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Quantitative Proteomics of Kinase Inhibitor Targets and Mechanisms

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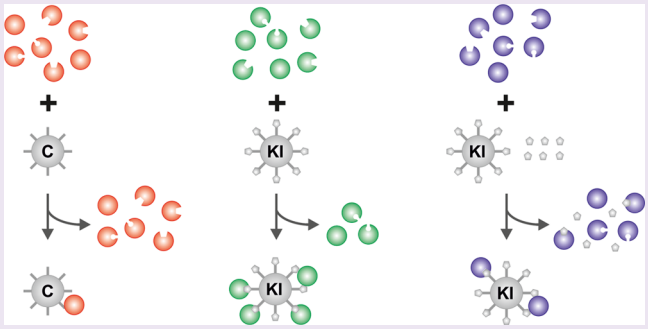
ABSTRACT: Small molecule inhibitors of protein kinases are key tools for signal transduction research and represent a major class of targeted drugs. Recent developments in quantitative proteomics enable an unbiased view on kinase inhibitor selectivity and modes of action in the biological context. While chemical proteomics techniques utilizing quantitative mass spectrometry interrogate both target specificity and affinity in cellular extracts, proteome-wide phosphorylation analyses upon kinase inhibitor treatment identify signal transduction pathway and network regulation in an unbiased manner. Thus, critical information is provided to promote new insights into mechanisms of kinase signaling and their relevance for kinase inhibitor drug discovery.



The protein kinase complement, also designated the kinome, comprises more than 500 distinct members and constitutes one of the largest enzyme superfamilies encoded by the human genome.[1] Protein kinases are the key regulators of phosphorylation-based signal transmission, which is critically involved in the regulation of nearly all biological processes in eukaryotic cells.[2] Moreover, as many members of the protein kinase superfamily have been implicated in the onset and progression of diseases such as human cancer, protein kinases have emerged as a major class of drug targets for pharmacological intervention.[3,4] Enormous efforts have been devoted to the development of small molecule drugs which directly interfere with the catalytic activities of disease-relevant protein kinases. However, although typically developed against individual or few targets, small molecule inhibitors of protein kinases can exhibit considerable cross-reactivities due to targeting of conserved structural elements such as the purine nucleotide-binding pockets of protein kinases.[5] This creates a strong rationale for the thorough assessment of kinase inhibitor selectivity, with the goal to identify additional targets pointing either to alternative therapeutic opportunities or to potential target-related toxicity. Also, small molecule kinase inhibitors have become highly popular tools in signal transduction and cell biology research over the past two decades, as they are easy-to-use reagents allowing for rapid, dose-dependent, conditional, and, in many cases, reversible modulation of cellular kinase activities. Again, target selectivity is a key issue, and how this translates into modes of action through catalytically active protein kinases in the biological system studied. Spurred by spectacular advances in mass spectrometry-based proteomics, various new technologies have been developed to assess protein kinase targets and signaling in an unbiased, sensitive, and quantitative manner.[6,7] These involve advanced implementations of chemical proteomics and large-scale phosphoproteomics, to quantify inhibitor interference on the level of cellular targets, substrates, and signaling pathways. This review focuses on such applications in kinase inhibitor research. For a more general discussion of the fields of chemical proteomics and phosphoproteomics, readers are referred to previous reviews.[6−12]

CHEMICAL PROTEOMICS WITH IMMOBILIZED KINASE INHIBITORS

The classical concept of protein affinity chromatography is essential to chemical proteomics procedures based on immobilized, low-molecular-weight compounds such as kinase inhibitors. Chemical proteomics combines the affinity capture of small molecule targets from cell or tissue extracts with protein identification by mass spectrometry (MS).[8] In contrast to drug selectivity testing on panels of recombinant enzymes,[5,13−15] chemical proteomics analysis is closely linked to the physiological environment as compounds are profiled against endogenously expressed, post-translationally modified full-length proteins in the presence of cellular cofactors and complex partners. To permit efficient affinity purification, small molecule kinase inhibitors have to retain their protein-binding properties upon immobilization. Therefore, derivatives with a suitable functional moiety, such as a primary amine function, are generated that can be covalently linked to chromatography beads exposing hydrophilic linker structures of about 10 atoms[6,8,9] (Figure 1). To select suitable points for attachment, knowledge of the likely spatial orientation of an inhibitor in the binding cavities of its potential target proteins is required. Often, positions for functional immobilization can be deduced

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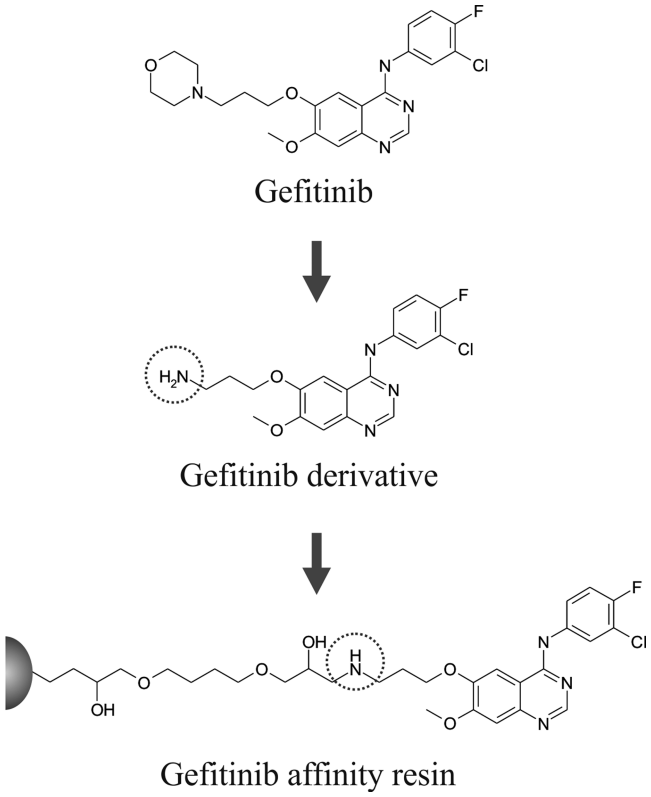

Figure 1. Immobilization of kinase inhibitors for chemical proteomics. The general strategy is exemplified by the clinical EGFR inhibitor gefitinib.[24] A kinase inhibitor derivative is synthesized with a functional moiety such as a primary amino function (encircled) at a position not engaged in target binding. In this example, the derivative is covalently coupled to epoxy-activated linker structures on the surface of chromatography beads.

from cocrystal structures. In the absence of such information, structure−activity relationship data from *in vitro* testing of small molecule derivatives against known kinase targets can be used to design linkable compound derivatives.

Chemical proteomics as an unbiased, postgenomic application in kinase research started about 15 years ago. Back then, Swanson et al. used affinity chromatography with immobilized bisindolylmaleimide, a prototypical protein kinase C inhibitor, followed by MS to identify ribosomal protein S6 kinase alpha-3 as Fos kinase from cell lysate fractions.[16] Meijer and colleagues developed immobilization strategies for purvalanol and other cyclin-dependent kinase (CDK) inhibitors to isolate and identify additional kinase and nonkinase targets from various biological sources.[17,18] Phenotypic screening of small molecules inducing neurogenesis in embryonic stem cells entailed the discovery of TWS119, and subsequent affinity enrichment with an immobilized compound variant led to the MS-based identification of glycogen synthase kinase 3 beta (GSK3β) as a relevant cellular target.[19] However, there was an initial bias toward the identification of highly abundant cellular kinase targets, such as CDKs, GSK3β, mitogen-activated protein kinases (MAPKs), or ribosomal S6 kinases.[20]

To address these limitations, we went through biochemical optimization to ensure specific target protein capture and elution, as well as compatibility with large amounts of starting material to boost sensitivity of chemical proteomics workflows.[21] Applied to the p38 inhibitor SB203580, this enabled the identification of new and unexpected kinase targets, such as the Ser/Thr kinases RIPK2/RICK, GAK, and CK1δ. These protein kinases exhibited nanomolar SB203580 sensitivity in *in vitro* activity assays similar to p38, thus qualifying them as potential cellular off-targets of a tool compound used in more than 5000 publications. Further chemical proteomics studies led to new insights into cellular kinase inhibitor selectivity. In particular, we identified high-affinity serine/threonine kinase targets for small molecules developed as tyrosine kinase antagonists.[22] For example, Aurora kinases and TANK-binding kinase 1 were identified as cellular targets of the angiogenesis inhibitor SU6668 and could be closely correlated with biological effects elicited by the drug.[23] Moreover, the aforementioned Ser/Thr kinase RIPK2/RICK was discovered as a cellular high-affinity target of the clinically approved epidermal growth factor receptor (EGFR) inhibitor gefitinib (Figure 1).[24] Notably, gefitinib potently suppressed RIPK2 kinase activity in cells and, as shown several years later, also RIPK2-mediated inflammatory responses in NOD2 hyperactivation states and an animal model of Crohn's Disease-like ileitis.[24−26] Thus, these data provide an example of how an off-target liability of a clinical drug, initially discovered by chemical proteomics, can point to alternative therapeutic applications and define starting points for target-based drug discovery.

Tyrosine kinase inhibitors have also been extensively analyzed in various chemical proteomics studies from the Superti-Furga group, in particular drugs targeting the oncogenic Bcr-Abl tyrosine kinase fusion protein in chronic myeloid leukemia (CML). These included imatinib and various second-line clinical inhibitors of imatinib-resistant Abl kinase mutants, such as dasatinib, bosutinib and nilotinib.[8] Similarities but also striking differences were observed for cellular selectivity profiles.[27,28] Notably, Bcr-Abl inhibitor affinity pull-downs revealed different target patterns and abundances in the K562 cell line and primary CML cells.[27] In a recent study, Rix and colleagues identified GSK3α and GSK3β as functionally relevant targets of the described c-Met inhibitor tivatinib.[29] Moreover, due to tremendous advances in MS technology sensitivity and systematic workflow optimization, chemical proteomics analysis has become feasible for sample amounts as little as 100 μg proteins, making it compatible with clinical applications such as target profiling in needle biopsies from tumor tissue.[28]

These results highlight the power of chemical proteomics to directly link drug selectivity studies to physiological target expression and binding competence, which can complement and significantly enhance kinase panel data toward a comprehensive understanding of biochemical and biological drug selectivity. Moreover, in contrast to kinase panels,[5,13−15] kinase inhibitor profiling by chemical proteomics can reveal off-target liabilities beyond the protein kinase superfamily. Nonprotein kinase targets might cause unintended side effects and have been identified in many studies.[18,23,27,28,30] For example, chemical proteomics of the CDK inhibitor roscovitine revealed pyridoxal kinase as a nonprotein kinase retained by inhibitor affinity beads.[18] Roscovitine treatment lowered pyridoxal-5′ phosphate levels in erythrocytes, demonstrating cellular suppression of this enzyme.[18] Moreover, off-targets do not necessarily exhibit kinase-similar binding modes and can require different spatial orientations of immobilized inhibitor ligands for affinity capture.[31]

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QUANTITATIVE CHEMICAL PROTEOMICS OF KINASE INHIBITORS

Initially, chemical proteomics studies utilized MS analysis for protein identification, which does not permit distinguishing between actual targets and nonspecific background binders *per se*. Although strategies were conceived to filter for likely targets, e.g. by removing highly abundant cellular proteins and frequently detected drug resin binders,[28] such candidates still required confirmation by orthogonal assays to determine their specificity and affinity.

This key issue could be addressed by quantitative MS, which allows for direct comparison of protein abundance in inhibitor affinity resin experiments. The most popular MS quantification techniques include stable isotope labeling with amino acids in cell culture (SILAC), the isobaric labeling reagents TMT and iTRAQ, and label-free quantification.[32] In SILAC, up to three cell populations are metabolically labeled with different isotopic variants of arginine and lysine until full incorporation into proteomes is achieved.[32] Upon digestion with trypsin, all proteolytically derived peptides (except the very C-terminal ones) contain at least one arginine or lysine residue. Labeled peptide variants coelute in liquid chromatography (LC)-MS experiments and are distinguishable by their metabolically introduced mass differences, and the ion intensities in MS spectra provide a quantitative measure for relative abundance in the compared samples. Amine-reactive TMT and iTRAQ reagents are attached after proteolytic digestion. While differentially labeled peptide variants are of identical mass, fragmentation generates distinguishable reporter ions which can be measured in MS/MS spectra. Finally, label-free quantification aligns and matches LC-MS data to directly compare peptide intensities across different experiments. Data analysis in quantitative proteomics has been streamlined and automatized through developments such as the widely used MaxQuant software.[33]

Two major concepts have emerged for quantitative chemical proteomics with immobilized protein kinase inhibitors—one focusing on target capture by a close analog of the compound of interest and the other using a mixture of immobilized nonselective kinase inhibitors for a broad enrichment of the expressed kinome.[8]

Identification of Specific Cellular Kinase Inhibitor Targets. The first concept builds on the "classical" drug affinity approach and was first introduced by Oda et al., who used quantitative MS to identify proteins bound to an immobilized bioactive compound compared to a control resin with an inactive analogue.[34] When later applied to kinase inhibitors, quantitative MS proved to be highly useful to control for binding specificity, for example by measuring the ratio of target bound to a kinase inhibitor resin compared to control beads devoid of an affinity ligand (Figure 2). This straightforward approach was used in a variety of SILAC-based studies, for kinase inhibitors such as the approved drug bosutinib, the p38 kinase antagonist SB202190, or the broad-spectrum kinase inhibitors K562a and CTx-0294885.[35−37] Kinase inhibitor molecules presented at the resin surface, in particular when immobilized a higher density, can confer hydrophobic properties to affinity beads. This can result in apparently selective binding, even without specific drug−target interactions.[36] This potential caveat is not critical in quantitative MS experiments measuring target protein displacement from drug resin by soluble, parental kinase inhibitors (Figure 2).[36,38,39] However, it

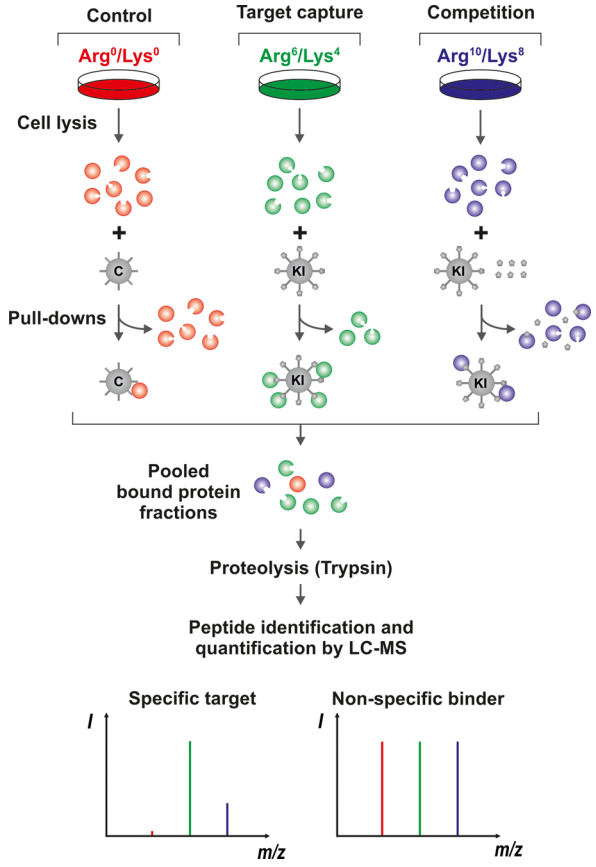

Figure 2. Quantitative proteomics concepts to identify specific kinase inhibitor targets. In this example, cultured cells are subjected to SILAC with light (Arg_0/Lys_0), intermediate (Arg_6/Lys_4), and heavy (Arg_{10}/Lys_8) isotopic variants of arginine and lysine to enable quantitative comparisons between three different binding experiments. Differentially labeled cell lysates are incubated with control beads (C) devoid of immobilized ligand, kinase inhibitor beads (KI), or kinase inhibitor beads in the presence of soluble compound. Resin-bound protein fractions are combined, digested, and measured by quantitative LC-MS analysis. Specific cellular targets are identified by characteristic SILAC patterns that reflect minimal protein binding to control beads and target competition by the soluble compound in kinase inhibitor affinity purifications. Alternative to SILAC, chemical isotope labeling or label-free quantification can be used in such comparative binding studies.

is noteworthy that even an excess of soluble compound might not always effectively compete with specific targets, for instance due to avidity effects in the case of targets or target complexes retained by multiple interactions. Moreover, quantitative MS methods such as SILAC allow for direct comparison of target spectra for different kinase inhibitor ligands, as shown for immobilized bosutinib and a close derivative, or for structurally related, nonspecific pyridopyrimidine inhibitors for kinome enrichment[35,40] Kuai et al. undertook quantitative affinity pull-downs using two affinity resins with K562a analogs that either potentiated or did not affect neuregulin-1-induced signaling and neuritogenesis in PC12 cells.[41] While most identified proteins showed binding ratios close to 1, the strategy identified a small set of protein kinases preferentially retained by a biologically active compound but not by an inactive, methylated K562a analog. Among those, adaptor-associated kinase 1 could be confirmed as the biologically relevant target.[41] These and other studies highlight how the specificity of protein kinase−small

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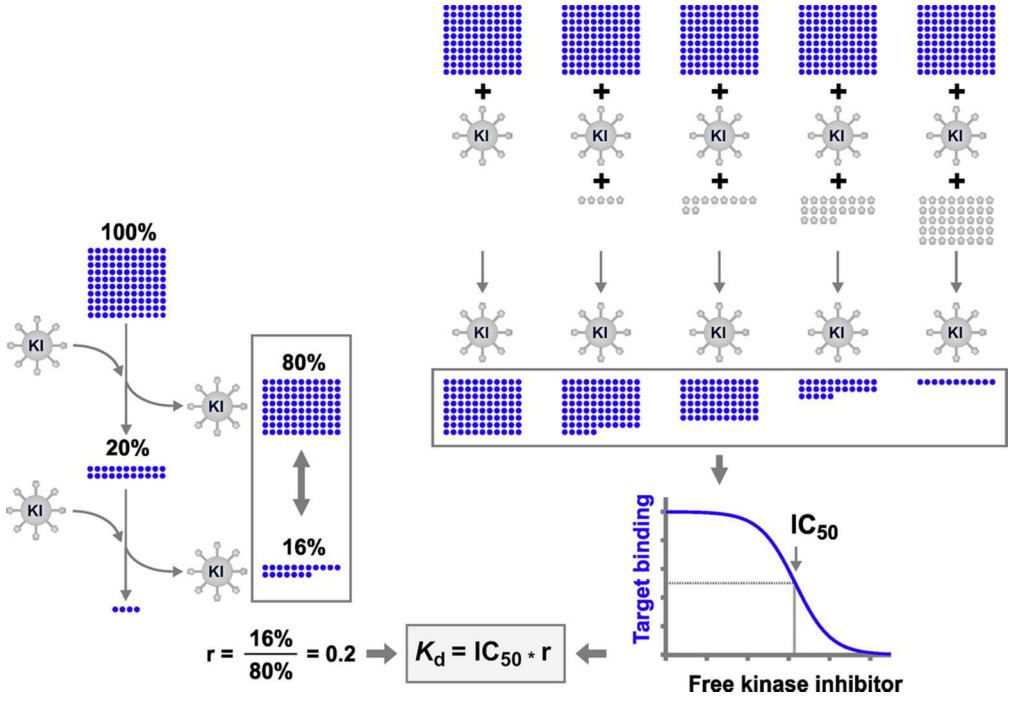

Figure 3. Schematic illustration of target affinity profiling in quantitative chemical proteomics. The distribution of a hypothetical kinase inhibitor target (depicted as blue dots) is shown for a consecutive binding experiment with kinase inhibitor (KI) beads and a parallel competition experiment with increasing concentrations of free kinase inhibitor. Quantitative comparisons are made for the affinity-purified target fractions enclosed by the two rectangles. Note that the ratio r = 0.2 indicates that 20% of the target remained in the supernatant after the first round of incubation with inhibitor beads. The ratio r corrects for target affinity to immobilized kinase inhibitor when multiplied with the IC_{50} from competition experiments. The resulting product represents the target-specific K_d value.

molecule interactions can be directly inferred from quantitative MS data in chemical proteomics studies.[42,43] Such experiments should always be done in replicates, to eliminate "false positive" targets due to occasional quantification errors.[36,40] When interpreting the data, it needs to be considered that both direct inhibitor targets, such as primarily protein kinases, and proteins associating with them exhibit similar, "specific" binding patterns. Thus, some downstream assessment is required to discern between likely direct and indirect targets, e.g. by mapping specific targets on known protein−protein interaction data retrieved from STRING or other resources.[44]

Quantitative Affinity Profiling of Cellular Kinase Targets. Even when specifically bound by a kinase inhibitor resin, targets may have very different affinities that determine whether they are likely to be relevant or not in cellular treatment conditions. We have previously developed a quantitative proteomics concept to determine target-specific affinities for immobilized ligands such as kinase inhibitors.[38] To account for this, we used SILAC-based MS to quantify target protein binding in two consecutive affinity purifications. The principle is depicted in Figure 3, assuming a hypothetical target of which 80% is retained in a first round of affinity purification. The supernatant fraction is then subjected to a second round of incubation with the same beads. There, 80% of the remaining 20%—corresponding to 16% of total cellular target in the original lysate—is bound. Relative amounts of target protein bound in the second versus the first affinity purification can then be quantitatively compared, resulting in a ratio r of 0.2. Notably, this ratio indicates the fraction of target that remained in the supernatant, whereas $(1 - r)$ indicates the fraction that was retained in the first purification step. This principle implemented in SILAC-based chemical proteomics allowed determination of target-specific affinities (K_d values) for immobilized compounds solely based on quantitative MS data. Moreover, as target affinities might be affected by compound modification and covalent attachment to beads, we quantitatively monitored the concentration-dependent effect of free drug on target protein binding to the kinase inhibitor beads, similar to earlier quantitative MS experiments by Bantscheff et al. (Figure 3).[39] Using the target-specific IC_{50} values for free compound—the concentrations reducing target binding by 50%—target-specific K_d values can be calculated according to the Cheng−Prusoff equation,[38] which can even be simplified to $K_d = IC_{50} \times r$ for all quantified targets (Figure 3). Notably, additional control incubations verified equilibrium binding conditions as well as molar excess of immobilized ligand over its cellular targets, which are requirements for such K_d value determinations.[38] This quantitative strategy determined target K_d values for gefitinib and SB203580 that were in good agreement with data from recombinant kinase assays, indicating that quantitative chemical proteomics can combine the unbiased, proteome-wide identification of specific drug targets with reliable affinity measurements.[38] The same approach was used for comparative target analyses of the clinical EGFR inhibitors gefitinib and erlotinib,[45,46] revealing protein kinases such as ARG, MAP3K1, and integrin-linked kinase as erlotinib-specific targets in pancreatic cancer cells in concordance with chemical proteomics data from lung cancer cell lines.[47] Recently, Colzani et al. applied SILAC-based competition and K_d value determination to profile known and new targets of the anticancer kinase inhibitor E-3810.[48] Of note, such quantitative chemical proteomics assays are not restricted to protein kinases and have found widespread

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commercial applications such as target deconvolution in drug discovery programs.

Broad Kinase Enrichment with Multiplexed Kinase Inhibitor Beads. A popular variant of chemical proteomics combines immobilized kinase inhibitors to capture a large fraction of the expressed kinome. To achieve this, inhibitor beads are selected which are as unselective and as complementary as possible with regard to their kinase binding properties. In an early implementation, immobilized bisindolylmaleimide X, a gefitinib derivative, the pyridopyrimidine compound PP58, and purvalanol B were combined for chromatographic enrichment of more than 140 distinct protein kinases from cell extracts.[49] Bantscheff et al. used structurally related as well as additional broad-spectrum compounds in a multiplexed kinase inhibitor resin ("kinobeads") to profile clinical Bcr-Abl inhibitors in leukemia cell lysates.[39] Imatinib, dasatinib, or bosutinib were added to cell lysate at different concentrations together with kinase inhibitor beads, and dose-dependent drug effects on resin binding were recorded by MS using iTRAQ quantification.[39] Alternatively, cells were treated with the drugs prior to lysis and kinase affinity purification. These experiments revealed various cellular dasatinib and bosutinib targets in addition to Bcr-Abl, but maybe the most important findings were the identification of discoidin domain receptor DDR1 and the oxidoreductase NQO2 as new high-affinity targets of imatinib.[39] The same chemical proteomics assay was also used in CDK inhibitor profiling studies in chronic lymphocytic leukemia cells, where it significantly contributed to the identification of CDK9 as the main target involved in pro-apoptotic drug effects, and in a study that revealed *Trypanosoma brucei* protein kinases as nanomolar targets of typical kinase inhibitors.[50,51] The initial "kinobeads" assay tended to underestimate target affinities, as evident from significantly higher IC_{50} values than measured *in vitro* for several high affinity kinase targets. Meanwhile, affinity correction for the influence of immobilized ligands[38] is also routinely applied in profiling assays using multiplexed kinase inhibitor resins (Figure 3). Examples are Cellzome's "kinobeads" or the KinAffinity beads developed by Evotec, which provide reliable apparent K_d values for target-specific affinities.[52−55] Drewes and colleagues used the approach to determine cellular kinase affinities for the nucleotide cofactors ATP, ADP, and GTP.[56] These data are of high relevance for pharmacological kinase inhibition, because cellular inhibitor potency depends on kinase-specific K_d values for both compound as well as ATP.[57] A key advantage of multiplexed kinase inhibitor resins is that they can be used off-the-shelf, without the need for linker derivative synthesis, testing, and immobilization. However, despite broad enrichment of an estimated 50 to 70% of the expressed kinome, a fraction including potentially relevant kinase targets is not covered. Various new affinity probes have been developed for under-represented kinases to increase kinome coverage when included in multiplexed kinase inhibitor resins.[40,53−55] For example, Küster and colleagues devised an affinity probe targeting Akt kinases, which have been notoriously absent in previous chemical proteomics studies despite their high biological relevance.[53]

Beyond kinase inhibitor profiling, mixtures of nonselective kinase inhibitor resins are valuable tools for cellular kinase biochemistry. Using a mixture of five immobilized kinase inhibitors, we purified and identified more than 200 protein kinases from S and M phase-arrested HeLa cells and quantified the cell cycle regulation of more than 1500 distinct kinase-derived phosphopeptides using SILAC.[58,59] The same kinase-selective proteomics workflow was also applied to analyze cellular imatinib targets and their downstream effectors, and to analyze kinases and their interaction partners in lipopolysaccharide-induced innate immune signaling.[60,61] Multiplexed kinase inhibitor beads were also used for large-scale quantitative kinome profiling across 34 head and neck squamous cell carcinoma cell lines[62] and the entire NCI-60 cell line panel.[63] Finally, the amount of kinase retained by an immobilized inhibitor is determined by both protein abundance and affinity. As the latter can change upon activation or inactivation, differential binding of a kinase to inhibitor resins may be observed.[30] Multiplexed kinase inhibitor beads were used to detect dynamic changes in the kinome that mediate resistance upon pharmacological MEK or BRAF inhibition.[64,65] While this represents an interesting approach, it remains to be evaluated how such results compare to large-scale proteomics and phosphoproteomics data of protein abundance and kinase/signaling pathway activities on a global scale.

Alternative Chemical Proteomics Approaches to Kinase Inhibitor Profiling. Most chemical proteomics applications in kinase inhibitor research utilized resin-immobilized compounds for target binding and assessment. On the basis of many successful studies this has emerged as a robust, reliable, and comprehensive approach feasible for a wide range of structurally diverse kinase inhibitors. An alternative kinome profiling technology makes use of desthiobiotin-tagged acyl-phosphates of ATP and ADP, which can covalently attach to conserved lysine residues in protein kinases.[66] These reactions are typically performed in cell lysates in the presence of different kinase inhibitor concentrations, followed by enrichment of desthiobiotinylated peptides or proteins and quantitative MS analysis.[66,67] Competition curves can be generated for kinase inhibitors that displace the reactive ATP probes from the ATP-binding site. When purifying desthiobiotinylated peptides, quantification may be less accurate, as often only a single peptide is analyzed per protein kinase, and such labeled peptides might be shared among related members of the protein kinase superfamily. Moreover, careful optimization of experimental conditions is needed as well as the development of targeted MS detection assays to maximize kinome coverage.[66−68] Potential advantages of covalent ATP probes compared to multiplexed kinase inhibitor beads are that targets do not need to be solubilized—they can be assayed in membrane preparations—and that different kinase domains on the same protein can be discerned.[66] Overall, the two approaches identify similar numbers of unique protein kinases[52,56,66,67] and can provide complementary results, such that multiplexed kinase inhibitor beads efficiently enrich for tyrosine kinases, whereas covalent ATP probes provide high coverage for STE family kinases.[68]

In addition to covalent ATP probes, several kinase inhibitor analogs have been developed that harbor photo-cross-linkable groups for covalent attachment as well as a clickable alkyne handle or a biotin moiety to enrich inhibitor-bound targets.[69−72] Several of these reagents are cell-permeable and can label targets inside live cells, which provides an interesting means to interrogate protein kinases in their native environment. Despite this conceptual advantage, known key targets captured by inhibitor resins are often not found, which indicates current limitations in target spectrum coverage by covalent inhibitor probes.[69,72] In contrast to reversible kinase

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inhibitors functionalized with photoreactive groups, a number of clinical kinase inhibitors can act irreversibly on their targets by covalent attachment to a noncatalytic cysteine at the active site. Cravatt and colleagues have presented a systematic cellular profiling approach for such covalent kinase inhibitors.[73] Of note, they also identified nonkinase off-targets with conserved active site cysteines and correlated proteomics results with selectivity windows for kinase-dependent mechanisms of action.[73]

Very recently, a proteomic version of the thermal shift assay was reported, which measures ligand-induced thermal stabilization of target proteins.[74,75] Savitski et al. performed thermal profiling in combination with quantitative MS for affinity profiling of different kinase inhibitors. While more than 50 targets could be delineated for the pan-specific kinase inhibitor staurosporine, the majority of high-affinity dasatinib targets were not identified. Thus, it remains to be seen whether further optimization can generally improve coverage for kinase inhibitor profiling. However, as thermal profiling can also be done on soluble proteins in living cells, it has the potential to complement established methods by analyzing drug engagement of on- and off-targets *in vivo*.

QUANTITATIVE PHOSPHOPROTEOMICS WITH KINASE INHIBITORS

While chemical proteomics allows interrogating target selectivity in the biological system, the mechanisms of action of kinase inhibitors *in vivo* are determined by a number of additional factors. Evidently, cellular activity of a target kinase is a prerequisite to modulate signaling. Next, only kinases affected by the applied compound concentrations in intact cells contribute to the drug mode of action. And, importantly, protein kinases act through their cell type-specific substrates and downstream signaling pathways. In addition to chemical proteomics of kinase inhibitors, the unbiased and comprehensive analysis of cellular phosphorylation changes upon drug interference provides highly complementary information. Such studies have become possible through spectacular advances in MS-based quantitative phosphoproteomics in recent years.[7,10,11] Often, cultured cells are treated with kinase inhibitors prior to lysis in buffer that preserves protein phosphorylation states. Upon proteolytic digestion, usually by trypsin, peptides are fractionated by strong cation exchange or high pH reversed phase chromatography to reduce sample complexity, followed by affinity purification of phosphorylated peptides with immobilized metal affinity chromatography (IMAC) or titanium dioxide beads (Figure 4).[7,10,11] Alternatively, antibodies recognizing phosphotyrosine or sequence-specific phosphoepitopes generated by certain Ser/Thr kinases are used to enrich phosphopeptide subsets directly from total lysate digests.[76] Phosphopetide-enriched fractions are then analyzed by high-resolution/high-accuracy tandem MS on sensitive instruments, such as orbitrap mass spectrometers, followed by MS data processing to identify and quantify phosphopeptides and the corresponding phosphorylation sites on proteins (Figuer 4).[7] While SILAC is most common in contemporary phosphoproteomics, chemical isotope labeling by various reagents, such as TMT, iTRAQ, or mTRAQ, and more recently label-free quantification have become useful options.[32,77−80] Modern phosphoproteomics workflow can identify in the range of 10 000 to 20 000 distinct phosphorylation sites within 1 or 2 days, or even close to 40 000 upon extensive sampling combined with the analysis of different

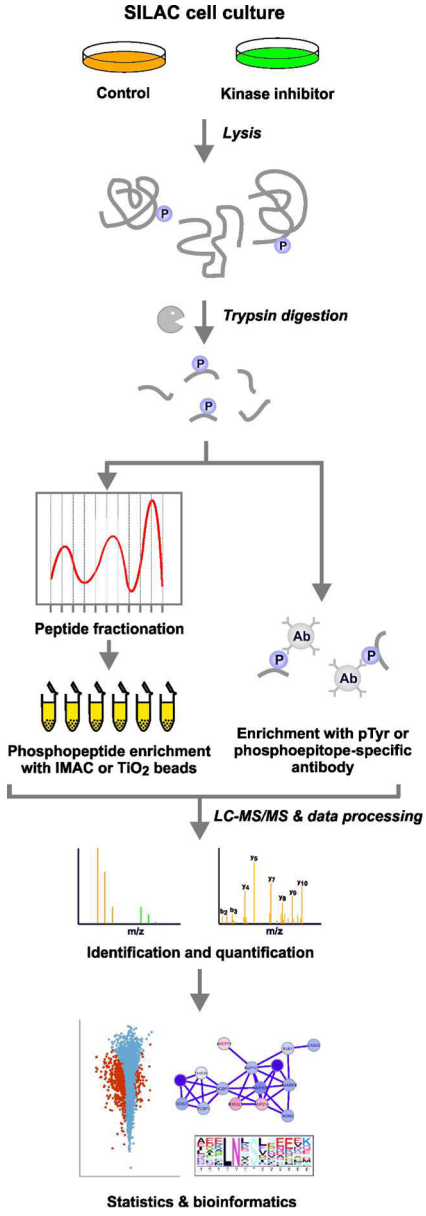

Figure 4. Typical quantitative phosphoproteomics workflow to study the cellular modes of action of a small molecule kinase inhibitor. Shown here is comparative SILAC quantification of phosphoproteomes in control- and kinase inhibitor-treated cultured cells. Alternatively, chemical isotope labeling or label-free quantification can be applied to identify kinase inhibitor-induced phosphorylation changes. After proteolytic digestion, peptides can be fractionated by chromatography prior to IMAC or TiO_2 phosphopeptide enrichment for global analysis of serine, threonine and tyrosine phosphorylation, or subjected to peptide immunoprecipitation to study phosphorylation on tyrosines or serine/threonine sites within certain sequence motifs.

treatment conditions.[79,81] Thus, discovery-type MS approaches enable far higher phosphoproteome coverage compared to immunodetection methods such as reverse phase protein arrays. In contrast, reverse phase protein arrays offer higher sensitivity and sample throughput for the targeted analysis of up to a few hundred phosphorylation sites, provided that specific and potent antibodies are available.[82]

To obtain reliable quantitative data for downstream bioinformatics and in-depth assessment of results, stringent statistical analysis is imperative in MS-based phosphoproteo-

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mics. Therefore, at least three replicate experiments should be performed to identify significant phosphorylation changes upon drug treatment, together with appropriate statistical approaches that correct for extensive multiple hypothesis testing in large-scale quantitative phosphoproteomics.[77,83]

Applied to kinase inhibitors, phosphoproteome regulation is often investigated upon preincubation with an inhibitor followed by an activating stimulus, such as growth factor treatment, or upon compound addition to cells harboring active kinases due to oncogenic mutations or cell cycle blockage. In the latter case, kinase inhibitor treatment should be long enough (e.g., 30 min) to allow for dephosphorylation of downstream signaling elements by cellular phosphatases. Moreover, kinase inhibitor treatment can affect cellular protein abundance, for instance by influencing phosphoprotein stability or mRNA synthesis. Protein expression changes are more pronounced upon longer treatment periods of several hours and may warrant concomitant proteome quantification to distinguish between changes of protein abundance, site-specific phosphorylation or a composite effect of both.[7]

Once significantly regulated phosphorylation sites and phosphoproteins are identified, various bioinformatics techniques for global data analysis can contribute to generate mode of action hypotheses. These typically test for enrichment in gene ontology terms or KEGG pathways that can point to biological or signaling processes associated with phosphoproteome regulation or include network analyses to reveal coordinated phosphoprotein regulation upon kinase inhibitor treatment. Further insights can be retrieved from sequence motif or clustering analyses of regulated phosphorylation sites, together with an in-depth literature review of prior knowledge.[7,10,11] Reported kinase autophosphorylation or substrate phosphorylation sites are of particular interest as read-outs for cellular kinase activity, as well as phosphorylation events functionally linked to the modulation of biological processes.

Case Studies for Kinase Inhibitor Phosphoproteomics. Quantitative analysis of antibody-purified phosphotyrosine peptides combined with small molecule inhibition has been used to study oncogenic tyrosine kinase signaling in the context of drug sensitivity and resistance mechanisms, as described for RTKs such as the EGFR and others.[84−87] Moreover, quantitative phosphotyrosine profiling has been performed to reveal direct tyrosine kinase substrates by comparison of *in vitro* and *in vivo* phosphorylation data.[88]

While the analysis of tyrosine phosphorylated peptides enables the rapid and sensitive analysis of the immediate consequences of tyrosine kinase inhibition, large-scale phosphoproteomics that also covers serine and threonine phosphorylation provides a global view, including downstream signaling pathways as shown for the clinical drugs dasatinib, lapatinib, and crizotinib.[77,89,90] Small molecule kinase inhibitors have been instrumental in large-scale phosphoproteomics studies of key signal transducers like MAPKs and mTOR. For example, MEK inhibitors such as U0126 or PD184352 were used to identify site-specific phosphorylations mediated by ERK MAP kinases in cells.[90,91] Data analysis comprised filtering for potential direct substrate sites matching the consensus motif of proline-directed ERK1/2 kinases, to provide starting points for validation of new cellular substrates such as the transcription factor JunB.[90,91] Moreover, phosphoproteomics experiments involving specific thiophosphorylation through analog-sensitive ERK2 mutant or employing ^{18}O-ATP in ERK1 kinase reactions were performed to screen for direct ERK substrates in cell lysates.[92,93] Merging of data from such orthogonal approaches can narrow down candidate lists to the most likely direct substrates *in vivo*.

Several phosphoproteomics studies used small molecule inhibitors to perturb and dissect mTor signaling, either by inhibiting mTORC1 with rapamycin and/or mTORC1/2 by ATP-competitive mTOR kinase inhibitors,[80,94,95] or by interfering with upstream signal transducers such as Akt or phosphatidylinositol-3 kinases.[76,81] Based on such phosphoproteomics data Grb10 was identified as the mTORC1 substrate that negatively regulates insulin signaling, and Akt was shown to directly regulate mTORC2 through modification of its core component SIN1.[80,81,94] These studies nicely demonstrated how unbiased phosphoproteome assessment upon kinase inhibitor treatment can trigger follow-up experiments to promote new mechanistic insights into kinase signaling.

Kinase inhibitors have been indispensible tools in the phosphoproteomic dissection of mitotic kinase signaling, as exemplified by studies on Aurora kinases and Polo-like kinase 1 (PLK1).[96,97] For example, the addition of PLK1 inhibitors such as BI2536 to human cells arrested in mitosis led to the identification of hundreds of candidate downstream targets. Many of them localized to key mitotic compartments such as the centrosome, kinetochore, or spindle apparatus and were regulated on phosphorylation sites matching the Plk1 consensus motif.[97−99] Of note, recent chemical proteomics on the PLK1 inhibitor BI2536 revealed death-associated protein kinase 2 as a novel high-affinity and functionally relevant target in mitosis.[100] This finding illustrates a general caveat of supposedly "specific" kinase inhibitors, which may have off-target liabilities that confound the assignment of cellular downstream targets. Chemical genetic approaches involving analog-sensitive kinase mutants offer a powerful alternative to dissect kinase signaling with high specificity.[101,102] In this approach, kinase mutants are generated which encode for a small amino acid residue (glycine or alanine) in the conserved "gatekeeper" position located at the ATP-binding site.[103,104] This modification opens up an adjacent hydrophobic pocket such that "bulky" kinase inhibitor analogs can be accommodated, while ATP binding and kinase activity is often preserved in the engineered kinases. Importantly, these "bulky" kinase inhibitors are specific for analog-sensitive mutants and have a minimal effect on naturally occurring kinases, due to steric interference with the side chain of a large amino acid at the "gatekeeper" position (in most cases threonine, methionine, phenylalanine, or leucine).[103,104]

We have applied this strategy for large-scale quantitative phosphoproteomics of cells expressing either inhibitor-sensitive PLK1 analog or, as a control, wild-type PLK1. Replicate quantifications in analog-treated mutant and wild-type PLK1 cells combined with stringent statistical analysis revealed almost 400 site-specific phosphorylations mediated by PLK1 with very high confidence.[105] Thus, chemical genetics approaches to kinase signaling have the unique strength of combining the specificity of genetics with the rapid, tunable, and conditional perturbation by small molecules, and ongoing developments in genome editing with engineered nuclease might facilitate future applications.[106]

Beyond signal transduction research, quantitative phosphoproteomics also supports the development of kinase-selective drugs for therapeutic use. In addition to unbiased mode of action analysis, typical applications include the (i) identification of specific pharmacodynamic read-outs for therapeutic kinase

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inhibition, to define target-specific, sensitive, and robust phosphorylation markers to monitor target engagement in preclinical and clinical development, (ii) phosphoproteomic comparisons under relevant cellular conditions, to inform the selection of lead compounds or preclinical candidates with maximal on- and minimal off-target activity, or (iii) monitoring phosphoproteome regulation in different biological models to shed light on factors underlying biological sensitivity or resistance to a kinase-selective drug.

Integrated Proteomics Approaches to Kinase Inhibitor Analysis. The combination of chemical and phosphoproteomics has considerable potential to promote a more systems-level view of kinase inhibitor targets and their cellular signaling functions. Superti-Furga and colleagues performed chemical proteomics profiling of the multitargeted kinase inhibitor dasatinib in lung cancer cells. In parallel, tyrosine phosphorylation changes upon exposure to the drug where quantified, with a focus on tyrosine kinase autophosphorylation sites as read-out for cellular activity.[84] Subsequent data integration revealed which dasatinib targets were expressed, present as active kinases, and inhibited in cells in a dose-dependent manner. Dasatinib-resistant mutants of these candidate tyrosine kinases were then tested in rescue experiments measuring viability and signaling in the presence of the drug, revealing Src family kinases and the EGFR as relevant targets for dasatinib action.[84] Integration of chemical proteomics, phosphoproteomics, and transcriptomics linked drug synergy between danusertib and bosutinib to MAPK pathway-dependent c-Myc regulation in CML cells harboring the drug-resistant BCR-ABL^{T315I} "gatekeeper" mutant.[107] We have pursued global phosphoproteomics in acute myeloid leukemia (AML) cells to investigate why gefitinib and erlotinib affect cell viability in the absence of their primary drug target, the EGFR.[46] These experiments revealed a drug-regulated phosphoprotein network comprising several cytoplasmic tyrosine kinases, of which Src family kinases and the tyrosine kinase BTK were identified as direct targets in an accompanying chemical proteomics analysis. In a recent report, Heck and colleagues combined kinome expression, drug target, and phosphotyrosine profiling in a comparative analysis of four tyrosine kinase inhibitors, which revealed striking differences in cellular target promiscuity and associated network regulation.[108]

Emerging Phosphoproteomics Concepts to Predict Kinase Inhibitor Sensitivity. In comparison to global protein or mRNA profiles, the phosphoproteome provides the most proximate read-out for cellular kinase activities. As functional linkage may be better reflected by direct post-translational modifications than by their aggregated downstream effects, the phosphoproteome could serve as a rich source for predictive markers correlated with therapeutic kinase inhibition. In principle, predictive phosphorylation sites may improve stratification based on genomic alterations,[109] such as activating mutations in oncogenic kinase targets, or even define alternative strategies to predict kinase inhibitor sensitivity when no genomic markers are available.

Cutillas and colleagues used phosphoproteomics data to classify hematological cancer cell lines and identify phosphorylation events associated with kinase inhibitor sensitivity.[110,111] Klammer et al. quantified the basal phosphoproteomes of 19 SILAC-encoded lung cancer cell lines. Subsequent feature selection and support vector machine training identified a signature of 12 phosphorylations sites, which predicted sensitivity or resistance to the kinase inhibitor dasatinib with high accuracy.[112] This concept was recently applied to human bone marrow-derived AML cells from patients treated with the FLT3 receptor tyrosine kinase inhibitor AC220 (quizartinib). A combination of five predictive phosphorylation sites was identified in a discovery phase involving 12 patients, and this phosphosignature correctly predicted treatment outcome in seven out of nine patients in an independent validation study.[113] Arguably, patient numbers were relatively low, and further work will be needed to validate such strategies in larger studies. Nevertheless, predictive phosphorylation markers might have significant potential in hematological cancers, to complement and enhance current patient stratification concepts based on genomic markers. Such concepts may also become applicable for solid tumors despite additional challenges, primarily due to more pronounced heterogeneity and ischemia-related effects on phosphoproteome measurements.[114] Additional filtering will be mandatory to identify stable phosphoepitopes, as demonstrated for PRAS phosphorylation on Thr-246 as readout for phosphatidylinositol-3 kinase pathway activity in tumor tissue.[115]

Conclusions and Perspective. Spectacular advances at the interface of quantitative proteomics and chemical biology have made it possible to link kinase inhibitors to their biological targets and signaling interference mechanisms by hypothesis-free approaches. These efforts provided numerous results that led to mechanistic insights and foster a systems-level view on the molecular pharmacology of protein kinase targets. Future developments will certainly address both the sensitivity and throughput of such studies, propelled by further increases in mass spectrometry performance,[116] new technologies for multiplexed quantification,[74,117] and streamlined workflows based on single-shot LC-MS analysis.[43,78] While most previous work in kinase inhibitor proteomics was carried out with cultured cells, the number of chemical proteomics and phosphoproteomics studies in animal or human tissue will likely increase, as well as translational applications in areas such as personalized medicine.[118,119] Thus, there are exiting research opportunities lying ahead to advance and apply knowledge about kinase inhibitor targets and mechanisms.

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Notes

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REFERENCES

(1) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science 298*, 1912−1934.

(2) Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. *Nature 411*, 355−365.

(3) Cohen, P. (2002) Protein kinases–the major drug targets of the twenty-first century? *Nat. Rev. Drug. Discovery 1*, 309−315.

(4) Zhang, J., Yang, P. L., and Gray, N. S. (2009) Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer 9*, 28−39.

(5) Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H., and Peterson, J. R. (2011) Comprehensive assay of kinase catalytic activity

reveals features of kinase inhibitor selectivity. *Nat. Biotechnol. 29*, 1039−1045.
(6) Cong, F., Cheung, A. K., and Huang, S. M. (2012) Chemical genetics-based target identification in drug discovery. *Annu. Rev. Pharmacol. Toxicol. 52*, 57−78.
(7) Roux, P. P., and Thibault, P. (2013) The coming of age of phosphoproteomics–from large data sets to inference of protein functions. *Mol. Cell. Proteomics 12*, 3453−3464.
(8) Rix, U., and Superti-Furga, G. (2009) Target profiling of small molecules by chemical proteomics. *Nat. Chem. Biol. 5*, 616−624.
(9) Bantscheff, M., Scholten, A., and Heck, A. J. (2009) Revealing promiscuous drug-target interactions by chemical proteomics. *Drug Discovery Today 14*, 1021−1029.
(10) Choudhary, C., and Mann, M. (2010) Decoding signalling networks by mass spectrometry-based proteomics. *Nat. Rev. Mol. Cell Biol. 11*, 427−439.
(11) Junger, M. A., and Aebersold, R. (2014) Mass spectrometry-driven phosphoproteomics: patterning the systems biology mosaic. *Wiley Interdiscip. Rev. Dev. Biol. 3*, 83−112.
(12) Lee, J., and Bogyo, M. (2013) Target deconvolution techniques in modern phenotypic profiling. *Curr. Opin. Chem. Biol. 17*, 118−126.
(13) Gao, Y., Davies, S. P., Augustin, M., Woodward, A., Patel, U. A., Kovelman, R., and Harvey, K. J. (2013) A broad activity screen in support of a chemogenomic map for kinase signalling research and drug discovery. *Biochem. J. 451*, 313−328.
(14) Davis, M. I., Hunt, J. P., Herrgard, S., Ciceri, P., Wodicka, L. M., Pallares, G., Hocker, M., Treiber, D. K., and Zarrinkar, P. P. (2011) Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol. 29*, 1046−1051.
(15) Kitagawa, D., Yokota, K., Gouda, M., Narumi, Y., Ohmoto, H., Nishiwaki, E., Akita, K., and Kirii, Y. (2013) Activity-based kinase profiling of approved tyrosine kinase inhibitors. *Genes Cells 18*, 110−122.
(16) Swanson, K. D., Taylor, L. K., Haung, L., Burlingame, A. L., and Landreth, G. E. (1999) Transcription factor phosphorylation by pp90(rsk2). Identification of Fos kinase and NGFI-B kinase I as pp90(rsk2). *J. Biol. Chem. 274*, 3385−3395.
(17) Knockaert, M., Gray, N., Damiens, E., Chang, Y. T., Grellier, P., Grant, K., Fergusson, D., Mottram, J., Soete, M., Dubremetz, J. F., Le Roch, K., Doerig, C., Schultz, P., and Meijer, L. (2000) Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. *Chem. Biol. 7*, 411−422.
(18) Bach, S., Knockaert, M., Reinhardt, J., Lozach, O., Schmitt, S., Baratte, B., Koken, M., Coburn, S. P., Tang, L., Jiang, T., Liang, D. C., Galons, H., Dierick, J. F., Pinna, L. A., Meggio, F., Totzke, F., Schachtele, C., Lerman, A. S., Carnero, A., Wan, Y., Gray, N., and Meijer, L. (2005) Roscovitine targets, protein kinases and pyridoxal kinase. *J. Biol. Chem. 280*, 31208−31219.
(19) Ding, S., Wu, T. Y., Brinker, A., Peters, E. C., Hur, W., Gray, N. S., and Schultz, P. G. (2003) Synthetic small molecules that control stem cell fate. *Proc. Natl. Acad. Sci. U.S.A. 100*, 7632−7637.
(20) Wan, Y., Hur, W., Cho, C. Y., Liu, Y., Adrian, F. J., Lozach, O., Bach, S., Mayer, T., Fabbro, D., Meijer, L., and Gray, N. S. (2004) Synthesis and target identification of hymenialdisine analogs. *Chem. Biol. 11*, 247−259.
(21) Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc. Natl. Acad. Sci. U.S.A. 100*, 15434−15439.
(22) Wissing, J., Godl, K., Brehmer, D., Blencke, S., Weber, M., Habenberger, P., Stein-Gerlach, M., Missio, A., Cotten, M., Muller, S., and Daub, H. (2004) Chemical proteomic analysis reveals alternative modes of action for pyrido[2,3-d]pyrimidine kinase inhibitors. *Mol. Cell. Proteomics 3*, 1181−1193.
(23) Godl, K., Gruss, O. J., Eickhoff, J., Wissing, J., Blencke, S., Weber, M., Degen, H., Brehmer, D., Orfi, L., Horvath, Z., Keri, G., Muller, S., Cotten, M., Ullrich, A., and Daub, H. (2005) Proteomic characterization of the angiogenesis inhibitor SU6668 reveals multiple impacts on cellular kinase signaling. *Cancer Res. 65*, 6919−6926.
(24) Brehmer, D., Greff, Z., Godl, K., Blencke, S., Kurtenbach, A., Weber, M., Muller, S., Klebl, B., Cotten, M., Keri, G., Wissing, J., and Daub, H. (2005) Cellular targets of gefitinib. *Cancer Res. 65*, 379−382.
(25) Tigno-Aranjuez, J. T., Benderitter, P., Rombouts, F., Deroose, F., Bai, X., Mattioli, B., Cominelli, F., Pizarro, T. T., Hoflack, J., and Abbott, D. W. (2014) In Vivo Inhibition of RIPK2 Kinase Alleviates Inflammatory Disease. *J. Biol. Chem. 289*, 29651−29664.
(26) Tigno-Aranjuez, J. T., Asara, J. M., and Abbott, D. W. (2010) Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. *Genes Dev. 24*, 2666−2677.
(27) Remsing Rix, L. L., Rix, U., Colinge, J., Hantschel, O., Bennett, K. L., Stranzl, T., Muller, A., Baumgartner, C., Valent, P., Augustin, M., Till, J. H., and Superti-Furga, G. (2009) Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia 23*, 477−485.
(28) Rix, U., Hantschel, O., Durnberger, G., Remsing Rix, L. L., Planyavsky, M., Fernbach, N. V., Kaupe, I., Bennett, K. L., Valent, P., Colinge, J., Kocher, T., and Superti-Furga, G. (2007) Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. *Blood 110*, 4055−4063.
(29) Remsing Rix, L. L., Kuenzi, B. M., Luo, Y., Remily-Wood, E., Kinose, F., Wright, G., Li, J., Koomen, J. M., Haura, E. B., Lawrence, H. R., and Rix, U. (2013) GSK3 alpha and beta are new functionally relevant targets of tivantinib in lung cancer cells. *ACS Chem. Biol. 9*, 353−358.
(30) Brehmer, D., Godl, K., Zech, B., Wissing, J., and Daub, H. (2004) Proteome-wide identification of cellular targets affected by bisindolylmaleimide-type protein kinase C inhibitors. *Mol. Cell. Proteomics 3*, 490−500.
(31) Missner, E., Bahr, I., Badock, V., Lucking, U., Siemeister, G., and Donner, P. (2009) Off-target decoding of a multitarget kinase inhibitor by chemical proteomics. *Chembiochem 10*, 1163−1174.
(32) Bantscheff, M., Lemeer, S., Savitski, M. M., and Kuster, B. (2012) Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal. Bioanal. Chem. 404*, 939−965.
(33) Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res. 10*, 1794−1805.
(34) Oda, Y., Owa, T., Sato, T., Boucher, B., Daniels, S., Yamanaka, H., Shinohara, Y., Yokoi, A., Kuromitsu, J., and Nagasu, T. (2003) Quantitative chemical proteomics for identifying candidate drug targets. *Anal. Chem. 75*, 2159−2165.
(35) Zhang, Y. X., Knyazev, P. G., Cheburkin, Y. V., Sharma, K., Knyazev, Y. P., Orfi, L., Szabadkai, I., Daub, H., Keri, G., and Ullrich, A. (2008) AXL is a potential target for therapeutic intervention in breast cancer progression. *Cancer Res. 68*, 1905−1915.
(36) Ong, S. E., Schenone, M., Margolin, A. A., Li, X., Do, K., Doud, M. K., Mani, D. R., Kuai, L., Wang, X., Wood, J. L., Tolliday, N. J., Koehler, A. N., Marcaurelle, L. A., Golub, T. R., Gould, R. J., Schreiber, S. L., and Carr, S. A. (2009) Identifying the proteins to which small-molecule probes and drugs bind in cells. *Proc. Natl. Acad. Sci. U.S.A 106*, 4617−4622.
(37) Zhang, L., Holmes, I. P., Hochgrafe, F., Walker, S. R., Ali, N. A., Humphrey, E. S., Wu, J., de Silva, M., Kersten, W. J., Connor, T., Falk, H., Allan, L., Street, I. P., Bentley, J. D., Pilling, P. A., Monahan, B. J., Peat, T. S., and Daly, R. J. (2013) Characterization of the novel broad-spectrum kinase inhibitor CTx-0294885 as an affinity reagent for mass spectrometry-based kinome profiling. *J. Proteome Res. 12*, 3104−3116.
(38) Sharma, K., Weber, C., Bairlein, M., Greff, Z., Keri, G., Cox, J., Olsen, J. V., and Daub, H. (2009) Proteomics strategy for quantitative protein interaction profiling in cell extracts. *Nat. Methods 6*, 741−744.
(39) Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Raida, M., Rau, C., Reader, V., Sweetman, G., Bauer, A., Bouwmeester, T., Hopf, C., Kruse, U., Neubauer, G., Ramsden, N., Rick, J., Kuster, B., and Drewes, G. (2007)

Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat. Biotechnol. 25*, 1035−1044.
(40) Oppermann, F. S., Gnad, F., Olsen, J. V., Hornberger, R., Greff, Z., Keri, G., Mann, M., and Daub, H. (2009) Large-scale proteomics analysis of the human kinome. *Mol. Cell. Proteomics 8*, 1751−1764.
(41) Kuai, L., Ong, S. E., Madison, J. M., Wang, X., Duvall, J. R., Lewis, T. A., Luce, C. J., Conner, S. D., Pearlman, D. A., Wood, J. L., Schreiber, S. L., Carr, S. A., Scolnick, E. M., and Haggarty, S. J. (2011) AAK1 identified as an inhibitor of neuregulin-1/ErbB4-dependent neurotrophic factor signaling using integrative chemical genomics and proteomics. *Chem. Biol. 18*, 891−906.
(42) Aye, T. T., Mohammed, S., Toorn, H. W., Veen, T. A., Heyden, M. A., Scholten, A., and Heck, A. J. (2008) Selectivity in enrichment of PKA RI and RII isoforms, their interactors using modified cAMP affinity resins. *Mol. Cell. Proteomics 8*, 1016−1028.
(43) Golkowski, M., Brigham, J. L., Perera, G. K., Romano, G. E., Maly, D. J., and Ong, S. E. (2014) Rapid profiling of protein kinase inhibitors by quantitative proteomics. *Medchemcomm 5*, 363−369.
(44) Szklarczyk, D., Franceschini, A., Kuhn, M., Simonovic, M., Roth, A., Minguez, P., Doerks, T., Stark, M., Muller, J., Bork, P., Jensen, L. J., and von Mering, C. (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res. 39*, D561−568.
(45) Conradt, L., Godl, K., Schaab, C., Tebbe, A., Eser, S., Diersch, S., Michalski, C. W., Kleeff, J., Schnieke, A., Schmid, R. M., Saur, D., and Schneider, G. (2011) Disclosure of erlotinib as a multikinase inhibitor in pancreatic ductal adenocarcinoma. *Neoplasia 13*, 1026−1034.
(46) Weber, C., Schreiber, T. B., and Daub, H. (2012) Dual phosphoproteomics and chemical proteomics analysis of erlotinib and gefitinib interference in acute myeloid leukemia cells. *J. Proteomics 75*, 1343−1356.
(47) Augustin, A., Lamerz, J., Meistermann, H., Golling, S., Scheiblich, S., Hermann, J. C., Duchateau-Nguyen, G., Tzouros, M., Avila, D. W., Langen, H., Essioux, L., and Klughammer, B. (2014) Quantitative chemical proteomics profiling differentiates erlotinib from gefitinib in EGFR wild-type non-small cell lung carcinoma cell lines. *Mol. Cancer Ther. 12*, 520−529.
(48) Colzani, M., Noberini, R., Romanenghi, M., Colella, G., Pasi, M., Fancelli, D., Varasi, M., Minucci, S., and Bonaldi, T. (2014) Quantitative chemical proteomics identifies novel targets of the anti-cancer multi-kinase inhibitor E-3810. *Mol. Cell. Proteomics 13*, 1495−1509.
(49) Wissing, J., Jansch, L., Nimtz, M., Dieterich, G., Hornberger, R., Keri, G., Wehland, J., and Daub, H. (2007) Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. *Mol. Cell. Proteomics 6*, 537−547.
(50) Kruse, U., Pallasch, C. P., Bantscheff, M., Eberhard, D., Frenzel, L., Ghidelli, S., Maier, S. K., Werner, T., Wendtner, C. M., and Drewes, G. (2011) Chemoproteomics-based kinome profiling and target deconvolution of clinical multi-kinase inhibitors in primary chronic lymphocytic leukemia cells. *Leukemia 25*, 89−100.
(51) Urbaniak, M. D., Mathieson, T., Bantscheff, M., Eberhard, D., Grimaldi, R., Miranda-Saavedra, D., Wyatt, P., Ferguson, M. A., Frearson, J., and Drewes, G. (2012) Chemical proteomic analysis reveals the drugability of the kinome of Trypanosoma brucei. *ACS Chem. Biol. 7*, 1858−1865.
(52) Delehouze, C., Godl, K., Loaec, N., Bruyere, C., Desban, N., Oumata, N., Galons, H., Roumeliotis, T. I., Giannopoulou, E. G., Grenet, J., Twitchell, D., Lahti, J., Mouchet, N., Galibert, M. D., Garbis, S. D., and Meijer, L. (2014) CDK/CK1 inhibitors roscovitine and CR8 downregulate amplified MYCN in neuroblastoma cells. *Oncogene 33*, 5675−5687.
(53) Pachl, F., Plattner, P., Ruprecht, B., Medard, G., Sewald, N., and Küster, B. (2013) Characterization of a chemical affinity probe targeting Akt kinases. *J. Proteome Res. 12*, 3792−3800.
(54) Ku, X., Heinzlmeir, S., Liu, X., Medard, G., and Kuster, B. (2014) A new chemical probe for quantitative proteomic profiling of fibroblast growth factor receptor and its inhibitors. *J. Proteomics 96*, 44−55.
(55) Ku, X., Heinzlmeir, S., Helm, D., Medard, G., and Kuster, B. (2014) New affinity probe targeting VEGF receptors for kinase inhibitor selectivity profiling by chemical proteomics. *J. Proteome Res. 13*, 2445−2452.
(56) Becher, I., Savitski, M. M., Savitski, M. F., Hopf, C., Bantscheff, M., and Drewes, G. (2013) Affinity profiling of the cellular kinome for the nucleotide cofactors ATP and ADP, and GTP. *ACS Chem. Biol. 8*, 599−607.
(57) Knight, Z. A., and Shokat, K. M. (2005) Features of selective kinase inhibitors. *Chem. Biol. 12*, 621−637.
(58) Daub, H., Olsen, J. V., Bairlein, M., Gnad, F., Oppermann, F. S., Korner, R., Greff, Z., Keri, G., Stemmann, O., and Mann, M. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol. Cell 31*, 438−448.
(59) Dulla, K., Daub, H., Hornberger, R., Nigg, E. A., and Korner, R. (2010) Quantitative site-specific phosphorylation dynamics of human protein kinases during mitotic progression. *Mol. Cell. Proteomics 9*, 1167−1181.
(60) Sharma, K., Kumar, C., Keri, G., Breitkopf, S. B., Oppermann, F. S., and Daub, H. (2010) Quantitative analysis of kinase-proximal signaling in lipopolysaccharide-induced innate immune response. *J. Proteome Res. 9*, 2539−2549.
(61) Breitkopf, S. B., Oppermann, F. S., Keri, G., Grammel, M., and Daub, H. (2010) Proteomics Analysis of Cellular Imatinib Targets and their Candidate Downstream Effectors. *J. Proteome Res. 9*, 6033−6043.
(62) Wu, Z., Doondeea, J. B., Gholami, A. M., Janning, M. C., Lemeer, S., Kramer, K., Eccles, S. A., Gollin, S. M., Grenman, R., Walch, A., Feller, S. M., and Kuster, B. (2011) Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. *Mol. Cell. Proteomics 10*, M111 011635.
(63) Moghaddas Gholami, A., Hahne, H., Wu, Z., Auer, F. J., Meng, C., Wilhelm, M., and Kuster, B. (2013) Global proteome analysis of the NCI-60 cell line panel. *Cell Rep. 4*, 609−620.
(64) Sos, M. L., Levin, R. S., Gordan, J. D., Oses-Prieto, J. A., Webber, J. T., Salt, M., Hann, B., Burlingame, A. L., McCormick, F., Bandyopadhyay, S., and Shokat, K. M. (2014) Oncogene mimicry as a mechanism of primary resistance to BRAF inhibitors. *Cell Rep. 8*, 1037−1048.
(65) Duncan, J. S., Whittle, M. C., Nakamura, K., Abell, A. N., Midland, A. A., Zawistowski, J. S., Johnson, N. L., Granger, D. A., Jordan, N. V., Darr, D. B., Usary, J., Kuan, P. F., Smalley, D. M., Major, B., He, X., Hoadley, K. A., Zhou, B., Sharpless, N. E., Perou, C. M., Kim, W. Y., Gomez, S. M., Chen, X., Jin, J., Frye, S. V., Earp, H. S., Graves, L. M., and Johnson, G. L. (2012) Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. *Cell 149*, 307−321.
(66) Patricelli, M. P., Nomanbhoy, T. K., Wu, J., Brown, H., Zhou, D., Zhang, J., Jagannathan, S., Aban, A., Okerberg, E., Herring, C., Nordin, B., Weissig, H., Yang, Q., Lee, J. D., Gray, N. S., and Kozarich, J. W. (2011) In situ kinase profiling reveals functionally relevant properties of native kinases. *Chem. Biol. 18*, 699−710.
(67) Xiao, Y., Guo, L., and Wang, Y. (2014) A targeted quantitative proteomics strategy for global kinome profiling of cancer cells and tissues. *Mol. Cell. Proteomics 13*, 1065−1075.
(68) Lemeer, S., Zorgiebel, C., Ruprecht, B., Kohl, K., and Kuster, B. (2013) Comparing immobilized kinase inhibitors and covalent ATP probes for proteomic profiling of kinase expression and drug selectivity. *J. Proteome Res. 12*, 1723−1731.
(69) Fischer, J. J., Dalhoff, C., Schrey, A. K., Graebner, O. Y., Michaelis, S., Andrich, K., Glinski, M., Kroll, F., Sefkow, M., Dreger, M., and Koester, H. (2011) Dasatinib and imatinib and staurosporine capture compounds - Complementary tools for the profiling of kinases by Capture Compound Mass Spectrometry (CCMS). *J. Proteomics 75*, 160−168.
(70) Ranjitkar, P., Perera, B. G., Swaney, D. L., Hari, S. B., Larson, E. T., Krishnamurty, R., Merritt, E. A., Villen, J., and Maly, D. J. (2012) Affinity-based probes based on type II kinase inhibitors. *J. Am. Chem. Soc. 134*, 19017−19025.

(71) Shi, H., Cheng, X., Sze, S. K., and Yao, S. Q. (2011) Proteome profiling reveals potential cellular targets of staurosporine using a clickable cell-permeable probe. *Chem. Commun. (Camb) 47*, 11306−11308.
(72) Shi, H., Zhang, C. J., Chen, G. Y., and Yao, S. Q. (2012) Cell-based proteome profiling of potential dasatinib targets by use of affinity-based probes. *J. Am. Chem. Soc. 134*, 3001−3014.
(73) Lanning, B. R., Whitby, L. R., Dix, M. M., Douhan, J., Gilbert, A. M., Hett, E. C., Johnson, T. O., Joslyn, C., Kath, J. C., Niessen, S., Roberts, L. R., Schnute, M. E., Wang, C., Hulce, J. J., Wei, B., Whiteley, L. O., Hayward, M. M., and Cravatt, B. F. (2014) A road map to evaluate the proteome-wide selectivity of covalent kinase inhibitors. *Nat. Chem. Biol. 10*, 760−767.
(74) Savitski, M. M., Reinhard, F. B., Franken, H., Werner, T., Savitski, M. F., Eberhard, D., Martinez Molina, D., Jafari, R., Dovega, R. B., Klaeger, S., Kuster, B., Nordlund, P., Bantscheff, M., and Drewes, G. (2014) Proteomics. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science 346*, 1255784.
(75) Martinez Molina, D., Jafari, R., Ignatushchenko, M., Seki, T., Larsson, E. A., Dan, C., Sreekumar, L., Cao, Y., and Nordlund, P. (2013) Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science 341*, 84−87.
(76) Moritz, A., Li, Y., Guo, A., Villen, J., Wang, Y., MacNeill, J., Kornhauser, J., Sprott, K., Zhou, J., Possemato, A., Ren, J. M., Hornbeck, P., Cantley, L. C., Gygi, S. P., Rush, J., and Comb, M. J. (2010) Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci. Signal 3*, ra64.
(77) Oppermann, F. S., Klammer, M., Bobe, C., Cox, J., Schaab, C., Tebbe, A., and Daub, H. (2013) Comparison of SILAC and mTRAQ quantification for phosphoproteomics on a quadrupole orbitrap mass spectrometer. *J. Proteome Res. 12*, 4089−4100.
(78) Lundby, A., Andersen, M. N., Steffensen, A. B., Horn, H., Kelstrup, C. D., Francavilla, C., Jensen, L. J., Schmitt, N., Thomsen, M. B., and Olsen, J. V. (2013) In vivo phosphoproteomics analysis reveals the cardiac targets of beta-adrenergic receptor signaling. *Sci. Signal 6*, rs11.
(79) Sharma, K., D'Souza, R. C., Tyanova, S., Schaab, C., Wisniewski, J. R., Cox, J., and Mann, M. (2014) Ultradeep human phosphoproteome reveals a distinct regulatory nature of tyr and ser/thr-based signaling. *Cell Rep. 8*, 1583−1594.
(80) Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villen, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science 332*, 1322−1326.
(81) Humphrey, S. J., Yang, G., Yang, P., Fazakerley, D. J., Stockli, J., Yang, J. Y., and James, D. E. (2013) Dynamic adipocyte phosphoproteome reveals that Akt directly regulates mTORC2. *Cell Metab. 17*, 1009−1020.
(82) Pierobon, M., Wulfkuhle, J., Liotta, L.,, Petricoin, E. (2014) Application of molecular technologies for phosphoproteomic analysis of clinical samples, *Oncogene* [Online early access]. DOI: 10.1038/onc.2014.16. Published Online: Mar 10, 2014.
(83) Klammer, M., Dybowski, J. N., Hoffmann, D., and Schaab, C. (2014) Identification of significant features by the Global Mean Rank test. *PLoS One 9*, e104504.
(84) Li, J., et al. (2010) A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. *Nat. Chem. Biol. 6*, 291−299.
(85) Yoshida, T., Zhang, G., Smith, M. A., Lopez, A. S., Bai, Y., Li, J., Fang, B., Koomen, J., Rawal, B., Fisher, K. J., Chen, A. Y., Kitano, M., Morita, Y., Yamaguchi, H., Shibata, K., Okabe, T., Okamoto, I., Nakagawa, K., and Haura, E. B. (2014) Tyrosine phosphoproteomics identifies both codrivers and cotargeting strategies for T790M-related EGFR-TKI resistance in non-small cell lung cancer. *Clin. Cancer Res. 20*, 4059−4074.
(86) Guo, A., Villen, J., Kornhauser, J., Lee, K. A., Stokes, M. P., Rikova, K., Possemato, A., Nardone, J., Innocenti, G., Wetzel, R., Wang, Y., MacNeill, J., Mitchell, J., Gygi, S. P., Rush, J., Polakiewicz, R. D., and Comb, M. J. (2008) Signaling networks assembled by oncogenic EGFR and c-Met. *Proc. Natl. Acad. Sci. U.S.A. 105*, 692−697.
(87) Tzouros, M., Golling, S., Avila, D., Lamerz, J., Berrera, M., Ebeling, M., Langen, H., and Augustin, A. (2013) Development of a 5-plex SILAC method tuned for the quantitation of tyrosine phosphorylation dynamics. *Mol. Cell. Proteomics 12*, 3339−3349.
(88) Xue, L., Geahlen, R. L., and Tao, W. A. (2013) Identification of direct tyrosine kinase substrates based on protein kinase assay-linked phosphoproteomics. *Mol. Cell. Proteomics 12*, 2969−2980.
(89) Imami, K., Sugiyama, N., Imamura, H., Wakabayashi, M., Tomita, M., Taniguchi, M., Ueno, T., Toi, M., and Ishihama, Y. (2012) Temporal profiling of lapatinib-suppressed phosphorylation signals in EGFR/HER2 pathways. *Mol. Cell. Proteomics 11*, 1741−1757.
(90) Pan, C., Olsen, J. V., Daub, H., and Mann, M. (2009) Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Mol. Cell. Proteomics 8*, 2796−2808.
(91) Courcelles, M., Fremin, C., Voisin, L., Lemieux, S., Meloche, S., and Thibault, P. (2013) Phosphoproteome dynamics reveal novel ERK1/2 MAP kinase substrates with broad spectrum of functions. *Mol. Syst. Biol. 9*, 669.
(92) Xue, L., Wang, P., Cao, P., Zhu, J. K., and Tao, W. A. (2014) Identification of Extracellular Signal-regulated Kinase 1 (ERK1) Direct Substrates using Stable Isotope Labeled Kinase Assay-Linked Phosphoproteomics. *Mol. Cell. Proteomics 13*, 3199−3210.
(93) Carlson, S. M., Chouinard, C. R., Labadorf, A., Lam, C. J., Schmelzle, K., Fraenkel, E., and White, F. M. (2011) Large-scale discovery of ERK2 substrates identifies ERK-mediated transcriptional regulation by ETV3. *Sci. Signal 4*, rs11.
(94) Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science 332*, 1317−1322.
(95) Chen, R. Q., Yang, Q. K., Lu, B. W., Yi, W., Cantin, G., Chen, Y. L., Fearns, C., Yates, J. R., 3rd, and Lee, J. D. (2009) CDC25B mediates rapamycin-induced oncogenic responses in cancer cells. *Cancer Res. 69*, 2663−2668.
(96) Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnad, F., Cox, J., Jensen, T. S., Nigg, E. A., Brunak, S., and Mann, M. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal 3*, ra3.
(97) Kettenbach, A. N., Schweppe, D. K., Faherty, B. K., Pechenick, D., Pletnev, A. A., and Gerber, S. A. (2011) Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells. *Sci. Signal 4*, rs5.
(98) Santamaria, A., Wang, B., Elowe, S., Malik, R., Zhang, F., Bauer, M., Schmidt, A., Sillje, H. H., Korner, R., and Nigg, E. A. (2011) The Plk1-dependent phosphoproteome of the early mitotic spindle. *Mol. Cell. Proteomics 10*, M110 004457.
(99) Grosstessner-Hain, K., Hegemann, B., Novatchkova, M., Rameseder, J., Joughin, B. A., Hudecz, O., Roitinger, E., Pichler, P., Kraut, N., Yaffe, M. B., Peters, J. M., and Mechtler, K. (2011) Quantitative phospho-proteomics to investigate the polo-like kinase 1-dependent phospho-proteome. *Mol. Cell. Proteomics 10*, M111 008540.
(100) Raab, M., Pachl, F., Kramer, A., Kurunci-Csacsko, E., Dotsch, C., Knecht, R., Becker, S., Kuster, B., and Strebhardt, K. (2014) Quantitative chemical proteomics reveals a Plk1 inhibitor-compromised cell death pathway in human cells. *Cell Res. 24*, 1141−1145.
(101) Holt, L. J., Tuch, B. B., Villen, J., Johnson, A. D., Gygi, S. P., and Morgan, D. O. (2009) Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science 325*, 1682−1686.
(102) Blasius, M., Forment, J. V., Thakkar, N., Wagner, S. A., Choudhary, C., and Jackson, S. P. (2011) A phospho-proteomic screen identifies substrates of the checkpoint kinase Chk1. *Genome Biol. 12*, R78.

(103) Koch, A., and Hauf, S. (2010) Strategies for the identification of kinase substrates using analog-sensitive kinases. *Eur. J. Cell. Biol. 89*, 184−193.
(104) Bishop, A. C., Ubersax, J. A., Petsch, D. T., Matheos, D. P., Gray, N. S., Blethrow, J., Shimizu, E., Tsien, J. Z., Schultz, P. G., Rose, M. D., Wood, J. L., Morgan, D. O., and Shokat, K. M. (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature 407*, 395−401.
(105) Oppermann, F. S., Grundner-Culemann, K., Kumar, C., Gruss, O. J., Jallepalli, P. V., and Daub, H. (2012) Combination of chemical genetics and phosphoproteomics for kinase signaling analysis enables confident identification of cellular downstream targets. *Mol. Cell. Proteomics 11*, O111 012351.
(106) Harrison, M. M., Jenkins, B. V., O'Connor-Giles, K. M., and Wildonger, J. (2014) A CRISPR view of development. *Genes Dev. 28*, 1859−1872.
(107) Winter, G. E., Rix, U., Carlson, S. M., Gleixner, K. V., Grebien, F., Gridling, M., Muller, A. C., Breitwieser, F. P., Bilban, M., Colinge, J., Valent, P., Bennett, K. L., White, F. M., and Superti-Furga, G. (2012) Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML. *Nat. Chem. Biol. 8*, 905−912.
(108) Giansanti, P., Preisinger, C., Huber, K. V., Gridling, M., Superti-Furga, G., Bennett, K. L., and Heck, A. J. (2014) Evaluating the promiscuous nature of tyrosine kinase inhibitors assessed in A431 epidermoid carcinoma cells by both chemical- and phosphoproteomics. *ACS Chem. Biol. 9*, 1490−1498.
(109) Kristensen, V. N., Lingjaerde, O. C., Russnes, H. G., Vollan, H. K., Frigessi, A., and Borresen-Dale, A. L. (2014) Principles and methods of integrative genomic analyses in cancer. *Nat. Rev. Cancer 14*, 299−313.
(110) Alcolea, M. P., Casado, P., Rodriguez-Prados, J. C., Vanhaesebroeck, B., and Cutillas, P. R. (2014) Phosphoproteomic analysis of leukemia cells under basal and drug-treated conditions identifies markers of kinase pathway activation and mechanisms of resistance. *Mol. Cell. Proteomics 11*, 453−466.
(111) Casado, P., Alcolea, M. P., Iorio, F., Rodriguez-Prados, J. C., Vanhaesebroeck, B., Saez-Rodriguez, J., Joel, S., and Cutillas, P. R. (2013) Phosphoproteomics data classify hematological cancer cell lines according to tumor type and sensitivity to kinase inhibitors. *Genome Biol. 14*, R37.
(112) Klammer, M., Kaminski, M., Zedler, A., Oppermann, F., Blencke, S., Marx, S., Muller, S., Tebbe, A., Godl, K., and Schaab, C. (2012) Phosphosignature Predicts Dasatinib Response in Non-small Cell Lung Cancer. *Mol. Cell. Proteomics 11*, 651−668.
(113) Schaab, C., Oppermann, F. S., Klammer, M., Pfeifer, H., Tebbe, A., Oellerich, T., Krauter, J., Levis, M., Perl, A. E., Daub, H., Steffen, B., Godl, K., and Serve, H. (2014) Global phosphoproteome analysis of human bone marrow reveals predictive phosphorylation markers for the treatment of acute myeloid leukemia with quizartinib. *Leukemia 28*, 716−719.
(114) Mertins, P., Yang, F., Liu, T., Mani, D. R., Petyuk, V. A., Gillette, M. A., Clauser, K. R., Qiao, J. W., Gritsenko, M. A., Moore, R. J., Levine, D. A., Townsend, R., Erdmann-Gilmore, P., Snider, J. E., Davies, S. R., Ruggles, K. V., Fenyo, D., Kitchens, R. T., Li, S., Olvera, N., Dao, F., Rodriguez, H., Chan, D. W., Liebler, D., White, F., Rodland, K. D., Mills, G. B., Smith, R. D., Paulovich, A. G., Ellis, M., and Carr, S. A. (2014) Ischemia in tumors induces early and sustained phosphorylation changes in stress kinase pathways but does not affect global protein levels. *Mol. Cell. Proteomics 13*, 1690−1704.
(115) Andersen, J. N., Sathyanarayanan, S., Di Bacco, A., Chi, A., Zhang, T., Chen, A. H., Dolinski, B., Kraus, M., Roberts, B., Arthur, W., Klinghoffer, R. A., Gargano, D., Li, L., Feldman, I., Lynch, B., Rush, J., Hendrickson, R. C., Blume-Jensen, P., and Paweletz, C. P. (201) Pathway-based identification of biomarkers for targeted therapeutics: personalized oncology with PI3K pathway inhibitors. *Sci. Transl. Med. 2*, 43ra55.
(116) Gallien, S., and Domon, B. (2014) Quantitative proteomics using the high resolution accurate mass capabilities of the quadrupole-orbitrap mass spectrometer. *Bioanalysis 6*, 2159−2170.
(117) Merrill, A. E., Hebert, A. S., MacGilvray, M. E., Rose, C. M., Bailey, D. J., Bradley, J. C., Wood, W. W., El Masri, M., Westphall, M. S., Gasch, A. P., and Coon, J. J. (2014) NeuCode labels for relative protein quantification. *Mol. Cell. Proteomics 13*, 2503−2512.
(118) Gridling, M., Ficarro, S. B., Breitwieser, F. P., Song, L., Parapatics, K., Colinge, J., Haura, E. B., Marto, J. A., Superti-Furga, G., Bennett, K. L., and Rix, U. (2014) Identification of kinase inhibitor targets in the lung cancer microenvironment by chemical and phosphoproteomics. *Mol. Cancer. Ther. 13*, 2751−2762.
(119) Demirkan, G., Yu, K., Boylan, J. M., Salomon, A. R., and Gruppuso, P. A. (2011) Phosphoproteomic profiling of in vivo signaling in liver by the mammalian target of rapamycin complex 1 (mTORC1). *PLoS One 6*, e21729.