# Where the (drug) action really is

## progress in systems-based analysis of CELLULAR SIGNALLING

Drug discovery currently focuses on targeted approaches, relying on validation of the target as a disease driver. However, the underlying biological complexity of disease often frustrates these attempts at therapeutic intervention, resulting in high failure rates due to lack of efficacy. Systems biology is being employed to understand the complexity of biological systems but has not been widely applied to understand the biology of cellular signalling, which is proximal to underlying mechanisms of disease and drug action, as the tools to acquire sufficient data breadth and quality have been lacking in the field. This article summarises recent developments in the analysis of cellular signalling, focusing on the analysis of protein phosphorylation and its potential applications to understanding disease states and the development of therapeutic strategies.

**By Dr Jeffrey A. Radding**

Much of the low hanging fruit has been picked from the drug discovery tree and efforts to improve or streamline the drug discovery and development process have failed to impact the unsustainable numbers of drug failures. However, the causes of drug attrition rates have changed over time. The major reason for compound failure in 1991 was poor PK/PD and bioavailability, accounting for nearly 40% of all terminated molecules. Pharmaceutical chemists addressed the PK/PD and bioavailability issues from 1991-2000 and shifted the primary cause of drug failures to lack of efficacy[1]. Further improvements on attrition rates in the pharmaceutical pipeline will require a thorough understanding of the relationship between a drug and its target protein in order to classify disease, understand mechanism-of-action, and predict therapeutic outcome. The biological complexity underlying disease makes this very challenging, requiring a systems approach for understanding disease mechanisms. By focusing on signal transduction networks, proximal to sites of therapeutic intervention, drug development can be improved by better linking compound activity with complex signalling behaviour that has been validated to be predictive of physiological outcome. Until recently, it has been difficult to obtain the breadth and quality of data on cell signalling networks that is needed to apply a systems approach to signal transduction network analysis.

The importance of target validation is illustrated by the success of Gleevec (imatinib), a protein kinase inhibitor drug, targeting the constitutively active BCR-Abl kinase, which leads to chronic myelogenous leukaemia (CML)[2]. By having a clear understanding that the drug target was the major, if not sole, driver of the disease state, Gleevec proved to be very successful at treating CML by inhibiting the activity of BCR-Abl. However, even Gleevec has been shown to have several activities, including activity against c-Kit and PDGF receptor. Moreover, very few diseases have single targets as major driving forces. The combination of multiple activities of compounds and multiple disease drivers requires different approaches to achieve therapeutic success.

Aberrant cellular signalling plays a critical role in tumour development and progression. Many oncogenes (gain-of-function mutations) and tumour suppressors (loss-of-function mutations) modulate cellular behaviour through a network of signalling events mediated by changes in protein and lipid phosphorylation[3]. Similarly, in diabetes, signalling plays a major role in regulating insulin sensitivity and metabolic homeostasis[4]. Both cancer and diabetes arise as a combination of underlying genetic components and external environmental influences that result in unique and multi-factorial alterations in signalling that characterise individual disease states. For example, the environmental influences in diabetes extend across multiple organ systems to include liver, kidney, skeletal muscle and adipose tissue influences upon each other. The critical implication of this biological complexity is that disease, and potentially optimal therapies for that disease, can be defined by the information flow through the signalling network under specific circumstances.

## Targeted therapy meets multiple disease drivers

Kinase inhibitor drug programmes represent approximately 30% of the current development pipeline, despite the fact that attrition in this class of compounds is higher than for other classes of molecules. These molecules are designed to block or inhibit enzymatic activity of kinases, which are the primary mediators of cellular signalling. The ATP-binding pocket represents a shared structural feature among kinases, which has been exploited for the development of both competitive and non-competitive molecules. Considerable effort has been expended to make kinase inhibitors 'specific' for given targets to avoid off-target effects, particularly those associated with specific toxicology. One paradigm of targeted therapy is that every disease has a single critical or major driver to it, and that drug specificity built towards this driver should result in significant efficacy.

As discussed above, it is unlikely that most disease states have single drivers associated with them. A case in point is the Her2/neu oncogene, which is a member of the ErbB family of receptor tyrosine kinases. High levels of Her2/neu expression in breast cancer are associated with poor prognosis and metastatic disease in about 30% of all breast cancers. A monoclonal antibody specifically targeting Her2/neu (trastuzumab) is used clinically, usually in an adjuvant setting for Her2+ amplified, node-positive breast cancer. Although clearly an important factor in breast cancer disease and progression, targeting of Her2/neu by trastuzumab, even in a patient population selected for Her2/neu over-expression, generally results in less than a 30% response rate to therapy as a single agent[5-7]. Without patient stratification, the small molecule inhibitors Tarceva (erlotinib) and Iressa (gefitinib), which inhibit the ErbB1 receptor tyrosine kinase, have struggled to demonstrate efficacy as single agents (tumour response rates of 8-15%) or in combination therapies in a variety of tumours[8-10]. For erlotinib, there is growing consensus that activating mutations or over-expression of the receptor represents biomarkers for patient selection and treatment outcome in NSCLC. These biomarkers may allow the selection of smaller patient populations with higher objective response rates to achieve statistical significance for approval[11]. This is contrasted with the >90% response rate for imatinib observed in CML. The limited success stories in these solid tumour diseases suggest that a specific drug target is not driving the disease in the majority of non-stratified patients and appropriate patient selection is critical for demonstrating drug efficacy.

Many companies are using pharmacogenomics approaches to select patients for clinical trials of new compounds based on genetic and gene expression signatures predictive of clinical outcomes. However, there are multiple mechanisms for regulating gene expression and it is important to determine which changes might be drivers of the disease versus inconsequential events. In the erlotinib trials, ErbB1 overexpression did not clearly correlate with outcome using FISH or IHC. Instead, it was the presence of activating mutations in ErbB1 along with the activation of a downstream target (Akt), detected by IHC, which correlated best with response to drug. The experience with erlotinib suggests that genetic

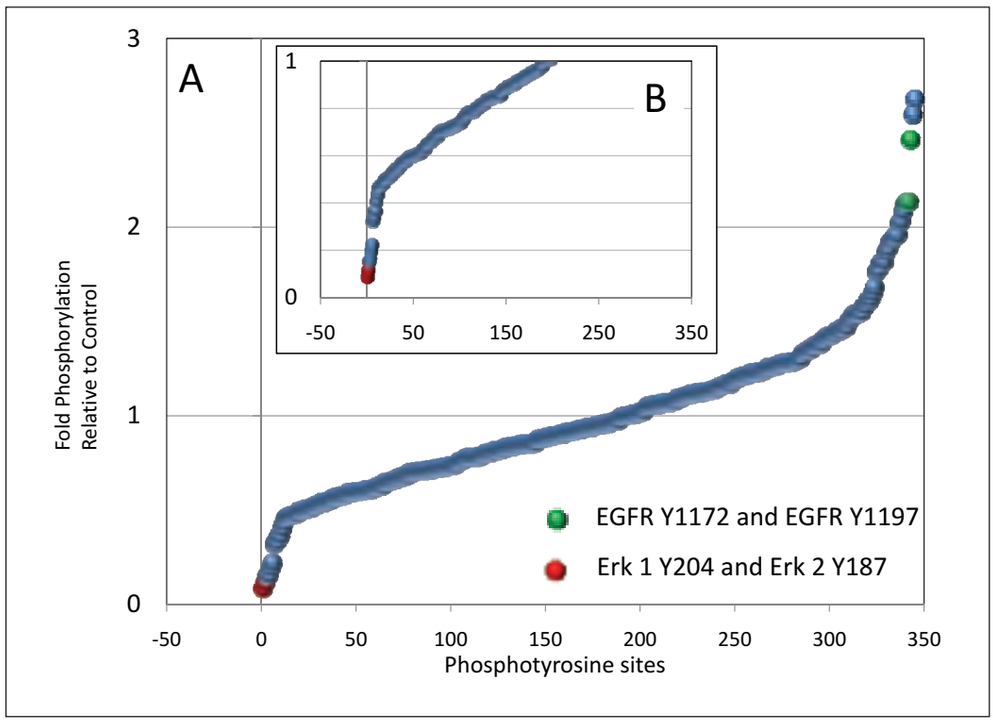



**Figure 1**
Global effect of Mek inhibition on EGF stimulated A431 cells

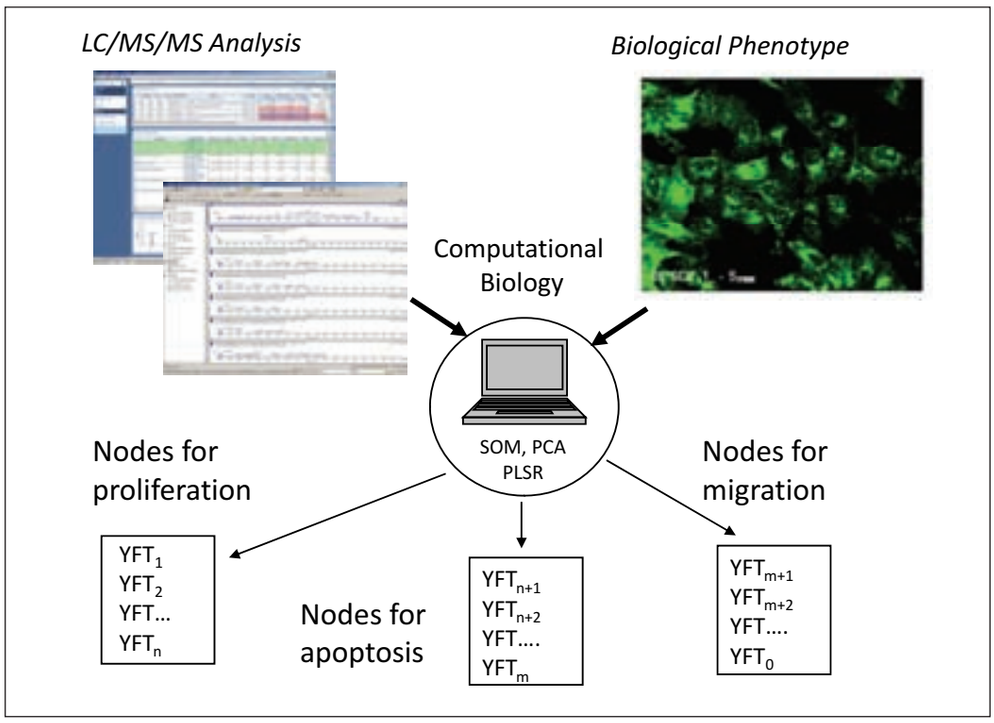
information and gene expression data by themselves are not sufficient to predict drug sensitivity, but functional data along the signalling network will be the most informative. Functional signalling information is particularly important when multiple disease drivers are involved.

Not only are there multiple disease drivers influencing cellular behaviour, but environmental factors also influence cellular signalling and the sensitivity to compounds. In recent studies of glioblastoma, co-activation of receptor tyrosine kinases (RTK) affected the response of tumour cells to targeted therapies. The important influence of environmental factors is observed in the differences that cultured cells show in RTK activation patterns compared to xenograft models derived from the same tumour cell lines and the profiles of RTK activation in primary glioblastomas[12]. Thus, the choice of appropriate therapy may have to be the result of profiling signalling responses with downstream effects to predict treatment outcomes.

With many drug discovery programmes focused on targeted therapies, the multiplicity of disease drivers may explain why drug development fails so often due to a lack of efficacy. Given that disease biology is considerably more complex than a targeted therapy paradigm would suggest, new approaches are needed to improve drug success. Several, recent reviews have championed a 'systems biology' approach to address this issue, focusing on genomic, proteomic and metabolomic technologies as their underlying platforms. Although cellular signalling has been a central theme in the study of disease for some time, systems biology approaches to studying signalling have been very limited. Systems biology approaches, at the signalling level, should be applied to understand disease states and to guide therapeutic development particularly where there are multiple disease drivers. Until recently, the inability to generate extensive, quantitative phosphorylation data has hampered this type of functional or systems biology approach. Innovations in quantitative phosphorylation methodologies are making it feasible to adopt these approaches to characterise disease and drug responses, as well as to stratify patients.

Initial forays into systems approaches to cellular signalling focused on traditional and labour-intensive assays for phosphorylation using standard lab methods such as Western blots and commercially available phospho-specific antibodies. Subsequent efforts have focused on the use of 'reverse-arrays', essentially micro-dot blots, where cellular lysates are immobilised on nitrocellulose in a microarray format and probed with commercially available antibodies[13]. Reverse arrays have achieved fairly wide use as they are relatively easy to construct

**Figure 2**
Q-SONAR™ analysis




and implement. Since the arrays are probed with single antibodies on complex mixtures of proteins, the specificity of each antibody becomes critical. In addition, the content probed on arrays is limited to our current knowledge and availability of appropriate reagents. Recent work has documented some of the difficulties in obtaining suitable antibodies, where only 12 of 61 commercial antibodies were suitable for use in a reverse array probing phosphorylation of the EGFR signalling pathway[14]. Reverse arrays, by nature, rely on relative comparisons between targets that are likely to have very different response factors. In addition, it is very difficult to standardise reverse-phase microarrays, and to compare signalling events on one site to another site on a different protein, or from one sample to another since there are no objective quantitative standards for either target. This is a significant issue when attempting to make meaningful comparisons across a range of targets via multiplexed antibody-based immunoassays.

## Signalling network discovery – unbiased, data driven

To approach cellular signalling at a systems level, a broad, unbiased, quantitative analysis of signalling events is required. Recent advances in mass spectrometry have provided the ability to measure protein phosphorylation sites in an unbiased manner[15-18]. Protein tyrosine phosphorylation has been most extensively studied using these methods for several reasons, including greater stability, reduced complexity and technical issues associated with the methodology. Using the incorporation of stable isotope labelling procedures, relative quantification can be achieved between different conditions. Stable isotope incorporation using iTRAQ labels (Applied Biosystems) provides the greatest flexibility for quantification as it can be applied to any sample, either cell culture or tissue. Several publications have highlighted the ability to use iTRAQ quantification with protein tyrosine phosphorylation analysis to examine cellular signalling as a function of different conditions. Numerous phosphotyrosine(pY ) sites were identified and signalling changes were correlated with phenotypes that ultimately led to suggestions for potential combination therapies that might be effective[19-21]. This type of analysis can be used for compound analysis, where phosphorylation changes are broadly profiled across the signalling network in an unbiased manner by sampling tyrosine phosphorylation and looking for on-target (**Figure 1B** inset, loss of ERK phosphorylation) and potential off-target effects (**Figure 1A**, elevated receptor phosphorylation). However, the unbiased and broad-based sampling of tyrosine phosphorylation is not intended for global analysis.

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Instead, it is designed to identify signalling network markers that reflect the cell state associated with drug treatment and changes in physiological outcome through a quantitative systems-oriented network-activity relationship (Q-SONAR™ Discovery, **Figure 2**). The quantitative data from such experiments can be analysed in a modelling framework, wherein multivariate data are utilised in a statistically driven framework, or in a more deterministic approach, if sufficient data and knowledge are available[22,23]. These models help define the key nodes in the network that are best correlated with particular cellular outcomes and they represent links between disease drivers and downstream effectors.

## Validation of key nodes – traditional protein immunoassays

One of the drawbacks for mass spectrometry-based discovery is that it does not lend itself to routine analysis due to the sophisticated equipment, required technical expertise and limited throughput. Routine analysis is more readily accomplished using immunoassay approaches. As discussed above, reverse-phase microarrays have significant limitations. Sandwich immunoassays provide much better specificity with comparable sensitivity. With an appropriate standard, sandwich immunoassays are quantitative and routinely used for clinical diagnostics. Traditional sandwich immunoassays are performed on intact proteins, which may have drawbacks, particularly in multiplexing analyses of post-translational modifications (PTM), such as phosphorylation. It is extremely difficult to find specific reagents for both capture and PTM detection, which do not cross-react with other sites, proteins or interfere with each other. This generally limits multiplexing capacity significantly to the detection of a few intracellular signalling proteins. One solution is to limit the number of antibodies by using pan-PTM antibodies, such as pan-pY, as a common detection reagent. However, in protein-based immunoassays, a pan-pY strategy makes it difficult to examine site specificity on proteins, where multiple phosphorylations correlate with different functions. Thus, important site-specific functional information can be lost in the translation from MS-based discovery to routine immunoassay analyses. For PTM quantification, particularly phosphorylation, it is extremely difficult and expensive to generate quantitative standards at the protein level. Thus, new

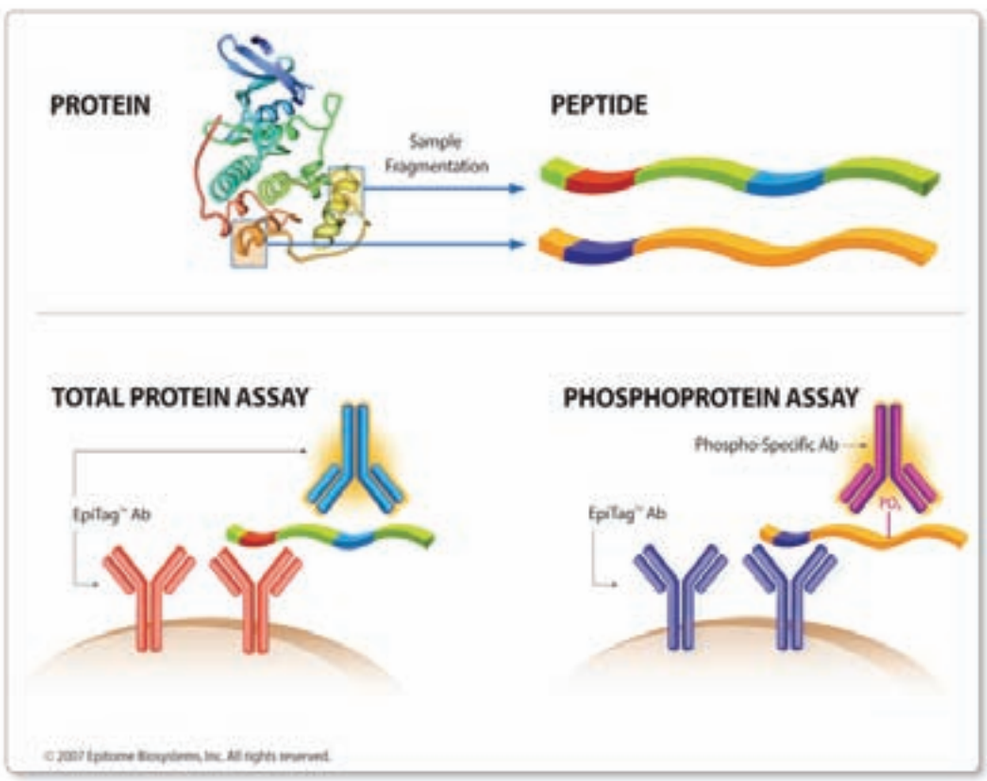


**Figure 3:** Principle of EpiTag™ assay strategy

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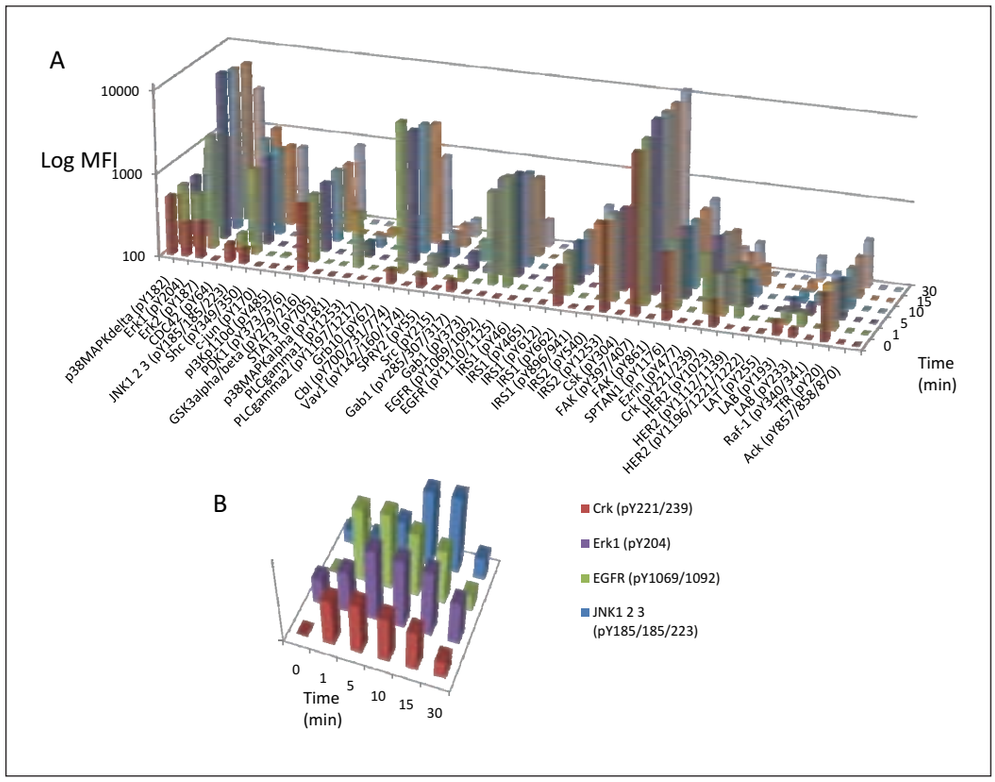


**Figure 4:** Ti-Tyr™ chip profiling of time course for growth factor stimulation

quantitative immunoassay approaches are needed that provide relevant information in a routine, multiplexed assay format.

## Validation of key nodes – peptide-based immunoassays provide site specificity and quantitative data

An alternative to protein-based sandwich immunoassays is the peptide-based immunoassay developed by Epitome Biosystems (EpiTag™ Assays). This peptide-based immunoassay relies on the *in silico* protein fragmentation pattern of the human proteome that is generated by specific proteases. Protein sites of interest are derived from the existing knowledge base or from data-driven MS discovery, and fragments containing these sites are further characterised for their immunoassay suitability. Bioinformatics is used to identify unique, linear sequences in peptides (EpiTag™ Sequences) or epitope tags that are used for antibody generation. Antibodies raised against these linear peptide sequences are characterised for specificity and sensitivity as potential capture reagents in the immunoassay. Capture antibodies are immobilised on a solid support (glass slides or beads) and assay performance is evaluated using synthetic peptide standards, with fully phosphorylated pY sites. For tyrosine phosphorylation measurements, the pan-pY strategy detection strategy described above is used to complete the sandwich assay (**Figure 3**).

Biological sample preparation for the peptide-based immunoassay is analogous to methodologies used for MS-based discovery, where lysates are denatured, reduced, alkylated and digested (using the protease selected from the *in silico* design) prior to analysis. This sample preparation method has beneficial aspects particularly with regard to sample stability, implementation of common assay conditions, ability to measure site-specific PTMs in multiplex, and the simultaneous measurement of total protein and PTMs in the same sample. This unique feature of these assays is due to the use of different fragments from the targeted protein for the total and PTM measurements. Two, EpiTag™ antibodies are generated to distinct regions on a fragment for a total protein assay, one antibody is used for peptide capture and the other for detection. The methodology described above has been used to develop bead-based assays for both total and phospho-proteins in the EGFR-MAPK pathway[24] and to develop a

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broad profiling chip, where EpiTag™ antibodies are used to quantitatively measure 46 different phosphorylation sites on 35 different proteins (Ti-Tyr™ Chip, **Figure 4**). The chip is formatted with 16 wells per chip, each containing all 46 antibodies arrayed in four sub-arrays in each well. Quantification of fluorescent signals is achieved using peptide standards for generation of calibration curves and subsequent interpolation of biological sample data.

## Translational impact of communication biomarkers

The peptide-based immunoassays allow access to a broad diversity of tyrosine phosphorylation sites. It becomes relatively facile to translate mass spectrometry-based discoveries into routine assays that can be performed at very high multiplex levels. The importance of this advance is in the ability to run many samples on a quantitative immunoassay platform that allows for rigorous data validation. As described above, initial model construction, either statistical or deterministic, can be generated from MS-based discovery data but validating the model requires high throughput and absolute quantitative measurements. Thus, key nodes involved in disease manifestation and progression can be identified and monitored temporally and under many experimental treatment conditions. This methodology is broadly applicable across the drug discovery and development continuum and can be translated to *in vivo* testing in a clinical setting. Here, a subset of MS signals now constitute a highly validated set of communication biomarkers, ready for implementation on an assay platform that is amenable to clinical evaluation of individual patient samples. Identification of these biomarkers will allow for both better patient stratification in clinical trials and will be useful for determining disease drivers and potential combination therapies to combat them. DDW

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