Critical Review

The Significant Others: Global Search for Direct Kinase Substrates Using Chemical Approaches

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Abstract

Protein kinases function as key signaling hubs in the intricate network of biochemical signaling processes in the living cell. More than two-thirds of the human proteome is estimated to be phosphorylated at ~960,000 phosphosites, which makes it challenging to identify the direct contribution of any desired kinase in generating this phosphoproteome. In this review, we discuss some of the methods that have been developed over the years for global identification of kinase substrates. The methods are essentially categorized into two classes, namely, (i) direct tagging of kinase substrates and (ii) indirect phosphoproteomics-based approaches. We discuss the advantages and limitations entailed to each of the method introduced, with a special emphasis on the analog-sensitive (as) kinase approach method. © 2019 IUBMB Life, 71(6):721–737, 2019

Keywords: chemical genetic; kinases; analog-sensitive; orthogonal ATP analogs; ASKA; KISS; PIKISS; proteomics; bump and hole approach; substrates

INTRODUCTION

Among the 200 different posttranslational modifications used in signal transduction, protein phosphorylation is arguably the most prominent form employed by the cell (1). Initially identified in glycogen phosphorylase as a regulatory mechanism for glycogen metabolism, protein phosphorylation affects nearly all aspects of cellular functions (2). The human kinome contains 535 protein kinases, representing 1.8% of human genes [www.kinase.com]. Eukaryotic protein kinases were predominantly known to phosphorylate serine, threonine, and tyrosine residues; however, recent studies have identified histidine kinases as new members of the eukaryotic kinome family (3). Among serine, threonine, and tyrosine residues, serine is the most abundantly phosphorylated residue in cells (86%), followed by threonine (12%) and tyrosine (2%) (4). It is estimated that 80–90% of the proteome is phosphorylated, making it a challenging endeavor to trace the substrates of each of the 535 human kinases (5). In addition, most of the proteins are phosphorylated at multiple sites, and often each phosphorylation site is associated with a specific biological function. Thus, most phosphoproteins integrate inputs from multiple kinases spanning diverse signaling cascades, resulting in biological outcomes that support each tissue's unique physiology.

Deregulated kinase signaling is implicated in more than 400 human diseases, making protein kinases the second largest class of drug targets after G protein-coupled receptors (6, 7). In fact, 85% of the kinome is believed to be deregulated in various human developmental defects and pathologies (http://diseases.jensenlab.org; (6)). In several diseases, direct targeting of the deregulated kinase(s) is highly efficacious as exemplified by the success of 50 FDA-approved kinase inhibitors to date (www.brimr.org). In others, it causes severe side effects due to the kinase's vital role in normal tissues. In such instances, targeting its pathological substrates provides a superior option for developing effective therapeutics and for combating collateral toxicity (8). Interestingly, recent advancements in mass spectroscopy have enabled rapid profiling of the phosphoproteome with accurate determination of phosphorylation sites (9, 10).

Abbreviations: *ASKA, Analog-sensitive kinase approach; A*TP, N-6-modified orthogonal ATP analogs; as, Analog-sensitive; EBV, Epstein–Barr virus; GPCR, G protein coupled receptors; HAKA-MS, Heavy ATP kinase assay combined with quantitative mass spectrometry; KISS, Kinase-interacting substrate screening; K-BILDS, Kinase-catalyzed biotinylation with inactivated lysates for discovery of substrates; LC–MS/MS, Liquid chromatography with tandem mass spectrometry; MS, Mass spectrometry; SILAC, Stable isotope labeling with amino acids in cell culture*

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However, it remains a challenge to dissect the contribution of a single kinase of interest in the generation of this phosphoproteome. A variety of chemical genetic approaches have been employed to characterize kinase–substrate relationships and to map their respective signaling pathways. This review will focus its discussion on several of such methods currently used in chemical biology.

We have organized various global substrate identification techniques into two categories—(i) direct tagging of kinase substrates and (ii) indirect phosphoproteomics-based approaches (Fig. 1). Direct approaches for identifying the substrates of a desired kinase often use in vitro kinase assays using peptide arrays, protein arrays, cell lysates, or permeabilized cells. Indirect approaches frequently employ specific inhibitor of a desired kinase in live cells, with an exception of chemical rescue methodology, which uses a kinase activator (section "Chemical Rescue of Crippled Kinase with Phosphoproteomics"), followed by global phosphoproteomics analysis using high-resolution mass spectroscopy (Fig. 1). Although a number of elegant approaches have been developed for both direct and indirect identification of kinase substrates, each methodology has its inherent advantages and limitations. We present a few of these methods, with special emphasis on the analog-sensitive kinase approach (ASKA) methodology.

DIRECT TAGGING OF KINASE SUBSTRATES

The biggest advantage of substrate-tagging approaches is that these methods uncover the direct targets of a desired kinase in the whole proteome. However, as ATP is a universal phosphodonor for all kinases, these methodologies often show high background phosphorylation in cell lysates or permeabilized cells resulting in many false positives. Furthermore, due to the impermeable nature of ATP, these assays are often conducted in vitro or in cell lysates and thus need to be coupled/complemented with cellular or in vivo validation. Several methodologies have been developed over the years to circumvent these issues, a few of which are discussed later (Fig. 1).

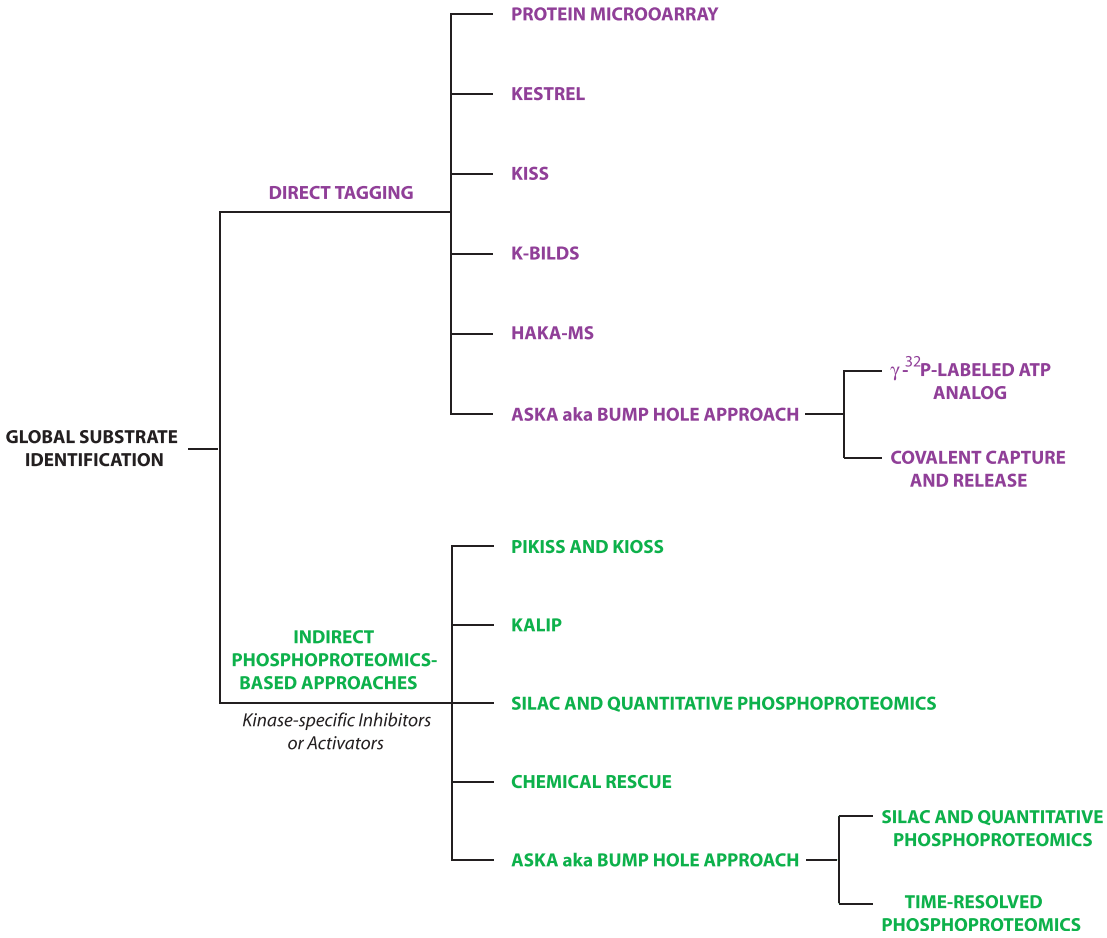

FIG 1 *Organization of various global substrate identification techniques into two categories—(i) direct tagging of kinase substrates and (ii) indirect phosphoproteomic-based approaches. Direct tagging often employs in vitro kinase assays using protein arrays, cell lysates or permeabilized cells. Indirect approaches use specific inhibition of a kinase of interest in intact cells, followed by global phosphoproteomics analysis and LC–MS/MS. In chemical rescue approach, however, the desired kinase is activated in intact cells followed by global phosphoproteomics analysis and LC–MS/MS.*

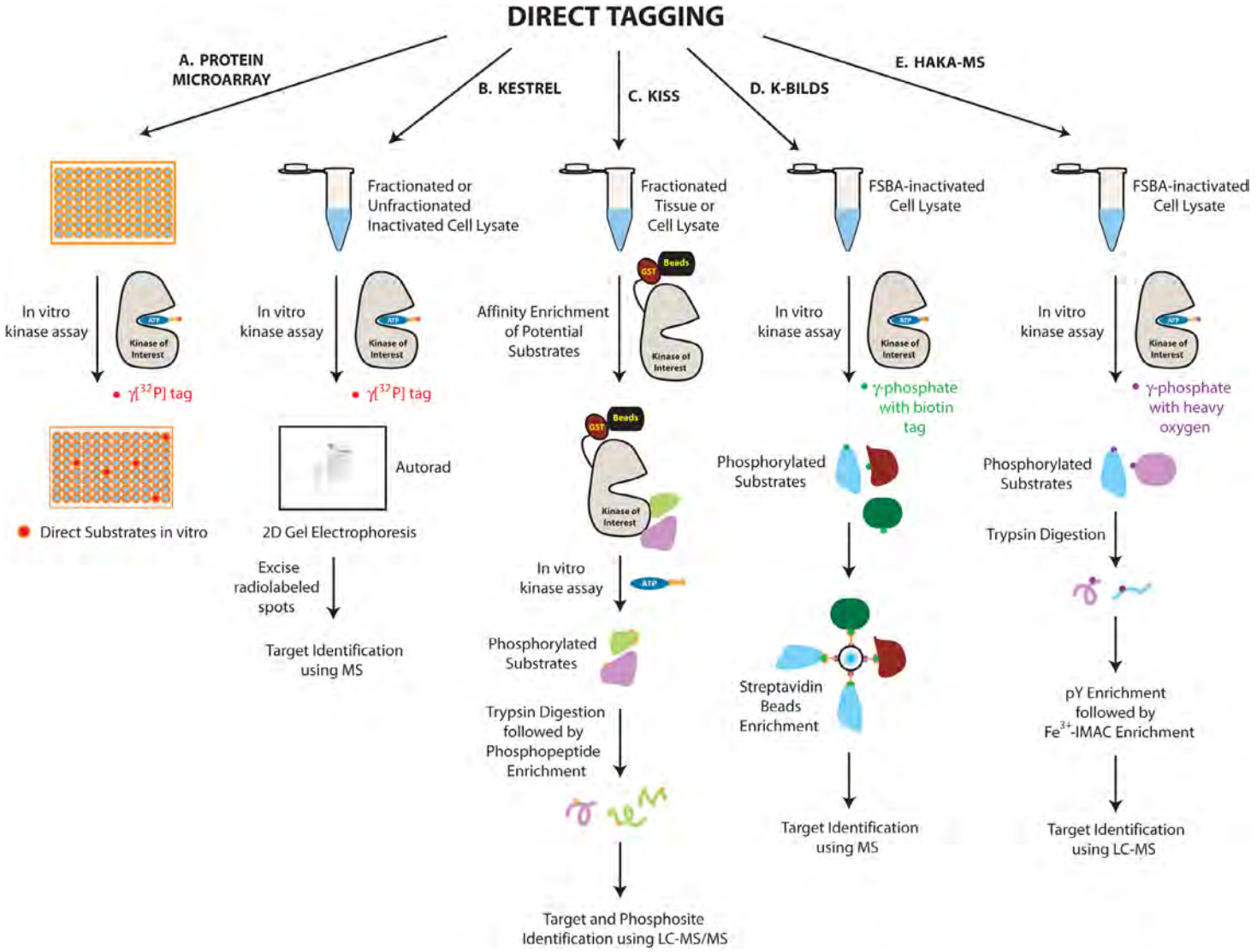

FIG 2

A few direct tagging approaches for kinase substrate identification on a global scale. A, Protein chips containing an array of immobilized substrates are incubated with a purified kinase of interest in the presence of $\gamma[^{32}P]$ ATP. The radiolabeled spots reveal the direct substrates of the desired kinase in vitro. B, In KESTREL approach, purified kinase of interest is incubated with inactive cell lysate in the presence of $\gamma[^{32}P]$ ATP. The proteins are separated on 1D or 2D gels, radiolabeled spots are excised and identified by mass spectrometry. C, In KISS approach, the cell lysate is added to a glutathione-sepharose affinity column containing the GST-tagged kinase of interest. The resulting kinase–substrate complexes are incubated with ATP to promote on-bead phosphorylation reactions, followed by tryptic digestion, phosphopeptide enrichment, and mass spectral analysis. D, K-BILDS approach utilizes biotinylated ATP, which when incubated with FSBA-treated cell lysate, transfers the biotin tag to putative substrates. Biotinylated substrates are affinity purified using streptavidin beads and analyzed using mass spectral analysis. E, HAKA-MS method uses ATP with heavy oxygen (O_{18}) at the γ-phosphate. The kinase of interest is incubated with FSBA-treated cell lysate using heavy ATP. Following trypsin digestion, phosphopeptides are enriched and the samples analyzed by LC–MS.

Protein Microarray

Ptacek et al. performed the first global analysis of yeast kinase substrates using proteome microarrays that included 4,400 yeast proteins in duplicate (11). In brief, protein chips containing an array of immobilized substrates are incubated with a purified kinase of interest in the presence of radiolabeled ATP (Fig. 2). As a control, an additional microarray is incubated in the absence of kinases to identify proteins that undergo autophosphorylation. This study led to the identification of >4,000 phosphorylation events involving 1,325 different proteins from 87 yeast protein kinases (11). Their study further revealed that kinases phosphorylate a distinct set of substrates, demonstrating that each kinase possesses a unique substrate recognition profile. As the identity and the corresponding location of all the proteins are predetermined and spotted on the protein chip, the candidate substrates of the kinase of interest can be identified with ease. The most noticeable advantage is that it allows the simultaneous interrogation of thousands of proteins in vitro in a

single biochemical assay. In addition, the increased availability of genome overexpression collections allows the analyses of substrate specificity of protein kinases on a global scale. Taking advantage of this method, Zhu et al. generated an Epstein–Barr virus (EBV) protein array to identify the direct substrates of BGLF4, an EBV protein kinase. This study uncovered multiple new BGLF4 substrates involved in lytic DNA replication, capsid assembly, and DNA packaging (12).

Subsequently, Newman et al. utilized human protein arrays and identified the substrates for 289 different human kinases. This information was used to construct a high-resolution map of phosphorylation networks that connected 230 kinases to 2,591 in vivo phosphorylation sites in 652 substrates (13). Protein microarray technology has also been applied to evaluate the global effects of PTEN on the phospho-kinome of glioma cells using lysates from whole cells and xenografts (14). Similarly, Boyle et al. screened a chip containing 2,400 human proteins using Abl and Arg kinases and identified cortactin as their direct substrate (15).

A major caveat of this method is that substrates identified in vitro may not necessarily represent bonafide in vivo substrates, as it cannot recapitulate the temporal and/or spatial expression profiles of kinases and substrates (Table 1). Substrates can be missed in the absence of other modifications such as priming phosphorylation, or due to the lack of other scaffolding proteins. Furthermore, the immobilization of candidate substrates can potentially alter their native structure and expose sites that are not accessible in vivo. Hence, further in vivo validations are required to preclude the generation of false-positive kinase–substrate relationships. Finally, overexpressing all the proteins of the proteome is a labor-intensive task and requires considerable time and effort.

Kinase Substrate Tracking Elucidation (KESTREL)

The kinase substrate tracking elucidation (KESTREL) method was developed by Cohen et al. for the identification of kinase substrates in cell extracts (16). This approach utilizes incubation of the cell extracts with high concentrations of an active kinase of interest along with [γ-^{32}P] ATP (Fig. 2). Prior to the kinase assay, ion-exchange chromatography is often performed on cell extracts to separate endogenous kinases from substrates, thereby increasing the concentration of available substrates and the overall likelihood of substrate identification (16). While several novel protein kinase substrates have been elucidated by the KESTREL method over the years, this approach is not free from limitations. Perhaps the most critical caveat of KESTREL is the high nonspecific background phosphorylation caused by endogenous kinase activity in the total cell extract. To circumvent this issue, Mn^{2+} is often added in place of Mg^{2+} to restrict the scope of kinases that can utilize Mn^{2+}-ATP (16). Heat-inactivation of the cell lysate was employed as another alternative means to prevent endogenous kinase and phosphatase activity (17). However, such measures only offer partial resolution as the use of Mn^{2+}-ATP is limited in its applicability and the introduction of a heat-inactivation step often cause structural alterations and/or denaturation of proteins in the cell extract. Finally, the KESTREL approach was a low throughput method that required months of laborious procedures for substrate identification and validation (Table 1).

Kinase-Interacting Substrate Screening (KISS)

To reduce the promiscuity displayed by kinases in an in vitro setting, Amano and Kaibuchi took advantage of affinity column chromatography to enrich kinase–substrate complexes, followed by kinase assays and subsequent identification using liquid chromatography with tandem mass spectrometry (LC–MS/MS). In this in vitro approach, termed kinase-interacting substrate screening (KISS), the cell or tissue lysates are passed through a glutathione-sepharose affinity column coated with the GST-tagged kinase of interest (Fig. 2). The resulting kinase-substrate complexes are incubated with ATP to promote on-bead phosphorylation reactions, followed by tryptic digestion, phosphopeptide enrichment, and mass spectral analysis (18). Through the KISS method, 356 phosphorylation sites of 140 proteins were identified as candidate substrates for Rho-associated kinase (ROCK2), some of which were further validated and demonstrated to interact with ROCK2 intracellularly (18). The KISS method has been further applied to identify the substrates of several other kinases including CaMK1, CDK5, and FYN (18). As such, the KISS method serves as a powerful tool to initially screen for substrate candidates and simultaneously identify their phosphorylation sites. Moreover, the entire process from sample preparation to LC–MS/MS analysis can be completed within a relatively short timeframe of 1 week (19). However, a major limitation entailed with KISS is that the isolation of substrates depends on often short-lived and unstable interactions with their respective kinases. Moreover, the reversible nature of protein phosphorylation makes the identification of phosphoproteins difficult even with the recent advances in mass spectrometry (MS). Finally, KISS is not free from the generic concerns associated with most in vitro kinase–substrate identification methods, in that its results alone cannot fully recapitulate what occurs within a cellular milieu (Table 1). Hence, results obtained from in vitro assays must be complemented with in vivo studies to validate its physiological relevance.

Kinase-Catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates (K-BILDS)

The utilization of ATP analogs is another widely used approach, occupying its own niche among the various methods in kinase-specific substrate identification. As such, a variety of ATP analogs have been developed to date, each of which possess modifications in the adenine base, ribose sugar, or the triphosphate region of the natural ATP molecule. Embogama and Pflum demonstrated the use of γ-phosphate-modified ATP-biotin analog and introduced a method termed kinase-catalyzed biotinylation with inactivated lysates for discovery of substrates (K-BILDS) (Fig. 2) (20). As the first step in the K-BILDS method, cell lysates are preincubated with FSBA (5′-(4-fluorosulfonylbenzoyl) adenosine hydrochloride), a pan-kinase inhibitor that irreversibly inactivates endogenous kinase activity. Excess FSBA is removed via dialysis

TABLE 1 ***Advantages and limitations of a few direct tagging approaches***

Method	*Advantages*	*Limitations*
Protein microarray	Minimal sample requirement	Loss of in vivo spatiotemporal expression profile
	Wide and high throughput screening of candidate substrates	Potential structural alteration resulting from immobilization
		Use of radioactive ATP
		Needs cellular or in vivo validation
KESTREL	Reduced background phosphorylation via use of Mn-ATP	Assays are conducted in vitro due to impermeable nature of ATP
		Use of radioactive ATP
		Low throughput
		Structural alterations following heat inactivation
		Requires cellular or in vivo validation
		Limited number of kinases use Mn^{2+} as the cofactor
KISS	Enriches kinase substrates via protein–protein interactions	Assays are conducted in vitro
	Simultaneous identification of phosphorylation sites	Requires cellular or in vivo validation
	Relatively rapid methodology	Isolation of substrates depends on their transient interaction with their respective kinases
K-BILDS	Precludes the use of radioactive ATP	Assays are conducted in vitro
	Rapid isolation of kinase substrates using streptavidin beads	Range of kinases applicable not yet determined
		FSBA partially inactivates endogenous kinases, resulting in background phosphorylation
		FSBA can nonspecifically label other proteins, thereby complicating MS analysis
		Requires cellular or in vivo validation
HAKA-MS	Labels the direct substrates of a kinase with a heavy mass tag	Assays are conducted in vitro due to impermeable nature of ATP
		FSBA partially inactivates endogenous kinases, resulting in background phosphorylation
		FSBA can nonspecifically label other proteins, thereby complicating MS analysis
		Requires cellular or in vivo validation
ASKA (γ -^{32}P ATP ANALOG)	Labels direct targets of a desired kinase with minimal background phosphorylation.	Use of radioactive ATP analog
	Most analog-sensitive kinases show high catalytic efficiency with orthogonal ATP analogs	Assays are conducted in cell lysates or permeabilized cells due to impermeable nature of ATP analog
	Has been used for >50 kinases	Requires cellular or in vivo validation
		In some cases, analog-sensitive mutations can be hypomorphic

(Continues)

TABLE 1 *(Continued)*

Method	*Advantages*	*Limitations*
ASKA (Covalent capture and release)	Identification of candidate substrates via nonendogenous thiophosphorylation	Assays are conducted in cell lysates or permeabilized cells due to impermeable nature of ATP analog
	Background phosphorylation is usually very low	Requires cellular or in vivo validation
	Rapid isolation of direct substrates of a desired kinase	In some cases, analog-sensitive mutations can be hypomorphic
	Identification of phosphorylation sites on candidate substrates	Many kinases show low catalytic efficiency with thio-ATP, compared to regular ATP

and exogenous active kinase and ATP-biotin are added to the lysates to promote kinase-catalyzed biotinylation of substrate proteins. The resulting biotinylated protein products are enriched via streptavidin resin, separated by SDS-PAGE and subsequently analyzed by LC–MS/MS. This method revealed 279 candidates of Protein Kinase A (PKA), including 56 known PKA substrates (20). Three novel PKA substrates (NASP, BAG3, and YWHAQ) were further validated, suggesting that K-BILDS method represents a promising tool for kinase–substrate identification compared to the traditional use of radiolabeled [γ-^{32}P] ATP (Table 1). Furthermore, streptavidin-mediated enrichment of phosphorylated proteins confers significant advantage in subsequent MS analysis. However, the range of kinases that can utilize ATP-biotin analogs as cosubstrates has yet to be determined. Nonetheless, this method represents a novel avenue that holds promise for further development.

Heavy ATP Kinase Assay Combined with Quantitative Mass Spectrometry

Recently, Müller et al. developed an approach named heavy ATP kinase assay combined with quantitative mass spectrometry (HAKA-MS), which utilizes an ATP with a heavy oxygen (O_{18}) at the γ-phosphate (21). Similar to previous studies, background phosphorylation by endogenous kinases is inhibited by treating the cell lysate with FSBA, followed by dialysis and in vitro kinase assay using constitutively active Abl kinase and heavy ATP. As a control, cell lysate alone and kinase-defective Abl with cell lysate are subjected to kinase assays. Following trypsin digestion, phosphopeptides are enriched using phosphotyrosine (pY) antibody. The eluted peptides are then labeled with isobaric amine-reactive tandem mass tag reagents, followed by a second enrichment step using immobilized metal-affinity chromatography (IMAC) column and the samples analyzed by LC–MS (Fig. 2). Using this approach, the authors identified 180 unique pY sites from 130 phosphoproteins targets of Abl kinase. Similar to the K-BILDS approach, HAKA-MS also labels the direct substrates of the kinase of interest with a unique tag, which are identified by MS. However, similar to other in vitro approaches, the candidate substrates identified need to be validated in cells to identify the bonafide cellular targets (Table 1).

ASKA (aka Bump and Hole Approach) for Direct Tagging of Kinase Substrates

The number of different approaches introduced thus far have employed unique methodologies to demolish endogenous kinase activity, such as heat inactivation, FSBA, or kinase-specific inhibitors. However, FSBA typically fails to completely inactivate all kinase activity in a lysate, resulting in background phosphorylation. Furthermore, FSBA is a highly reactive compound that can nonspecifically label other proteins, thus complicating MS analysis and potentially affect substrates in a way that could hinder their phosphorylation (Table 1). The use of highly specific kinase inhibitors may not always be a viable option and a comprehensive screening of compound libraries is a labor-intensive and time-consuming task.

In this regard, analog-sensitive chemical genetic approaches provide an elegant means to bypass these critical hurdles and, thus, have served as a valuable tool in the identification of direct kinase substrates. In 1997, Shokat et al. developed the currently well-known ASKA technology (22). The ASKA method has two major components, the first of which is the use of a modified ATP analog (orthogonal ATP or A*TP) or an orthogonal inhibitor. The second is an analog-sensitive kinase, which bears a corresponding hole in the active site that accommodates the orthogonal ATP analog with the bump (Fig. 3A,B).

Orthogonal ATP Analogs. Specifically, the ATP molecule is modified to carry a sterically "bulky" group (bump) at the N-6-position of the adenine ring, thereby rendering it orthogonal to all protein kinases except for the analog-sensitive mutant kinase of interest. Initially, a number of orthogonal ATP analogs were synthesized including benzyl, cyclopentyl, cyclohexyl, phenethyl, and napthyl (22), however, almost all analog-sensitive kinases generated in the last two decades show highest catalytic activities with either N-6-benzyl ATP or N-6-phenethyl ATP, thereby precluding the need to screen a library of N-6-modified ATP analogs (Fig. 3B). These orthogonal ATP analogs have a [^{32}P]-labeled gamma phosphate "tag," which allows

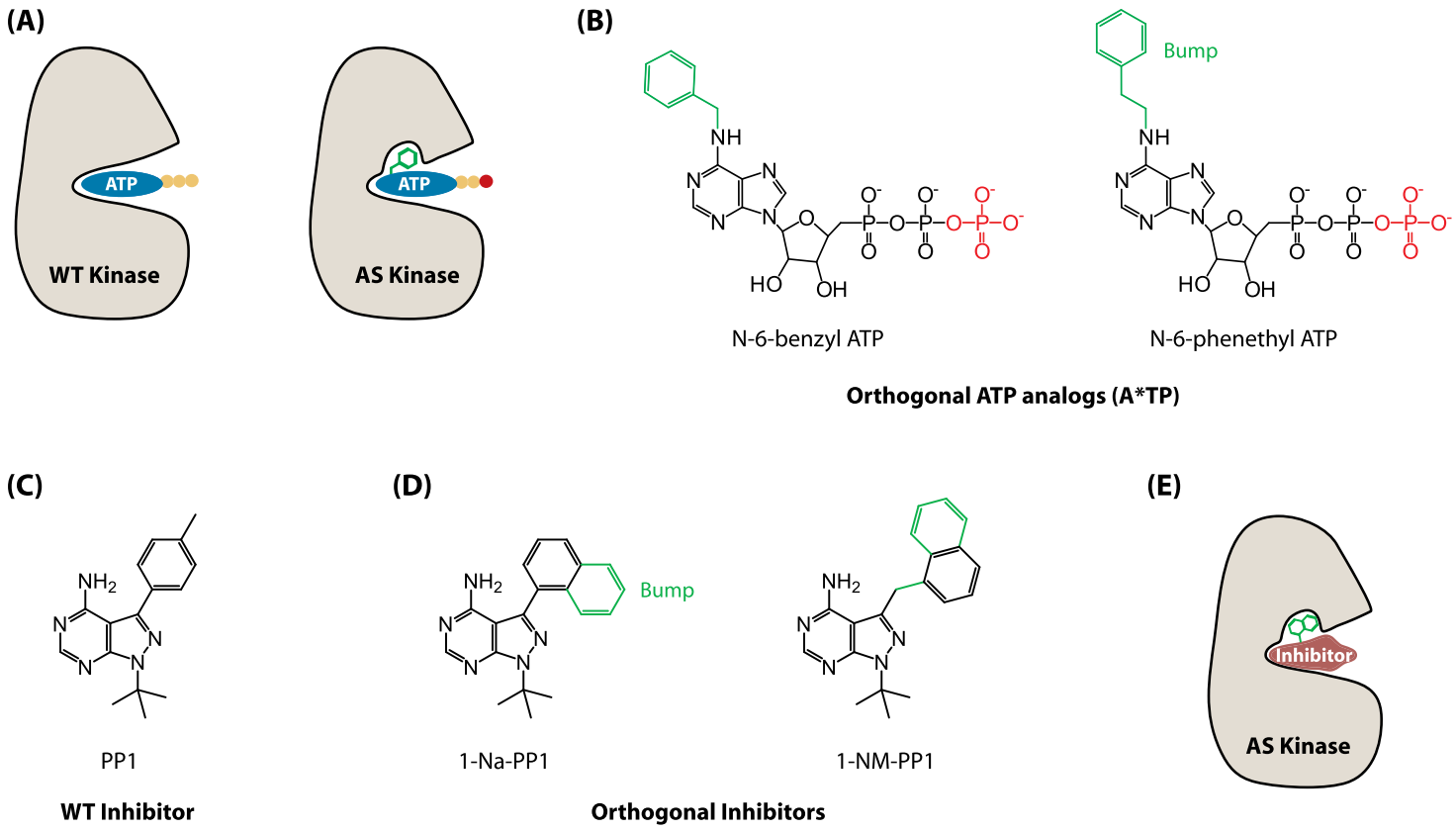

FIG 3

*ASKA aka bump and hole approach: A, Analog sensitive kinases possess a pocket in the active site that is generated using a functionally silent truncation of the gatekeeper residue (additional mutations may be required in some cases). This hole allows them to preferentially accept an orthogonal ATP analog (A*TP) carrying a steric bulk at the N-6 position (shown in green) or orthogonal inhibitors such as shown in (D). B, To date, all reported analog-sensitive kinases show highest catalytic efficiency with either N-6-benzyl ATP (left) or N-6-phenethyl ATP (right). Red phosphate shows radiolabeled [γ-^{32}P] phosphate, which serves as the tag to label the direct targets of the kinase of interest. C, 4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) inhibits several WT kinases. D, Orthogonal inhibitors 1-Na-PP1 and 1-NM-PP1 are derivatives of PP1, which contain bulky groups (bump, shown in green). E, Orthogonal inhibitors inhibit analog-sensitive kinases with extremely high affinity and specificity.*

for unbiased labeling of the direct substrates of an analog-sensitive kinase of interest on a proteome-wide scale (Table 1). Importantly, as the ATP analog is orthogonal, FSBA is not needed, which offers a significant advantage to the ASKA method.

Orthogonal Inhibitors. The "bump and hole approach" also makes analog-sensitive kinases susceptible to small molecules possessing a large hydrophobic group designed to complement the pocket of the analog-sensitive kinases. These inhibitors were generated by substituting the phenyl group on the Src family-specific inhibitor PP1 (4-[p-tert-butyl]benzamido-1-tert-butyl-3-phenylpyrazolo[3,4-d]pyrimidine, Fig. 3C) with either a naphthyl (1-Na-PP1) or a naphthylmethyl group (1-NM-PP1) (Fig. 3D). The steric bulk of the naphthyl ring renders these inhibitors highly orthogonal to all wild-type kinases (23). Thus, these inhibitors inhibit the engineered kinases with extremely high specificity and temporal control and have been used for more than 50 analog-sensitive kinases to date. Orthogonal inhibitors thus serve as invaluable tools for identifying the downstream targets (including direct substrates) of any desired kinase of interest in vivo on a proteome-wide scale (section "ASKA Coupled with Quantitative Phosphoproteomics and Time-Resolved Phosphoproteomics").

Generation of the Analog-Sensitive Kinase via Gatekeeper Mutation. as1 and as2 kinases. The analog-sensitive mutation takes advantage of the high degree of conservation in the ATP-binding site of protein kinases. Specifically, a space-creating mutation is introduced at the gatekeeper residue, which is conserved as a large hydrophobic residue within the ATP-binding pocket across the entire kinome (Fig. 4A,B). The side chain of the gatekeeper residue is in close proximity of the N-6-position of ATP, therefore, truncating the side chain to either glycine (aka analog-sensitive as1 kinase) or alanine (aka analog-sensitive as2 kinase) creates a large pocket. Thus, only the analog-sensitive kinase is able to utilize the ATP analogs with the bulky group at the N-6-position (24). Finally, the space-creating mutation is often functionally silent, and does not impede the functions of engineered kinases in the cells.

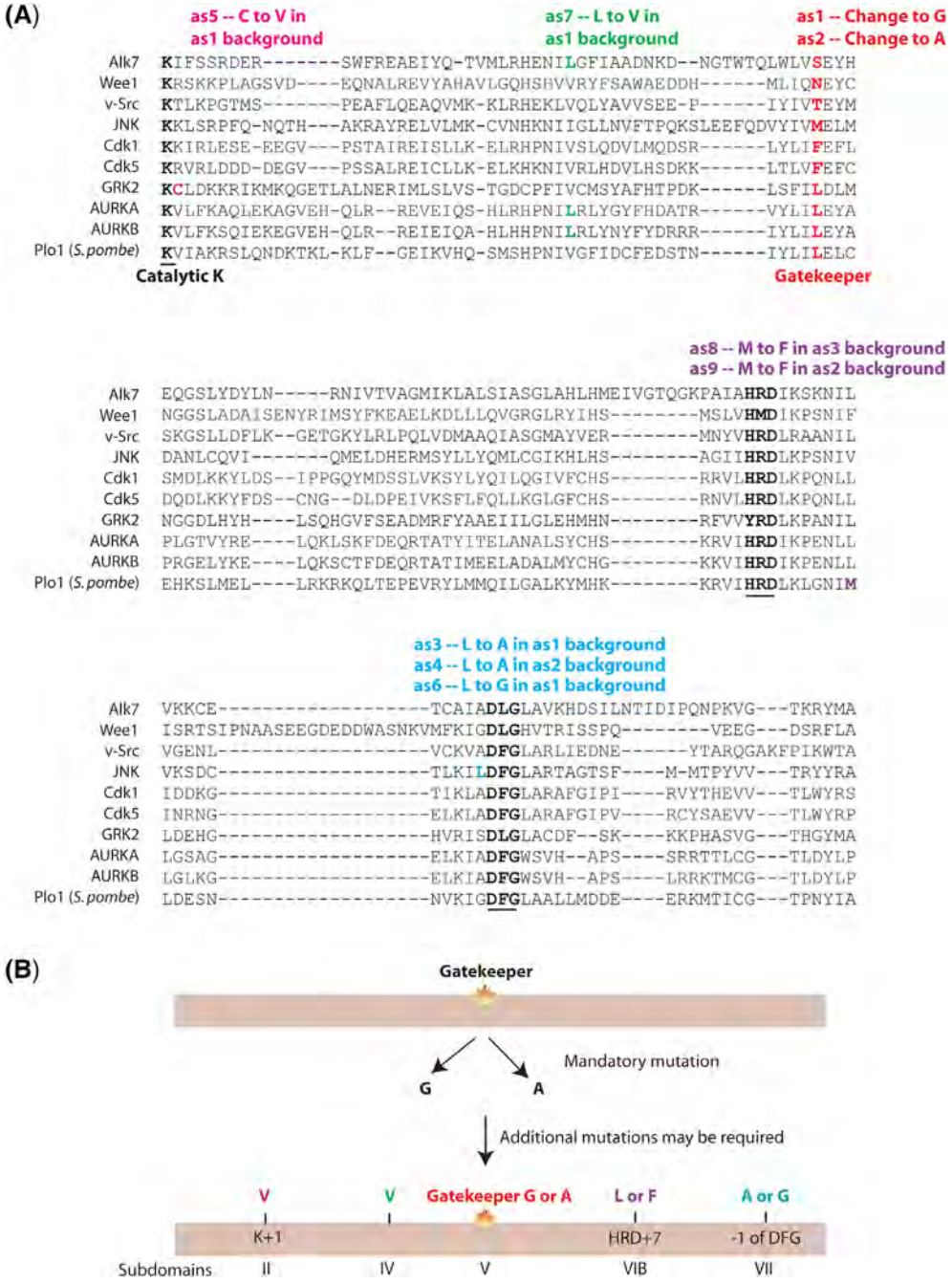

FIG 4

A, Sequence alignment of a few representative kinases showing the gatekeeper residue along with other analog-sensitive sites. All analog-sensitive kinases require a mandatory gatekeeper mutation to either a glycine (as1) or an alanine (as2) (shown in red). Several kinases may require additional mutations in the active site to either rescue the loss of kinase activity due to the gatekeeper mutation and/or to increase the catalytic efficiency of the mutant kinases with ATP analogs or orthogonal inhibitors. At −1 position of the DFG motif, an A or G is preferred by analog-sensitive kinases (shown in blue). Similarly, L or F is favored at +2 position after HRDLKxxN motif (shown in purple). We have observed that a valine is highly preferred in subdomain IV, ~15 residues upstream of the gatekeeper residue (shown in green). A cysteine to valine mutation may be required next to the catalytic lysine in some cases to confer analog sensitivity (dark pink color). B, Generation of analog-sensitive kinases: The first step is to generate gatekeeper mutation by making a glycine mutation (as1). In case the as1 mutation renders the kinase less active, then as2 mutant is chosen, in which gatekeeper residue is instead mutated to an alanine. Many as1 and as2 kinases show high catalytic efficiency with orthogonal ATP analogs and orthogonal inhibitors. In case as1 and as2 kinases are less active or do not efficiently accept ATP analogs and inhibitors, additional mutations are introduced as listed above either in as1 or as2 background. The kinase domain shows the preferred residues of analog-sensitive kinases at various positions in the ATP-binding site. These include a valine at +1 position of the catalytic lysine, a valine in subdomain IV which is 15 amino acids upstream of the gatekeeper residue in v-Src (details in Fig. 4A in green), a leucine or a phenyalanine at HRD +7 position, and an alanine or glycine at −1 position of the DFG motif.

Multiple kinases including v-Src-as1, c-Src-as1, Hog1-as1, Cdk5-as1, and Cdc28-as1 have been generated which display high catalytic efficiency with either N-6-benzyl or N-6-phenethyl ATP analogs (Fig. 3B) (25–30). In particular, the Cdk family members are highly amenable to the as1 mutation and largely retain their catalytic activity in cells. In other cases, substitution of the gatekeeper residue with a glycine significantly perturbs the active site, resulting in an inactive or hypomorphic as1 kinase. In such cases, an alanine mutation is preferable (as2 kinase), which creates a sufficiently large pocket allowing it to specifically label the substrates using N-6-benzyl or N-6-phenethyl ATP analogs. This latter method was employed in developing the analog-sensitive protein kinase Cδ that can utilize N-6-benzyl ATP and is vulnerable to inhibition by PP1 analogs (Fig. 3D) (31).

Secondary mutations along with mandatory gatekeeper mutation. While the gatekeeper mutation is mandatory for generating analog-sensitive kinases, in several instances, additional mutations are required either to rescue the activity of a hypomorphic as1 or as2 kinase, or to further tailor the pocket for rendering them sensitive to orthogonal ATP analogs and orthogonal inhibitors (Fig. 4A,B).

as3, as4, as5, and as6 kinases. A few kinases require an additional sensitizing mutation immediately amino-terminal to the conserved DFG motif (−1 position from DFG motif) in kinase subdomain VII (Fig. 4A, B). An alanine or a glycine at this position is favored for both rescuing the activity and sensitizing the analog-sensitive kinases to orthogonal ATP analogs. Thus, as1 and as2 mutations along with an alanine substitution at this position are named as as3 and as4, respectively. Similarly, a glycine substitution at −1 position of DFG motif in as1 background was named as6 kinase.

The as3 mutation was first introduced in JNK (M108G, L168A aka JNK-as3), which enabled the mutant kinase to accommodate [γ-^{32}P]-N-6-phenethyl ATP without affecting its substrate recognition ability. This study identified heterogeneous nuclear ribonucleoprotein K Protein (hnRNP K) as a direct JNK target. JNK phosphorylates hnRNP K at 216 and 353, which increases its transcriptional activity (32). In another study, GRK2-as1 kinase displayed drastically reduced activity, which was rescued by mutating the cysteine residue immediately adjacent to the catalytic lysine to a valine (as5 kinase) (Fig. 4A, B). Most analog-sensitive kinases prefer β-branched amino acids at this position (33).

as7 kinases. In our laboratory, Aurora A-as1 and Aurora B-as1 kinases showed comparable activities with those of corresponding wild-type kinases, however, did not accept N-6-modified ATP analogs or orthogonal inhibitors efficiently. Crystal structure analysis revealed that L194 in Aurora A (and L154 in Aurora B) was within 5Å of N-6 position of ATP. In the case of v-Src and c-Src (the original kinases using which this method was developed), this residue was a valine, which prompted us to mutate L194 in Aurora A and L154 in Aurora B to a valine. This second mutation rendered both Aurora A (L194V, L210G) and Aurora B (L138V, L154G) kinases highly receptive to N-6-modified ATP analogs (named as analog-sensitive-7 aka as7) and 1-NM-PP1 (34). Despite two mutations in the active site, both Aurora A-as7 and Aurora B-as7 are highly active in cells and in vitro; and show very high catalytic efficiency with N-6-phenethyl ATP analog. Using Aurora A-as7 kinase, we have identified several direct targets including PHLDA1, LIMK2, ALDH1A1, and TWIST1 in cell lysates (8, 34–36).

This additional as7 mutation was further used for Alk7 kinase, which sensitized it to orthogonal inhibitor 2-Na-PP1 (section "Orthogonal Inhibitors"). Specific inhibition of Alk7-as7 kinase using 2-Na-PP1 in mice increased adipocyte lipolysis and β-adrenergic signaling, but reduced diet-induced weight gain, fat accumulation, and adipocyte size (37). These findings suggest that as7 mutation has the potential to sensitize the analog-sensitive kinases to both orthogonal ATP analog as well as orthogonal inhibitors. Most importantly, many kinases which have not been hitherto amenable to analog-sensitive technology can be rendered sensitive to orthogonal ATP analogs using this secondary leucine to valine mutation (Fig. 4).

as8 and as9 mutations. In addition to as3 mutation, some kinases such as plo1 (*Schizosaccharomyces pombe*), required an additional mutation at the bottom of the ATP-binding pocket. In the primary sequence, this residue is at +2 position after HRDLKxxN motif in subdomain VIb in c-Src and v-Src kinases (Fig. 4). It is a valine in c-Src and v-Src, but a methionine in plo1, Orb5, and Wee1 kinases. A mutation of methionine to valine (M to V) sensitized plo1-as3, Orb5-as3, and Wee1-as3 kinases to orthogonal small molecule inhibitors (named as analog-sensitive plo1-as8, Orb5-as8, and Wee1-as8, respectively) (38). Similarly, methionine to valine mutation in as2 background was coined as9 (39). The authors reported that 5–9% of kinases harbor a methionine at this position in key model organisms, hinting at its broader applicability across the kinase family.

Identification of the Direct Substrates on a Proteome-Wide Scale Using ^{32}P-Labeled Orthogonal ATP Analog and Analog-Sensitive Kinase (Original ASKA). To identify the substrates of a desired kinase, analog-sensitive kinases can be expressed endogenously in cells or recombinant kinases could be produced in heterologous systems and then used to tag its direct substrates in cell or tissue lysates. As the ATP analog is not cell permeable, it necessitates the usage of lysates, which if needed can be fractionated prior to kinase assays. Fractionation leads to protein enrichment, which aids in subsequent identification of kinase targets by mass spectrometry. Kinase assays are conducted in lysates for 10–15 min to label the direct targets of the analog-sensitive kinases using [^{32}P]-labeled ATP analog. Proteins are separated by 2D gel electrophoresis, hot spots are excised and identified by mass spectrometry (Fig. 5). This method was initially developed for v-Src, and

subsequently applied to numerous kinases. For v-Src-as1, N-6-(cyclopentyl) ATP was identified as the most optimal ATP analog using a small library of ATP analogs, however, subsequent studies revealed N-6-benzyl ATP and N-6-phenethyl ATP to be superior to *N*-6-(cyclopentyl) ATP (24, 25). Ubersax et al. utilized this approach for yeast Cdk1-as1/Clb2 complex and identified 200 Cdk1 substrates, many of which were confirmed to be phosphorylated in vivo (30). We have identified many substrates of Cdk5-as1/p25 in mouse brain lysates, which uncovered several molecular mechanisms by which deregulated Cdk5 promotes neurodegenerative pathways in Alzheimer's disease pathogenesis (40–44). Similarly, this approach revealed oncogenic targets of Aurora A kinase in breast and pancreatic cancer, which unraveled the mechanism of Aurora A-induced epithelial to mesenchymal transition and cancer stem cell formation in highly aggressive cancers (35, 36). As such, the ASKA method represents a powerful tool for identifying kinase-specific substrates. Perhaps the most prominent feature of the ASKA method is that it eliminates concerns pertaining to background phosphorylation and provides certainty that the phosphoproteins are direct substrates of the kinase of interest. However, a major challenge facing this approach is the difficulty associated with the intracellular delivery of bulky ATP analogs. Therefore, putative targets identified using the chemical genetic approach needs to be validated in cells or in vivo similar to in vitro screens (Table 1).

Identification of the Direct Substrates on a Proteome-Wide Scale Using Covalent Capture-and-Release with Analog-Sensitive Kinase (New ASKA). The original ASKA approach is one of most widely used method for global search of kinase substrates. However, the constraint to use freshly synthesized [γ^{32}P] ATP analog (N-6-benzyl ATP or N-6-phenethyl ATP) for substrate labeling is often a limiting factor. To circumvent this requirement, Shokat et al., building upon their ASKA method, developed a chemical approach that employs ATPγ-S analog, instead of [γ^{32}P] ATP analog (45). Typically the cell lysate is incubated with the engineered analog-sensitive kinase of interest along with either N-6-(benzyl)ATPγ-S or N-6-(phenethyl)ATPγ-S to promote thiophosphorylation reactions (Fig. 5). As thiophosphorylation is not an endogenous posttranslational modification, this feature is ingeniously exploited to "capture" thiophosphorylated kinase substrates from the cellular milieu using two complementary approaches. In the first method, thiophosphorylated kinase substrates are specifically alkylated using *p*-nitrobenzyl mesylate (PNBM), which distinguishes thiophosphorylated candidate substrates from other endogenous peptides containing cysteine residues (45). This mode of selectivity can be attributed to the fact that alkylation of the former results in thiophosphate esters while the latter leads to thioethers (45). The candidate proteins then can be immunoprecipitated via thiophosphate ester-specific antibodies and identified by mass spectrometry (Fig. 5).

This approach however, does not readily identify the phosphorylation site of the kinase of interest. Hence, in the second approach, the thiophosphorylated substrates are subjected to trypsin digestion, followed by incubation with iodoacetyl-agarose resin. This resin "captures" all thiol-containing peptides in the protein mixture, including the target substrate peptides and other cysteine and methionine bearing peptides. All other unbound peptides are subsequently washed away. The beads are next treated with Oxone, which oxidizes sulfur atom in the thiophosphate ester to yield sulfoxide. At this step, peptides bound via thiophosphate ester linkages are "released" from the beads by spontaneous hydrolysis while those containing cysteine thioether linkages are left bound to the beads. The enriched proteins are then analyzed by mass spectrometry (Fig. 5). Using this method, Shokat et al. successfully identified more than 70 substrates of the Cdk1-cyclin B substrates along with their phosphorylation sites (46). Subsequently, Banko et al. utilized this approach in permeabilized cells overexpressing energy-sensing analog-sensitive AMP-activated protein kinase (AMPK-as1) and identified 28 previously unknown substrates (47). Similarly, Schaffer et al. utilized this approach to uncover 50 AMPK substrates along with their phosphorylation sites in permeabilized AMPK-as1-expressing U2OS cells (48).

This method offers several advantages over the original ASKA method. First, covalent capture and release method alleviates the need for any 1D or 2D gel separation, thereby making this method amenable to high-throughput screening of global kinase substrates. Second, due to the enrichment step, the probability of identifying low abundance kinase substrates is increased. Third, ATPγ-S analogs are more stable against hydrolysis by ATPase, an important benefit when whole cell lysate or permeabilized cells are used for conducting global search of kinase substrates (49). Fourth, thiophosphorylated proteins and peptides are more resistant to phosphatases compared to normal phosphorylated proteins and peptides, resulting in increased signal and higher sensitivity in LC–MS/MS (50). Fifth, this method does not require radioactive ATP analog. Finally, one of the biggest advantage of this method is identification of phosphorylation sites on putative kinase substrates (Fig. 5; Table 1).

While efficacious, this method also inherently shares some of the disadvantages associated with ATP analogs in that they are primarily restricted to in vitro applications due to their poor cell-permeability. In addition, the use of ATPγ-S analogs in an in vivo setting raises the issue of cellular toxicity as thiophosphorylation is resistant to phosphatase activity, further rendering in vivo application of this method problematic. More importantly, many kinases show poor catalytic efficiency with ATPγ-S, thereby somewhat limiting the applicability of this approach across the kinome (Table 1). However, for many kinases, substituting $MgCl_2$ (or $MnCl_2$) with $NiCl_2$ in the kinase buffer remarkably enhances the K_{cat} of kinases with thio-ATP (51), which could be employed with capture and release ASKA methodology. Despite these limitations, thio-ATP shows very low background signal from endogenous kinases, making this method a popular choice for kinase–substrate identification on a global scale.

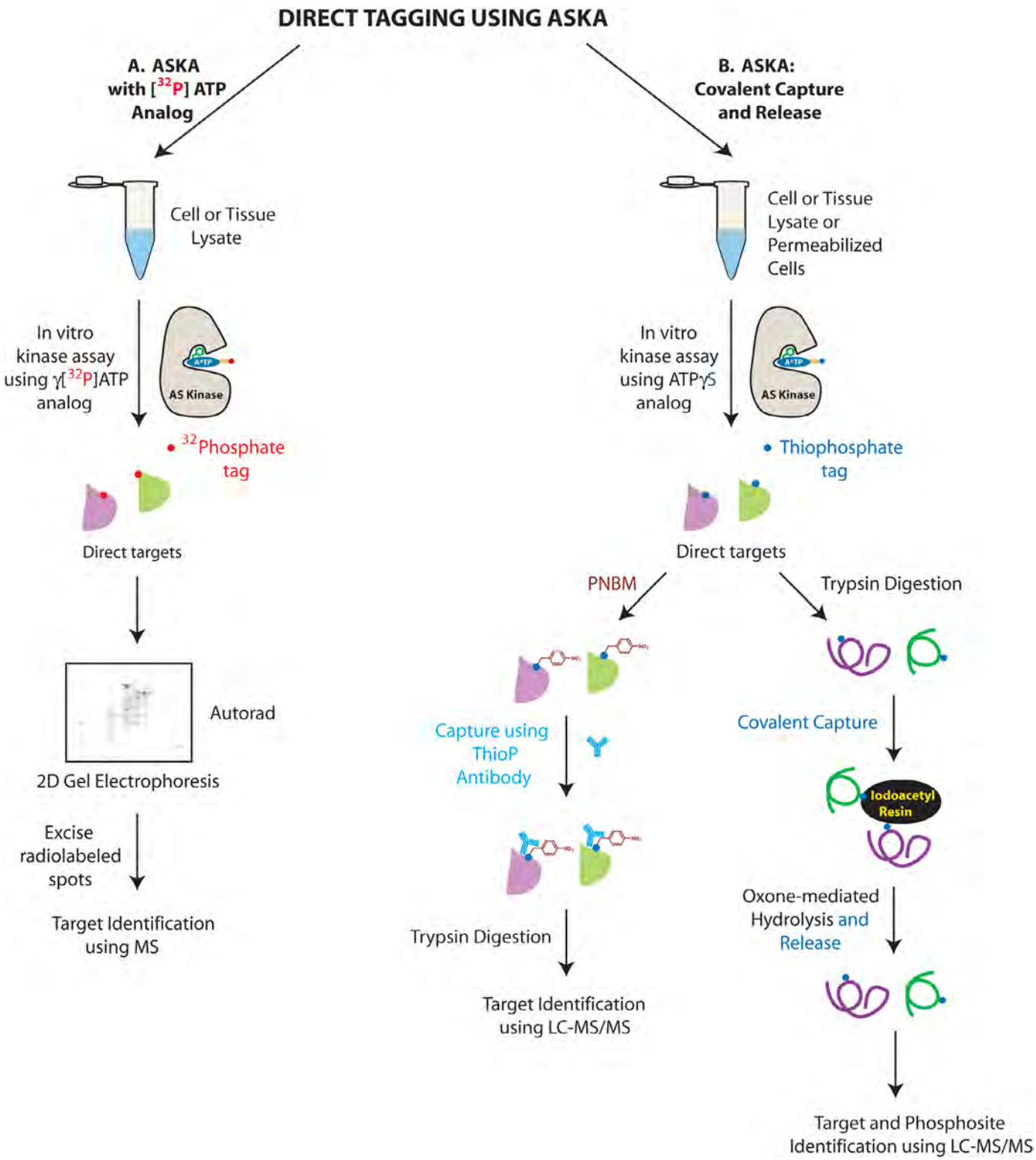

FIG 5 *Global tagging of kinase substrates using ASKA methods. A, Original ASKA approach using γ[^{32}P] labeled ATP analog (N-6-benzyl ATP or N-6-phenethyl ATP). The cell or tissue lysate is incubated with analog-sensitive kinase and γ[^{32}P] labeled ATP analog. Alternatively, analog-sensitive kinase is expressed endogenously in cells and the corresponding cell lysate is used to identify its direct targets. Proteins are separated by 2D gel electrophoresis, radiolabeled spots are excised and identified by mass spectrometry. B, Covalent capture and release ASKA technology uses ATPγ-S analog, instead of [γ^{32}P] ATP analog. The lysate is incubated with the engineered analog-sensitive kinase of interest along with N-6-(benzyl)ATPγ-S or N-6-(phenethyl)ATPγS analogs to tag the direct substrates of the desired kinase. The thiophosphorylated substrates from the cellular milieu are captured using two complementary approaches. In the first method, thiophosphorylated substrates are alkylated using PNBM, followed by immunoprecipitation using thiophosphate ester-specific antibody and identified by mass spectrometry. In the second approach, the thiophosphorylated substrates are trypsin digested and then captured using iodoacetyl-agarose resin. The beads are next treated with Oxone, which spontaneously hydrolyzes peptides bound via thiophosphate ester linkages. The enriched proteins are then analyzed by mass spectrometry.*

INDIRECT APPROACHES FOR IDENTIFYING KINASE SUBSTRATES ON A PROTEOME-WIDE SCALE

Unlike direct substrate tagging approaches, indirect kinase substrate identification approaches usually employ intact cells, thus, the kinase of interest and the substrates are in their native environment with proper spatial and temporal context, which increases the probability of identifying their true cellular partners. The desired kinase is selectively inhibited (or activated), and then the resulting phosphoproteome is analyzed by LC–MS/MS using vehicle-treated phosphoproteome as a control (Fig. 1). In addition, these approaches are often accompanied by substrate enrichment techniques using phosphorylated motifs binding domains, for example, kinases belonging to CDK family, MAPK family, JNK and GSK3 phosphorylate SP/TP motifs. As WW domain of PIN1 binds phosphorylated SP/TP sites, global search for aforementioned kinase families are often coupled with substrate enrichment strategies using WW domain (52) (Fig. 6).

However, these approaches face three major challenges: (i) It is hard to activate/inhibit one kinase of interest with very high specificity in the presence of numerous other kinases in the cellular milieu. In this regard, the ASKA methodology allows for selective inhibition of any kinase of interest with extremely high specificity. (ii) As the phosphoproteome is analyzed downstream of kinase inhibition, the substrates identified are often indirect downstream targets of the kinase of interest, instead of being their direct partners (addressed in time resolved phosphoproteomics; substrates dephosphorylated with fast kinetics are most likely to be direct). (iii) Instead of directly tagging the substrates of a kinase, the targets are identified following subtractive comparisons with controls (Table 2).

Phosphatase Inhibitor and Kinase Inhibitor Substrate Screening and Kinase-Oriented Substrate Screening (PIKISS)

Kaibuchi et al. developed an in vivo approach to probe kinase-specific substrates named as the phosphatase inhibitor and kinase inhibitor substrate screening (PIKISS). Later this approach gave rise to kinase-oriented substrate screening (KIOSS) method, in which additional reagents such as kinase activators and receptor agonists were added (53, 54). These approaches have three major components that differ from its in vitro counterpart KISS (section “Kinase-Interacting Substrate Screening”). First, it is conducted in intact cells; second, the method involves the treatment of kinase-specific inhibitors, kinase activators, and/or phosphatase inhibitors prior to protein extraction; third, phosphoproteins are enriched using specific phospho-binding motifs, which largely filters the background signal (53, 54).

For example, for identifying Rho-kinase substrates, HeLa cells were treated with Rho-kinase-specific inhibitor Y-27632 and phosphatase inhibitor calyculin A (53). Resulting phosphoproteins were enriched by affinity chromatography using beads coated with 14-3-3 proteins that bind to phosphorylated serine and threonine residues with K or R at −3 position (R/KXXpS/T) (55), similar to the consensus sequence phosphorylated by Rho kinase (56). Tryptic digestion and phosphopeptide enrichment were followed by LC-MS/MS for quantification of protein phosphorylation and identification of phosphorylation sites. Candidate substrates were determined by the careful comparison of the phosphopeptide ion peaks exhibited from samples treated with or without the kinase inhibitor and phosphatase inhibitor, respectively. Using this method, Kaibuchi et al. identified 297 phosphorylation sites of 121 proteins as candidate substrates for Rho-kinase (53). However, it is worth noting that this approach requires the use of highly specific kinase inhibitors, which are not always available. Second, although enrichment strategy largely removes background phosphorylation by other kinases, many kinases still share similar consensus motifs, which may result in false positives (Rho kinase, PKC, and PKA phosphorylate similar consensus sequences). Most importantly, this approach identifies downstream targets of Rho kinase, which may or may not be its direct substrates (Table 2).

Kinase Assay Linked with Phosphoproteomics (KALIP)

Xue et al. developed an approach termed kinase assay linked with phosphoproteomics (KALIP) that incorporates an in vitro kinase reaction with endogenous kinase-dependent phosphoproteomics to identify direct substrates of kinases (57). The in vitro component of this method introduces a unique step to prepare a pool of dephosphorylated peptides derived from formerly phosphorylated proteins. In the first step, cells are exposed to a phosphatase inhibitor to increase overall phosphorylation. Phosphoproteins are subsequently purified via antibody-based immunochromatography and FSBA is added to eliminate endogenous kinase activity. This step is followed by tryptic digestion and dephosphorylation by phosphatase treatment to generate a pool of candidate substrate peptides (57). The candidate peptides are then phosphorylated in vitro by adding the protein kinase of interest. The resulting newly phosphorylated peptides are enriched and analyzed by MS. The major feature of KALIP is that a separate in vivo phosphoproteomics screening is conducted in parallel to its in vitro counterpart. Specifically, in vivo kinase-dependent phosphorylation events are determined by using wild-type cells and those in which the kinase of interest is inhibited. Cross-referencing the results obtained from the in vitro kinase assay and in vivo phosphoproteomics then enables the identification of direct physiological substrates of the kinase of interest (57). As such, the essence of this approach is that it serves as a link to the gap between in vitro phosphorylation and in vivo phosphorylation events. Using this method, Xue et al. identified 64 and 23 direct substrates of spleen tyrosine kinase in B cells and breast cancer cells, respectively (57). While this method attempts to minimize the discrepancies between in vitro and in vivo results, it nonetheless suffers from some of the general concerns shared by other in vitro approaches. For example, the cell lysis step inherently results in the loss of information pertaining to localization of proteins. Furthermore, this method may not be applicable

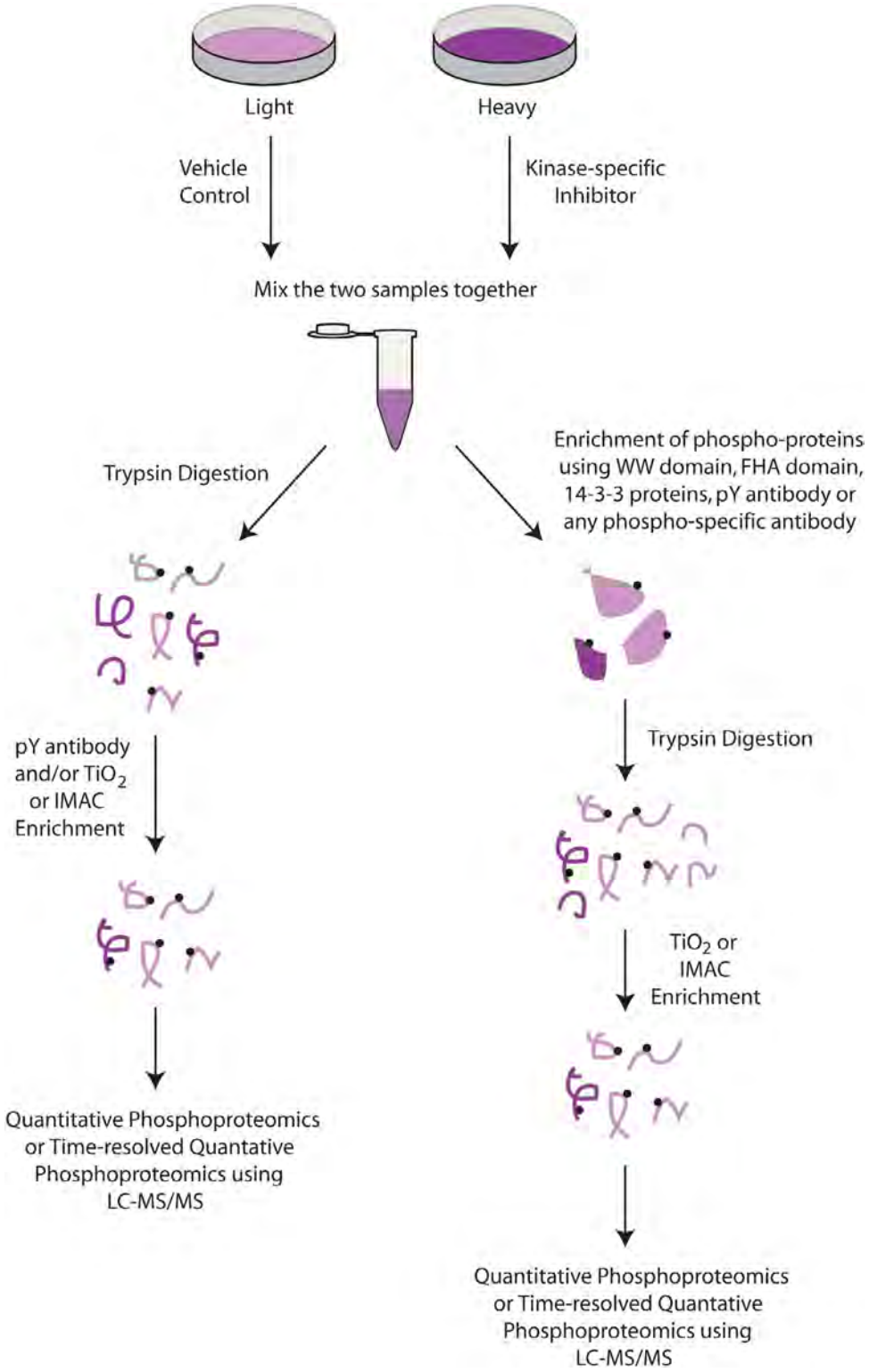

FIG 6

SILAC-based phosphoproteomic approaches for global identification of kinase substrates. In SILAC, a set of cells is isotopically labeled with heavy isotope, while keeping the other set in regular media (light). Light and heavy cells are treated with either vehicle or a kinase inhibitor, respectively. Following treatment, light and heavy cells are mixed, lysed, trypsinized, and the phosphopeptides are enriched and subsequently analyzed by LC–MS/MS (left panel). Alternatively, after mixing heavy and light cells, specific phosphoproteins can be enriched using different phospho-motif binding proteins or phosphospecific antibodies, followed by trypsin digestion, phosphopeptide enrichment and LC–MS/MS analysis. Phosphoproteins can also be enriched in the absence of SILAC labeling. Vehicle or inhibitor-treated cells are lysed (without mixing), phosphoproteins are enriched using different phospho-motif binding proteins, followed by trypsin digestion and LC–MS/MS analysis.

TABLE 2 *Advantages and limitations of indirect phosphoproteomics-based approaches*

Method	*Advantages*	*Limitations*
PIKISS and KIOSS	Phosphorylation occurs in intact cells	Most kinase inhibitors exhibit off-target effects
	Phosphoprotein enrichment using specific domains	Identifies both direct and indirect targets of the desired kinase
	Simultaneous identification of phosphorylation sites	Kinase stimulators influence many kinases in the pathway and are not kinase-specific
		Kinase substrates are identified following subtractive comparison with controls
KALIP	Cross-referencing of in vitro and in vivo candidate substrates	May miss the substrates that require priming phosphorylation
SILAC and quantitative phosphoproteomics	Rapid and global identification of kinase substrates phosphorylated or dephosphorylated in live cells	Most kinase inhibitors exhibit off-target effects
		Identifies both direct and indirect targets of the desired kinase
		Kinase substrates are identified following subtractive comparison with controls
Chemical rescue	Tagging of direct substrates of a desired kinase in live cells in a temporal manner	Labels both direct and indirect targets of the desired kinase
		Off target effect of 20 mM imidazole in cells
ASKA coupled with quantitative phosphoproteomics and time-resolved phosphoproteomics	Extremely specific and potent inhibitors for analog-sensitive kinases	Identifies both direct and indirect targets of the desired kinase, even with time-resolved phosphoproteomics
	Rapid and global identification of kinase substrates in live cells	Kinase substrates are identified following subtractive comparison with controls

for identifying the direct substrates of kinases that require priming phosphorylation (Table 2).

Stable Isotope Labeling with Amino Acids (SILAC) in Cell Culture and Quantitative Phosphoproteomics

One of the commonly used approaches to identify the downstream targets of a particular kinase is to conduct quantitative phosphoproteomics in intact cells with or without a kinase inhibitor. This approach is often coupled with stable isotope labeling with amino acids in cell culture (SILAC), where a set of cells is isotopically labeled with heavy arginine and/or heavy lysine, while keeping the other set in regular media (light). Light and heavy cells are treated with either vehicle or a kinase inhibitor, respectively. Following treatment, light and heavy cells are mixed, lysed, trypsinized, and the phosphopeptides are enriched and subsequently analyzed by LC–MS/MS. Because SILAC labeling allows mixing of the cells soon after the treatment, it drastically reduces false positives arising due to experimental errors in subsequent processing steps. The phosphopeptides from differentially labeled samples can then be precisely quantified, which in turn reveals the phosphoproteome regulated by that kinase (Fig. 6).

An alternative approach to conventional SILAC is to methylate the trypsinized peptides from the control and kinase inhibitor-treated cells using light and heavy methanol respectively, followed by IMAC enrichment and subsequent LC–MS identification. Using this approach, Grosstessner-Hain et al. identified 401 proteins as the downstream targets of PLK1 kinase (58). Many of these proteins are known to be involved in mitotic processes suggesting these may be true partners of PLK1 in cells. Furthermore, as this approach utilizes intact cells, the substrates that are phosphorylated are likely to be bonafide downstream effectors of PLK1. However, similar to

most indirect approaches, SILAC-based approaches also suffer from promiscuity of kinase inhibitors and identification of indirect downstream effectors of the kinase, along with its direct substrates.

Chemical Rescue of Crippled Kinase with Phosphoproteomics

Cole and colleagues developed an elegant alternative methodology to specifically activate a desired kinase using a small molecule in cells. The resulting phosphorylated targets were subsequently analyzed using SILAC-based phosphoproteomics. A kinase-defective c-Src (R390A) mutant was generated, which could be activated ~100-fold using imidazole. c-Src mutant was stably expressed in Src, Yes, and Fyn triple knockout mouse embryonic fibroblasts, and the cells were isotopically labeled. c-Src was subsequently activated using imidazole and phosphoproteins were enriched and analyzed by quantitative phosphoproteomics (59). This study identified 29 potential novel c-Src substrates including C3G, a Rap1 guanine exchange factor. The authors further revealed the spatiotemporal dynamics of C3G activation by c-Src, thereby highlighting the potential of chemical rescue methodology in identifying the bonafide substrates of a desired kinase (59). Cole et al. recently extended this approach to activate a catalytically-defective Abl kinase (R367A) using imidazole and rapidly accessed tyrosine phosphorylation at the outset of signaling using quantitative proteomics (60). Thus, the chemical rescue method is useful in identifying the direct substrates of a desired kinase in a native environment in cells. Nevertheless, similar to any global phosphoproteomics screen, the possibility of identifying downstream indirect substrates of Abl or c-Src kinase cannot be excluded regardless of the rapid activation and isolation of the phosphorylated targets. Furthermore, activation using 20 mM imidazole could potentially perturb other signaling cascades resulting in background phosphorylation/dephosphorylation events (Table 2).

ASKA Coupled with Quantitative Phosphoproteomics and Time-Resolved Phosphoproteomics

One of the major challenges of indirect kinase substrate identification is the off-target effects of kinase inhibitors, which can be effectively eliminated using the ASKA methodology. As discussed in section "Generation of the Analog-Sensitive Kinase via Gatekeeper Mutation," the endogenous kinase needs to be replaced with the analog-sensitive kinase using CRISPR or specific shRNA knockdown in cells. AS kinases can then be specifically inhibited using extremely specific orthogonal inhibitors (section "Orthogonal Inhibitors") in cells, and phosphoproteome analyzed. Holt et al. combined ASKA with SILAC approach and identified 547 phosphorylation sites on 308 Cdk1 substrates in yeast (61).

Another challenge arising from kinase inhibitor strategy is to discriminate between the direct targets of a desired kinase versus phosphorylation events that are mediated by its downstream kinases. Kanshin et al. thus combined ASKA with quantitative time-resolved phosphoproteomics to identify numerous direct targets of Cdc28 and Snf1 kinases in the *Saccharomyces cerevisiae*. This study suggested that both under physiological conditions and upon inhibitor treatment, direct kinase substrates are more rapidly dephosphorylated compared to indirect substrates. Thus, using acute time-series, the direct substrates of the desired kinase can be identified on a global scale with relatively high specificity (62).

CONCLUSION

Deregulated kinase activity is a major cause of a myriad of pathological conditions and continues to be an area of active research. Advances in the field of proteomics, protein engineering, genetics, mass spectrometry, and chemical approaches have led to the advent of various methods to investigate intracellular signaling pathways in both health and diseases. Nevertheless, sorting out physiological and pathological kinase substrates and delineating how these perturbations at the molecular level manifest in a disease phenotype remains a great challenge. The number of approaches highlighted in this review has greatly enhanced our knowledge of kinase signaling pathways and fueled the advances in our understanding of the pathophysiology of various diseases. To realize the full potential of these technologies, it is crucial to understand the advantages and disadvantages entailed to each of these approaches and determine which method is best tailored to address a specific problem. This would greatly aid us in our investigation of the complex intracellular signaling networks and ultimately, facilitate the development of novel therapeutic strategies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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