a particular method for the generation of reference methylomes, although Harris *et al.*[1] suggest the possibility of hybrid methods and show improved results for MeDIP-seq integrated with MRE-seq (based on methylation-sensitive restriction).

Although the two studies[1,2] have successfully resolved many long-standing questions in the epigenomics community, several challenges remain. The most pressing concern is that a full methylome analysis should include mC and hmC in addition to mCG, although the biological functions of these modifications have yet to be determined. Another challenge is that bisulfite-based methods (the current gold standard of methylation analysis) cannot distinguish between methylation and hydroxymethylation[8], which has implications for all bisulfite-based data already deposited in public databases.

As the International Human Epigenome Consortium gears up to generate 1,000 reference epigenomes, the participating laboratories will undoubtedly use different methylome analysis methods. It will therefore be important to develop a procedure for assigning quality values to the methylation status of each cytosine. A similar metric proved to be very helpful in the assembly and use of the draft sequence of the human genome. For the future, there are great expectations that one day we will be able to read the different forms of DNA methylation directly using methods such as nanopore[9] and single-molecule, real-time[10] sequencing. For now, however, with careful management, our current technology is adequate to move 'AHEAD'.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

1. Harris, R.A. *et al. Nat. Biotechnol.* **28**, 1097–1105 (2010).
2. Bock, C. *et al. Nat. Biotechnol.* **28**, 1106–1114 (2010).
3. Jones, P.A. & Martienssen, R. *Cancer Res.* **65**, 11241–11246 (2005).
4. Satterlee, J. *Nat. Biotechnol.* **28**, 1039–1044 (2010).
5. Li, N. *et al. Methods* published online, doi:10.1016/j.ymeth.2010.04.009, 27 April 2010.
6. Robinson, M. *et al. Epigenomics* **2**, 587–598 (2010).
7. Lister, R. *et al. Nature* **462**, 315–322 (2009).
8. Huang, Y. *et al. PLoS ONE* **5**, e8888 (2010).
9. Clarke, J. *et al. Nat. Nanotechnol.* **4**, 265–270 (2009).
10. Flusberg, B.A. *et al. Nat. Methods* **7**, 461–465 (2010).

Tracing cancer networks with phosphoproteomics

David B Solit & Ingo K Mellinghoff

A mass-spectrometry approach for identifying downstream events in cancer signaling pathways may help to tailor therapies to individual patients.

The clinical success of kinase inhibitors such as Gleevec (imatinib) has provided a glimpse of what can be achieved by targeting the signaling pathways involved in the growth of cancer cells[1]. But these signal-transduction networks are still poorly understood, hampering efforts to apply this paradigm more broadly to patients with advanced cancer. Two recent studies, by Moritz *et al.*[2] and Andersen *et al.*[3], show how this challenge might be addressed with 'compound-centric' phosphoproteomics. The findings, reported in *Science Signaling*[2] and *Science Translational Medicine*[3], not only provide new insights into the signaling circuitry responsible for cell proliferation but may also be of value in identifying the greatest vulnerabilities of particular tumor cells and, therefore, in optimizing therapies for individual patients.

David B. Solit and Ingo K. Mellinghoff are in the Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, New York, USA.
e-mail: solitd@mskcc.org or mellingi@mskcc.org

Many human cancers have alterations in the phosphatidylinositol-3-OH kinase (PI(3)K) pathway, which has become an area of particular interest in drug development[4]. Rapamycin (Rapamune, sirolimus), an immunosuppressive agent that inhibits the kinase mTOR ('mammalian target of rapamycin') and is used clinically in organ transplantation, was the first inhibitor of a PI(3)K signaling intermediate to enter broad clinical testing for cancer[5]. But despite compelling preclinical results (particularly in models with aberrant PI(3)K pathway activation) and modest efficacy in patients with renal cell carcinoma, the overall clinical success of rapamycin in oncology has been disappointing. These failures may be due in part to activation of AKT and MAPK by de-inhibition of negative feedback loops[6,7] and to redundant regulation of key downstream effectors of transformation by parallel signaling pathways[8]. In many respects, this experience exemplifies the challenges of targeting a signaling network that is insufficiently understood.

The two new studies[2,3] used global mass spectrometry (MS)-based approaches to identify substrates of serine (Ser)/threonine (Thr) kinases downstream of receptor tyrosine kinases (RTKs), RAS, PI(3)K and mTOR. Selected members of the signaling network comprising RAS, PI(3)K and the mTOR-containing complexes TORC1 and TORC2 (ref. 9) are shown in **Figure 1a**. Each of these core signaling pathways activates kinases that phosphorylate their substrates in a context-specific manner, depending on the amino acids flanking the phosphorylation site. Both studies[2,3] used phosphomotif-specific antibodies for immunoaffinity purification before MS analysis and quantified the effects of various pathway inhibitors on the newly identified Ser/Thr-phosphorylation sites using an approach based on stable isotope labeling with amino acids in cell culture (SILAC)[10] (**Fig. 1b**).

Moritz *et al.*[2] identified >300 substrates in three human cancer cell lines with mutations in either epidermal growth factor (*EGFR*), hepatocyte growth factor receptor (*MET*) or platelet-derived growth factor receptor α (*PDGFRA*); almost half of these substrates were identified for the first time. Phosphorylation of 21 proteins decreased significantly in all three cell lines after inhibition of the oncogenic RTK. The targets include the previously reported Akt-RSK-S6 kinase substrates glycogen synthase kinase 3A and B, ribosomal protein S6 (RPS6) and the proline-rich Akt1 substrate (PRAS40).

The study by Andersen *et al.*[3] focused on the PI(3)K branch of the network and used a PTEN-deficient human prostate cancer cell line, a broader immunoaffinity purification scheme (enriching for AKT substrates, MAPK substrates and PDK1-docking motifs) and a different set of pathway inhibitors (targeting PDK1, AKT and both PI3K and mTOR). The authors identified 375 nonredundant phosphopeptides, of which about a quarter showed a substantial change in phosphorylation in response to pathway perturbation. Some proteins (e.g., RPS6 and PRAS40) showed decreased phosphorylation in response to all three pathway inhibitors, whereas others showed more selective responses to particular inhibitors (e.g., RPS6KA6 for the PDK1 inhibitor). The authors then focused on PRAS40 and showed that its phosphorylation at Thr246 positively correlates with phosphorylation of

© 2010 Nature America, Inc. All rights reserved.

AKT at Ser473 and with the sensitivity of cancer cell lines to an allosteric AKT inhibitor.

Until now, the technical challenges of MS-based detection of phosphopeptide substrates have limited our ability to detect Ser/Thr phosphorylation events in cancer. Beyond providing new information about the signaling circuitry downstream of Akt, MAPK, RSK and S6K, these pioneering studies[2,3] open the entire space of Ser/Thr protein phosphorylation for further study. Nonetheless, it remains possible that some of the observed drug-induced phosphorylation changes represent 'off-target' effects. Additional, confirmatory experiments involving genetic approaches and more specific compounds will be needed before we can revise our picture of the RTK/RAS/PI3K/mTOR network.

The studies[2,3] are equally promising from the perspective of clinical drug development. First, they document the effects of compounds on a large number of phosphorylation events, which can be quantified in clinical tumor samples using various antibody-based proteomic assays. Such information can guide dosing decisions with molecularly targeted therapies during early clinical drug evaluations and help to prevent drug development resources from being wasted on compounds that do not achieve sufficient target inhibition in tumor tissue[11]. Second, especially if linked to the detection of phosphotyrosine protein modifications in the same sample[3], compound-centric phosphoproteomics may uncover unexpected effects of the drug on upstream or parallel signaling networks that mediate drug resistance, identify mechanisms of 'off-target' drug toxicity or suggest new opportunities for combination therapies[12].

Thus far, kinase inhibitor therapy has been most successful for cancers with an activating mutation that can be readily identified in routinely collected clinical samples using genomic assays. Examples include non-small cell lung cancers with mutations in the EGFR kinase domain or melanomas with BRAF mutations. It remains unclear which mutations predict responsiveness to PI(3)K/mTOR pathway inhibitors. Moreover, signaling through this pathway can be deregulated by many molecular alterations. This genomic complexity represents a rate-limiting step in the further development of PI(3)K pathway inhibitors and has spurred interest in transcriptional[13] or proteomic markers of aberrant pathway activation.

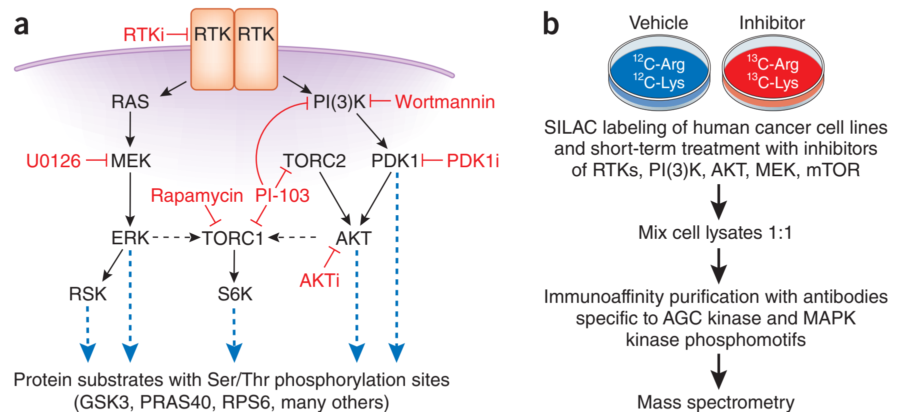

Figure 1 Identification of (Ser)/(Thr) phosphorylation substrates in core cancer signaling pathways. (**a**) Probing the RAS, PI(3)K and mTOR signaling pathways with inhibitors. Members of the RAS-PI(3)K-mTOR signaling network[9] are shown in black and inhibitors used by Moritz *et al.*[2] and Andersen *et al.*[3] are shown in red. ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI(3)K, phosphatidylinositol-3-OH-kinase; PRAS40, proline-rich AKT1 substrate 1; RPS6, ribosomal protein S6; RSK, ribosomal S6 kinase; RTK, receptor tyrosine kinase; S6K, p70 ribosomal protein S6 kinase; TORC1/2, mammalian target of rapamyin complex 1/2. (**b**) SILAC-based mass spectrometry[10] to quantify inhibitor-induced changes in Ser/Thr phosphorylation. Cancer cell lines are grown either in 'light' medium containing the normal forms of the amino acids lysine ($^{12}C_6$-Lys) and arginine ($^{12}C_6$-Arg) or in 'heavy' medium containing $^{13}C_6$-Lys and $^{13}C_6$-Arg. After short-term treatment with inhibitor, cells are lysed and lysates pooled before immunoaffinity purification with antibodies specific to phosphomotifs of interest. Inhibitor-induced changes in phosphorylation patterns are quantified by comparing protein abundance using the light and heavy peaks in the mass spectra. AGC, cAMP (adenosine 3′,5′-monophosphate)-dependent, cGMP (guanosine 3′,5′-monophosphate)-dependent, and protein kinase C; MAPK, mitogen-activated protein kinase.

It remains to be seen whether at least a subset of cancers display 'pathway addiction' as opposed to 'oncogene addition' to particular components within the PI(3)K pathway. Perhaps the combination of a robust pathway-activation marker (e.g., phospho-PRAS40^{Thr246}) with focused mutational analysis (e.g., mutational profiling of *PIK3CA*) will offer a reasonable compromise for patient stratification into PI(3)K/AKT inhibitor trials, as suggested by Anderson *et al.*[3]. Clearly, much work remains to be done to realize the clinical potential of genomics and proteomics. Nonetheless, these two studies[2,3] represent outstanding examples of hypothesis-driven biomarker discovery, which, once validated in a broader genetic context, are likely to produce new pharmacological opportunities for disrupting cancer-associated signaling networks.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Varmus, H. *Science* **312**, 1162–1165 (2006).
2. Moritz, A. *et al. Sci. Signal.* **3**, ra64 (2010).
3. Andersen, J.N. *et al. Sci. Transl. Med.* **2**, 43ra55 (2010).
4. Courtney, K.D., Corcoran, R.B. & Engelman, J.A. *J. Clin. Oncol.* **28**, 1075–1083 (2010).
5. Sabatini, D.M. *Nat. Rev. Cancer* **6**, 729–734 (2006).
6. Cloughesy, T.F. *et al. PLoS Med.* **5**, e8 (2008).
7. Carracedo, A. *et al. J. Clin. Invest.* **118**, 3065–3074 (2008).
8. She, Q.B. *et al. Cancer Cell* **18**, 39–51 (2010).
9. Shaw, R.J. & Cantley, L.C. *Nature* **441**, 424–430 (2006).
10. Mann, M. *Nat. Rev. Mol. Cell Biol.* **7**, 952–958 (2006).
11. Sawyers, C.L. *Nature* **452**, 548–552 (2008).
12. Wallin, J.J. *et al. Sci. Transl. Med.* **2**, 48ra66 (2010).
13. Saal, L.H. *et al. Proc. Natl. Acad. Sci. USA* **104**, 7564–7569 (2007).

© 2010 Nature America, Inc. All rights reserved.