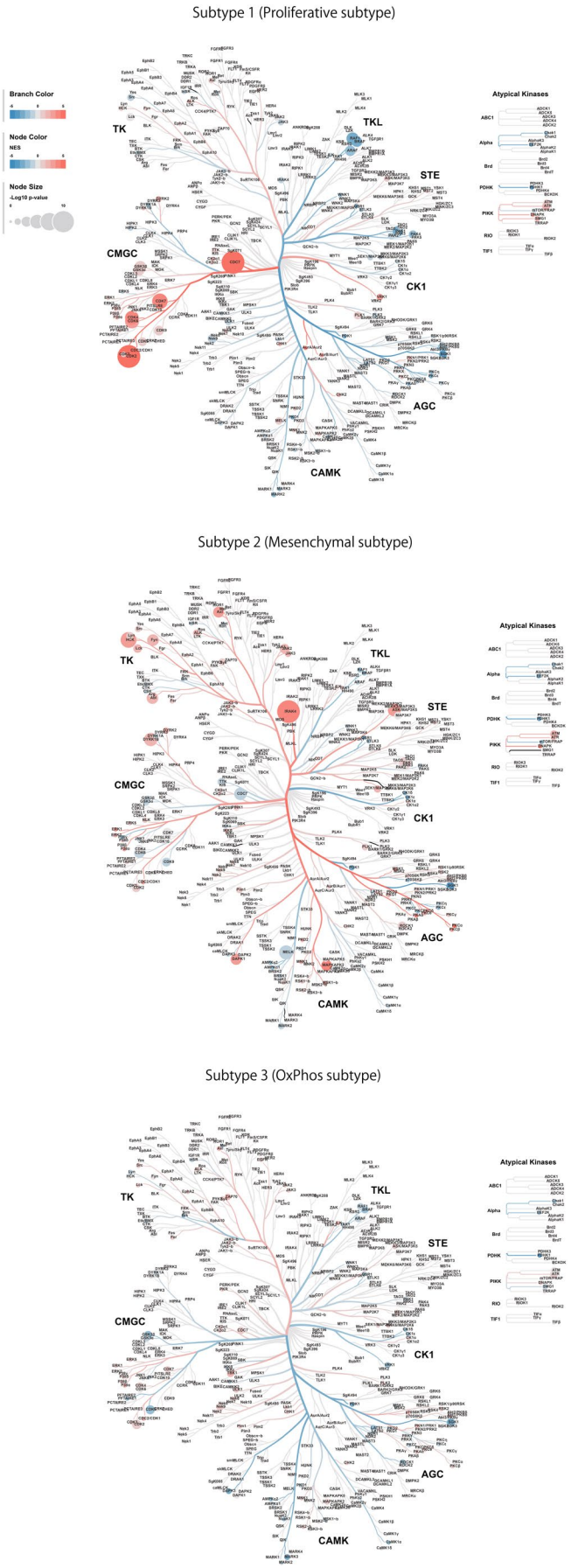

FIGURE 3 | Kinase activity profiles of treatment-naive gastric cancer phosphoproteomic subtypes [16].

into the molecular tumor board workflow of the Germany-wide INFORM (for children with relapsed cancers) and MASTER (for young adults with refractory cancers and patients with rare tumors) registry studies was established [75]. This project demonstrates that phosphoproteome analysis possesses the reproducibility, throughput, and sensitivity required for routine implementation. The proteomics data and real-world data that will accumulate going forward will provide valuable insights not only for diagnostics but also for the development of novel therapies.

4 | Conclusion

MS-based phosphoproteomics has achieved remarkable depth and precision, enabling large-scale profiling of phosphorylation events. These advances provide unprecedented opportunities to interrogate cancer signaling networks and identify therapeutic vulnerabilities. Remaining challenges include incomplete kinase–substrate databases and the need for integration with single-cell and spatial proteomics. The incorporation of artificial intelligence–driven network analysis is expected to further accelerate progress. Collectively, these developments are expected to establish phosphoproteomics as a cornerstone for precision oncology, rational drug development, and mechanistic exploration of cancer biology.

Author Contributions

Hirokazu Shoji: conceptualization, writing – review and editing. **Narikazu Boku:** conceptualization, writing – review and editing. **Jun Adachi:** conceptualization, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

1. D. Hanahan, "Hallmarks of Cancer: New Dimensions," *Cancer Discovery* 12, no. 1 (2022): 31–46, https://doi.org/10.1158/2159-8290.CD-21-1059.

2. D. Hanahan and R. A. Weinberg, "Hallmarks of Cancer: The Next Generation," *Cell* 144, no. 5 (2011): 646–674, https://doi.org/10.1016/j.cell.2011.02.013.

3. D. Hanahan and R. A. Weinberg, "The Hallmarks of Cancer," *Cell* 100, no. 1 (2000): 57–70, https://doi.org/10.1016/s0092-8674(00)81683-9.

4. J. M. Golas, K. Arndt, C. Etienne, et al., "SKI-606, a 4-Anilino-3-Quinolinecarbonitrile Dual Inhibitor of Src and Abl Kinases, Is a Potent Antiproliferative Agent Against Chronic Myelogenous Leukemia Cells in Culture and Causes Regression of K562 Xenografts in Nude Mice," *Cancer Research* 63, no. 2 (2003): 375–381.

5. B. J. Druker, S. Tamura, E. Buchdunger, et al., "Effects of a Selective Inhibitor of the Abl Tyrosine Kinase on the Growth of Bcr–Abl Positive Cells," *Nature Medicine* 2, no. 5 (1996): 561–566, https://doi.org/10.1038/nm0596-561.

6. F. A. Shepherd, J. Rodrigues Pereira, T. Ciuleanu, et al., "Erlotinib in Previously Treated Non–Small-Cell Lung Cancer," *New England Journal of Medicine* 353, no. 2 (2005): 123–132, https://doi.org/10.1056/NEJMoa050753.

7. M. Hidalgo, L. L. Siu, J. Nemunaitis, et al., "Phase I and Pharmacologic Study of OSI-774, an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, in Patients With Advanced Solid Malignancies," *Journal of Clinical Oncology* 19, no. 13 (2001): 3267–3279, https://doi.org/10.1200/JCO.2001.19.13.3267.

8. R. Roskoski, Jr., "Properties of FDA-Approved Small Molecule Protein Kinase Inhibitors: A 2025 Update," *Pharmacological Research* 216 (2025): 107723, https://doi.org/10.1016/j.phrs.2025.107723.

9. G. Manning, D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam, "The Protein Kinase Complement of the Human Genome," *Science* 298, no. 5600 (2002): 1912–1934, https://doi.org/10.1126/science.1075762.

10. B. Anderson, P. Rosston, H. W. Ong, M. A. Hossain, Z. W. Davis-Gilbert, and D. H. Drewry, "How Many Kinases Are Druggable? A Review of Our Current Understanding," *Biochemical Journal* 480, no. 16 (2023): 1331–1363, https://doi.org/10.1042/BCJ20220217.

11. M. Liu, Y. Wang, Y. Zhang, et al., "Landscape of Small Nucleic Acid Therapeutics: Moving From the Bench to the Clinic as Next-Generation Medicines," *Signal Transduction and Targeted Therapy* 10, no. 1 (2025): 73, https://doi.org/10.1038/s41392-024-02112-8.

12. G. Zhong, X. Chang, W. Xie, and X. Zhou, "Targeted Protein Degradation: Advances in Drug Discovery and Clinical Practice," *Signal Transduction and Targeted Therapy* 9, no. 1 (2024): 308, https://doi.org/10.1038/s41392-024-02004-x.

13. C. Dumontet, J. M. Reichert, P. D. Senter, J. M. Lambert, and A. Beck, "Antibody-Drug Conjugates Come of Age in Oncology," *Nature Reviews Drug Discovery* 22, no. 8 (2023): 641–661, https://doi.org/10.1038/s41573-023-00709-2.

14. P. Mertins, F. Yang, T. Liu, et al., "Ischemia in Tumors Induces Early and Sustained Phosphorylation Changes in Stress Kinase Pathways but Does Not Affect Global Protein Levels," *Molecular & Cellular Proteomics* 13, no. 7 (2014): 1690–1704, https://doi.org/10.1074/mcp.M113.036392.

15. S. von der Heyde, N. Raman, N. Gabelia, et al., "Tumor Specimen Cold Ischemia Time Impacts Molecular Cancer Drug Target Discovery," *Cell Death & Disease* 15, no. 9 (2024): 691, https://doi.org/10.1038/s41419-024-07090-x.

16. H. Shoji, H. Hirano, Y. Nojima, et al., "Phosphoproteomic Subtyping of Gastric Cancer Reveals Dynamic Transformation With Chemotherapy and Guides Targeted Cancer Treatment," *Cell Reports* 43, no. 10 (2024): 114774, https://doi.org/10.1016/j.celrep.2024.114774.

17. H. Hirano, Y. Abe, Y. Nojima, et al., "Temporal Dynamics From Phosphoproteomics Using Endoscopic Biopsy Specimens Provides New Therapeutic Targets in Stage IV Gastric Cancer," *Scientific Reports* 12, no. 1 (2022): 4419, https://doi.org/10.1038/s41598-022-08430-7.

18. Y. Abe, H. Hirano, H. Shoji, et al., "Comprehensive Characterization of the Phosphoproteome of Gastric Cancer From Endoscopic Biopsy Specimens," *Theranostics* 10, no. 5 (2020): 2115–2129.

19. Z. A. Knight, B. Schilling, R. H. Row, D. M. Kenski, B. W. Gibson, and K. M. Shokat, "Phosphospecific Proteolysis for Mapping Sites of Protein Phosphorylation," *Nature Biotechnology* 21, no. 9 (2003): 1047–1054, https://doi.org/10.1038/nbt863.

20. Y. Oda, T. Nagasu, and B. T. Chait, "Enrichment Analysis of Phosphorylated Proteins as a Tool for Probing the Phosphoproteome," *Nature Biotechnology* 19, no. 4 (2001): 379–382, https://doi.org/10.1038/86783.

21. H. Block, B. Maertens, A. Spriestersbach, et al., "Immobilized-Metal Affinity Chromatography (IMAC): A Review," *Methods in Enzymology* 463 (2009): 439–473, https://doi.org/10.1016/S0076-6879(09)63027-5.

22. N. Sugiyama, T. Masuda, K. Shinoda, A. Nakamura, M. Tomita, and Y. Ishihama, "Phosphopeptide Enrichment by Aliphatic Hydroxy Acid-Modified Metal Oxide Chromatography for Nano-LC-MS/MS in Proteomics Applications," *Molecular & Cellular Proteomics* 6, no. 6 (2007): 1103–1109, https://doi.org/10.1074/mcp.T600060-MCP200.

23. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff, and T. J. Jorgensen, "Highly Selective Enrichment of Phosphorylated Peptides From Peptide Mixtures Using Titanium Dioxide Microcolumns," *Molecular & Cellular Proteomics* 4, no. 7 (2005): 873–886, https://doi.org/10.1074/mcp.T500007-MCP200.

24. A. Sano and H. Nakamura, "Chemo-Affinity of Titania for the Column-Switching HPLC Analysis of Phosphopeptides," *Analytical Sciences* 20, no. 3 (2004): 565–566, https://doi.org/10.2116/analsci.20.565.

25. M. W. Pinkse, P. M. Uitto, M. J. Hilhorst, B. Ooms, and A. J. Heck, "Selective Isolation at the Femtomole Level of Phosphopeptides From Proteolytic Digests Using 2D-NanoLC-ESI-MS/MS and Titanium Oxide Precolumns," *Analytical Chemistry* 76, no. 14 (2004): 3935–3943, https://doi.org/10.1021/ac0498617.

26. I. Kuroda, Y. Shintani, M. Motokawa, S. Abe, and M. Furuno, "Phosphopeptide-Selective Column-Switching RP-HPLC With a Titania Precolumn," *Analytical Sciences* 20, no. 9 (2004): 1313–1319, https://doi.org/10.2116/analsci.20.1313.

27. M. C. Posewitz and P. Tempst, "Immobilized Gallium(III) Affinity Chromatography of Phosphopeptides," *Analytical Chemistry* 71, no. 14 (1999): 2883–2892, https://doi.org/10.1021/ac981409y.

28. Y. Abe, M. Nagano, A. Tada, J. Adachi, and T. Tomonaga, "Deep Phosphotyrosine Proteomics by Optimization of Phosphotyrosine Enrichment and MS/MS Parameters," *Journal of Proteome Research* 16, no. 2 (2017): 1077–1086.

29. Y. Abe, M. Nagano, T. Kuga, et al., "Deep Phospho- and Phosphotyrosine Proteomics Identified Active Kinases and Phosphorylation Networks in Colorectal Cancer Cell Lines Resistant to Cetuximab," *Scientific Reports* 7, no. 1 (2017): 10463, https://doi.org/10.1038/s41598-017-10478-9.

30. J. Rush, A. Moritz, K. A. Lee, et al., "Immunoaffinity Profiling of Tyrosine Phosphorylation in Cancer Cells," *Nature Biotechnology* 23, no. 1 (2005): 94–101, https://doi.org/10.1038/nbt1046.

31. Y. Bian, L. Li, M. Dong, et al., "Ultra-Deep Tyrosine Phosphoproteomics Enabled by a Phosphotyrosine Superbinder," *Nature Chemical Biology* 12, no. 11 (2016): 959–966, https://doi.org/10.1038/nchembio.2178.

32. P. Mertins, L. C. Tang, K. Krug, et al., "Reproducible Workflow for Multiplexed Deep-Scale Proteome and Phosphoproteome Analysis of Tumor Tissues by Liquid Chromatography-Mass Spectrometry," *Nature Protocols* 13, no. 7 (2018): 1632–1661, https://doi.org/10.1038/s41596-018-0006-9.

33. S. E. Ong, B. Blagoev, I. Kratchmarova, et al., "Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics," *Molecular & Cellular Proteomics* 1, no. 5 (2002): 376–386.

34. P. L. Ross, Y. N. Huang, J. N. Marchese, et al., "Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-Reactive Isobaric Tagging Reagents," *Molecular & Cellular Proteomics* 3, no. 12 (2004): 1154–1169, https://doi.org/10.1074/mcp.M400129-MCP200.

35. A. Thompson, J. Schafer, K. Kuhn, et al., "Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS," *Analytical Chemistry* 75, no. 8 (2003): 1895–1904, https://doi.org/10.1021/ac0262560.

36. A. Hogrebe, L. von Stechow, D. B. Bekker-Jensen, B. T. Weinert, C. D. Kelstrup, and J. V. Olsen, "Benchmarking Common Quantification Strategies for Large-Scale Phosphoproteomics," *Nature Communications* 9, no. 1 (2018): 1045, https://doi.org/10.1038/s41467-018-03309-6.

37. L. Yi, C. F. Tsai, E. Dirice, et al., "Boosting to Amplify Signal With Isobaric Labeling (BASIL) Strategy for Comprehensive Quantitative Phosphoproteomic Characterization of Small Populations of Cells," *Analytical Chemistry* 91, no. 9 (2019): 5794–5801, https://doi.org/10.1021/acs.analchem.9b00024.

38. J. Adachi, R. Narumi, and T. Tomonaga, "Targeted Phosphoproteome Analysis Using Selected/Multiple Reaction Monitoring (SRM/MRM)," *Methods in Molecular Biology* 1394 (2016): 87–100, https://doi.org/10.1007/978-1-4939-3341-9_7.

39. R. Narumi, T. Murakami, T. Kuga, et al., "A Strategy for Large-Scale Phosphoproteomics and SRM-Based Validation of Human Breast Cancer Tissue Samples," *Journal of Proteome Research* 11, no. 11 (2012): 5311–5322, https://doi.org/10.1021/pr3005474.

40. S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner, and S. P. Gygi, "Absolute Quantification of Proteins and Phosphoproteins From Cell Lysates by Tandem MS," *Proceedings of the National Academy of Sciences of the United States of America* 100, no. 12 (2003): 6940–6945, https://doi.org/10.1073/pnas.0832254100.

41. K. Kalogeropoulos, S. Savickas, A. M. Haack, et al., "High-Throughput and High-Sensitivity Biomarker Monitoring in Body Fluid by Fast LC SureQuant IS-Targeted Quantitation," *Molecular & Cellular Proteomics* 23, no. 12 (2024): 100868, https://doi.org/10.1016/j.mcpro.2024.100868.

42. E. Assarsson, M. Lundberg, G. Holmquist, et al., "Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity, Specificity, and Excellent Scalability," *PLoS One* 9, no. 4 (2014): e95192, https://doi.org/10.1371/journal.pone.0095192.

43. L. Gold, D. Ayers, J. Bertino, et al., "Aptamer-Based Multiplexed Proteomic Technology for Biomarker Discovery," *PLoS One* 5, no. 12 (2010): e15004, https://doi.org/10.1371/journal.pone.0015004.

44. A. Makarov, "Electrostatic Axially Harmonic Orbital Trapping: A High-Performance Technique of Mass Analysis," *Analytical Chemistry* 72, no. 6 (2000): 1156–1162, https://doi.org/10.1021/ac991131p.

45. J. E. Syka, J. J. Coon, M. J. Schroeder, J. Shabanowitz, and D. F. Hunt, "Peptide and Protein Sequence Analysis by Electron Transfer Dissociation Mass Spectrometry," *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 26 (2004): 9528–9533, https://doi.org/10.1073/pnas.0402700101.

46. P. Skowronek, M. Thielert, E. Voytik, et al., "Rapid and In-Depth Coverage of the (Phospho-)proteome With Deep Libraries and Optimal Window Design for Dia-PASEF," *Molecular & Cellular Proteomics* 21, no. 9 (2022): 100279, https://doi.org/10.1016/j.mcpro.2022.100279.

47. F. Meier, A. D. Brunner, M. Frank, et al., "diaPASEF: Parallel Accumulation-Serial Fragmentation Combined With Data-Independent Acquisition," *Nature Methods* 17, no. 12 (2020): 1229–1236, https://doi.org/10.1038/s41592-020-00998-0.

48. N. M. Lancaster, P. Sinitcyn, P. Forny, et al., "Fast and Deep Phosphoproteome Analysis With the Orbitrap Astral Mass Spectrometer," *Nature Communications* 15, no. 1 (2024): 7016, https://doi.org/10.1038/s41467-024-51274-0.

49. P. Bortel, I. Piga, C. Koenig, C. Gerner, A. Martinez-Val, and J. V. Olsen, "Systematic Optimization of Automated Phosphopeptide Enrichment for High-Sensitivity Phosphoproteomics," *Molecular & Cellular Proteomics* 23, no. 5 (2024): 100754, https://doi.org/10.1016/j.mcpro.2024.100754.

50. C. R. Weisbrod, J. K. Eng, M. R. Hoopmann, T. Baker, and J. E. Bruce, "Accurate Peptide Fragment Mass Analysis: Multiplexed Peptide Identification and Quantification," *Journal of Proteome Research* 11, no. 3 (2012): 1621–1632, https://doi.org/10.1021/pr2008175.

51. L. C. Gillet, P. Navarro, S. Tate, et al., "Targeted Data Extraction of the MS/MS Spectra Generated by Data-Independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis," *Molecular & Cellular Proteomics* 11, no. 6 (2012): 016717, https://doi.org/10.1074/mcp.O111.016717.

52. V. Demichev, C. B. Messner, S. I. Vernardis, K. S. Lilley, and M. Ralser, "DIA-NN: Neural Networks and Interference Correction Enable Deep Proteome Coverage in High Throughput," *Nature Methods* 17, no. 1 (2020): 41–44, https://doi.org/10.1038/s41592-019-0638-x.

53. J. K. Eng, A. L. McCormack, and J. R. Yates, "An Approach to Correlate Tandem Mass Spectral Data of Peptides With Amino Acid Sequences in a Protein Database," *Journal of the American Society for Mass Spectrometry* 5, no. 11 (1994): 976–989, https://doi.org/10.1016/1044-0305(94)80016-2.

54. D. N. Perkins, D. J. Pappin, D. M. Creasy, and J. S. Cottrell, "Probability-Based Protein Identification by Searching Sequence Databases Using Mass Spectrometry Data," *Electrophoresis* 20, no. 18 (1999): 3551–3567, https://doi.org/10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2.

55. S. A. Beausoleil, J. Villen, S. A. Gerber, J. Rush, and S. P. Gygi, "A Probability-Based Approach for High-Throughput Protein Phosphorylation Analysis and Site Localization," *Nature Biotechnology* 24, no. 10 (2006): 1285–1292, https://doi.org/10.1038/nbt1240.

56. J. V. Olsen, B. Blagoev, F. Gnad, et al., "Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks," *Cell* 127, no. 3 (2006): 635–648, https://doi.org/10.1016/j.cell.2006.09.026.

57. J. Cox and M. Mann, "MaxQuant Enables High Peptide Identification Rates, Individualized p.p.b.-Range Mass Accuracies and Proteome-Wide Protein Quantification," *Nature Biotechnology* 26, no. 12 (2008): 1367–1372, https://doi.org/10.1038/nbt.1511.

58. R. Bruderer, O. M. Bernhardt, T. Gandhi, et al., "Extending the Limits of Quantitative Proteome Profiling With Data-Independent Acquisition and Application to Acetaminophen-Treated Three-Dimensional Liver Microtissues," *Molecular & Cellular Proteomics* 14, no. 5 (2015): 1400–1410, https://doi.org/10.1074/mcp.M114.044305.

59. B. MacLean, D. M. Tomazela, N. Shulman, et al., "Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments," *Bioinformatics* 26, no. 7 (2010): 966–968, https://doi.org/10.1093/bioinformatics/btq054.

60. B. M. Invergo, B. Petursson, N. Akhtar, et al., "Prediction of Signed Protein Kinase Regulatory Circuits," *Cell Systems* 10, no. 5 (2020): 384–396, https://doi.org/10.1016/j.cels.2020.04.005.

61. F. Luo, M. Wang, Y. Liu, X. M. Zhao, and A. Li, "DeepPhos: Prediction of Protein Phosphorylation Sites With Deep Learning," *Bioinformatics* 35, no. 16 (2019): 2766–2773, https://doi.org/10.1093/bioinformatics/bty1051.

62. M. Mischnik, F. Sacco, J. Cox, et al., "IKAP: A Heuristic Framework for Inference of Kinase Activities From Phosphoproteomics Data," *Bioinformatics* 32, no. 3 (2016): 424–431, https://doi.org/10.1093/bioinformatics/btv699.

63. S. Muller-Dott, E. J. Jaehnig, K. P. Munchic, et al., "Comprehensive Evaluation of Phosphoproteomic-Based Kinase Activity Inference," *Nature Communications* 16, no. 1 (2025): 4771, https://doi.org/10.1038/s41467-025-59779-y.

64. W. Jiang, E. J. Jaehnig, Y. Liao, et al., "Deciphering the Dark Cancer Phosphoproteome Using Machine-Learned Co-Regulation of Phosphosites," *Nature Communications* 16, no. 1 (2025): 2766, https://doi.org/10.1038/s41467-025-57993-2.

65. M. Haines, J. R. Thorup, S. Gohsman, et al., "High-Throughput Proteomic and Phosphoproteomic Analysis of Formalin-Fixed Paraffin-Embedded Tissues," *Molecular & Cellular Proteomics* 24, no. 9 (2025): 101044, https://doi.org/10.1016/j.mcpro.2025.101044.

66. C. Friedrich, S. Schallenberg, M. Kirchner, et al., "Comprehensive Micro-Scaled Proteome and Phosphoproteome Characterization of Archived Retrospective Cancer Repositories," *Nature Communications* 12, no. 1 (2021): 3576, https://doi.org/10.1038/s41467-021-23855-w.

67. S. Vasaikar, C. Huang, X. Wang, et al., "Proteogenomic Analysis of Human Colon Cancer Reveals New Therapeutic Opportunities," *Cell* 177, no. 4 (2019): 1035–1049, https://doi.org/10.1016/j.cell.2019.03.030.

68. D. J. Clark, S. M. Dhanasekaran, F. Petralia, et al., "Integrated Proteogenomic Characterization of Clear Cell Renal Cell Carcinoma," *Cell* 179, no. 4 (2019): 964–983, https://doi.org/10.1016/j.cell.2019.10.007.

69. K. Krug, E. J. Jaehnig, S. Satpathy, et al., "Proteogenomic Landscape of Breast Cancer Tumorigenesis and Targeted Therapy," *Cell* 183, no. 5 (2020): 1436–1456, https://doi.org/10.1016/j.cell.2020.10.036.

70. K. B. Emdal, N. Palacio-Escat, C. Wigerup, et al., "Phosphoproteomics of Primary AML Patient Samples Reveals Rationale for AKT Combination Therapy and p53 Context to Overcome Selinexor Resistance," *Cell Reports* 40, no. 6 (2022): 111177, https://doi.org/10.1016/j.celrep.2022.111177.

71. R. Beekhof, A. Bertotti, F. Bottger, et al., "Phosphoproteomics of Patient-Derived Xenografts Identifies Targets and Markers Associated With Sensitivity and Resistance to EGFR Blockade in Colorectal Cancer," *Science Translational Medicine* 15, no. 709 (2023): eabm3687.

72. A. Valles-Marti, G. Mantini, P. Manoukian, et al., "Phosphoproteomics Guides Effective Low-Dose Drug Combinations Against pancreatic Ductal Adenocarcinoma," *CellRep* 42, no. 6 (2023): 112581.

73. D. Gunji, Y. Abe, S. Muraoka, et al., "Longitudinal Phosphoproteomics Reveals the PI3K-PAK1 Axis as a Potential Target for Recurrent Colorectal Liver Metastases," *Cell Reports* 43, no. 12 (2024): 115061.

74. H. Rodriguez, J. C. Zenklusen, L. M. Staudt, J. H. Doroshow, and D. R. Lowy, "The Next Horizon in Precision Oncology: Proteogenomics to Inform Cancer Diagnosis and Treatment," *Cell* 184, no. 7 (2021): 1661–1670, https://doi.org/10.1016/j.cell.2021.02.055.

75. J. W. Annika Schneider, B. J. Cecilia, S. Amirhossein, et al., "Therapeutic implications of phosphoproteomics in molecular cancer diagnostics," *ASCO Annual Meeting* (2024): 3068.