Mass Spectrometry-based Expression Profiling of Clinical Prostate Cancer

Michael E. Wright‡§, David K. Han¶, and Ruedi Aebersold§

The maturation of MS technologies has provided a rich opportunity to interrogate protein expression patterns in normal and disease states by applying expression protein profiling methods. Major goals of this research strategy include the identification of protein biomarkers that demarcate normal and disease populations, and the identification of therapeutic biomarkers for the treatment of diseases such as cancer (Celis, J. E., and Gromov, P. (2003) Proteomics in translational cancer research: Toward an integrated approach. Cancer Cell 3, 9–151). Prostate cancer is one disease that would greatly benefit from implementing MS-based expression profiling methods because of the need to stratify the disease based on molecular markers. In this review, we will summarize the current MS-based methods to identify and validate biomarkers in human prostate cancer. Lastly, we propose a reverse proteomic approach implementing a quantitative MS research strategy to identify and quantify biomarkers implicated in prostate cancer development. With this approach, the absolute levels of prostate cancer biomarkers will be identified and quantified in normal and diseased samples by measuring the levels of native peptide biomarkers in relation to a chemically identical but isotopically labeled reference peptide. Ultimately, a centralized prostate cancer peptide biomarker expression database could function as a repository for the identification, quantification, and validation of protein biomarker(s) during prostate cancer progression in men. Molecular & Cellular Proteomics 4:545–554, 2005.

Prostate cancer (PCa) is the most commonly diagnosed cancer among men in the United States, where ∼200,000 men are diagnosed with PCa every year, causing an estimated 30,000 deaths annually (1). However, the indolent nature of early-stage, prostate-localized PCa makes it a highly curable disease, provided that it is detected at an early stage (2). Prostate-specific antigen (PSA) screening is the most commonly used diagnostic serum biomarker for detecting PCa in men (3). While PSA levels can detect PCa in men, this biomarker lacks specificity, as infections of the prostate gland, such as prostatitis, can also elevate PSA levels in the absence of cancer (4, 5). Lack of tumor specificity makes it very difficult to determine how to treat early-stage PCa in men based on PSA levels alone. Furthermore, the anatomical location of the prostate gland in the male urinary tract makes it very difficult to monitor PCa progression in a noninvasive manner over time (6). This forces most early-stage PCa patients to undergo aggressive treatments such as surgical removal of the prostate gland (radical prostatectomy) or localized radiation therapy (6). Early detection by PSA screening in combination with aggressive treatment regimens has most likely contributed to the 100% 5-year survival rates of patients treated with early-stage PCa (7). However, the risk that early-stage PCa will develop into significant PCa is unknown but believed to be quite low (7). Due to a lack of accurate biomarkers that detect, monitor, and quantify significant PCa and reliably distinguish it from more benign disease, many early-stage PCa patients are treated as patients harboring significant PCa. This has put an undue burden, physically and emotionally, on early-stage PCa patients because the invasive treatments substantially impact the quality of life (8). This problem can be tackled if two critical issues are appropriately addressed. First, the correct population of PCa patients needs to be targeted and monitored over time to determine the level of significant PCa in men. This issue is beyond the scope this review and has been covered in greater depth elsewhere (9). Second, it has to be assessed whether clinicians possess the right analytical tools to identify and monitor biomarkers during PCa tumorigenesis. New MS-based methods have been used to identify biomarkers to detect, predict, and treat significant PCa in men (10). This review will summarize the various MS-based expression platforms used to study prostate carcinoma in clinical samples. We will discuss limitations of existing methods and also highlight new applications of proteomic technologies to PCa research.

THE PROSTATE CANCER DILEMMA AND ENTRY POINTS OF MS-BASED EXPRESSION PROTEOMICS

Currently, specific biomarkers that reliably detect early PCa cells localized within the prostate gland have not been iden-
Early detection of prostate cancer (PCa) is a critical issue in clinical practice. While PSA levels above 4 ng/ml are routinely detected by PSA screening, false-negative results are common. Inadequate biomarkers to detect and predict significant PCa lead to invasive treatment of early-stage localized PCa in men. Developing robust biomarkers to detect and predict significant PCa will improve the treatment regime of early-stage localized PCa.

For example, PSA levels less than 4 ng/ml typically represent patients that lack PCa, while PSA levels above 10 ng/ml generally represent histologically confirmed PCa. Inadequate biomarkers to detect and predict significant PCa will result in invasive treatment regimens patients undergo when diagnosed with early-stage prostate localized PCa (Fig. 1). These biomarkers would usher in a long-awaited improvement in the management strategy of early-stage PCa patients.

The capacity to identify clinically relevant PCa biomarkers using MS-based expression methods will be benchmarked by the ability to define PCa along the tumorigenic pathway (Fig. 2). Obtaining unique protein signatures in normal prostate epithelium, BPH, localized, metastatic, and androgen-refractory PCa may help to identify biomarkers capable of diagnosing, monitoring, and possibly treating PCa at different stages of disease progression. The most obvious and readily available sources of biological material to identify stage-specific biomarkers reside in the serum, proximal fluids, and disease tissue of PCa patients. However, identifying and characterizing proteins from these sources has been quite difficult for biomarker discovery in PCa research and cancer research in general. To date, the most robust and well-established analytical method for detecting biomarkers in serum and disease tissue utilizes antibody-based detection methods such as the ELISA method. Developing a robust antibody reagent to detect a specific biomarker is difficult and a time-consuming process. The maturation of high-resolution analytical instruments like the mass spectrometer has generated tremendous interest in using this tool in the clinical setting to detect and monitor protein biomarkers in serum, proximal fluids, and tissues in humans. Despite the increased sensitivity of present day mass spectrometers, it is still a challenge to detect low-abundance protein biomarkers in serum, proximal fluids, and tissues. Serum has a dynamic protein expression range of 10 orders of magnitude dominated by albumin and immunoglobulins that represent greater than 80% of the total protein content. Thus, low abundance biomarkers are contained in the thousands of proteins that represent the remainder of the total serum protein mass. This poses a significant obstacle for detecting biomarkers by current mass spectrometrometric methods. In addition, PCa is a very heterogeneous disease in which tissue biopsies are small and contain a mixture of cell types. This also poses an obstacle to biomarker identification by MS-based methods because the tumor cells and their target molecules will be present at low levels, which makes their identification and quantification more difficult. Thus purification strategies that can selectively reduce the complexity of protein samples without diluting the biomarkers, as well as enrichment methods for isolating biomarkers present at low levels in small amounts of dissected tissue will be a necessary addition to mass spectrometry platforms used to identify biomarkers in clinical PCa.

**EXPRESSION PROFILING OF PCA TISSUE, FLUIDS, AND SERUM USING TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)**

Historically, 2-DE has been the tool of choice to resolve complex protein mixtures and to detect differences in protein expression patterns between normal and diseased tissue. As shown in Fig. 3, differentially expressed proteins ob-

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**Fig. 1. Three-dimensional representation of the PCa dilemma in men.** Inadequate biomarkers to detect and predict significant PCa leads to invasive treatment of early-stage localized PCa in men. Developing robust biomarkers to detect and predict significant PCa will improve the treatment regime of early-stage localized PCa.
served between normal and tumor samples are separated by 2-DE and detected by protein staining and differential pattern analysis. Selected proteins are then usually identified by MS methods (peptide fingerprinting, MS; tandem mass spectrometry, MS/MS) (19). Prior to the development of routine MS and MS/MS protein identification methods, many 2-DE studies were unable to identify the proteins that changed in their abundance between samples as observed by 2-DE (20). Several reports characterizing protein differences between normal and disease prostate belong to this category (21–23). These early studies detected protein 2-DE expression differences between samples from normal individuals and individuals affected by BPH and PCa. Samples analyzed were urine, prostatic fluids, and tissue biopsies. A 22-kDa protein with an isoelectric point (pI) of 4 was consistently detected in the prostatic fluid and urine of PCa patients (21). Fourteen proteins isolated from the nuclear matrix of prostatic tissues were differentially expressed in normal, BPH, and PCa samples (23). A more recent 2-DE analysis that used MS methods to identify differentially expressed proteins reported that surgically resected metastatic PCa tumors contained significantly higher levels of calreticulin, proliferating cell nuclear antigen, heat-shock protein 90, GST pi, superoxide dismutase, triose phosphate isomerase, oncoprotein 18, and elongation factor 2 when compared with BPH, while cytokeratin 18 and tropomyosins-1 and -2 were decreased in PCa (24, 25). Whether and how these protein expression changes effect PCa progression and whether these proteins are reliable markers for tumor progression or classification is at present not known. However, the fact that many of these proteins tend to represent highly expressed proteins in cells and tissues suggests that they might not be specific biomarkers for PCa. Another recent 2-DE study found that 20 proteins were lost in malignant tumor tissue when compared with normal prostate tissue isolated from 34 radical prostatectomy cases (26). The most biologically notable proteins identified were NEDD8, calponin, and follistatin-related protein, proteins not previously known to be expressed in normal prostate tissues. The biological significance of these observations also awaits future investigations. In general, using 2-DE methods to study PCa biomarker expression in clinical samples has been difficult due to the inherent limitations of 2-DE methodology. First, the hydrophobic, insoluble nature of membrane and membrane-associated proteins makes them incompatible with the buffers of the 2-DE system. Therefore this class of proteins tends to be significantly underrepresented in 2-DE studies (27). Invariably, due to limited dynamic range of the gel method, the most-abundant soluble proteins are typically visualized and detected by 2-DE methods. This impacts biomarker discovery and characterization in PCa in several ways. First, it severely limits the ability to detect and exploit differences in cell-surface receptor membrane protein expression that may occur between normal, BPH, and PCa tissues. Second, despite recent advances in IEF methods (28), 2-DE gel protein patterns are notoriously difficult to reproduce between laboratories (27). To address these shortcomings, a newer method referred to as DIGE, which incorporates fluorescent cyanine dyes (Cy3 and Cy5) into the proteins prior to 2-DE, has been developed (29). With this method, two protein samples differentially labeled with different fluorescent stains are processed in a single 2-DE gel so that the relative signal intensity of the different fluorescent labels can be detected by spectral analysis and, based on the ratio of signal intensities, the relative abundance of each protein in the two samples can be quantified. 2-DIGE alleviates the pattern reproducibility problem but not the other problems associated with 2-DE. Specifically, the method still requires large amounts of starting material (40–100 µg of total protein to generate upward of ~500

![Fig. 2. Expression protein profiling during PCa tumorigenesis. Secretory epithelium (EP), basal epithelium (BC). Androgen-refractory PCa develops in advanced metastatic prostate disease.](image-url)
spots/gel) to visualize adequate number of silver-stained proteins in the gel (27). Unfortunately, normal and disease prostate epithelia are heterogeneous tissues that lack “pure cell populations” (16). Methods such as laser capture microdissection (LCM), a robust method that uses a laser beam to selectively and efficiently remove target cells from surrounding cells and tissues has been used with great success to select homogeneous cell populations (30). Although LCM has been used with great success in procuring pure populations in PCa tissues (31, 32), this technology is not without limitations when used in conjunction with 2-DE. For example, LCM studies typically report extracting 50,000–70,000 cells from microdissected tissues resulting in approximately 40–80 μg of total protein (33–35). Assuming a 25-kDa biomarker is expressed at 1,000 copies/cell (low levels) and subjected to 2-DE, it would amount to running approximately ~2.9 pg (120 amol) of the target biomarker into the gel. The limit of detection for a silver-stained protein spot in a 2-DE gel is around ~1 ng (36). Thus, the biomarker would be ~2–3 orders of magnitude below visual detection by 2-DE staining methods. Even optimistically assuming very small sample losses during the complex 2-DE process, and assuming the biomarker was detectable in the gel, detecting 120 amol using a standard mass spectrometer would be very challenging. Thus, 2-DE methods may not represent the optimal proteomic platform to detect and study PCa biomarkers residing in tissues. Using 2-DE methods to identify PCa biomarkers in serum has been met with little success. The very broad, dynamic protein expression range of serum samples has severely limited the use and success of 2-DE methods for detecting PCa biomarkers. In contrast, 2-DE methodology has and will continue to be a powerful platform for identifying potential biomarkers that replicate PCa progression using in vitro and xenograft model systems (37–39). These experimental systems can generate abundant amounts of protein, which is typically not the case when clinical samples are utilized.

**EXPRESSION PROFILING OF PCA TISSUE, FLUIDS, AND SERUM USING SELDI MS**

A new MS-based proteomic approach termed SELDI coupled to the mass spectrometer (TOF mass analyzer) has sparked tremendous excitement as a diagnostic tool in cancer research (40). The SELDI approach involves extracting proteins/peptides from tissue and/or serum and applying them to an affinity capture surface located on a chip (e.g. metal affinity, IMAC-Cu; hydrophobic, C16/H4; weak cation exchange, WCX2) that selectively binds a specific subset of proteins (Fig. 2). Nonbound proteins are washed away, the captured proteins are ionized by MALDI MS, and their unique masses are recorded in a high-resolution TOF mass spec-
trometer. The SELDI-TOF method generates signatures of thousands of potential protein peaks that investigators use to compare pattern differences between normal and disease samples (41, 42). Computer algorithms are subsequently used to analyze and select “discriminatory” peaks that separate normal and diseased populations (43). It has been stated that the SELDI-TOF proteomic platform will revolutionize modern medicine and function as a rapid, robust diagnostic and prognostic tool in cancer research (10). The most notable report of the SELDI-TOF approach was reported in ovarian cancer (43). This study identified diagnostic peak patterns that identified 100% of all ovarian cancer samples and correctly assigned 95% of healthy and benign subjects correctly. As anticipated, the discriminatory power of the SELDI approach has sparked tremendous excitement in its use as a diagnostic tool in detecting PCa in men (44–49). These PCa studies have identified discriminate serum peaks (m/z) capable of distinguishing between normal, BPH, and PCa patients with sensitivities ranging from 63 to 100% and specificities ranging from 38 to 100%. Interestingly, as reviewed in greater detail by Diamandis (50), these PCa studies share very little, if any, overlap in the discriminate peaks to distinguish normal, BPH, and PCa samples even though the same chromatographic affinity surface was used to perform the SELDI-TOF analyses (46, 47). These studies highlight just a few of the underlying issues plaguing the SELDI-TOF proteomic platform that have not been satisfactorily resolved (50). First, research groups need to independently confirm the discriminate peaks reported by other research groups using the same sample preparation and analytical procedures. Second, a standardized procedure for comparing algorithms between research groups should be agreed upon so that discriminate peaks generated between research groups can be compared and validated with consistency. However, a more fundamental weakness of these SELDI-TOF studies lies in the fact that the chemical identity of the discriminating peaks used to separate normal and diseased populations are largely unknown (45–48). These discriminate peaks may separate normal and diseased populations based upon experimental bias caused by differences in how the samples were processed (51, 52). Thus, elucidating the exact sequence identity of “potential” protein/peptide peaks will undoubtedly bring overdue uniformity to this proteomic approach, which is necessary if biomarkers are to be validated as diagnostic and prognostic tools in PCa. For example, many SELDI-TOF studies have found discriminate peaks in serum samples whose protein constituents range more than 10 orders of magnitude in concentration (15). Analytically it is a tremendous challenge to detect clinically interesting biomarkers represented at concentrations of <1 ng/ml in serum that also contains highly abundant proteins at concentrations of 30–50 mg/ml. For example, assume 10 µl of serum (SELDI-TOF studies typically use 1–10 µl of serum), equivalent to 800 µg of total protein, is applied to a chromatographic affinity chip and processed for SELDI-TOF analysis. If the biomarker of interest is present at a concentration of ~1 ng/ml (PSA concentration in serum ~1 ng/ml) (11), the sample would contain 8 pg of target for mass spectrometer detection. Assuming the biomarker is fully retained on the chromatographic surface and ionized into the mass spectrometer for MS or MS/MS detection, ~320 amol of the biomarker would be available for detection. Detection of 320 amol of target would be feasible if it were directly infused into the mass spectrometer. However, detecting this target among the other protein peak masses that are bound to the chromatographic chip would be a difficult even using the most sensitive commercially available mass spectrometers. Thus, adopting methods that fractionate and effectively remove high-abundance proteins from serum prior to the affinity chromatography step is expected to increase the chance of detecting low-abundance biomarkers by the SELDI-TOF method (discussed in greater detail below). Based upon the concentration of known cancer biomarkers in serum (PSA 1 ng/ml), it is highly unlikely that the discriminatory peaks reported to date that distinguishing normal, BPH, and PCa samples represent traditional, low-abundant protein biomarkers routinely used to detect cancer in the clinic (45–49). Until the sequence identity of “discriminate” peaks are verified by MS/MS methods, great caution should be exercised in drawing biological inference from the results of these studies. However, a recently published SELDI-TOF study using radical prostatectomy samples found that the mature form of secreted growth differentiation factor 15 (GDF15) may represent an early-stage PCa biomarker (53). The authors reported this as the only single consistent protein change that was detected from 22 patient samples. Independent confirmation of these results is highly anticipated. Unlike this example, most SELDI-TOF studies fail to confirm the identity of the “discriminatory” peaks. Thus, until standards for sample processing, validation of known clinically relevant biomarkers, and unified protocols for identifying the discriminatory peaks are needed implemented, this methodology will lack uniformity in its current format. If these issues are not adequately addressed, the SELDI-TOF method will probably be unable to reach the high expectations of becoming a robust, high-throughout analytical method for diagnosing and characterizing cancer in the clinic as previously anticipated (51, 52, 54).

**EXPRESSION PROFILING OF TISSUES AND FLUIDS USING MULTIDIMENSIONAL LC METHODS**

Historically, 2-DE has been the separation tool of choice for resolving complex protein mixtures isolated from serum and tissue of normal and disease individuals (17). However, gel-free proteomic approaches that incorporate multiple steps of LC to reduce the protein and peptide complexity prior to protein identification by LC-MS/MS methods have gained broader acceptance over the past several years (19, 55). For example, a number of groups have described the fractionation procedures for whole intact proteins before the diges-
differentially expressed cell-surface proteins in normal and have been pioneered using different cellular states. These isotopic labeling approaches identification and accurate quantification of proteins between of stable isotopes into proteins allows for the simultaneous protein expression in clinical samples because the proteins are being analyzed. Recently, multidimensional chromatography has been enhanced by the incorporation of stable isotope labeled proteins directed at the selective isolation of proteins or peptides in complex samples. These methods have in common that the mass spectrometer is focused on the analysis of the targeted analyte and in the process ignores the complex matrix of peptides that are unrelated to the targeted protein. Such methods that also have the potential to determine the absolute quantity of an analyte may become increasingly important to monitor the levels of previously discovered biomarkers during PCa tumorigenesis and in oncology in general. In the AQUA method, short synthetic peptides that are chemically identical to the native target peptide but labeled with stable isotope tags serve as internal standards to precisely and accurately quantify the absolute levels of the protein after proteolysis using selected reaction monitoring in a tandem mass spectrometer (68). For example, AQUA peptides representing a specific biomarker can be added to resected normal, BPH, and PCa tissues and the abundance of the corresponding native protein can be monitored during PCa tumorigenesis. In principal, the VICAT method is very similar to the AQUA method, except the VICAT reagent reacts with cysteine-containing peptides and thus provides another level of enrichment and quantification of proteins. Multidimensional LC methods have also been used to reduce protein complexity in serum samples in the effort to identify biomarkers (69, 70). However, in the cases where the complexity of the samples is such that detectable levels of protein of interest cannot be analyzed, a pre-fractionation or pre-enrichment step will be necessary. For example, a recent study used centrifugal ultrafiltration of serum to analyze the low-molecular-weight serum proteome (69). This strategy effectively removed the highly abundant albumin and immunoglobulin proteins from the MS analysis, which resulted in the identification of over 340 human serum proteins. Another separation strategy called the “glycocapture method” has been developed that allows for the selective enrichment of glycosylated proteins in serum, cells, or tissue (70). For example, proteins normally found in serum are glycosylated on asparagine residues (N-linked glycosylation) (71). This physical property of secreted proteins was recently exploited in a newly developed glyco-capture method that successfully purified glycosylated proteins away from albumin in serum (70). The glyco-capture method was adapted so that isotopic labels were introduced into the glycopeptides after glyco-capture, which allowed for the simultaneous protein identification and quantification by LC-MS/MS (70). This method has great potential and several obvious advantages in biomarker discovery and validation in PCAs. First, albumin, the predominant protein component in serum is effectively left behind by the glycoprotein purification step, which effectively removes a large contaminant from the sample and increases the chance of detecting low-abundant biomarker proteins in the samples. Second, isotopic labeling of glycopeptides could facilitate biomarker quantification in patient samples under different disease states. The glyco-capture method could also be applied to microdissected tissue specimens because many cell-surface proteins also undergo
N-linked glycosylation. Traditional PCa biomarkers such as PSA, which is N-linked glycosylated, should be detectable and quantifiable using this methodology. An early successful example of this MS-based research approach has recently been described (72). The authors developed a high-throughput proteomic screening method implementing the power of the MALDI-TOF/TOF mass spectrometer to identify and quantify glycocaptured peptides in human serum. In short, isotopically labeled reference glycopeptides were synthesized and spiked into serum samples so that the absolute abundance of the particular glycopeptide and thus protein was determined in serum. This strategy shows great promise for the rapid and accurate screening of complex protein samples for the presence and quantity of selected proteins and demonstrates the feasibility to detect and quantify targeted proteins in a complex system using a high-throughput MS-based platform. Future studies that employ this methodology using tissue and serum samples to monitor the expression pattern of known biomarkers and identify new biomarkers in PCa samples are greatly anticipated.

FUTURE OF PCA RESEARCH USING MS-BASED EXPRESSION PROFILING

The maturation of MS technologies has given clinical scientists a powerful analytical tool to study human disease (19). There is a high likelihood that MS-based technologies and approaches will play an increasing role in biomarker identification and validation in cancer, and especially PCa. The increased sensitivity of mass spectrometers over the past several years will inevitably expedite the identification and validation of critical biomarkers involved in PCa. However, two issues, one inherent to PCa tumorigenesis, stand to block this goal from being fully being reached. First, it is very difficult to obtain sufficient amounts of diseased prostate tissue to perform in-depth protein biomarker discovery and validation using a MS-based expression method. If this issue is solved and the large majority of proteins that are expressed in different stages of PCa can be identified, one could envision a global prostate expression library that could be a basis for rational and targeted treatment regimen. Second, the broad dynamic protein expression range inherent to serum has acted as a barrier in biomarker discovery in cancer research (15). Thus, we believe utilizing MS-based expression profiling approaches to identify potential biomarkers using well-characterized in vitro and animal xenograft models that mimic human PCa development disease will play an important role in this process (Fig. 4). Integration of basic and clinic research programs will help fuel the translational research pipeline and increase the speed of biomarker discovery and validation in PCa research (Fig. 4). For example androgens, which function through the androgen receptor (AR), are critical in PCa development (73). The critical role AR plays in the PCa development is demonstrated by the fact that androgen deprivation
therapy has been the foundation for treating advanced PCa (74). Recent studies have shown that reactivation of AR-mediated cell growth pathways is a major mechanism propelling the growth of androgen-refractory PCa (75, 76). Thus carefully defining AR-regulated cell growth pathways may identify potential biomarkers that change in expression as PCa transitions to the androgen-refractory state (73, 77, 78). Expression profiling studies that detect potentially interesting biomarkers in model in vitro and xenograft PCa systems can be probed directly in PCa tissue and serum samples. This research strategy is analogous to the “reverse genetic” approach commonly used by molecular biologist to study a gene(s) function. Here the gene of interest is mutated and studied to see what role it plays in normal development and disease. Here we apply an analogous “reverse proteomic” approach in which a selected group of potential protein biomarkers are followed using “AQUA-like” technologies so that their expression levels can be monitored and correlated in normal and diseased states in tissue and serum samples during PCa tumorigenesis. Essentially a prostate peptide biomarker database can be developed and implemented to help detect and monitor the expression levels of potential biomarkers during prostate progression in cancer patients. Ideally, this approach may address some of the traditional problems that plague clinical proteomics today, which include low levels of protein material extracted from diseased tissue, and dealing with the enormous protein complexity problems presented by serum. Using a directed proteomic approach employing AQUA, VICAT, or alternative quantitative MS methods would hopefully extract known biologically relevant needle(s) out of the haystack as opposed to identifying unknown needle(s) in the haystack. Only time will determine whether this approach may also lead to better diagnostic, prognostic, and therapeutic treatments for significant PCa in men.

§ To whom correspondence should be addressed: Michael E. Wright, UC Davis Genome Center, Department of Pharmacology and Toxicology, University of California Davis School of Medicine, Davis, CA. E-mail: mewright@ucdavis.edu. Ruedi Aebersold, Institute for Molecular Systems Biology, ETH-Zuerich, and Faculty of Natural Sciences, University of Zurich, Switzerland. E-mail: aebersold@biotech.biol.ethz.ch.

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