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Current Opinion in Pharmacology

The active kinome: The modern view of how active protein kinase networks fit in biological research

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Abstract

Biological regulatory networks are dynamic, intertwined, and complex systems making them challenging to study. While quantitative measurements of transcripts and proteins are key to investigate the state of a biological system, they do not inform the "active" state of regulatory networks. In consideration of that fact, "functional" proteomics assessments are needed to decipher active regulatory processes. Phosphorylation, a key post-translation modification, is a reversible regulatory mechanism that controls the functional state of proteins. Recent advancements of high-throughput protein kinase activity profiling platforms allow for a broad assessment of protein kinase networks in complex biological systems. In conjunction with sophisticated computational modeling techniques, these profiling platforms provide datasets that inform the active state of regulatory systems in disease models and highlight potential drug targets. Taken together, system-wide profiling of protein kinase activity has become a critical component of modern molecular biology research and presents a promising avenue for drug discovery.

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Current Opinion in Pharmacology 2022, **62:**117–129

This review comes from a themed issue on **Neurosciences: Advances in the field**

Edited by **Sinead O'Donovan, Robert Mccullumsmith,** and **Rammohan Shukla**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.coph.2021.11.007

Keywords

Post-translational modification, Bioinformatics, PamChip, PamStation, Protein array.

Introduction

Interconnectivity of biological regulation

Complex biological entities that resulted from millions of years of evolution orchestrate intertwined, dynamic, and elastic biological systems. Regulation of these elaborate systems is even more convoluted and difficult to untangle because it involves many factors operating on different molecular levels. Due to these regulatory factors, the amount of information that can be extrapolated from one molecular domain to another is limited.

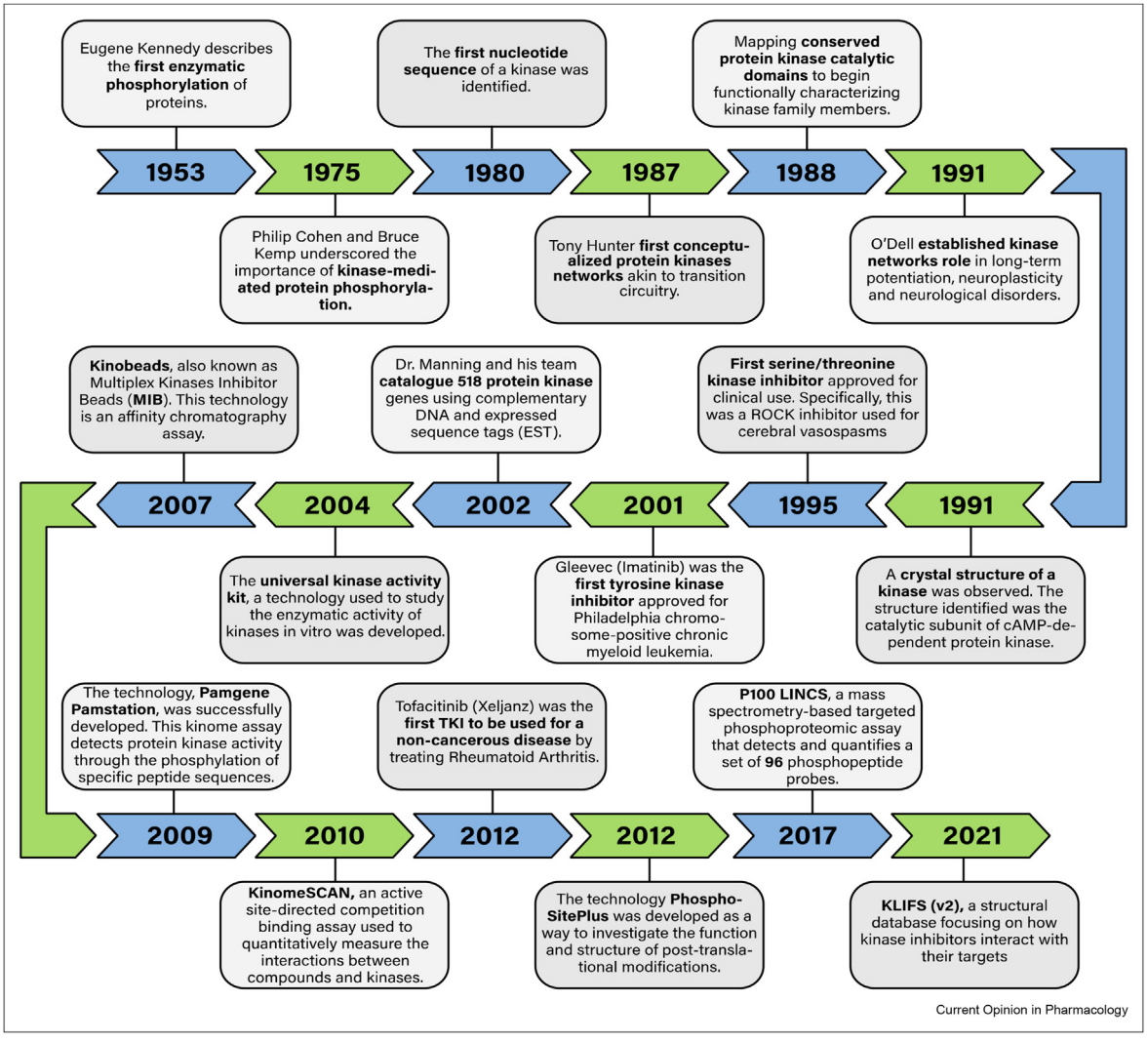
Even though theories of how molecular systems interface are fairly developed, the means of practically measuring these active molecular domains is lagging. Thus, assumptions must be made that may not reflect the true nature of how biological systems are regulated. A useful example relates to studies of mRNA expression. It is a common practice to use mRNA expression as a proxy for protein abundance. However, several studies showed that only ~40% of protein abundance

can be explained by mRNA expression levels, while the rest is explained by other factors including post-translational modifications (PTMs) [1,2]. Extending this example, protein abundance does not necessarily reflect protein activity. A recent study analyzed 150 tumor samples and found that phosphorylation at specific phosphosites and overall kinase abundance are generally uncorrelated [3]. Taken together, these concepts highlight how assumptions about biological regulation may confound biomedical research.

In summary, we posit that simple quantitative measures of gene expression levels, including mRNA and protein, fall well short of what is needed to understand changes in biological regulation. For this reason, acquisition of "functional" data, in conjunction with gene expression levels, is essential to understand the active state of regulation in complex biological systems.

While high-throughput measurements of genomic, transcriptomic, and proteomic substrates are well-established, high-throughput assays of "functional" proteomics are not prevalent. This is due to the paucity of highly adopted technologies tailored to "functional" proteomics, as well as the high cost associated with utilizing existing platforms.

Figure 1



Kinase Research Timeline. A historical timeline of events related to protein kinases. Early discoveries of the structure and mechanisms involving kinases were essential to understand how kinases operate. This timeline also shows the first efforts of cataloguing all protein kinases, mapping their active domains, and first approved kinase inhibitors. The figure also dates technologies and databases that were developed to expand kinase research by making it easier to quantify kinase enzymatic activity and degree of phosphorylation in a high-throughput fashion.

As one examines the suite of functional protein types, protein kinases rank above the others in terms of influence due to the high interactivity, ubiquitous expression, and functional relevance of these enzymes.

Biological context of protein kinases

Biological systems are comprised of complex protein interactions that power the underlying functionalities of all physiologic processes. Thousands of proteins have evolved to work in concert with other proteins to construct localized subnetworks that drive generalized and specialized biological functions. Some protein families are among the most interconnected proteins, including transcription factors and chaperone proteins [4]. However, in several large interactome (protein–protein interactions) studies, protein kinases are consistently found at the top of the most interactive biological entities [4,5]. Supporting this notion, there are over 500 protein kinases in the human genome which phosphorylate ~30% of all cellular proteins [6]. As a result, kinases are a central part of several dense, interconnected, and complex regulatory networks. Protein kinases have been the center of tremendous research efforts that span several decades to investigate their structures and functions (Figure 1) [7–13].

The complexity of the active kinome

The active kinome is the broad-based activity of the complete set of protein kinases. It is a dynamic system that is context dependent, multivariate, and responsive to endogenous and exogenous stressors [14]. The nature of dynamic regulation mechanisms governed by protein kinase interactions is well established for certain protein kinase cascades [15]. However, crosstalk mechanisms between kinase cascades are more challenging to study because these mechanisms deviate from the relatively simplistic linear signal transduction flow. Crosstalk mechanisms connect multiple linear cascades to act as interconnected subnetworks in which modulation in one part of a network can have secondary or compensatory effects in other parts of a network [16,17]. These secondary or compensatory effects present a major challenge for protein kinase drug discovery, as acquired drug resistance is a common response to many protein kinase inhibitors [18]. In order to investigate these crosstalk mechanisms, the active role of protein kinase networks in biological systems must be assessed using holistic measurements to fully map and model protein kinase interactions. Interestingly, the term "kinome" has become more favored compared to "kinase cascades" in recent years (Figure 2).

In this review, we examine the past and current trends of kinase research and highlight the importance of studying protein kinases as networks with the aid of recent advancements of high-throughput active kinome technologies and computational modeling.

The landscape of protein kinase research

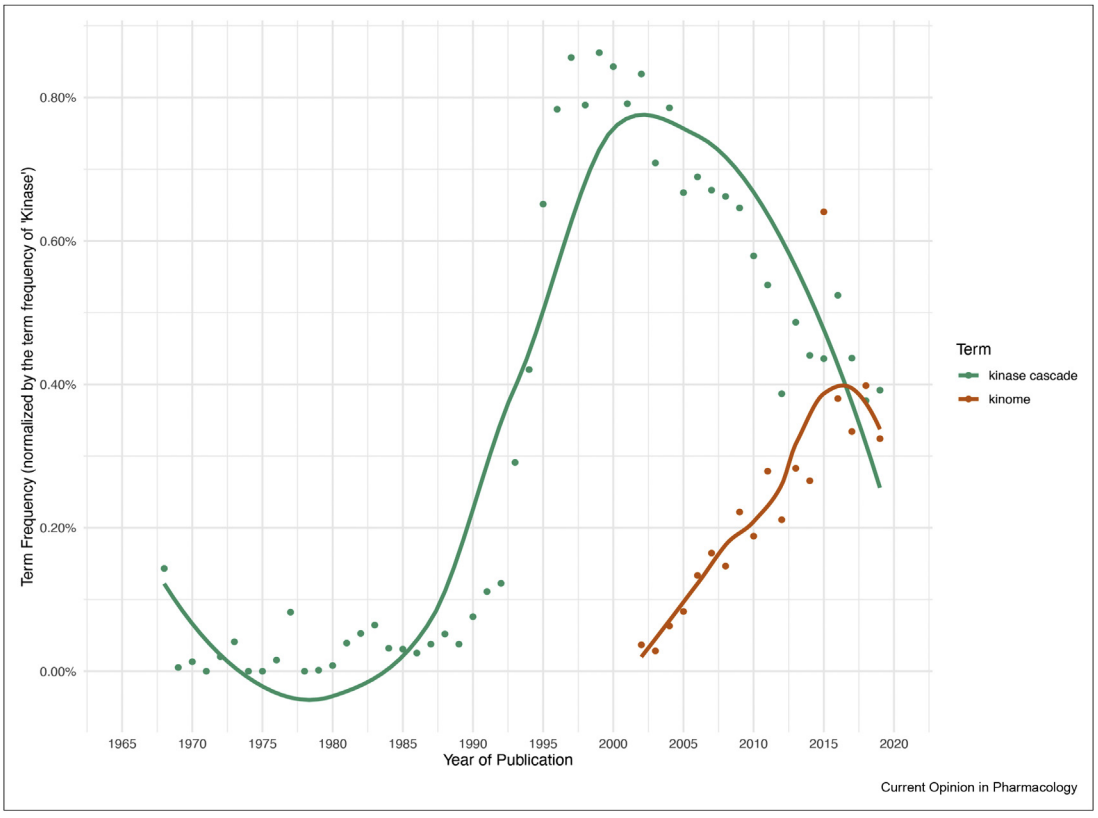
Historically, the study of protein kinases was directed first towards their involvement in protein kinase cascades as the mechanism of transducing and amplifying extracellular stimuli via activation of receptors coupled to protein kinases [19,20]. Protein kinase cascades, intrinsically necessary for signal amplification because of protein kinase bioenergetic constraints, were viewed later as distinct from protein kinase networks, the interactions serving as the primary mechanism by which a cell regulates physiologic processes [20,21]. In this fashion, protein kinase networks were first conceptualized in 1987, in a groundbreaking paper comparing protein kinase networks to transistor circuitry, the "signal" being protein phosphorylation and the "gain" being regulation of protein kinase activity within the network [21]. Indeed, the notion of protein kinase networks as a primary regulatory mechanism in cells prompted landmark discoveries of many complex physiologic processes. For example, the widely cited work published in 1991 by O'Dell & colleagues established kinase networks as essential to long-term potentiation, a process now understood to be a molecular correlate of learning and memory, critical for neuroplasticity, neuronal development, and pathophysiology of neurological disorders [22–26].

As new innovations and discoveries are made in the field of biology, research on kinase networks has also evolved. A study from 2014 used a bioinformatic approach to investigate protein kinase networks, finding that the majority of research on the subject was focused on protein–protein interactions instead of kinase-substrate interactions, which would provide more information regarding kinase function [27]. Following up on this work, we used a text mining approach to identify changes in word usage over time in kinase research. The word 'kinase' was used as the query and four groups of 5000 documents were generated over four different date ranges (2001–2005, 2006–2010, 2011–2015, and 2016–2020). Examining the patterns of word usage, there is a shift from focusing on individual nodes of particular kinases or a cascade towards a focus on the holistic view of kinase networks (Figure 3).

Perturbed protein kinase networks

Perturbations in protein kinase signaling pathways have long been implicated in many diseases, especially cancer [28,29]. While some cancers are caused by specific mutations in a given protein kinase, mutations in genes related to how a protein is phosphorylated occur in approximately 90% of tumors [30]. Mutations in these genes, also known as phosphorylation-related single nucleotide variants (SNVs), map to "network rewiring mutations" that change how protein kinases interact [30,31]. As such, rather than an inherent mutation in one coding region of a particular kinase, variations in

Figure 2



The Frequency patterns of "Kinase Cascade" and "Kinome" in kinase research. Using Google Books Ngram English 2019 corpus, the term frequencies of "kinase cascade" and "kinome" averaged by year and normalized by the frequency of the term "kinase."

phosphorylation regions may rewire the interaction network of a specific protein kinase.

Changes in the interaction network of a protein kinase may explain why inhibition of an individual protein kinase may not be an effective therapeutic strategy [32,33]. Broadly inhibiting protein kinases and phosphorylation targets which are highly perturbed in cancer may correct for aberrations in other cellular signaling networks, reversing pathologic cellular states.

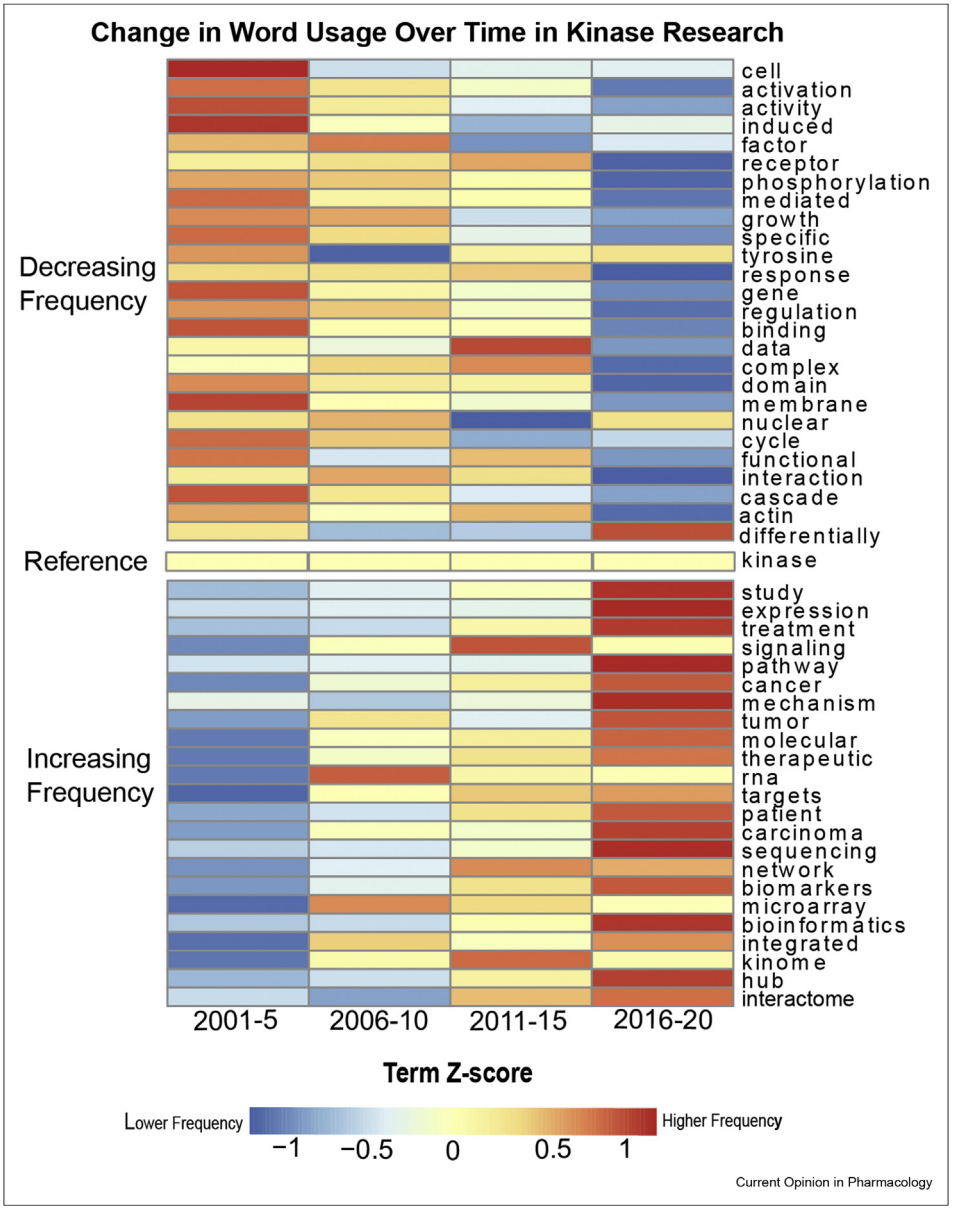
Recent work in hepatocellular carcinoma exemplifies how kinase network studies are more informative than isolated studies focused on a particular kinase. Because most hepatocellular carcinoma drugs target protein kinases, researchers posit kinase-dependent signaling networks as drivers of hepatocellular carcinoma progression [34]. Integrating active kinome data resulting from treatments with approximately 300 kinase inhibitors across 17 hepatocellular carcinoma cell lines, investigators revealed kinase networks that promote drug resistance and epithelial—mesenchymal transition [34]. These data also identified novel anti-cancer pharmacotherapeutic targets that provide urgently needed new possibilities for the development of effective hepatocellular carcinoma drugs [34].

Protein kinases as drug targets

To date, over 73 small molecule protein kinase inhibitors have been approved by the FDA, with many more undergoing clinical trials (Table 1) [35,36]. In the year 2020 alone, eight orally active kinase inhibitors were approved, and 175 other compounds are currently in clinical trials around the world [37]. Protein kinase inhibitors typically reduce the activity of kinases involved in known cancer-causing signaling pathways. Unfortunately, gene and protein expression are not sufficient to fully understand how these disease pathways may be rescued by targeted therapies [38]. Assessments of functional proteomics, kinase enzymatic activity, and post-translational modifications are needed to understand drug response and sensitivity.

Kinase inhibitors developed for a single target may have promiscuous structural moieties that allow for docking at the ATP-binding pocket of many kinases. This is indeed a common finding, with previously assumed single-kinase inhibitors typically exhibiting varying

Figure 3



Semantics shifts in the historical landscape of protein kinase research. A heatmap presenting the trends in kinase research queried from PubMed. Each row represents the number of occurrences of a particular term within each date range and the values are normalized relative to each term. Negative values (blue) indicate that the term occurs less frequently in the group compared to others while positive values (red) indicate that it occurs more frequently. Terms are organized relative to overall trends, with the top group decreasing in usage over time and the bottom group increasing in usage over time. The middle row represents the query term, "kinase", and serves as a reference marker for the other terms. In order to detect a change in word usage, a semi-curated list of fifty terms composed of the most common words and terms deemed relevant to kinases were filtered and presented in this figure. A clear pattern can be seen when examining the change in word usage: terms such as "hub," "network," and "bioinformatics" increase in frequency over time, indicating that bioinformatic and high-throughput data-driven methods are becoming increasingly prevalent. Interestingly, words such as "phosphorylation," "cascades," and "activity" appear to decrease over time, suggesting that there is less of a focus on the individual nodes of a particular kinase or cascade and more of a focus on the holistic view of kinase networks.

Table 1

FDA approved kinase inhibitors by drug name, approval year, and primary target.

Drug (Code) Trade name	Year approved	Primary targets
Fasudil (HA 1077)	1995	ROCK1/2
Sirolimus (AY 22989) Rapamycin	1999	FKBP12/mTOR
Imatinib (STI571) Gleevec	2001	BCR-Abl
Gefitinib (ZD1839) Iressa	2003	EGFR
Erlotinib (OSI-774) Tarceva	2004	EGFR
Sorafenib (BAY 43-9006) Nexavar	2005	VEGFR1/2/3
Dasatinib (BMS-354825) Sprycel	2006	BCR-Abl, SRC
Sunitinib (SU11248) Sutent	2006	VEGFR1/2/3, PDGFR
Lapatinib (GW572016) Tykerb	2007	EGFR, ERBB2
Nilotinib (AMN107) Tasigna	2007	BCR-Abl, KIT, PDGFR
Temsirolimus (CCI-779) Torisel	2007	FKBP12/mTOR
Everolimus (RAD001) Afinitor	2009	FKBP12/mTOR
Pazopanib (GW786034) Votrient	2009	VEGFR1/2/3
Crizotinib (PF 2341066) Xalkori	2011	ALK, ROS1, MET
Ruxolitinib (INCB-018424) Jakafi	2011	JAK1/2/3, TYK
Vandetanib (ZD6474) Zactima	2011	VEGFR2
Vemurafenib (PLX-4032) Zelboraf	2011	B-Raf
Axitinib (AG-013736) Inlyta	2012	VEGFR1/2/3
Bosutinib (SKI-606) Bosulif	2012	BCR-Abl
Cabozantinib (BMS-907351) Cometriq	2012	RET, VEGFR2
Ponatinib (AP 24534) Iclusig	2012	BCR-Abl
Regorafenib (GSK2118436) Tafinlar	2012	VEGFR1/2/3
Tofacitinib (CP-690550) Tasocitinib	2012	JAK3
Afatinib (BIBW 2992) Tovok	2013	ERBB1/2/4
Dabrafenib (GSK2118436) Tafinlar	2013	B-Raf
Ibrutinib (PCI-32765) Imbruvica	2013	BTK
Trametinib (GSK1120212) Mekinist	2013	MEK1/2
Ceritinib (LDK378) Zykadia	2014	ALK
Idelalisib (GS1101) Zydelig	2014	PI3K
Nintedanib (BIBF-1120) Vargatef	2014	FGFR1/2/3
Alectinib (CH5424802) Alecensa	2015	ALK, RET
Cobimetinib (GDC-0973) Cotellic	2015	MEK1/2
Lenvatinib (AK175809) Lenvima	2015	VEGFR, RET
Osimertinib (AZD-9292) Tagrisso	2015	EGFR T970M
Palbociclib (PD-0332991) Ibrance	2015	CDK4/6
Olmutinib (BI1482694) Tagrisso	2016	EGFR
Abemaciclib (LY2835219) Verzenio	2017	CDK4/6
Acalabrutinib (ACP-196) Calquence	2017	BTK
Brigatinib (AP 26113) Alunbrig	2017	ALK
Copanlisib (BAY 806946)	2017	PI3K
Midostaurin (CPG 41251) Rydapt	2017	Flt3
Neratinib (HKI-272) Nerlynx	2017	ERBB2
Ribociclib (LEE011) Kisqali	2017	CDK4/6
Netarsudil (AR11324) Rhopressa	2017	ROCK1/2
Baricitinib (LY 3009104) Olumiant	2018	JAK1/2
Binimetinib (MEK162) Mektovi	2018	MEK1/2
Dacomitinib (PF-00299804) Visimpro	2018	EGFR, ERBB2, ERBB4
Duvelisib (IPI 145) Copiktra	2018	PI3K
Encorafenib (LGX818) Braftovi	2018	B-Raf
Fostamatinib (R788) Tavalisse	2018	Syk
Gilteritinib (ASP2215) Xospata	2018	Flt3
Larotrectinib (LOXO-101) Vitrakvi	2018	TRKA/B/C
Lorlatinib (PF-06463922) Lorbrena	2018	ALK
Alpelisib (BYL719) Piqray	2019	PI3K
Entrectinib (RXDX-101) Ignyta	2019	TRKA/B/C, ROS1
Erdafitinib (JNJ-42756493) Balversa	2019	FGFR1/2/3/4
Fedratinib (TG101348) Inrebic	2019	JAK2
Pexidartinib (PLX3397) Turalio	2019	CSF1R
Upadacitinib (ABT-494) Rinvoq	2019	JAK1
Zanubrutinib (BGB3111) Brukinsa	2019	BTK
Avapritinib (BLU285) Ayvakit	2020	PDGFRα
Capmatinib (INC-280) Tabrecta	2020	c-MET
Pemigatinib (INCB054828) Pemazyre	2020	FGFR2
Pralsetinib (Blu-667) Gavreto	2020	RET
Ripretinib (DCC-2618) Qinlock	2020	Kit, PDGFRα
Selpercatinib (CEGM9YBNG) Retevmo	2020	RET
Selumetinib (AZD6224) Koselugo	2020	MEK1/2
Tucatinib (ONT-380) Tukysa	2020	ERBB2
Infigratinib (BGJ 398) Truseltiq	2021	FGFR
Tepotinib (EMD 1214063) Tepmetko	2021	MET
Tivozanib (AV951) Fotivda	2021	VEGFR
Trilaciclib (G1T28) Cosela	2021	CDK4/6
Umbralisib (TGR 1202)	2021	PI3K, CK1

affinity towards multiple kinases [39]. This variability in docking affinity for several kinases may provide advantageous effects on signaling networks if developed with toxicity and kinome signaling in mind. Many kinase inhibitors fail early stage clinical trials due to the side-effects many of these drugs pose to patients. High-throughput active kinome screening methods are needed to determine the effects kinase inhibitors confer to signaling networks in tandem with toxicity determination in-vivo.

Current high-throughput active kinome profiling technologies

Most of the publicly available datasets that inform kinome signatures are mass spectrometry (MS) derived

signatures of phosphopeptides. However, new high-throughput kinome technologies are being developed and adopted by researchers seeking to profile "functional" kinome activity signatures (Table 2). One of these technologies is the Kinobead platform, also called multiplex kinase inhibitor beads (MIBs). MIBs are an enrichment tool that is utilized to determine the degree of protein expression and phosphorylation of kinases. MIBs are mostly used to determine the selectivity of kinase inhibitors against a large set of kinases within biological samples. This platform takes advantage of the conserved ATP binding domains in kinases for broad kinome enrichment. It works by coating a bead surface with broad-spectrum of promiscuous kinase inhibitors that target the ATP-binding sites of protein kinases, capturing hundreds of kinases. This process results in strong kinome enrichment from complex biological samples such as cell or tissue lysates. MIBs are often coupled with liquid chromatography—mass spectrometry (LC-MS) in order to quantify protein kinase expression levels [40,41]. The combination of MIBs and LC-MS allows for abundance measurement of protein kinase activity following different biochemical manipulations. These manipulations are often set up with the purpose of deconvoluting kinase inhibitor targets [42]. For example, a sample is treated with either a vehicle control or a kinase inhibitor at different concentrations. The inhibitor will compete with MIBs on kinase binding sites resulting in lower enrichment of the compound's targets compared to the control samples. As a result, kinases with lower abundance are mapped as targets for the tested compound. While this platform offers a powerful technique to deconvolute a compound's targets within complex biological samples, it has some limitations. These limitations include 1) it does not directly assess protein kinase activity, 2) only kinases that exhibit a conformation change (typically phosphorylation) are bound by these beads, and 3) the platform relies on well characterized protein kinase inhibitors, requiring some a priori knowledge of targets. A priori knowledge of the kinase inhibitors used with MIBs is needed to assess which kinases these inhibitors fail to capture.

A similar assay is KiNativ. Instead of using a coated bead to capture kinases, it uses biotinylated acyl-phosphates of ATP and ADP soluble probes. These probes modify the conserved lysine residues in the ATP binding site found in most kinases [43]. Similar to MIBs, when coupled with mass spectrometry (MS), these tagging probes allow for identification and abundance measurement of hundreds of kinases. While also a powerful technique, it has the same limitations of MIBs. Neither of these platforms directly assess protein kinase activity, and they must be coupled with MS for quantification of protein levels.

Another active kinome assay is the KINOMEscan platform. KINOMEscan is a competitive binding assay, designed to test and quantify the binding characteristics of test compounds with a large panel of purified kinases. This platform can test binding characteristics of 489 kinases with multiple panels available of different families, groups, and combinations of kinases, designed for specific and broad studies. In this assay, DNA-tagged purified kinases bind to immobilized ligands which are positioned on a solid support (beads). In the presence and absence of a test compound, the degree of binding of kinases to ligands is compared to assess the selectivity of the compound. Screening is performed by measuring the amount of captured kinase using qPCR that detects a tagged DNA in each kinase. The results of the assay can be used to determine the degree of selectivity of the tested inhibitors, as well as the dissociation constant (Kd) which is calculated by measuring the amount of kinase captured as a function of the concentrations of the inhibitor [44–46]. While a valuable kinase profiling technology, KINOMEscan is a biochemical assay of isolated protein kinases outside cellular context; it thus does not account for cellular localization, kinase activity, or regulation via other domains or protein—protein interactions present in complex biological systems. This limitation is what constitutes the main difference between KINOMEscan and assays like Kinobeads and KiNativ. Despite these limitations, KINOMEscan is a promising platform that may be used for an initial broad assessment of kinase inhibitor selectivity.

The reverse phase protein array (RPPA) is another high-throughput technology that can be utilized for profiling kinome signatures. RPPA is a protein array composed of immobilized cell or tissue lysate printed on nitrocellulose-coated slides. Utilizing automated staining systems, these slides are probed with approximately 500 validated primary antibodies followed by biotinylated secondary antibodies [47]. The slides are scanned and analyzed using different software tools for high-throughput assessment of protein expression and post-translational modification. RPPA is a highly sensitive functional proteomics platform that provides derived data on post-translational modifications beyond phosphorylation, such as acetylation and glycosylation. The major limitations of this platform include the need for highly specific primary antibodies and the labor-intensive process of sample array printing. However, various improvement and automation features for this platform are under development to streamline the RPPA pipeline and workflow [47].

The Library of Integrated Network-based Cellular Signatures (LINCS) program developed an assay, called the P100, to measure a set of 96 phosphopeptide probes [48]. It was designed to overcome the challenges of the

Table 2

Summary table of high-throughput kinome profiling techniques.

Active Kinome Profiling Technologies

Technique	Description	Advantages	Limitations	Barriers to Entry
Mass Spectrometry (MS)	• Measures mass spectra of phosphopeptides • Involves phosphopeptide fragmentation • Often coupled with chromatography to determine expression	• Widespread data and tools availability • Ease of integration with other techniques	• Does not directly measure kinase activity. • Reproducibility across samples.	• Core based (local core at University, requires buying a Mass Spectrometer)
Multiplex Kinase Inhibitor Beads (MIBS) or Kinobeads	• Coupled with MS to determine the degree of protein expression and phosphorylation levels. • Often used to determine the selectivity of kinase inhibitors in a biological context. • Uses a set of beads coated with kinase inhibitors that target the ATP-binding domains of kinases passed over it.	• Results in strong kinome enrichment. • Allows for deconvolution of kinase inhibitor targets with biochemical manipulations.	• Relies on a priori knowledge of well characterized kinase inhibitors. • Biased toward kinases that exhibit conformational changes. • Does not directly measure kinase activity.	• The enrichment process is followed by MS • Core based (local core at University, requires buying a Mass Spectrometer)
KiNativ	• Similar to Kinobead/MIBS, except it uses biotinylated acyl-phosphate probes. • Probes modify conserved lysine residues in the kinase ATP binding site. • Measures the identification and abundance of kinases that are being assayed.	• Generates data with high fidelity. • Can identify and measure abundance for most of the kinome.	• Does not directly measure kinase activity. • Must be coupled with LC-MS for quantification of protein levels.	• Fee for service (i.e., samples sent away to the company)
KINOMEscan	• Competitive binding assay that uses test compounds to analyze target kinases. • Determines the status of ATP-competitive inhibitors. • Calculates the dissociation constant of a particular compound.	• Broad applications as well as specific applications due to its diverse array of settings. • Covers 489 kinases, which is a substantial amount of the human kinome.	• Independent of a cellular context, meaning it does not account for localization, regulation, or activity. • Compounds being tested must be known. • Does not directly measure kinase activity.	• Fee for service (i.e., samples sent away to the company)
Reverse Phase Protein Array (RPPA)	• Profiles kinome signatures using immobilized cell lysate on slides. • Slides are probed with primary antibodies followed by biotinylated secondary antibodies. • Computer scans each slide to detect protein expression and post-translational modification patterns.	• Highly sensitive, provides information on other post-translational modifications besides phosphorylation. • Platform is high-throughput with improvements under active development to streamline the analysis	• Highly specific primary antibodies are required. • Workflow includes a laborious sample-array printing process. • Does not directly measure kinase activity.	• Core based (local core at University, requires buying a pin-based contact printer and signal scanner)
P100	• Measures a set of 96 phosphopeptide probes using targeted MS. • Measurements are stored as standardized phosphoproteomic signatures and stored in a database.	• Same set of peptides can be measured across multiple different samples for reproducibility.	• Does not directly measure kinase activity. • Biased towards STKs with a limited amount of PTKs.	• Large publicly available database • Requires expertise for targeted enrichment of peptides followed by MS

PamChip	• Signature library is stored in the LINCS database. • Composed of a chip containing ~13 residue long reporter peptides. • A sample (recombinant kinase, cell culture, tissue lysate, etc.) is placed on the chip, where kinases present in the sample phosphorylate the reporter peptides. • Degree of phosphorylation is measured by fluorescent antibodies.	• Hundreds of signatures publicly available for different chemical perturbagens. • Assay can be completed in a relatively short amount of time. • Images can be taken at multiple different time points, allowing for real-time analysis of kinase activity.	• Reporter peptides can be phosphorylated by several different kinases, requiring sophisticated deconvolution methods to process the data. • Peptides on the chips are pre-selected, meaning that not all possible targets for a particular kinase may be accounted for.	• Two options: 1) fee for service (i.e., samples sent away to the company), 2) core based (local core at University, requires buying the PamStation12)	

MS technologies, most notably the inability to reproducibly measure the same phosphopeptides across multiple samples. This targeted MS approach utilizes standardized phosphoproteomic signatures that can be easily compared against each other. The LINCS project used this assay to generate hundreds of cell-culture derived phosphoproteomic signatures following exposure to different chemical perturbagens. All of these signatures are publicly available under the iLINCS portal (http://www.ilincs.org/). There are some limitations with the P100 assay, including that it does not directly measure kinase activity and is primarily comprised of serine/threonine peptides, leaving the tyrosine subkinome unexplored. While these are important limitations, the P100 approach has a high potential to accelerate drug discovery based on "functional" profiling.

The development of peptide array platforms such as the PamGene Ser/Thr (STK) and phospho-tyrosine (PTK) chips permits simultaneous profiling of kinase activity for hundreds of protein kinases in complex biological samples. The PamGene platform is a well-established, highly cited, microarray technology for multiplex kinase activity profiling [49–54]. The PamChip is composed of a grid of reporter peptides that can be phosphorylated by kinases present in the sample. These reporter peptides are ~13aa long and immobilized on a three dimensional (3D) structure. This design allows for higher concentration of peptides which will increase the sensitivity of the signal and reduce noise. Each reporter peptide contains a single or multiple phosphorylation sites that can be phosphorylated by multiple upstream kinases. Once these sites are phosphorylated, they are detected by fluorescently labelled anti-phospho antibodies. To maximize binding kinetics and reduce reaction time, the samples are pumped back and forth through the array throughout the duration of the assay. As a result, the assay profiling can be complete in under two hours. During that time, the LED imaging system captures continuous images of the fluorescent antibodies allowing real time measurement of relative phosphorylation intensities at each reporter peptide. The PamGene platform has been successfully deployed across multiple research domains and generated actionable scientific insight in neuroscience, cancer biology, and anti-cancer drug development. The PamGene system does not require isolation or immobilization of the protein kinase for assay specificity. This permits assessment of almost any biological sample, where the net effects of all the interactions between kinases and kinase regulating molecules are reflected in the results. One limitation of the PamGene platfrom is that reporter peptides on the STK and PTK chips may be phosphorylated by multiple protein kinases, requiring sophisticated bioinformatic software packages to process kinase

substrate associations for accurate upstream kinases analyses. This platform has other limitations, including the pre-selection of printed peptides on the chips and the split of Ser/Thr and Tyr substrates into two different chips. Other limitations include non-specific binding of antibodies and the required deconvolution methods to map upstream kinases to explain the variation of the phosphorylation signals. Even though these are notable limitations, the PamGene peptide array provides a powerful high-throughput profiling technique to measure kinome activity in complex biological samples.

All of these platforms generate rich and high-dimensional datasets that need to be expertly analyzed to model complex kinase networks. Unfortunately, due to the novelty and low adoption of kinome-based technologies, widespread kinome analytic efforts lag behind the well-established genomic, transcriptomic and proteomic based analyses. However, due to the nature of kinases interactivity, high-throughput kinome signatures provide ideal datasets for computational network modeling.

Active kinome computational modeling

Kinase networks and their complex nature make them an attractive target for computational modeling [55]. The availability of the mathematical and computational tools allows researchers to develop kinase network models that are more complex and able to provide a wider array of testable hypotheses.

For computational modeling to generate accurate and usable models, it is necessary for a database of kinase knowledge to exist. Savage and Zhang have recently published a comprehensive list of these resources [55]. They categorize these data sources to include databases that have information about Kinases (e.g., KinWeb [56], KinBase [57], KinaseNET), databases on phosphorylation sites (e.g., PhosphoPep [58], Phospho.ELM [59], PHOSIDA [60]) and resources for kinase substrate and site prediction (e.g., PHOSIDA predictor [60], GPS [61], DeepPhos [62]). These databases have become essential to the modeling of kinase networks.

The attempts to model kinase networks can be broadly categorized, based on the focus of the approach, into two categories: targeted, and general. The targeted approaches tend to focus on specific pathways and networks and aim to study how such networks and pathways are affected in different states of perturbation. For example, this modeling approach was used to identify the role of JAK/STAT pathway in inflammatory cascades that lead to oncogenesis [63]. The general approaches, on the other hand, expand the focus to include all kinases that are part of the biological sample and aims to identify both known and novel kinase networks.

The more recent but welcome trend has been aimed towards global profiling of the active kinome. These global or general approaches look at the total complement of protein kinase activity in a given biological sample. These approaches, alongside traditional biochemical assays, can be used both for hypothesis generation and confirmation. These approaches usually rely on mass spectrometry data. Three approaches that should be mentioned here are Phosphomatics [64], PhosphoPICK [65] and PHONEMeS [66]. These approaches all use the data obtained from mass spectrometry to identify kinase-substrate phosphorylation events. These events are then put in a putative order by taking into account other data like known pathways, protein abundance, transcriptomic data and existing expert knowledge. There are also tools like CARNIVAL, which rely on multiple data sources, including the differentially expressed genes and transcription factor binding sites as input to generate networks [67,68].

There are other promising tools that do not necessarily rely on mass spectrometry data for causal modeling, such as Bayesian networks. Bayesian networks are graphical models that allow for learning of the dependence structure between variables while keeping the network as simple as possible [69,70]. There are multiple techniques to infer networks from data, including score-based methods that allow for assigning a value to the network and then noting the effect of changing a link on the network score and constraint-based methods that use statistical tests to identify conditionally independent nodes and identify linkages.

Score-based Bayesian network modeling was the approach used by Sachs et al. [71,72], as it was particularly suited to the problem of inferring kinase networks given the constraints of Bayesian networks and the availability of multi-parameter single cell flow cytometry data. The strength of this approach was the insight that each cell could be considered as a separate sample. This allowed Sachs et al. to overcome the limitation of sample size. This approach, along with perturbation-based modeling, has enabled scientists to identify key connections in the active kinome and their functional implication [73]. This has also allowed for further innovation, utilizing more advanced techniques, such as piece-wise linear regression [74].

However, Bayesian networks are not the only way to identify kinase interaction networks. Other approaches can include applying novel techniques and algorithms or devising novel ways to deploy already established techniques. Hijazi et al. have utilized bespoke algorithms, using existing phosphoproteomic databases to predict novel interactions and validate them in cancer cell lines [75]. Their approach relies on generating kinase-substrate signatures using kinase inhibitors as modulators. Their algorithm, called Expectancy of Being

Downstream Target (EBDT), uses existing knowledge to overcome the problem of kinase and kinase inhibitor promiscuity. The EBDT Algorithm is a multistage process that is seeded by two kinds of data: The fold-change value of each phosphosite and the compound inhibitor specificities. In essence, EBDT first identifies the inhibitors for a specific kinase, and then compares that to the activity in the form of log fold change for each phosphosite. Intuitively, if inhibition of a kinase causes a corresponding decrease in the phosphorylation of a given phosphosite, it is likely to be a downstream target of that kinase. This algorithm iterates over the input data of phosphosite changes and kinase inhibitor profiles to generate the network.

Astl and Verkhivker used molecular dynamic simulation and selective allosteric modulation to infer the kinase network implicated in the modulation of ABL kinase [76]. They utilized molecular dynamic simulations of ABL kinase in complex with both activators and inhibitors. This approach was augmented by using selective in silico perturbation of individual residues to identify regulatory events. Network analysis was done both at the individual protein level by representing the protein structure as a network with residues as nodes, and at the community level by including related proteins. Other approaches use similar methods of molecular dynamics and ordinary differential equations to identify links between kinases [77,78].

Computational modeling provides a convenient way to visualize networks of kinases and their interactions. Despite their shortcomings, computational modeling of kinase networks is a promising new direction for kinome research.

Conclusions

Given the complexity of biological systems, deciphering regulatory biological networks of complex diseases is needed to understand disease pathogenesis and predict potential drug targets. The limited success of untangling these regulatory networks solely on gene expression measurements highlights a need for holistic "functional" molecular assessments to unravel the active components within these intricate networks. Since phosphorylation is a key post-translation modification mechanism that plays a critical role in many cellular regulatory processes, studying the active kinome is essential to provide a more sophisticated representation of regulatory processes. As the complexity of the active kinome has become more apparent, profiling technologies that assess hundreds of protein kinases simultaneously have been developed and adopted to extend our knowledge of the active kinome role in complex disease models and drug responses. While studying single protein kinases will remain essential in biochemical confirmation/validation studies, omics techniques that assess the active kinome represent the future for this growing field. Combined with computational modeling, as well as bioinformatic drug discovery tools, the active kinome provides a novel vehicle for a deeper understanding of biological systems and a promising new avenue for drug target discovery.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by The National Institute of Mental Health [grant numbers MH107487, MH121102]; National Institute of Health [grant number AG057598].

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