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Understanding and exploiting substrate recognition by protein kinases

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Summary

Protein kinases play a virtually universal role in cellular regulation and are emerging as an important class of new drug targets, yet the cellular functions of most human kinases largely remain obscure. Aspects of substrate recognition common to all kinases in the ATP nucleotide binding site have been exploited in the generation of analog specific mutants for exploring kinase function and discovering novel protein substrates. Likewise, understanding interactions with the protein substrate, which differ substantially between kinases, can also help to identify substrates and to produce tools for studying kinase pathways, including fluorescent biosensors. Principles of kinase substrate recognition are particularly valuable in guiding bioinformatics and phosphoproteomics approaches that impact our understanding of signaling pathways and networks on a global scale.

Introduction

Eukaryotic organisms dedicate some 2% of their genes to encode protein kinases, underscoring the widespread importance of protein phosphorylation in the regulation of myriad cellular actions [1]. Phosphorylation on serine, threonine and tyrosine can modulate protein function in many ways: by controlling subcellular localization, by acting as a tag for protein degradation or stabilization, by triggering the assembly of multiprotein complexes, or by allosterically regulating biochemical activity (e.g. activation or repression of an enzyme or transcription factor). Through regulated protein phosphorylation, processes as diverse and proliferation, migration, differentiation, and cell death are thereby subject to control by protein kinases. Each of these processes is deregulated in cancer, and protein kinases are frequently implicated as culprits in neoplastic initiation and progression. The clinical success of the BCR-Abl tyrosine kinase inhibitor imatinib in treating chronic myeloid leukemia was hailed as a triumph in “bench to bedside” translational medicine, and has led to a flood of pharmaceutical industry activity aimed at therapeutic targeting of kinases [2]. In addition to ten protein kinase inhibitors that have received regulatory approval in the United States, there are nearly 100 others in clinical trials. Aside from oncology, kinases are also being targeted in other disease areas, including diabetes, inflammatory disease, and neurodegenerative disease.

The choice of a kinase as a drug target requires a thorough understanding of its role in disease and in normal physiology. Among the challenges facing researchers in the field are to better understand where and when a kinase is activated, how such regulation occurs, how the kinase impacts physiology at the cellular and organismal level, and perhaps most critically, which proteins serve as substrates of the kinase to carry out its downstream effects. Elucidating such

mechanistic details for each of the over 500 human kinases is a formidable task. To address these questions, new “kinomic” methods and approaches have emerged that allow global analysis of kinase activities and targets, including focused RNAi screening, kinome-wide inhibitor profiling, mass spectrometry (MS)-based phosphoproteomics, and analysis of proteome microarrays [3–6]. In this review, I will illustrate ways in which a detailed understanding of protein kinase-substrate interactions can help to elucidate cellular signaling pathways, particularly in the identification of new kinase substrates. I will give special attention to chemical and proteomic tools and approaches described within the last two years that have the capacity for large scale analysis of kinase function.

Reengineering the nucleotide binding pocket

All eukaryotic protein kinases share a common overall fold, comprising a β -sheet rich small N-terminal lobe and a mostly helical large C-terminal lobe (Figure 1A). The ATP binding site is at the interface between the two lobes, and most protein kinase inhibitors typically bind at this site as well in a manner competitive with ATP. The adenine moiety of ATP sits within a hydrophobic pocket in the small lobe, with the N⁶ and sometimes N¹ nitrogens making hydrogen bonds to the kinase. In work carried out over the last decade, Shokat and co-workers have elaborated a strategy for exploiting conserved features of the adenine binding site to engineer kinase mutants specific for modified substrates and inhibitors [7,8]. For most kinases, mutation of the so-called gatekeeper residue (equivalent to Thr338 of human c-Src) to glycine or alanine produces an analog specific (AS) allele that can use non-natural ATP analogs bearing bulky residues at the N⁶ position (e.g. N⁶-benzyl-ATP) as a substrate (Figure 1B). Wild-type (WT) kinases, conversely, cannot use the modified nucleotide due to steric clashes with the gatekeeper. Similarly, AS mutants are uniquely inhibited by analogs of kinase inhibitors with appropriately placed bulky groups. These tools have seen increasing utilization as a means to identify protein substrates of kinases and to pharmacologically target a single kinase. Using WT kinases, labeling of substrates *in vitro* within a complex mixture such as a cell or tissue extract is problematic due to background from the endogenous kinases present [9]. However, incubation of an AS kinase mutant with radiolabeled N⁶-benzyl-ATP (or a similar analog) in a complex protein mixture results in exclusive labeling of direct substrates of the kinase, since endogenous WT kinases cannot use the analog as a substrate.

Identifying the protein substrates detected using AS kinases generally requires biochemical purification and can pose a challenge, particularly if the targets are present at low levels (see for example [10]). Some recent innovations that have facilitated identification of substrates tagged using AS kinases include the use of antibodies to phosphotyrosine (pTyr) to affinity purify tyrosine kinase substrates [11] and an *S. cerevisiae* strain collection expressing each protein with an affinity tag from its endogenous locus to pick out substrates of yeast kinases [12]. In addition, the Shokat laboratory has found that most AS kinases tested can use ATP- γ -S analogs as the substrate, which yields thiophosphorylated rather than phosphorylated protein products. Subsequent alkylation of the incorporated thiophosphate group with a thiol-reactive electrophilic tag marks direct substrates of the kinase with a unique epitope (Figure 1C) [13, 14••]. Though the electrophilic tag used also reacts with cysteine residues present in most proteins, by using high affinity antibodies raised against the full epitope (including the thiophosphate moiety), direct substrates can be specifically immunopurified and identified by MS. The group has validated this approach by isolating a known substrate of the Erk2 mitogen-activated protein kinase (MAPK) from permeabilized embryonic fibroblasts expressing an Erk2 AS allele from the endogenous locus. Interestingly, Zhou and co-workers have reported that at low pH (<4.0), electrophiles such as iodoacetamido-biotin react selectively with thiophosphate over cysteine thiols, allowing site-selective tagging of thiophosphorylated kinase substrates with biotin [15], which could allow for highly efficient purification on immobilized streptavidin. Further, Green and Pflum have shown that several kinases can use

ATP biotinylated at the γ position as a substrate, thus transferring phosphobiotin directly to substrates [16]. Though the range of kinases that can accept biotin-ATP as a substrate and precise catalytic parameters for its use have not been determined, this approach holds promise as a generally accessible way to identify new kinase substrates through direct labeling.

Interactions with the protein substrate

The protein substrate binding site has been structurally characterized for a number of kinases through X-ray crystallography in complex with pseudosubstrate inhibitors and short peptide substrates [17–21]. The repertoire of kinase-peptide complex structures is rapidly expanding through the use of high affinity bisubstrate analogs, in which ATP is tethered to the phosphorylation site of a peptide substrate [22–25] (see [26] for a discussion of general features of substrate binding). Interactions with residues surrounding the phosphorylation site vary considerably between kinases, reflecting differences in sequence specificity (Figure 2). This selectivity at the active site of the kinase is reflected in specific sequence motifs found at phosphorylation sites in its protein substrates, and provides one mechanism by which different kinases phosphorylate distinct protein substrates. Protein kinase phosphorylation motifs can be experimentally determined by screening of either immobilized [27–29] or solution phase [30,31] peptide arrays. Peptide libraries immobilized on beads have recently been described for profiling tyrosine kinase specificity [32•]. A pool of beads, each bearing an individual peptide, is treated with the kinase. Phosphorylated beads are then detected using alkaline phosphatase-conjugated anti-pTyr antibodies and then manually segregated from the larger pool of unphosphorylated beads, followed by MS peptide sequencing. The method could theoretically be modified to incorporate fluorescence detection, allowing automated bead selection and higher throughput [33]. Unfortunately, the lack of suitably general antibodies for detecting pSer and pThr [34] restricts the approach to tyrosine kinases.

Peptide-based kinase biosensors

Peptide screening approaches often produce optimized substrates with high catalytic efficiency and selectivity for a particular kinase. One exciting application of such substrates is in the generation of genetically encoded kinase biosensors, which allow kinase activity to be monitored dynamically in living cells with spatiotemporal resolution using fluorescence microscopy [35]. Typically kinase biosensors are fusion proteins incorporating a pair of green fluorescent protein variants (e.g. YFP and CFP) that exhibit fluorescence resonance energy transfer (FRET). A phosphorylation site for a given kinase and a phosphopeptide binding module are installed in between the two fluorescent proteins (Figure 3A). Phosphorylation of the sensor triggers an intramolecular interaction between the phosphopeptide binding domain and the phosphorylated sequence. By altering the interfluorophore distance and geometry, this changes the extent of FRET between the two fluorescent proteins. Biosensors of this type have now been developed for multiple kinases, and have recently helped to elucidate novel regulatory mechanisms for PKA and for PKD [36,37]. Optimization of the PKA biosensor by Zhang and coworkers produced a reagent suitable for small molecule inhibitor screening, thus expanding the repertoire of cell based assays for protein kinase signaling pathways [38•].

Several groups have described synthetic peptides that bind divalent metal ions in a phosphorylation-dependent manner, with phosphate providing an essential ligand to coordinate the metal; incorporation of chelation-sensitive fluorophores into such peptides creates “chemosensors” that have altered fluorescence upon phosphorylation in the presence of the appropriate metal ions [39,40]. An interesting elaboration on this strategy has been the design of a kinase-inducible domain (Figure 3B) [41•]. A minimal EF hand motif peptide was modified to replace an essential metal-coordinating glutamate residue with serine; in this context only the phosphorylated peptide binds metal ions tightly and becomes structured. Incorporating tryptophan residues into the peptide to absorb light produced phosphorylation-dependent

fluorescence when luminescent lanthanides were used as the metal ion. Inherent sequence flexibility within the system allows the generation of such substrates for many kinases with distinct phosphorylation motifs, and the exclusive use of naturally occurring amino acids means that these domains have the capacity to be genetically encoded and used in living cells.

Motif-based approaches to the identification of novel kinase substrates

Armed with a consensus phosphorylation motif, bioinformatics tools can be used to scan proteomes for sequences likely to be phosphorylated by a particular kinase [42,43]. A drawback of this approach is the high rate of false positives: predicted sites may be buried within the protein and thus inaccessible to the kinase, the kinase and substrate may not co-localize, and authentic protein substrates may require docking or scaffolding interactions that occur distal to the site of phosphorylation. Approaches to filter out such false positives include artificial neural networks trained on experimentally verified phosphorylation sites [44] and consideration of intrinsic disorder [45] or evolutionary conservation of the surrounding sequence [46]. Some protein kinase families, such as cyclin dependent kinases (CDKs) and MAPKs, often phosphorylate substrates at multiple sites that are clustered within proteins, which can have predictive value [47–49].

Protein kinases with close homology that belong to a common family tend to have similar if not identical phosphorylation site motifs (see for example [21]), and there can also be considerable overlap in sequence specificity between families (both CDKs and MAPKs share a minimal phosphorylation consensus of Ser/Thr-Pro, for example). Substrate selection by protein kinases *in vivo* can involve interactions distal to the phosphorylation site mediated by scaffolds, docking sites, or separate protein interaction domains [50,51]. Thus, it can be difficult to unambiguously match a specific kinase to a known or predicted phosphorylation site. Indeed, for kinases with relatively simple phosphorylation motifs, computer database searching has failed to identify known substrates [52,53]. Linding *et al.* have taken a computational approach to this problem, termed NetworKIN [54,55••]. The approach integrates an algorithm that matches sites to known phosphorylation motifs (from 20 kinase families, 112 human kinases in all) with a protein interaction network compiled from various literature sources, including curated pathway databases and co-expression data. Using NetworKIN, the authors found that they could not only increase the confidence level of kinase-substrate predictions made based on phosphorylation motifs alone, but could also assign sites to specific members of a kinase family. Among a set of predictions made in a model of the mammalian DNA damage response (DDR), the authors validated several new kinase targets in cultured cells. As our knowledge of kinase phosphorylation motifs becomes more complete, this approach will no doubt become even more powerful as a means to globally predict cellular phosphorylation networks.

Large scale MS-based phosphoproteomics efforts have begun to catalog thousands of *in vivo* phosphorylation sites and to follow quantitatively changes in phosphorylation levels in response to cellular stimuli [4,56]. Consideration of experimentally determined consensus phosphorylation motifs has been used to suggest likely phosphorylating kinases [57–60]. A recent example targeting the eukaryotic DNA damage response (DDR) illustrates the converse: using consensus phosphorylation motifs to direct phosphoproteomics to define protein kinase targets [61••]. The DDR is a conserved pathway that is essential for activating processes that occur in response to damaged DNA, including cell cycle arrest, gene transcription, and DNA repair. Two related serine-threonine kinases, ATM and ATR, are essential upstream components of the DDR that function to relay the signal to a diverse group of downstream effectors. ATM and ATR possess a unique phosphorylation motif, requiring a glutamine residue immediately downstream of the phosphorylated serine or threonine. The authors took advantage of this unique consensus in a motif-directed phosphoproteomics approach. Protein extracts from cells treated with ionizing radiation were digested with trypsin to produce short

peptides, from which a subset of the phosphopeptides was affinity purified using antibodies that recognize proteins and peptides phosphorylated within an S/T-Q sequence. This analysis spectacularly identified some 900 phosphorylation sites on 700 proteins, increasing the number of known ATM/ATR substrates approximately 30-fold. Enriching for peptides phosphorylated within a particular consensus sequence almost certainly facilitated identification of low abundance phosphorylation sites, and this strategy will likely be useful for identifying targets of other protein kinases as well.

Conclusions

Though a great deal of new information regarding protein kinase signaling networks has been uncovered in recent years, clearly the current state of knowledge is only the tip of the iceberg. That the majority of phosphorylation sites uncovered by each successive quantitative phosphoproteomics study are novel suggests that thousands, perhaps tens of thousands more sites exist in humans in excess of our current tally. For most of the ones catalogued thus far, the responsible kinases and the functional significance for cellular regulation remain obscure, and this fraction is only likely to increase as phosphoproteomes become more fully defined. The methods described here will hopefully help narrow this growing gap in our knowledge.

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References

1. Manning G, Plowman GD, Hunter T, Sudarsanam S. Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci* 2002;27:514–520. [PubMed: 12368087]
2. O'Dwyer ME, Mauro MJ, Druker BJ. STI571 as a targeted therapy for CML. *Cancer Invest* 2003;21:429–438. [PubMed: 12901289]
3. Moffat J, Sabatini DM. Building mammalian signalling pathways with RNAi screens. *Nat Rev Mol Cell Biol* 2006;7:177–187. [PubMed: 16496020]
4. Schmelzle K, White FM. Phosphoproteomic approaches to elucidate cellular signaling networks. *Curr Opin Biotechnol* 2006;17:406–414. [PubMed: 16806894]
5. Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol* 2005;23:329–336. [PubMed: 15711537]
6. Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, et al. Global analysis of protein phosphorylation in yeast. *Nature* 2005;438:679–684. [PubMed: 16319894]
7. Elphick LM, Lee SE, Gouverneur V, Mann DJ. Using chemical genetics and ATP analogues to dissect protein kinase function. *ACS Chem Biol* 2007;2:299–314. [PubMed: 17518431]
8. Knight ZA, Shokat KM. Features of selective kinase inhibitors. *Chem Biol* 2005;12:621–637. [PubMed: 15975507]
9. Cohen P, Knebel A. KESTREL: a powerful method for identifying the physiological substrates of protein kinases. *Biochem J* 2006;393:1–6. [PubMed: 16336195]
10. Larochelle S, Batliner J, Gamble MJ, Barboza NM, Kraybill BC, Blethrow JD, Shokat KM, Fisher RP. Dichotomous but stringent substrate selection by the dual-function Cdk7 complex revealed by chemical genetics. *Nat Struct Mol Biol* 2006;13:55–62. [PubMed: 16327805]
11. Boyle SN, Koleske AJ. Use of a chemical genetic technique to identify myosin IIb as a substrate of the Abl-related gene (Arg) tyrosine kinase. *Biochemistry* 2007;46:11614–11620. [PubMed: 17892306]

12. Dephoure N, Howson RW, Blethrow JD, Shokat KM, O'Shea EK. Combining chemical genetics and proteomics to identify protein kinase substrates. *Proc Natl Acad Sci U S A* 2005;102:17940–17945. [PubMed: 16330754]
13. Allen JJ, Lazerwith SE, Shokat KM. Bio-orthogonal affinity purification of direct kinase substrates. *J Am Chem Soc* 2005;127:5288–5289. [PubMed: 15826144]
14. Allen JJ, Li M, Brinkworth CS, Paulson JL, Wang D, Hubner A, Chou WH, Davis RJ, Burlingame AL, Messing RO, et al. A semisynthetic epitope for kinase substrates. *Nat Methods* 2007;4:511–516. [PubMed: 17486086] In an extension of their AS kinase mutant approach, the authors describe what appears to be a general way to tag direct substrates of kinases with an epitope to allow for immunoaffinity purification. This advance could well sidestep the main shortcoming of using AS kinases with ATP analogs: the difficulty in identifying, as opposed to merely detecting, the substrates.
15. Kwon SW, Kim SC, Jaunbergs J, Falck JR, Zhao Y. Selective enrichment of thiophosphorylated polypeptides as a tool for the analysis of protein phosphorylation. *Mol Cell Proteomics* 2003;2:242–247. [PubMed: 12734387]
16. Green KD, Pflum MK. Kinase-catalyzed biotinylation for phosphoprotein detection. *J Am Chem Soc* 2007;129:10–11. [PubMed: 17199263]
17. Knighton DR, Zheng JH, Ten Eyck LF, Xuong NH, Taylor SS, Sowadski JM. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 1991;253:414–420. [PubMed: 1862343]
18. Lowe ED, Noble ME, Skamnaki VT, Oikonomakos NG, Owen DJ, Johnson LN. The crystal structure of a phosphorylase kinase peptide substrate complex: kinase substrate recognition. *Embo J* 1997;16:6646–6658. [PubMed: 9362479]
19. Brown NR, Noble ME, Endicott JA, Johnson LN. The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. *Nat Cell Biol* 1999;1:438–443. [PubMed: 10559988]
20. Yang J, Cron P, Good VM, Thompson V, Hemmings BA, Barford D. Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* 2002;9:940–944. [PubMed: 12434148]
21. Bullock AN, Debreczeni J, Amos AL, Knapp S, Turk BE. Structure and substrate specificity of the Pim-1 kinase. *J Biol Chem* 2005;280:41675–41682. [PubMed: 16227208]
22. Parang K, Till JH, Ablooglu AJ, Kohanski RA, Hubbard SR, Cole PA. Mechanism-based design of a protein kinase inhibitor. *Nat Struct Biol* 2001;8:37–41. [PubMed: 11135668]
23. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 2006;125:1137–1149. [PubMed: 16777603]
24. Levinson NM, Kuchment O, Shen K, Young MA, Koldobskiy M, Karplus M, Cole PA, Kuriyan J. A Src-like inactive conformation in the abl tyrosine kinase domain. *PLoS Biol* 2006;4:e144. [PubMed: 16640460]
25. Cheng KY, Noble ME, Skamnaki V, Brown NR, Lowe ED, Kontogiannis L, Shen K, Cole PA, Siligardi G, Johnson LN. The role of the phospho-CDK2/cyclin A recruitment site in substrate recognition. *J Biol Chem* 2006;281:23167–23179. [PubMed: 16707497]
26. Bose R, Holbert MA, Pickin KA, Cole PA. Protein tyrosine kinase-substrate interactions. *Curr Opin Struct Biol* 2006;16:668–675. [PubMed: 17085043]
27. Schutkowski M, Reimer U, Panse S, Dong L, Lizcano JM, Alessi DR, Schneider-Mergener J. High content peptide microarrays for deciphering kinase specificity and biology. *Angew Chem Int Ed* 2004;43:2761–2674.
28. Rodriguez M, Li SS, Harper JW, Songyang Z. An oriented peptide array library (OPAL) strategy to study protein-protein interactions. *J Biol Chem* 2004;279:8802–8807. [PubMed: 14679191]
29. Shults MD, Kozlov IA, Nelson N, Kermani BG, Melnyk PC, Shevchenko V, Srinivasan A, Musmacker J, Hachmann JP, Barker DL, et al. A multiplexed protein kinase assay. *Chembiochem* 2007;8:933–942. [PubMed: 17471478]
30. Hutti JE, Jarrell ET, Chang JD, Abbott DW, Storz P, Toker A, Cantley LC, Turk BE. A rapid method for determining protein kinase phosphorylation specificity. *Nature Methods* 2004;1:27–29. [PubMed: 15782149]

31. Fujii K, Zhu G, Liu Y, Hallam J, Chen L, Herrero J, Shaw S. Kinase peptide specificity: improved determination and relevance to protein phosphorylation. *Proc Natl Acad Sci U S A* 2004;101:13744–13749. [PubMed: 15356339]
- 32•. Kim YG, Shin DS, Kim EM, Park HY, Lee CS, Kim JH, Lee BS, Lee YS, Kim BG. High-throughput identification of substrate specificity for protein kinase by using an improved one-bead-one-compound library approach. *Angew Chem Int Ed Engl* 2007;46:5408–5411. [PubMed: 17554743] This paper provides the first example of bead-immobilized peptide libraries being used to determining kinase specificity.
33. Garske AL, Denu JM. SIRT1 top 40 hits: use of one-bead, one-compound acetyl-peptide libraries and quantum dots to probe deacetylase specificity. *Biochemistry* 2006;45:94–101. [PubMed: 16388584]
34. Panse S, Dong L, Burian A, Carus R, Schutkowski M, Reimer U, Schneider-Mergener J. Profiling of generic anti-phosphopeptide antibodies and kinases with peptide microarrays using radioactive and fluorescence-based assays. *Mol Divers* 2004;8:291–299. [PubMed: 15384422]
35. Zhang J, Allen MD. FRET-based biosensors for protein kinases: illuminating the kinome. *Mol Biosyst* 2007;3:759–765. [PubMed: 17940658]
36. Zhang J, Hupfeld CJ, Taylor SS, Olefsky JM, Tsien RY. Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. *Nature* 2005;437:569–573. [PubMed: 16177793]
37. Kunkel MT, Toker A, Tsien RY, Newton AC. Calcium-dependent regulation of protein kinase D revealed by a genetically encoded kinase activity reporter. *J Biol Chem* 2007;282:6733–6742. [PubMed: 17189263]
- 38•. Allen MD, DiPilato LM, Rahdar M, Ren YR, Chong C, Liu JO, Zhang J. Reading dynamic kinase activity in living cells for high-throughput screening. *ACS Chem Biol* 2006;1:371–376. [PubMed: 17163774] By reengineering a PKA biosensor, the authors succeed in generating a cell based assay suitable for high-throughput screening.
39. Chen CA, Yeh RH, Lawrence DS. Design and synthesis of a fluorescent reporter of protein kinase activity. *J Am Chem Soc* 2002;124:3840–3841. [PubMed: 11942815]
40. Shults MD, Janes KA, Lauffenburger DA, Imperiali B. A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat Methods* 2005;2:277–283. [PubMed: 15782220]
- 41•. Balakrishnan S, Zondlo NJ. Design of a protein kinase-inducible domain. *J Am Chem Soc* 2006;128:5590–5591. [PubMed: 16637598] The authors describe peptides that adopt a folded structure in response to phosphorylation in the presence of metal ions. Possible applications include new kinase biosensors and as tunable components in engineered cellular systems.
42. Yaffe MB, Leparé GG, Lai J, Obata T, Volinia S, Cantley LC. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat Biotechnol* 2001;19:348–353. [PubMed: 11283593]
43. de Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res* 2006;34:W362–365. [PubMed: 16845026]
44. Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 1999;294:1351–1362. [PubMed: 10600390]
45. Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z, Dunker AK. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* 2004;32:1037–1049. [PubMed: 14960716]
46. Budovskaya YV, Stephan JS, Deminoff SJ, Herman PK. An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* 2005;102:13933–13938. [PubMed: 16172400]
47. Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO. Targets of the cyclin-dependent kinase Cdk1. *Nature* 2003;425:859–864. [PubMed: 14574415]
48. Moses AM, Heriche JK, Durbin R. Clustering of phosphorylation site recognition motifs can be exploited to predict the targets of cyclin-dependent kinase. *Genome Biol* 2007;8:R23. [PubMed: 17316440]

49. Chang EJ, Begum R, Chait BT, Gaasterland T. Prediction of cyclin-dependent kinase phosphorylation substrates. *PLoS ONE* 2007;2:e656. [PubMed: 17668044]
50. Remenyi A, Good MC, Lim WA. Docking interactions in protein kinase and phosphatase networks. *Curr Opin Struct Biol* 2006;16:676–685. [PubMed: 17079133]
51. Ubersax JA, Ferrell JE Jr. Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol* 2007;8:530–541. [PubMed: 17585314]
52. Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AE, Yaffe MB. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol Cell* 2005;17:37–48. [PubMed: 15629715]
53. Rennefahrt UE, Deacon SW, Parker SA, Devarajan K, Beeser A, Chernoff J, Knapp S, Turk BE, Peterson JR. Specificity profiling of Pak kinases allows identification of novel phosphorylation sites. *J Biol Chem* 2007;282:15667–15678. [PubMed: 17392278]
54. Linding R, Juhl Jensen L, Pasulescu A, Olhovsky M, Colwill K, Bork P, Yaffe MB, Pawson T. NetworKIN: a resource for exploring cellular phosphorylation networks. *Nucleic Acids Res.* 2007
- 55••. Linding R, Jensen LJ, Ostheimer GJ, van Vugt MA, Jorgensen C, Miron IM, Diella F, Colwill K, Taylor L, Elder K, et al. Systematic discovery of in vivo phosphorylation networks. *Cell* 2007;129:1415–1426. [PubMed: 17570479] Taking phosphorylation motif searching to the next level by integrating “contextual” information derived from a virtual protein interaction network, NetworKIN predicts kinase-substrate relationships at much higher confidence than by sequence matching alone.
56. Witze ES, Old WM, Resing KA, Ahn NG. Mapping protein post-translational modifications with mass spectrometry. *Nat Methods* 2007;4:798–806. [PubMed: 17901869]
57. Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol Cell Proteomics* 2005;4:310–327. [PubMed: 15665377]
58. Schwartz D, Gygi SP. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nat Biotechnol* 2005;23:1391–1398. [PubMed: 16273072]
59. Brinkworth RI, Munn AL, Kobe B. Protein kinases associated with the yeast phosphoproteome. *BMC Bioinformatics* 2006;7:47. [PubMed: 16445868]
60. Smolka MB, Albuquerque CP, Chen SH, Zhou H. Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. *Proc Natl Acad Sci U S A* 2007;104:10364–10369. [PubMed: 17563356]
- 61••. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lereenthal Y, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 2007;316:1160–1166. [PubMed: 17525332] In a phosphoproteomic tour-de-force, the authors identify nearly 1000 new phosphorylation sites triggered by the DNA damage response, all probably direct substrates of the ATM and ATR.
62. Favelyukis S, Till JH, Hubbard SR, Miller WT. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. *Nat Struct Biol* 2001;8:1058–1063. [PubMed: 11694888]
63. Xu W, Doshi A, Lei M, Eck MJ, Harrison SC. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell* 1999;3:629–638. [PubMed: 10360179]
64. Witucki LA, Huang X, Shah K, Liu Y, Kyin S, Eck MJ, Shokat KM. Mutant tyrosine kinases with unnatural nucleotide specificity retain the structure and phospho-acceptor specificity of the wild-type enzyme. *Chem Biol* 2002;9:25–33. [PubMed: 11841936]

Figure 1.

Protein kinase structure and nucleotide binding pocket. A. Structure of IGF1R tyrosine kinase domain (green) in complex with a nucleotide analog (yellow) and peptide substrate (cyan), PDB code 1K3A [62]. B. The X-ray crystal structures of WT Src bound to AMP-PNP (a non-hydrolyzable ATP analog, top, PDB code 2SRC [63]) and AS mutant Src with in complex with N⁶-benzyl-ADP (bottom, PDB code 1KSW [64]) are shown. The gatekeeper residue (Thr338 in WT Src, mutated to Gly in AS Src) is indicated in magenta. The benzyl group of the bulky ATP analog (green sticks) is accommodated in the mutant but would clash with the gatekeeper in the WT enzyme. C. Epitope tagging of direct kinase substrates in two steps. Antibodies raised against a *p*-nitrobenzylthiophosphate hapten specifically recognize the final adduct.

Figure 2.

The protein substrate binding site. Structures of two protein kinases (green), Pim1 (top, PDB code 2BIL [21]) and Cdk2 (bottom, PDB code 1GY3 [19]), in complex with their respective peptide substrates (cyan). In both frames the phosphoacceptor residue is in magenta. For Pim1, the kinase makes an extensive network of polar contacts to residues upstream of the phosphorylation site, while Cdk2 interacts principally downstream of the phosphoacceptor. Note for example the ion pair between a pThr residue in the kinase activation loop and a lysine residue in the bound peptide. For clarity the cyclin subunit has been removed.

Figure 3. Fluorescent kinase biosensors. A. Multidomain genetically encoded biosensors incorporating fluorescent proteins. B. Kinase inducible domain that folds in response to phosphorylation in the presence of metal ions to produce a fluorescent signal.