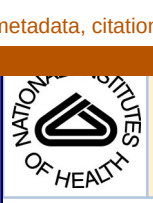

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Assessing adaptation of the cancer kinome in response to targeted therapies

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Abstract

Cancer cells are dependent on protein kinase signaling networks to drive proliferation and to promote survival, and accordingly kinases continue to represent a major target class for development of anti-cancer therapeutics. Kinase inhibitors nevertheless have yielded only limited success with many different malignancies due to the inability of single agents to sustain a durable clinical response. Cancer cell kinomes are highly resilient and able to bypass targeted kinase inhibition leading to tumor resistance. A novel platform has been developed to analyze the activity of the expressed kinome using Multiplexed Inhibitor Beads (MIBs), which consist of Sepharose beads with covalently immobilized inhibitors that preferentially bind activated kinases. Coupling MIB capture with mass spectrometry (MIB/MS) allows simultaneous determination of the activity of over 75% of the expressed kinome facilitating high throughput assessment of adaptive kinase responses resulting from deregulated feedback and feed-forward regulatory mechanisms. The adaptive response frequently involves transcriptional upregulation of specific kinases that allow bypass of the targeted kinase. Understanding how the kinome reprograms to targeted kinase inhibition will allow novel therapeutic strategies to be developed for durable clinical responses.

Studies both in cell culture and in patients have identified predominant modes of adaptive resistance to targeted kinase inhibition. Mutation of the targeted kinase itself is one such mechanism and is classically exemplified by imatinib resistance stemming from kinase domain mutation of BCR-ABL in leukemia[1]. Resistance to gefitinib and erlotinib, ATP-competitive inhibitors of EGFR, commonly occurs by T790M mutation of EGFR in non small-cell lung cancer (NSCLC)[2–4], whereby the mutation increases the ATP affinity of EGFR, effectively competing with the inhibitors. In addition to substitution mutations, genomic amplification of the targeted kinase or pathway members of the targeted kinase leading to increased expression is a prototypical mode of acquired resistance to targeted kinase inhibition. This has been observed in gastric cancer cell lines and tumor tissue as well as in lung cancer[5], where resistance to MET inhibitors was accompanied by MET amplification and subsequent MET expression and phosphorylation[6,7]. In melanoma cells harboring activating V600E BRAF mutations, acquired resistance to BRAF inhibitor can be mediated by amplification of BRAF[8]. A recent report describes the combination of aforementioned modes of resistance to kinase inhibition in a melanoma patient treated with both the MEK inhibitor trametinib and the BRAF inhibitor dabrafenib[9]. This patient's

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melanoma progressed, despite the combination kinase inhibitor therapy due to the acquisition of both a MEK2 Q60P mutation and concurrent BRAF genomic amplification.

In contrast to resistance mechanisms that occur as a result of direct genetic modification of the targeted kinase or targeted kinase pathway, this review will focus on the utilization of alternative kinase networks that circumvent the action of the initial kinase inhibition, in a process that we refer to as "kinome reprogramming."[10]. In BRAF V600E melanoma cells resistant to BRAF inhibitor, a receptor tyrosine kinase antibody array revealed upregulation of IGF1R which drove downstream PI3K kinase signaling[11]. Targeting the IGF1R/PI3K pathway concurrently with MEK inhibition drove apoptosis in the BRAF resistant line, illustrating the shift to dependence on AKT signaling during the course of acquired resistance. Also invoking receptor tyrosine kinase activation as a mechanism of adaptive response, AKT inhibition was shown to perturb feedback regulation and increase HER3, IGF1R and insulin receptor transcription[12]. Concomitant HER kinase inhibition and AKT inhibition in xenograft models synergized to reduce tumor volume. Similarly, activation of Src family kinases (Lyn, Hck) have been shown to facilitate resistance to imatinib in both cell models and patients with chronic myelogenous leukemia (CML)[13,14]. Hence kinase inhibitors that effectively target both BCR-Abl and Src family kinases (dasatinib) are being used as first line treatments for CML.

These examples illustrate the remarkable resiliency of the cancer kinome in averting the growth suppressive effects of a single kinase inhibitor, and even upon dual kinase inhibition[9]. There would be thus great power in defining the response of the expressed kinome for each tumor type/kinase inhibitor pair, to maximize the potential for the rational design of drug combinations as well as to define subnetworks of kinases involved in the adaptive response.

We have developed a proteomic approach to assess the behavior of a large fraction of the kinome in one assay. Our strategy, multiplexed inhibitor beads coupled to quantitative mass spectrometry (MIB/MS), is comprised of layered Sepharose-immobilized kinase inhibitors[10] (Figure 1). Layering the column with beads conjugated to kinase inhibitors capable of differentially binding kinases in the chromatography column, rather than simply mixing the different beads, maximizes the total number of kinases detected by quantitative mass spectrometry. Having very broad pan kinase inhibitors at the bottom of the column and more specific inhibitors layered near the top of the column is designed to capture many metabolic and highly abundant kinases at the top of the column. This further acts to prevent saturation and loss of binding of less abundant signaling kinases—allowing broad-acting inhibitor-bead conjugates at the bottom of the column to capture a larger spectrum of kinases.

Type I kinase inhibitors, with affinity for the ATP binding pocket of the active conformation of kinases, are employed for MIB/MS. This is an important distinction, as it allows the assessment of the activation state of the kinome as opposed to solely determining protein expression levels. We have extensively validated the preferential MIB binding of the activated state of kinases[10]. There is currently no other methodology to probe the active state of kinases at a kinome-wide scale with the ability to measure activation dynamics

including both inhibition and activation of specific kinases. MIB/MS thus provides an unmet level of analysis that is not achieved with mRNA measurements or kinase antibody arrays. A powerful approach is to use RNA deep sequencing (RNA-seq) in conjunction with MIB/MS (Figure 1) for the determination of baseline kinome expression levels and to assess whether there are accompanying transcript changes in response to drug treatment for kinase networks of interest.

In our initial studies we demonstrated the MIB capture of ~50–60% of the kinome in triple-negative breast cancer (TNBC) cell lines as defined by deep sequencing of transcript levels. The eluted kinases represented all phylogenetic kinase families, and many could be classified as understudied and part of the “untargeted” kinome[15,16]. Capturing these understudied kinases by MIB/MS is a key advantage of the methodology as there continues to be a thrust in industry and academics for the development of novel kinase inhibitors, yet the majority of resources have been hitherto aimed at a small fraction of the kinome. We have since refined our MIB/MS protocol by optimizing the kinase inhibitor composition with advances in mass spectrometry. These refinements have resulted in our ability to capture what we estimate represents ~75–80% of the expressed kinome in cell lines and tumors.

In the context of TNBC we were able to characterize the activation state of the kinome following 4 or 24 hr MEK inhibition in cell lines of the mesenchymal claudin-low subtype[17]. ERK1/2 remained inhibited during the initial 4 hours of MEK inhibition but reactivation was observed at 24 hours. MIB/MS defined a signature of MEK inhibition in SUM159PT claudin-low TNBC cells. In addition to increased ERK binding resulting from escaped MEK inhibition and consequential loss of cyclin-dependent kinase (CDK) binding, we observed increased MIB binding of the receptor tyrosine kinases (RTKs) AXL, DDR1, PDGRFB and the non-receptor kinases FAK2, JAK1. The upregulated RTKs were confirmed as active by tyrosine phosphorylation analysis using RTK arrays and western blotting. Critically, we were able to show that the adaptive response of MEK inhibitor and induced RTK upregulation was not limited to cell lines. Tumors from the genetically engineered mouse model (GEMM) C3 TAg[18] profile with similar gene expression as seen in human TNBC tumors. Upregulation of PDGFRB was seen by MIB/MS in C3 TAg tumors treated with MEK inhibitor, and a cell line (T2) derived from a C3 TAg tumor correspondingly displayed increased MIBs binding of PDGFRB and DDR1. We confirmed the physiological relevance of RTK upregulation in the adaptive response to MEK inhibition by showing that individual siRNA knockdown of each of the RTKs synthetically enhanced the growth suppression caused by MEK inhibition.

The characterization of the adaptive response by MIB/MS culminated in elucidation of a logical rationale for a second pharmacological agent for co-targeting with MEK inhibitor. Sorafenib, a kinase inhibitor with activity toward multiple RTKs implicated in the reprogramming response, PDGFRB, DDR2 and CSF1R (reviewed in[19]), was chosen for co-treatment with MEK inhibitor. The AZD6244/sorafenib combination blocked ERK activation and PDGFRB induction in the T2 C3 TAg cell line with a concomitant growth arrest. C3 TAg tumors dually treated with AZD6244 and sorafenib showed greater

regression and apoptosis than tumors from mice subjected to MEK inhibitor treatment alone (77% vs. 30% of mice).

As our MIB/MS approach revealed a role for RTK activation in the adaptive response, we pursued further mechanistic insights to better define the nature of this response. In addition to increased RTK activation demonstrated by increased MIB binding, there was a corresponding transcriptional upregulation of the same RTK cohort and their ligands. ERK stabilizes c-MYC from proteosomal degradation by direct phosphorylation of Serine 62[20] and c-MYC has been shown to associate with the PDGFRB promoter to negatively regulate transcription[21]. MEK inhibition caused both c-MYC protein and mRNA loss, suggesting that the RTK response was due to loss of c-MYC serving as a suppressor of transcription. Accordingly, c-MYC or ERK1/2 siRNA knockdown phenocopied the MEK inhibitor induced adaptive response of RTK upregulation. Expression of mutant (T58A) c-MYC resistant to proteosomal degradation[22] attenuated the RTK upregulation. Collectively the data suggested a model whereby acute MEK inhibition results in loss of ERK-mediated protein stabilization of c-MYC and consequential loss of repressive transcriptional regulation of RTKs. Transcriptional upregulation of cytokines (EGFR, Gas6, PDGFB, PDGFD) accompanied the RTK upregulation, indicative of autocrine loops contributing to the defined adaptive response.

MIB/MS can also afford mechanistic insight into acquired resistance where a lack of specific reagents exists to study the role of a given kinase. As an illustration, we were able to characterize feedback regulation of MEK by ERK2 following prolonged treatment with MEK inhibitor, whereby MEK2 but not MEK1 escaped AZD6244 treatment to reactivate ERK1/2. As the phosphorylated sequences in the activation loop of MEK1 and MEK2 are identical (SMANS), phospho-specific antibodies do not discriminate the two proteins, yet MEK2 vs. MEK1 enrichment was detected in the MIB/MS spectra. Mechanistically, MEK-inhibitor induced RTK upregulation drove Ras activation of Raf kinase, culminating in the activation loop serine phosphorylation of MEK1/2. Phosphorylation of the activation loop serines in MEK1 and MEK2 diminishes the affinity for the allosteric MEK inhibitor AZD6244 nearly 20-fold[23,24], effectively reducing the efficacy of MEK inhibitors. There is also feedback inhibition of MEK1 by ERK1/2 catalyzed phosphorylation of threonine 292 of MEK1. MEK2 lacks this threonine residue[25]—allowing MEK2 but not MEK1 to escape from prolonged MEK inhibition.

MIB/MS as a tool to study the adaptive response has been proven useful in other malignancies. In chronic myelogenous leukemia (CML) cell lines either resistant or sensitive to the BCR-ABL inhibitor imatinib were subjected to MIB/MS analysis[14]. A signature of activated kinases upregulated in the imatinib-resistant cell line was identified including, as validation, Lyn, a Src-family kinase previously shown to contribute to imatinib resistance[13,26,27]. Additionally, MEK2 and IKKα were identified as kinases statistically enriched in the resistant cell line. Analogous to our rational design of the MEK inhibitor plus sorafenib RTK inhibitor combination in TNBC cell lines, MIB/MS identified the strategy of employing MEK plus IKK inhibitors concurrently in imatinib-resistant CML cell lines which resulted in apoptosis induction.

We have described herein the utility of our chemical proteomics approach to rationally predict combination therapies with efficacy in cell lines and in mouse models of tumorigenesis. The reprogramming observed in TNBC and in CML exemplifies the dynamic nature of kinome and underscores the importance of being able to rapidly assess system-wide changes in a single experiment. It is becoming increasingly clear that the resiliency of the kinome is capable of averting the initial success of a single kinase inhibitor and has the potential to eventually overcome combination therapies. The kinome activation state will have to be probed at each stage of this acquired resistance to begin to unravel the hierarchies of kinase signaling pathways leading to adaptive resistance for a given cancer.

In this context, we anticipate that MIB/MS approach for “kinotyping” will be informative for pre-clinical models and in the clinic with patients (Figure 1). Preclinically, cell lines, including drug-resistant lines, will continue to be of great use for initial assessment of kinome reprogramming induced by single kinase inhibitors or by combinatorial strategies. Once modes of adaptive resistance are defined by MIB/MS and a given kinase network is implicated, this network can be targeted by siRNA approaches in the same cell line to aid further predictions of which inhibitors might be efficacious at the next iteration of the kinome response. Secondly, murine models will continue to be a mainstay for in vivo validation of kinome reprogramming assessed in cell culture. We have shown that GEMMs yield concordant reprogramming signatures with those observed in cell lines. Going forward, patient-derived xenograft (PDX) models are being employed by our laboratory for MIB/MS assessment of kinome activation, and represent an important model for human tumors that allows study of treatment with therapeutic agents.

Finally, there must be a transition to the utilization of human tissues for studying the role of the kinome as a whole in acquired resistance. Matched tumor/normal tissue can reveal key differences in the activation signature of the kinome and point to kinase pathways that may be relevant in the adaptive response. We envision that significant progress will be made with window of opportunity clinical trials whereby patient tumor tissue is isolated prior to and after kinase inhibitor treatment. Quantitative MIB/MS can be used to evaluate naïve needle biopsied tumor and the response of the tumor kinome to drug treatment—allowing reprogramming to be assessed and future combination therapies to be designed in the clinical realm.

References

1. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001; 293:876–880. [PubMed: 11423618]
2. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong K-K, Tenen DG, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res. 2005; 65:7096–7101. [PubMed: 16103058]
3. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A. 2005; 102:7665–7670. [PubMed: 15897464]
4. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med. 2005; 2:e73. [PubMed: 15737014]

5. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007; 316:1039–1043. [PubMed: 17463250]
6. Lee J, Seo JW, Jun HJ, Ki C-S, Park SH, Park YS, et al. Impact of MET amplification on gastric cancer: possible roles as a novel prognostic marker and a potential therapeutic target. Oncol Rep. 2011; 25:1517–1524. [PubMed: 21424128]
7. Kawakami H, Okamoto I, Arao T, Okamoto W, Matsumoto K, Taniguchi H, et al. MET amplification as a potential therapeutic target in gastric cancer. Oncotarget. 2013; 4:9–17. [PubMed: 23327903]
8. Shi H, Moriceau G, Kong X, Lee M-K, Lee H, Koya RC, et al. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. Nat Commun. 2012; 3:724. [PubMed: 22395615]
9. Villanueva J, Infante JR, Krepler C, Reyes-Uribe P, Samanta M, Chen H-Y, et al. Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma. Cell Rep. 2013; 4:1090–1099. [PubMed: 24055054]
10. Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, Zawistowski JS, et al. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. Cell. 2012; 149:307–321. [PubMed: 22500798]
11. Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer Cell. 2010; 18:683–695. [PubMed: 21156289]
12. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, et al. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011; 19:58–71. [PubMed: 21215704]
13. Ito T, Tanaka H, Kimura A. Establishment and characterization of a novel imatinib-sensitive chronic myeloid leukemia cell line MYL, and an imatinib-resistant subline MYL-R showing overexpression of Lyn. Eur J Haematol. 2007; 78:417–431. [PubMed: 17432977]
14. Cooper MJ, Cox NJ, Zimmerman EI, Dewar BJ, Duncan JS, Whittle MC, et al. Application of multiplexed kinase inhibitor beads to study kinome adaptations in drug-resistant leukemia. PloS One. 2013; 8:e66755. [PubMed: 23826126]
15. Fedorov O, Müller S, Knapp S. The (un)targeted cancer kinome. Nat Chem Biol. 2010; 6:166–169. [PubMed: 20154661]
16. Knapp S, Arruda P, Blagg J, Burley S, Drewry DH, Edwards A, et al. A public-private partnership to unlock the untargeted kinome. Nat Chem Biol. 2013; 9:3–6. [PubMed: 23238671]
17. Perou CM. Molecular stratification of triple-negative breast cancers. The oncologist. 2011; 16(Suppl 1):61–70. [PubMed: 21278442]
18. Maroulakou IG, Anver M, Garrett L, Green JE. Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. Proc Natl Acad Sci U S A. 1994; 91:11236–11240. [PubMed: 7972041]
19. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther. 2008; 7:3129–3140. [PubMed: 18852116]
20. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. 2000; 14:2501–2514. [PubMed: 11018017]
21. Oster SK, Marhin WW, Asker C, Facchini LM, Dion PA, Funa K, et al. Myc is an essential negative regulator of platelet-derived growth factor beta receptor expression. Mol Cell Biol. 2000; 20:6768–6778. [PubMed: 10958674]
22. Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, et al. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol. 2004; 6:308–318. [PubMed: 15048125]
23. Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, et al. GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic

properties for sustained in vivo pathway inhibition. Clin Cancer Res Off J Am Assoc Cancer Res. 2011; 17:989–1000.

24. Sheth PR, Liu Y, Hesson T, Zhao J, Vilenchik L, Liu Y-H, et al. Fully activated MEK1 exhibits compromised affinity for binding of allosteric inhibitors U0126 and PD0325901. Biochemistry (Mosc). 2011; 50:7964–7976.
25. Eblen ST, Slack-Davis JK, Tarcsafalvi A, Parsons JT, Weber MJ, Catling AD. Mitogen-activated protein kinase feedback phosphorylation regulates MEK1 complex formation and activation during cellular adhesion. Mol Cell Biol. 2004; 24:2308–2317. [PubMed: 14993270]
26. Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R, et al. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood. 2003; 101:690–698. [PubMed: 12509383]
27. Wu J, Meng F, Lu H, Kong L, Bornmann W, Peng Z, et al. Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells. Blood. 2008; 111:3821–3829. [PubMed: 18235045]
28. Chartier M, Chénard T, Barker J, Najmanovich R. Kinome Render: a stand-alone and web-accessible tool to annotate the human protein kinome tree. PeerJ. 2013; 1:e126. [PubMed: 23940838]

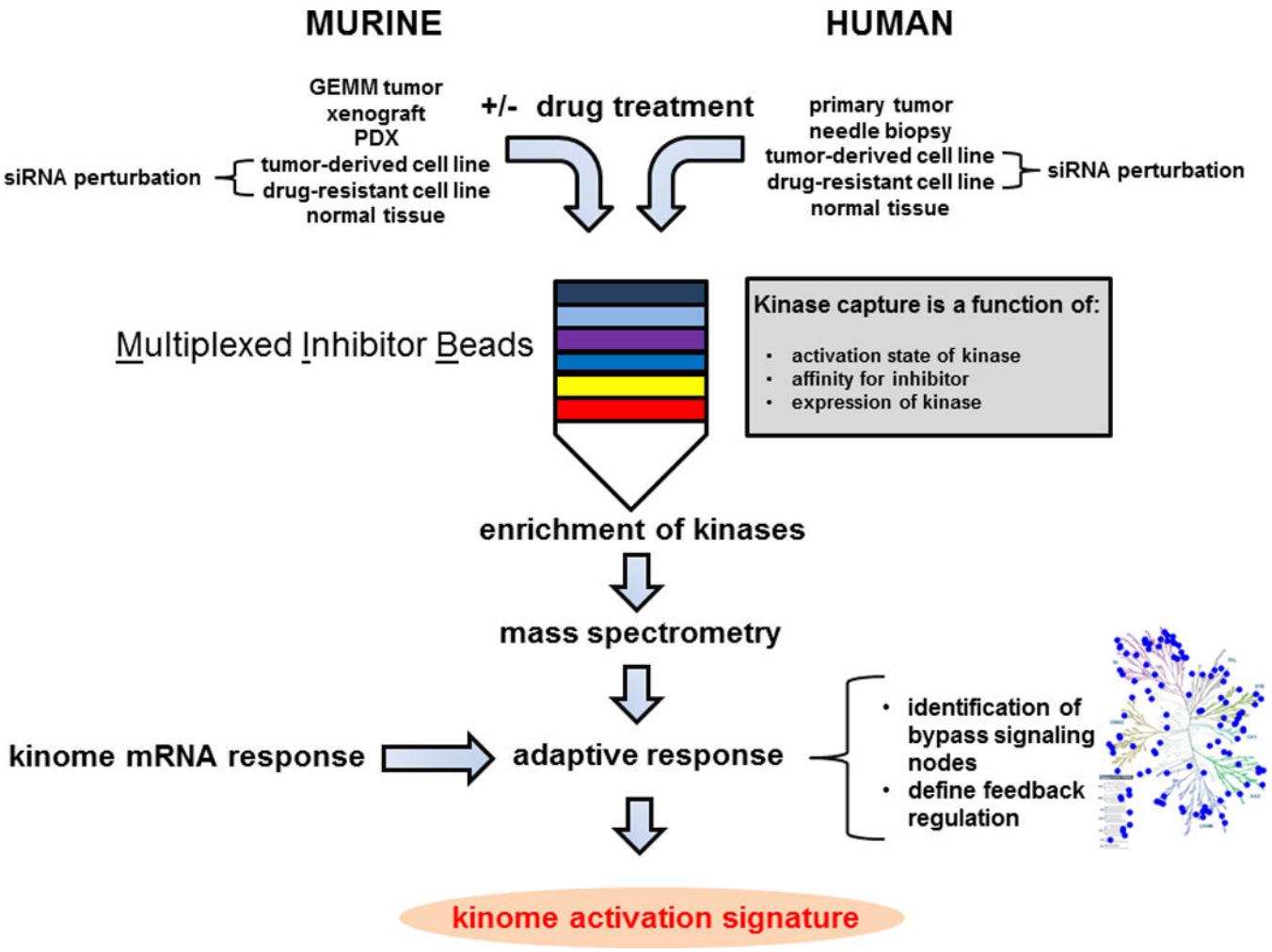

Figure 1. Multiplexed Inhibitor Beads coupled to mass spectrometry as a tool to study adaptive responses in cancer

A MIB chromatography column consists of Sepharose-conjugated Type I kinase inhibitors, layered with increasing broadness of action toward the bottom of the column. Current and potential sources of MIB/MS sample input in context of drug treatment are presented. The kinome phylogenetic tree image was produced using Kinome Render[28] whereby the original tree image is reproduced courtesy of Cell Signaling Technology, Inc.