REVIEW

Using Small Molecules and Chemical Genetics To Interrogate Signaling Networks

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ABSTRACT The limited clinical success of therapeutics targeting cellular signaling processes is due to multiple factors, including off-target effects and complex feedback regulation encoded within the signaling network. To understand these effects, chemical proteomics and chemical genetics tools have been developed to map the direct targets of kinase inhibitors, determine the network-level response to inhibitor treatment, and to infer network topology. Here we provide an overview of chemical phosphoproteomic and chemical genetic methods, including specific examples where these methods have been applied to yield biological insight regarding network structure and the system-wide effects of targeted therapeutics. The challenges and caveats associated with each method are described, along with approaches being used to resolve some of these issues. With the broad array of available techniques the next decade should see a rapid improvement in our understanding of signaling networks regulation and response to targeted perturbations, leading to more efficacious therapeutic strategies.

Virtually all cellular responses to environmental perturbations are controlled in part by tightly regulated protein phosphorylation signaling networks. Aberrant activation of kinases or loss of phosphatase activity underlies many major human diseases including cancer, autoimmunity, and diabetes (*1*–*3*). The early discovery of the transforming capability of constitutively active tyrosine kinases has led to extensive research on dysregulated signaling in cancer (*4*). As a result, increased expression or activation of kinases has been found to play a causative role in many cancers, including the BCR-ABL fusion protein in chronic myeloid leukemia (CML), B-Raf in melanoma, the receptor tyrosine kinase (RTK) EGFR in lung cancer and glioblastoma, and HER2 in breast cancer (*5, 6*). Because kinase-activated signaling networks are associated with increased proliferative, migratory, and invasive phenotypes, small molecule kinase inhibitors are a major area of pharmaceutical research and development (*7*).

The theory of oncogene addiction suggests that targeted inhibition or removal of the transforming "driver" mutation should block malignant phenotypes and lead to apoptosis by eliminating proliferation and survival signals, while having a minimal effect on normal tissues. Unfortunately, clinical results with targeted kinase inhibitors as monotherapies for cancer treatment have been largely disappointing because of confounding issues including lack of specificity, primary resistance because of compensating or redundant signals, rapid development of acquired resistance, and toxicity (*7*). To develop more effective cancer therapeutics it is necessary to address these issues by determining the complete target spectrum of the clinical molecules and to couple this information to a comprehensive understanding of signaling networks across tissue types and disease states.

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Chemical biology provides a wide array of tools to interrogate interactions between small molecules and biological systems. In this review, we highlight techniques in chemical biology that have demonstrated the greatest utility for understanding phosphorylation signaling networks and therapeutic response to kinase inhibitors. We focus on chemical proteomics and phosphoproteomics, two methods that probe signaling networks at a system-wide level. With these techniques it is now possible to identify the spectrum of targets for each small molecule and measure their respective interaction strengths and inhibition kinetics. Since therapeutic effect is a combination of on- and off-target effects, comprehensively mapping interactions can identify clinically important or potentially toxic off-target effects prior to *in vivo* testing.

Signaling networks are dynamic, and feedback processes and adaptation can lead to unexpected or undesirable effects including therapeutic resistance. Therefore, mapping downstream signaling processes and adaptive responses is also important for understanding the effects of targeted therapeutics. A range of chemical biology techniques have been developed for systemic analysis of signaling networks, including multiplexed or high-throughput assays for activity of key signaling nodes (*8–10*) and mass spectrometry-based approaches that probe signaling networks on a global scale (*11, 12*). These approaches are providing new insight into signaling networks, including their function, topology, and response to chemical perturbations. With these tools, including the latest technical developments in chemical phosphoproteomics, systemic effects of kinase inhibitors can be quantified with site-specific resolution across the signaling network (reviewed in ref 13). Global analysis of phosphorylation following treatment with targeted inhibitors is rapidly increasing our understanding of how cells adapt to loss of oncogenic signals by altering signaling. Activated pathways may be critical in evolution of a resistant state and may serve as combinatorial therapeutic targets. Often these experimental strategies are combined with increasingly sophisticated computational analysis to generate or test specific hypotheses based on system-wide measurements (*14*).

Despite rapid advancements in methodology and instrumentation, we identify three primary experimental challenges in using chemical biology to understand phosphorylation signaling networks:

1. Lack of specificity of most kinase and phosphatase inhibitors limits their usefulness in both research and clinical settings. Off-target effects must be considered when designing and interpreting experiments meant to provide mechanistic insights.
2. Even specific inhibition of a signaling node leads to complex downstream effects through largely uncharacterized interactions and feedback regulation. Predicting how cells will respond to targeted perturbations requires high quality, quantitative, temporal data across cell types and conditions. Progress in microarray and mass spectrometry technologies have enabled systems-level quantitative analysis, but all techniques still require extensive validation to ensure high quality data.
3. Although many important signaling pathways have been mapped, most network topology is still uncharacterized. Studies to identify direct interactions among kinases, substrates, and regulators are still routinely identifying novel interactions. Recent developments in chemical genetics have enabled unbiased and sensitive identification of kinase substrates, but high-throughput studies remain stalled by limitations of protein engineering and analytical technology.

Biochemical Characterization of Kinase Inhibitors. Most kinase inhibitors bind to the ATP-binding pocket of the kinase in an active conformation (Type I inhibitors). However, since the structures of ATP-binding pockets are strongly conserved across most kinases, ATP-competitive inhibitors tend to have a broad range of off-target interactions. Inhibitors that stabilize inactive kinase conformations (Type II inhibitors) and allosteric inhibitors (Type III inhibitors) provide opportunities for greater specificity but are difficult to develop and do not completely eliminate off-target binding (*7*). Off-target interactions can contribute to clinical toxicity, and therefore a range of methods have been developed to comprehensively profile kinase–inhibitor interactions. Frequently used approaches include immobilizing small molecules to capture kinase targets (*15*), parallel kinase activity assays (*16*), and interrogation of systemic readouts of biological activity such as transcript profiling (*17*). These techniques have been recently reviewed by Rix *et al.* (*18*) and will not be elaborated here.

Limits of Biochemical Characterization. Biochemical analysis of an inhibitor typically determines only the binding constant (K_D) or the potency of inhibition (IC_{50}) for each kinase. These values are important for understanding inhibitor specificity but they do not describe

the biological consequences of on- or off-target inhibition. Feedback processes in many signaling networks allow the network to adjust rapidly to maintain downstream signaling levels following inhibition of a central node. In addition, our incomplete knowledge of signaling network structure means that even highly specific inhibitors can have unpredictable effects on signaling and resulting phenotypes (Figure 1). For example, B-Raf inhibition in the presence of activated NRAS has recently been shown to increase signaling by ERK1/2 through compensatory activation of Raf-1 (*19*).

A priori prediction of feedback-mediated effects can be especially challenging because of cell-type and context-specific interactions: emerging data indicates that signaling networks are differentially "wired" in different cell types and disease contexts (*20*) and can be "rewired" in response to exogenous stimuli (*21*). A quantitative and mechanistic understanding of signaling processes in a variety of contexts will be critical for predicting and interpreting complex signaling responses and their resulting phenotypes. However, despite extensive research, detailed mechanisms underlying the connection between signaling perturbations and simple phenotypes such as migration and survival are still incompletely understood. The role of signaling processes in more complicated phenotypes such as sensitivity or resistance of a tumor to targeted therapeutics depends not only on the immediate signaling network but also on interactions with the microenvironment and the larger organ system. Our understanding of these higher-order processes is rudimentary at best; this knowledge gap directly limits our ability to predict response to therapeutic treatments in complex, physiologically relevant settings.

System-Level Approaches to Study of Kinase Signaling. To begin to unravel complex signaling processes, systems-wide approaches to interrogate signaling networks have been developed. When the key nodes in a signaling network are known, antibodies targeted against particular post-translational modifications (PTMs) can be used to measure the state of the system, frequently in high-throughput or multiplexed formats (*22–24*). Although these assays can be very informative, many antibodies are not specific for a single PTM and may therefore yield false-positive results, as demonstrated by Sevecka *et al.* (*25*). Additionally, the range of possible downstream effects is often unknown, and targeted assays are often unavailable. Proteomic strategies using affinity enrichment for PTMs along with quantitative mass spectrometry allow signaling networks to be interrogated without prior knowledge of which nodes or pathways may be perturbed. For the most part, mass spectrometric analysis of signaling networks has focused on phosphorylation-mediated signaling (phosphoproteomics) due to the importance of these networks in regulating biology and to the ready availability of several affinity enrichment methods (*26–28*). Strategies to isolate other PTMs such as methylation (*29*), lysine acetylation (*30, 31*), and ubiquitination (*32, 33*), along with S-nitrosylation (*34, 35*), are less developed but beginning to enter common use.

Chemical Phosphoproteomics: Systems-Level Analysis of Kinase Inhibitors. Global phosphoproteomic analysis uses affinity enrichment of phosphorylated peptides to provide a broad and unbiased view of the signaling network, including phosphorylation of serine, threonine, and tyrosine residues (*26*) (*36–38*). In these experiments cell lysates are digested to peptides and phosphorylated peptides are enriched using approaches such as immobilized metal affinity chromatography or metal oxide affinity chromatography. Mass spectrometric analysis of peptide fragmentation patterns allow specific phosphorylated peptides to be identified by statistical comparison to peptides from *in silico* digest of the proteome (*39*). These experiments routinely identify hundreds to thousands of phosphorylated peptides.

Unfortunately, this breadth tends to come at the cost of limited depth, as abundant proteins are more likely to be detected by global analysis, while many important signaling proteins are present only at low levels. Even so, this approach has been used to interrogate the effects of kinase inhibitors (Figure 2), as demonstrated by Pan *et al.* (*40*). In this study, global phosphoproteomics was performed to ana-

KEYWORDS

Affinity enrichment: capturing proteins or peptides by their affinity for bait molecules anchored on a solid surface

Analogue-sensitive kinase: kinases with ATP-binding pockets engineered to accept bulky ATP analogues

Chemical genetics: genetic engineering to create a specific interaction between a protein of interest and a bio-orthogonal small molecule

Chemical proteomics: characterizing interactions between proteins and small molecules by using affinity purification coupled to mass spectrometry for protein identification and measurement

Kinase substrate: molecules, usually proteins, that are phosphorylated by a particular kinase

Phosphoproteomics: identifying and quantifying phosphorylation across a large set of proteins by combining affinity enrichment for phosphopeptides with identification by mass spectrometry

Signaling network: a diverse set of proteins involved in cellular information processing; signaling molecules are often highly connected and information is processed through a wide range of regulatory mechanisms

Stable isotopic labeling: using chemically identical tags with distinct isotope distributions to enable accurate quantitative measurements by mass spectrometry

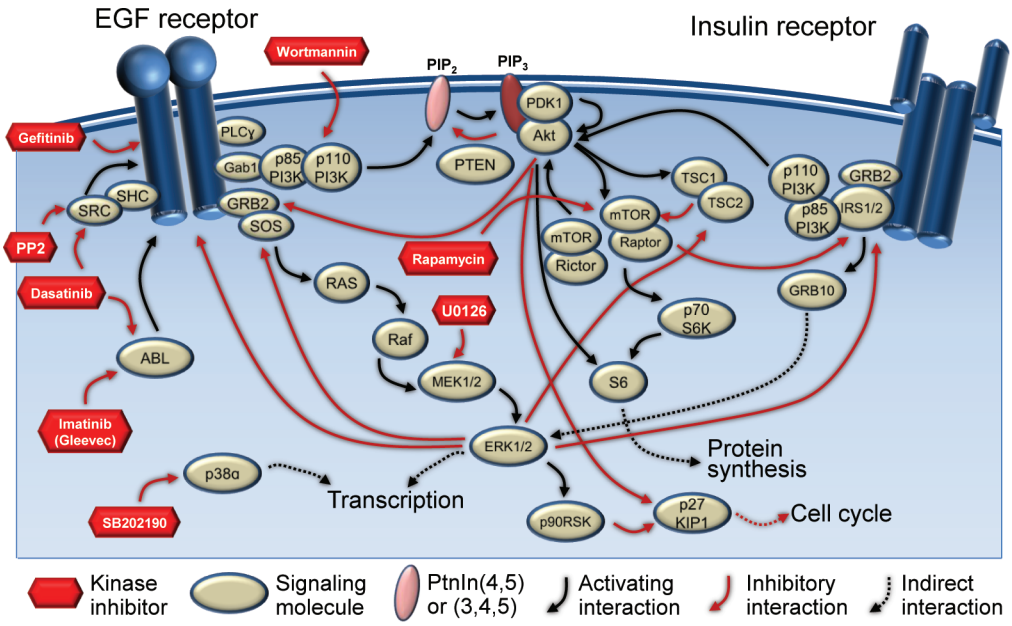

Figure 1. Schematic diagram showing core elements of the ERK1/2 and Akt signaling pathways downstream of EGF receptor and insulin receptor. Selected feed-forward and feedback processes are emphasized to highlight the connected nature of the signaling network. Kinase inhibitors referenced in the text are shown along with their primary targets.

lyze the effects of U0126, a MEK1/2 inhibitor, and SB202190, a p38α inhibitor, on EGFR signaling in HeLa cells and to quantify the effects of dasatinib, an inhibitor of ABL and SRC family kinases, on the BCR-ABL signaling in a leukemia cell line. Of the several thousand sites identified in this study, approximately 500 increased after EGF treatment and 200–300 decreased with inhibitor co-treatment. Amino acid motifs around dynamic phosphorylation sites were queried to determine the percentage of affected sites matching the motif of the targeted kinase, but this data has to be carefully considered given the approximately 1% false positive identification rate (FPR) of the methods used in this manuscript. With this FPR, 5000 peptide identifications corresponds to approximately 50 false positive identifications. Because incorrectly identified peptides will tend to be weaker signals, they are also likely to be among the most variable in the data set and could constitute a significant percentage of peptides that seem to be affected by the inhibitors. When drawing biological conclusions, it is important to be aware of possible identification errors, and we recommend that peptide assignments important for biological conclusions be verified by manual inspection and comparison to synthetic peptides.

Chemical Phosphoproteomics: Targeted Approaches. Relative to global analysis, targeted phosphoproteomics techniques enable more in-depth characterization of a subset of the phosphoproteome, allowing for quantification of effects of small molecule inhibitors on low-level phosphorylation sites. This approach has been critical for the analysis of tyrosine kinase inhibitors, as tyrosine phosphorylation represents a small part (<1%) of total phosphorylation in mammalian cells (*41*). The most common method for targeted phosphoproteomics uses pan-specific antibodies targeting a subset of the phosphoproteome, such as proteins or peptides containing phosphotyrosine or phosphorylation in the context of specific amino acid motifs (*42, 43*). Alternate methods for targeted phosphoproteomics approaches include those based on affinity for small molecules, such as ATP or kinase inhibitors (*44, 45*).

In a recent example of targeted chemical phosphoproteomics, Moritz *et al.* developed antibodies recognizing phosphorylation in the context of the RXRXXS/T motif common to basophilic kinases including Akt, RSK, and p70 S6K (*46*). They used peptide immunoprecipitation followed by mass spectrometry analysis optimized for basic peptides to identify hundreds of basophilic phosphorylation sites affected by treatment with gefitinib, SU11274, and imatinib (inhibitors of activated EGFR, c-Met, and PDGFR, respectively), as well as U0126, wortmannin, and rapamycin (inhibitors of MEK1/2, PI3K, and mTOR). This analysis identified phosphorylation sites downstream of activated RTKs in several cancer cell lines and signaling nodes where tyrosine and basophilic phosphorylation are likely to interact. Im-

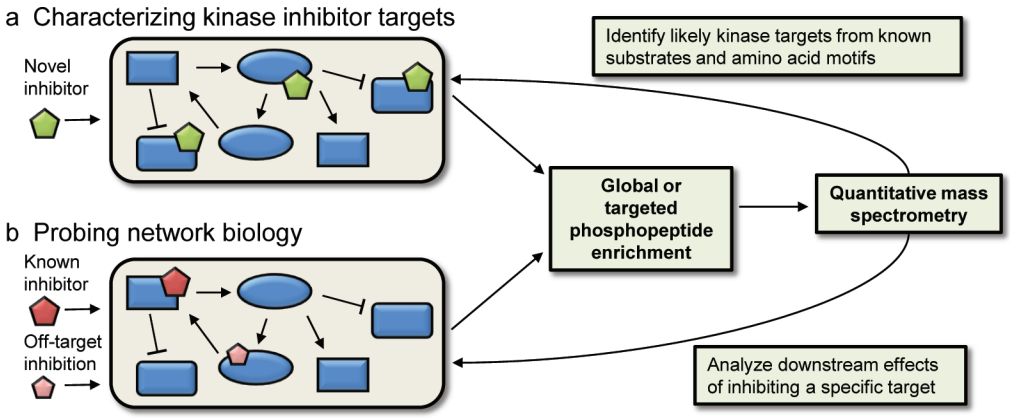

Figure 2. A shared workflow for quantitative phosphoproteomics can be applied to characterizing novel kinase inhibitors and to probing network biology by targeted inhibition with well-known inhibitors. In each case analysis and interpretation of the data must be considered in the context of confounding network or off-target effects.

portantly, the authors note that their basophilic phosphorylation sites are not necessarily direct substrates of any particular basophilic kinase, even when a site responds to a particular targeted inhibitor. Demonstration of a direct kinase substrate relationship requires both a direct physical interaction, often shown biochemically, as well as an *in vivo* functional relationship.

Phospho-motif antibodies recognizing basophilic phosphorylation sites were also used in a separate study to investigate the effects of inhibitors of Akt, MEK1/2, and PDK1, along with enrichment of proline-directed phosphorylation (PXS/TP) and binding sites for PDK1 (*47*). Following mass spectrometry-based identification of phosphorylation sites, phosphospecific antibodies were used to show that phosphorylation of Thr246 on PRAS40 is correlated with activated Akt in a cohort of breast and lung cancer cell lines and that phosphorylated PRAS40 is a more sensitive marker than phosphorylated AKT to identify upregulated PI3K signaling in tumors by immunohistochemistry.

High quality quantitative data is critical for phosphoproteomics, as important signaling processes can be mediated by relatively small changes, often on the order of tens of percent. To achieve accurate quantification, labeling with stable isotopes, *e.g.*, stable isotope labeling with amino acids in cell culture (SILAC) (*48*) or isobaric tags for relative and absolute quantitation (iTRAQ) (*49*), can be incorporated into phosphoproteomics experiments, enabling acquisition of quantitative data reflecting the effect of small molecules on the signaling network. These strategies allow conditions to be multiplexed (generally 2- or 3-plex for SILAC and up to 8-plex for iTRAQ) and compared directly in a single experiment, effectively eliminating much of the variability from preparation, chromatography, and mass spectrometry. The degree of accuracy afforded by stable isotope labeling is important for characterizing the system-level effects of small molecules and necessary if chemical proteomics data is going to be incorporated into mathematical models or analyzed by most types of statistical or machine-learning methods.

In a recent application of quantitative, targeted chemical phosphoproteomics, Li *et al.* identified several novel direct kinase targets of dasatinib through a combination of chemical proteomics and quantitative targeted phosphoproteomics (*45*). In the first part of this study, direct targets of dasatinib were identified through affinity enrichment of proteins bound to dasatinib-coated beads. In the second phase of the study, targeted analysis of tyrosine phosphorylation was first used to identify tyrosine phosphorylated kinases in multiple cell lines and then to quantify the effect of dasatinib treatment on the kinase phosphorylation sites. Overall, dasatinib treatment led to decreased phosphorylation on 18 tyrosine kinases, including direct targets and multiple downstream kinases, providing some information regarding the complexity and connectivity of the signaling network. This combined approach was able to identify novel off-target direct targets of a well-studied inhibitor, as shown by the finding that EGFR is directly inhibited by dasatinib, a result that

was strengthened through multiple confirmatory biological experiments.

Quantitative analysis of the tyrosine phosphoproteome has been used to characterize tyrosine signaling downstream of EGFR and in the context of oncogenic EGFR in model systems for glioblastoma and non-small cell lung cancer (*43, 50, 51*). These analyses revealed unexpected crosstalk between EGFR and c-Met, two oncogenic RTKs, and found that combined inhibition of c-Met and EGFR could generate a synergistic response compared to EGFR inhibition alone. In addition to direct signaling mechanisms, phosphorylation profiling has been used to understand genetic mechanisms leading to acquired resistance to targeted inhibitors. For example, Engelman *et al.* also used phosphoprotein microarrays to find that acquired resistance to targeted inhibition in EGFR-driven lung cancer can occur by amplification of c-Met leading to sustained activation of survival signaling (*52*).

Quantitative tyrosine phosphoproteomics has also been applied to understand the effects of tyrosine kinase inhibitors by mapping changes in signaling following treatment with imatinib (Gleevec) (*53*), genistein (*54*) or the Src-family inhibitor PP2 (*55*). Interestingly, treatment with tyrosine kinase inhibitors often causes a small fraction of tyrosine phosphorylation sites to increase, probably as a result of activation of another kinase or inactivation of a phosphatase. More detailed understanding of the signaling network topology should provide insight into the mechanisms underlying these nonintuitive changes in phosphorylation.

Computational Analysis of Phosphoproteomic Data. Although phosphoproteomic analysis can reveal unexpected downstream effects, it provides very little information about the underlying interactions and molecular mechanisms. Also, because most phosphorylation sites lack known function, it is often unclear whether changes in phosphorylation will have biological consequences or whether these sites may simply be "bystander" effects of altered kinase activity (*56*). To address these issues, a variety of computational approaches have been developed to link systems-level phosphorylation data to other biological processes and cellular responses.

To infer functional consequences by linking phosphoproteomics data with biological responses, it is necessary to have quantitative phosphorylation and phenotypic data gathered across a shared set of biological conditions, although not at the same time-points since signaling should precede phenotypic responses. Although most quantitative data is relative (*i.e.*, fold change between conditions) there is also interest in incorporating information about absolute phosphorylation stoichiometry (*57*). In the absence of prior knowledge about a phosphorylated protein, phosphorylation data can be linked to phenotypic outcomes by looking for correlated signals, typically through multivariate regression such as partial least squares regression (PLSR) or nonlinear machine-learning approaches like self-organizing maps or decision trees (reviewed in ref 58, some examples in refs 21 and 59). This type of data-driven modeling can suggest unexpected hypotheses but it does not incorporate existing knowledge and provides minimal mechanistic insight.

Other computational approaches use systems-level data to build or elaborate on network models representing functional or biochemical interactions among signaling molecules. These methods generally start from existing information, such as databases of protein–protein interactions, kinase–substrate relationships, or rate constants of biochemical processes, and test the predictive ability of each model (*58*). In some cases, physical models of some well-studied signaling networks have been represented by coupled differential equations and used to predict phenotypic sensitivity to inhibition of selected nodes in the network. For instance, Merrimack Pharmaceuticals used sensitivity analysis with a physical model of ErbB receptor-family signaling to identify ErbB3 (HER3) as a key node in regulating system-level behavior (*60, 61*). An antibody antagonist for HER3 is now entering phase II clinical trials. These types of models generally work with only a small part of the data that falls within our limited knowledge of signaling processes.

Chemical Genetics for Engineered Specificity. Although it is possible to link phosphorylation sites to phenotype, it is still challenging to relate altered phosphorylation to a particular kinase or phosphatase, due to limited knowledge of signaling network connectivity (*62*). To address this challenge and define direct kinase–substrate relationships, chemical genetic techniques have been developed in which the kinase or substrate has been mutated to introduce interactions with small molecules that do not interact with the complex background of biochemical activity in the cell (Figure 3). For instance, Blair *et al.* engineered EGFR and c-Src to be irre-

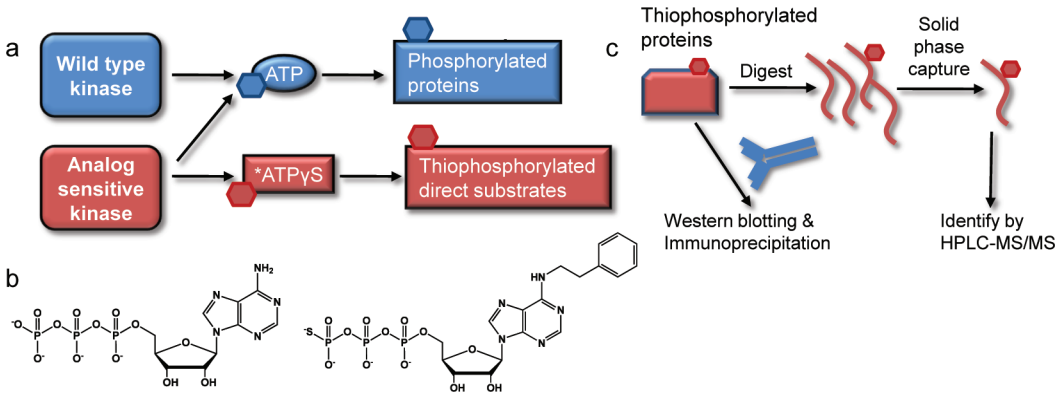

Figure 3. Chemical genetics approaches for kinase substrate identification. (a) Analogue sensitive kinases are used with labeled ATP analogues to selectively tag direct substrates with ^{32}P phosphate or thiophosphate. (b) Structures of ATP and a γ-thio labeled example of a bulky ATP analogue used in chemical genetics (N^6-(2-phenylethyl)-ATPγS). (c) Antibodies recognizing alkylated thiophosphate or solid-phase capture of thiophosphorylated peptides can be used to identify tagged proteins.

versibly inhibited by 6-acrylamido-4-anilinoquinazoline and used a fluorescent form of the inhibitor to study the fraction of active signaling molecules required to initiate downstream signals (*63*), while Maly *et al.* replaced a particular serine/threonine phosphorylation site with a cysteine and then treated with an ATP analogue to covalently cross-link the substrate to its kinase (*64*). Perhaps the most powerful of these chemical genetics approaches was developed by the Shokat lab in 1997 (*65*). In this approach, structural analysis was used to design a mutation in the ATP-binding site of v-Src, thereby altering the structure to accommodate a bio-orthogonal ATP analogue (in this case *N6*-benzyl adenosine triphosphosate). To differentiate substrates of the mutated, analogue sensitive (AS) kinase from substrates of other kinases, a radiolabeled phosphate was included in the terminal position of the ATP analogue, thereby resulting in the transfer of radiolabeled phosphate to the kinase substrate. In addition to radiolabeling of direct substrates, the altered ATP-binding pocket of the AS-kinase rendered it sensitive to inhibition by a variety of bio-orthogonal ATP analogue inhibitors that have minimal affinity for endogenous ATP-binding pockets (*66, 67*).

The AS-kinase approach has several advantages over other chemical methods: it allows direct kinase substrates to be definitively tagged, it bypasses the need to design and validate a specific inhibitor for every kinase, and it ensures a limited and consistent set of off-target effects since the same inhibitor can be used for different AS-kinases. Engineering inhibitor sensitivity into specific kinases can be particularly useful to differentiate roles of related kinases, such as the various Src-family kinases. AS alleles have been used to parse out the transcriptional and apoptotic roles of JNK1 and JNK2 following stimulation by tumor necrosis factor α and in p53-induced senescence (*68, 69*). The AS-kinase approach was also used to identify a role for polo-like kinase 1 (PLK1) in positioning RhoA during cytokinesis, by replacing endogenous PLK1 with an analogue sensitive allele for targeted inhibition (*70*).

Discovering Network Topology by Chemical Genetics. In addition to studying the phenotypic effects of targeted inhibition of a selected kinase, the AS-kinase approach has been also been used to identify substrates for multiple different kinases, including v-Src (*71*), JNK2 (*72*), PKA (*73*), CDK1 (*74*), and ERK2 (*75*). All of these studies were relatively low-throughput, as they relied on radiolabeled ATP-analogues to label substrates, which were then identified through MS analysis of bands cut from 1D or 2D electrophoresis gels. Without the ability to selectively enrich radiolabeled substrates in a complex background of phosphoproteins and non-phosphoproteins, analysis of many of the bands resulted in identification of multiple different proteins, from which the true substrate had to be determined. Although over 40 AS-kinases have been developed, successful substrate identification using these kinases was limited by the inability to selectively enrich substrates and by challenges associated with designing

and validating AS-kinases that retain enough activity to function in their biological context (*76*).

Over the past two years the substrate enrichment problem has been partially solved by replacing the ^{32}P radiolabel on the ATP analogue with thiophosphate, thereby providing a chemical handle for detection of substrate proteins by immunoblotting and immunoprecipitation (*77, 78*). Solid-phase capture methods have also been reported recently; in these methods the thiophosphorylated peptides from AS-kinase substrates are covalently captured and subsequently eluted for identification by HPLC–MS/MS (*79, 80*). In each case dozens of substrates of cyclin-dependent kinases were identified in yeast and human cells; recent work in our lab has extended the approach developed by the Shokat group to identify over 100 substrates of ERK2 (manuscript in preparation). A remarkable result from these AS-kinase based substrate identification experiments is that only a small fraction (~10–20%) of the substrates have been previously reported. Given that dozens of substrates have been already been identified for many of these kinases by other methods, including classical biochemistry experiments, the AS-kinase results can be extrapolated to suggest that each of these kinases may have hundreds of substrates. In fact, compared to systems-level phosphoproteomic profiling experiments, the AS-kinase experiments interrogate a much smaller part of the signaling network but reveal a similar level of complexity. These experiments suggest that signaling networks are much more highly connected than had been previously recognized and that a tremendous amount of mechanistic detail remains to be discovered at the level of direct interactions among signaling molecules, regulators and effector proteins.

Although chemical genetics is a powerful, unbiased strategy for discovering signaling network topology, there are a number of challenges and caveats to be considered. First, ATP-analogues are not readily taken up by cells, and therefore labeling of kinase substrates typically occurs in cell lysates, either from cells expressing the AS-kinase or by exogenous addition of AS-kinase to the lysate. Since the reaction occurs outside the normal cellular environment, loss of biological context, including subcellular localization, can lead to spurious interactions, while the dilution of scaffold and substrate molecules may cause some substrates to be missed. Second, bio-orthogonal ATP analogues are not perfectly specific for the AS-kinase, leading to background substrate labeling due to endogenous wild-type kinases in the cell lysate. Depending on the cellular context and the expression and activity level of the AS-kinase, the level of nonspecific labeling can make a substantial contribution to total labeling, significantly altering the results of the assay. It is important to include an appropriate negative control (*e.g.*, replacement of the AS-kinase with an equivalent amount of wild-type kinase). Third, many substrates are present at very low levels, such that identification is limited by the sensitivity of even the latest generation of mass spectrometers. Further improvements in the protocol for substrate enrichment combined with more sensitive instrumentation will greatly expand the range of applications for AS-kinases.

Clinical Relevance of Chemical Phosphoproteomics. An increasing number of therapeutics target dysregulated kinase signaling, especially in cancer. Imatinib (Gleevec), developed to target BCR-ABL for treatment of CML, is the most successful clinical example of a targeted kinase inhibitor. Treatment with imatinib leads to almost complete remission in most susceptible cases of CML, although acquired resistance and recurrence remain problematic (*81*). Targeted inhibitors for other kinases including EGFR, other RTKs, Src, B-Raf, PI3K, MEK, and Akt are a major area of pharmaceutical development (*7*). Unfortunately these therapeutics have, for the most part, had a limited impact on disease progression and overall patient survival in the clinic. Even tumors that respond to initial treatment often develop resistance within a few months, either by acquiring a mutation in the drug target or by activating compensatory pathways (*50, 82–84*). One striking example comes from glioblastoma, where increased expression and/or mutation of EGFR occurs in approximately 50% of cases. However, treatment with EGFR inhibitors has shown only modest improvement in progression-free survival (*85*), perhaps due to activation of alternate pathways, including PTEN/AKT/mTOR (*86, 87*). Combining EGFR inhibitors with mTOR pathway inhibitors has shown improved response in *in vitro* and preclinical models, but this has not translated to human tumors for several reasons, including negative feedback regulation in which inhibition of mTOR can actually increase signaling through AKT (*88*). Targeted therapeutics have encountered the same challenges in melanoma, non-small cell lung cancer, and pancreatic cancer, among others (*89, 90*). It is clear that a systems-level understanding of the complex regulatory circuits governing signaling re-

sponses to therapeutic inhibition is required to overcome these adaptive mechanisms.

Future Directions. Through chemical proteomics and chemical genetics, it is now possible to dissect signaling networks, identifying substrates for selected kinases and targets for small molecule kinase inhibitors. Results from these experiments have shown that signaling processes are highly interconnected and are subject to feedback and regulation through interrelated temporal, spatial, and biochemical mechanisms.

Connecting this information with phenotypic and clinical responses remains an extraordinary challenge, one that has proven far more difficult than originally hoped. Since phosphoproteomic experiments have already demonstrated impressive breadth and depth of coverage, simply increasing the number of sites identified through developments in instrumentation and enrichment protocols is not likely to yield useful biological insights. Instead, more sophisticated strategies, both experimental and computational, to elucidate signaling mechanisms and define complex responses to targeted perturbations, are likely to be more fruitful. Specifically, experiments that provide high quality, quantitative temporal signaling data across multiple conditions will be especially useful for generating hypotheses and testing specific mechanisms. Quantitative and mechanistic understanding will eventually allow development of co-treatment strategies and clinical interventions targeting redundant or compensatory signaling nodes.

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