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INVITED REVIEW

Analysing signalling networks by mass spectrometry

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Abstract Sequence analysis of the human genome and the association of genetic aberrations with diseases have provided a rough framework whereby the impact of individual genotypes can be assessed. To fully understand the effect of individual and co-occurring genetic aberrations, as well as their individual and collected contribution to the development of diseases, it is critical to analyse the matching proteome and to determine how the organisation, expression level and function of protein networks are affected. Sensitive mass spectrometric platforms in combination with innovative workflows allow qualitative and quantitative analyses of the cellular as well as the extracellular proteome. Importantly, in addition to specifically identifying the content of the proteome, several aspects of the proteomic organisation can be analysed including protein complexes, protein modifications, enzymatic activities and subcellular/organelle localisation. Together, these measurements will provide novel insight into the biological effect of disease-causing mutations ultimately coupling genotype and phenotype.

Keywords Signal transduction · Mass spectrometry · Protein kinase signalling

Introduction

Recent advancements in mass spectrometric (MS) platforms and workflows for proteomics have permitted proteome-wide qualitative and quantitative analyses of several parameters defining protein function including abundance, modification and stability. In addition, protein–protein interactions and the formation of protein networks are now readily interrogated with recent studies comprising analysis of individual protein distribution between distinct complexes. One of the unique capabilities of MS-based proteomics is the ability to address biological samples in a hypothesis-free (known as discovery-based) or hypothesis-directed (known as directed or targeted analysis) manner, underscoring the versatility and complementarity of these approaches. In this review, we will provide recent examples where MS-based proteomics have provided unique insight into the cellular organisation and function of proteins with a particular emphasis on cellular signalling. Furthermore, we will discuss how proteomics can be used as an analytical tool to determine the impact of genetic aberrations on the functional proteome.

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Mass spectrometry based proteomics

Protein identification and quantification

To delineate the impact of individual genetic changes on cellular function, it is important to have a basic understanding of the composition of the cellular proteome. This should include a description of expression level, modification, stability and localisation of individual proteins as well as their splice variants. Since MS can identify and quantify protein abundance in both a hypothesis free and in a targeted manner, it provides an eminent tool for global expression analysis (Domon and Aebersold 2010) (Fig. 1). In undertaking a hypothesis-free approach, also known as discovery-based proteomics, the samples analysed on the mass spectrometer are subjected to identification, which is dependent on the relative abundance of individual peptides.

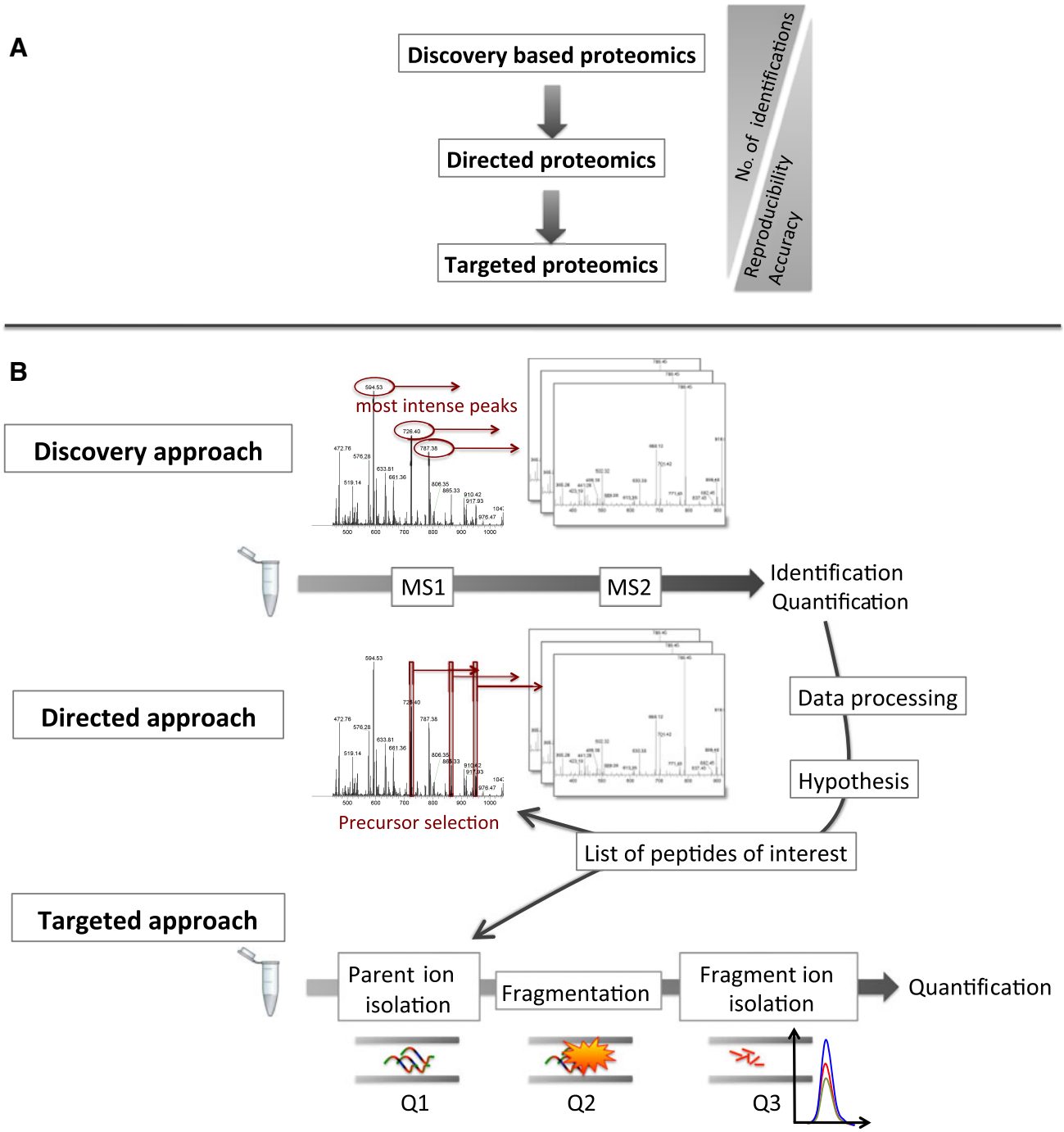

Fig. 1 Hypothesis-free and hypothesis-based mass spectrometric approaches. The number of identified peptides and the accuracy of their quantification depend on the chosen approach (**a**). A hypothesis-free discovery-based approach allows the identification of numerous peptides. Targeting peptides of interests by a directed or targeted approach reduces the number of identifications while increasing the accuracy of their quantification and the reproducibility of the experiments. **b** The discovery approach involves MS/MS analysis of the most abundant peptides in the sample, whereas the directed approach only triggers fragmentation for a defined list of peptides of interest. The targeted approach, here exemplified using a triple quadrupole, includes isolation of peptides of interest in the first quadrupole (Q1), fragmentation in the second (Q2) and isolation of specific fragment ions in the third (Q3) for identification and quantification

Therefore, for a given set of peptides observed in the mass analyser, selection of individual peptides for subsequent isolation and identification is dependent on their relative abundance. As such, while discovery-based MS is an eminent tool for studying novel cellular effects where limited prior knowledge is available, coverage of lower

abundance peptides and proteins can be sparse. In contrast, MS can also be used to analyse specific peptides and proteins of interest, known as directed or targeted analysis. In this case, prior knowledge of the analyte and a defined hypothesis of the biological system interrogated are required. Since other peptides and proteins present in the sample are not analysed in parallel, target analysis has the distinct advantages of increased sensitivity and dynamic range for quantification. For more in depth description of discovery-based and targeted analysis, the reader is referred to recent excellent reviews (Lange et al. 2008; Domon and Aebersold 2010; Steen and Mann 2004).

Discovery-based proteomics

Using these orthogonal approaches, MS-based proteomics can readily be applied to the identification and quantification of global differences in the composition of the cellular proteome (Fig. 2). An illustration of the power of discovery-based analysis was recently provided by de Godoy and colleagues (de Godoy et al. 2008) where the proteomic composition of haploid and diploid yeast was compared. Here, the authors identified several differentially expressed proteins of the mating response, thereby showcasing the ability of mass spectrometry to directly map pathways that are impacted by genetic differences with limited prior knowledge. Other types of genetic perturbations have also been successfully interrogated by discovery-based proteomics. For example, global analysis of cells expressing individual microRNAs revealed widespread, albeit minor changes, on protein expression and synthesis (Selbach et al. 2008; Baek et al. 2008). In addition, changes in the proteomic composition of cells have been identified following disruption of single genes by RNAi (Bonaldi et al. 2008) and have recently been extended to the analysis of genetic perturbations in an organismal fashion in mouse (Krüger et al. 2008), *D. melanogaster* (Sury et al. 2010) and *C. elegans* (Dong et al. 2007). From a disease perspective this is very powerful since genomic alterations now can be linked to differences in protein expression level. For instance, MS-based expression analysis has been used to identify changes in the level of proteins concurrent with amplification of specific genomic regions (Geiger et al. 2010), suggesting that direct links between genetic aberrations and disease-causing alterations in the proteome can be identified.

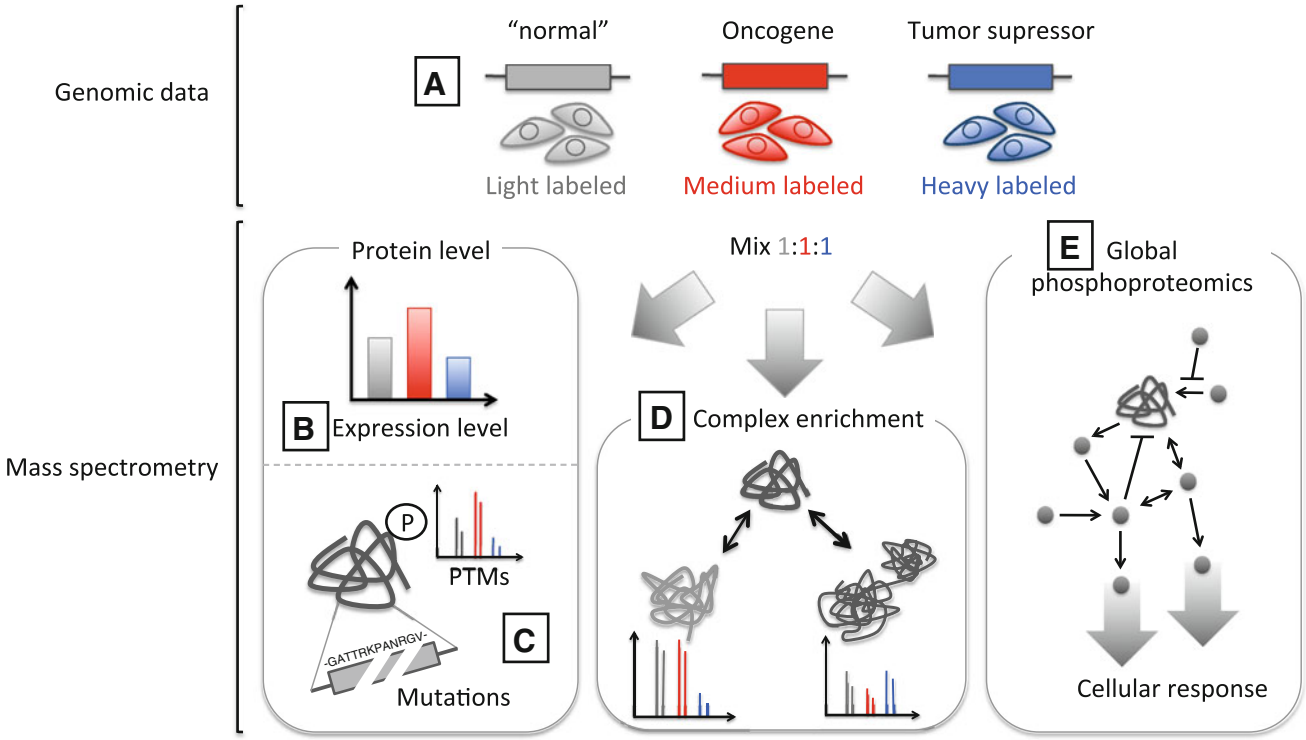

Fig. 2 Linking genotype to proteomic alterations. In order to fully understand the impact of disease-causing genetic aberrations, the effect on protein expression, function and modifications must be identified and integrated. MS analysis of labelled cells (**a**) allows relative quantification at the protein level (**b**), mutation identifications and analysis of post-translational modifications of a protein (**c**). Characterisation of protein complexes can be performed by enrichment of a bait protein followed by analysis of its associated partners (**d**). Signalling networks are constructed by integration of these data in response to a stimulus and in function of time, and then refined with data from in vitro and in vivo studies (**e**)

Targeted proteomics

While mass spectrometers have become increasingly more sensitive and sample preparations more sophisticated, the dynamic range of the proteome, and especially the human proteome, still represents a major obstacle to high reproducible and in-depth expression analysis (Anderson and Anderson 2002). To overcome these challenges, proteomics methodologies have been developed for directed and targeted analysis (Domon and Aebersold 2010). In contrast to discovery-based analysis, these approaches require prior knowledge of the peptides and their fragmentation patterns before implementation. The increased specificity arises as a consequence of selected scanning for specific combinations of precursor and fragment ions (known as transitions) on dedicated MS platforms such as triple quadrupoles. This results in increased dynamic range for quantification (Lange et al. 2008). Targeted analysis has widespread applications in the analysis of proteins, their abundance, interactions, half-life and modifications. A recent example highlights the feasibility of these studies where targeted analysis was used to identify and quantify the level of metabolic enzymes in yeast. Impressively, the authors identified and quantified proteins down to below 50 copies/cell (Picotti et al. 2009). The specificity and sensitivity of targeted analysis has had a significant impact in biomarker research. Importantly, the robustness of this workflow also limits inter-laboratory variability in identification and quantification of peptides with CVs below 20 % (Prakash et al. 2010), thereby ensuring that clinical relevant observations can be validated across institutions. Thus, following the initial discovery-based analysis, targeted analysis offers a robust way to validate an initial hypothesis on a larger scale. Furthermore, targeted analysis has recently proved useful in high-density analysis of protein–protein interactions (Bisson et al. 2011) and phospho-proteomics analysis (Wolf-Yadlin et al. 2007), illustrating that cellular signalling now can be analysed in a high content and reproducible manner by MS.

The cumbersome process of identifying and validating suitable peptides for targeted analysis has limited proteome-wide analysis. To overcome this hurdle, Picotti et al. developed and optimised a workflow for high throughput establishment and evaluation of peptide transitions for targeted analysis. Here, synthetic versions of predicted proteotypic peptides (Picotti et al. 2010) were analysed by MS on targeted- and discovery-based platforms. This workflow makes it feasible to generate transitions for approximately 100 peptides/h of instrument time. Importantly, although this allows the generation of a large number of transitions, synthesis of isotopically labelled peptides is still costly. Directed analysis therefore offers a link between discovery and targeted proteomic applications, where peptides of interest are repeatedly analysed using discovery-based platforms or where only peptides of predefined mass and elution profiles are subjected to analysis (Fig. 1). This can now be conducted at increased reproducibility, where recent studies obtained ~70 % overlap between replicate analyses (Schmidt et al. 2008). Peptides of interest can therefore be identified using discovery platforms, and subsequently analysed at higher reproducibility by directed analyses. This will then be followed by validation and quantification in targeted analyses (Schmidt et al. 2008; Domon and Aebersold 2010). Together, these workflows have the potential to increase our analysis of the proteomic alterations resulting from genomic aberrations. This has important implications in biomarker detection and validation, which recently was illustrated by targeted analysis of activating mutations in Ras (Wang et al. 2011) and changes in plasma biomarkers (Lopez et al. 2010, 2011; Rifai et al. 2006).

Determining the components of cell signalling complexes

Identifying and analysing protein–protein interactions

Organisation of proteins in complexes impacts multiple cellular functions by controlling the specificity and fidelity of signalling processes (Pawson and Nash 2003). Therefore, by determining the components of protein complexes and the dynamics by which they are rearranged, their association with specific cellular processes can be delineated. Furthermore, analysis of genetic aberrations and their effects on protein interactions as well as their cellular function, will allow us to better understand the molecular events underpinning disease phenotypes (Fig. 2).

Mapping the cellular organisation of proteins is a major undertaking, which relies on several technological platforms to identify protein complexes, binary interactions as well as their dynamic regulation (Gavin et al. 2006; Ho et al. 2002; Walhout et al. 2000; Tarassov et al. 2008; Barrios-Rodiles et al. 2005). The application of mass spectrometry to the analysis of protein complexes has had a major impact on our understanding of the cellular organisation (Gavin et al. 2006; Ho et al. 2002; Krogan et al. 2006). Typically, proteins of interest are purified using antibodies targeting endogenously expressed proteins or by isolating ectopically expressed and tagged versions of target proteins (Gavin et al. 2006; Ho et al. 2002). Co-purified partners are then identified and quantified by MS. Notably, several workflows exist, each offering specific advantages and disadvantages (Gingras et al. 2007).

In a recent example, mass spectrometric identification of protein interactions was used to generate a high-density map of kinase interactions in yeast (Breikreutz et al. 2010). Here, the authors expressed tagged versions of kinases and phosphatases from an inducible promoter followed by affinity purification and identification of interacting proteins. Although high levels of bait protein expression would be anticipated to result in elevated numbers of false-positive interactions, this approach appeared to work advantageously for identifying kinase and phosphatase interactions. This is evident by the finding that several of the identified interacting proteins were substrates in vitro. Furthermore, several interactions were functionally validated. Since kinase/phosphatase–substrate interactions are transient by nature, inducible expression of the baits could give rise to a more efficient capture of target proteins. This underscores the importance of selecting the correct purification strategy, which depends on the class of proteins studied. Interestingly, the authors observed a high level of kinase–kinase interactions as well as a high number of phosphorylation sites on the identified kinases, supporting that kinase–kinase network is critical to signalling fidelity. This is further corroborated by the observation that yeast kinases have a higher than average number of phosphorylation sites (Chi et al. 2007) and that a disruption of kinases has system-wide effects on phosphorylation-dependent signalling (Bodenmiller et al. 2010). Affinity purification coupled with MS-based identification of interactions has furthermore been used for large-scale analysis of protein–protein interactions in Human (Ewing et al. 2007) as well as in *D. melanogaster* (Guruharsha et al. 2011). Furthermore, this approach can also be used in more selected manner to study specific cellular protein classes or signalling networks (e.g. Friedman et al. 2011; Sowa et al. 2009).

Stoichiometry of protein interactions: towards interaction networks

An added benefit of using affinity purification coupled with mass spectrometry to define molecular complexes is that the stoichiometric composition can be addressed. Examples of this type of approach include the analysis of the PP2A complex (Wepf et al. 2009), ubiquitin ligase and deubiquitinating enzymes (Behrends et al. 2010; Sowa et al. 2009; Bennett et al. 2010), the nuclear pore complex (Alber et al. 2007a, b) and transcriptional co-activator complexes (Malovannaya et al. 2011). As an example, Wepf et al. established a methodology whereby the relative abundance of individual proteins can be determined within as well as between protein complexes. Here, the absolute amount of selected components were initially determined and subsequently used as 'internal standards' to determine the relative abundance of interaction partners between complexes (Wepf et al. 2009). Alternatively, the abundance of individual components can be directly determined using stable isotope-labelled peptide standards such as AQUA or QconCat (Gerber et al. 2003; Pratt et al. 2006), which readily provide information of stoichiometry (Behrends et al. 2010; Sowa et al. 2009; Bennett et al. 2010). Finally, the relative amount of each of the identified interaction partners can be determined by correlating the co-precipitation 'efficiency' between purifications. This becomes powerful when multiple antibodies are used to purify the target of interest and was recently used to deconvolute interaction networks of transcriptional co-activator complexes (Malovannaya et al. 2011). Together, these approaches each provide valuable insight into the macromolecular assembly of protein complexes. Importantly, as determination of stoichiometry and absolute quantification become standard in these analyses, meta-analysis can be readily conducted to investigate the impact of cellular stimulation and mutational status.

Post-translational modifications in cellular function

Mass spectrometry for global analysis of post-translational modifications

Cellular information processing is crucial for the cell to swiftly respond to changes in the extracellular environment as well as for fine-tuning intracellular processes such as progression through the cell cycle, migration or apoptosis. Cellular response to these signals can be controlled through multiple processes such as by adjustment of mRNA abundance, translation, protein stability, localisation and interactions. In addition, to ensure rapid and reversible regulation of the cellular response, post-translational modifications have been adapted as a way to interpret multiple and sometimes conflicting signals. To date, more than 200 possible protein modifications have been identified, generating a high-dimensional signalling space for the cell to exploit (Jensen 2006). Some of the better-described protein modifications include glycosylation, acetylation, phosphorylation and ubiquitination. Importantly, these modifications can control protein function in a combinatorial fashion, whereby the cellular response can integrate information such as signal intensity and duration. As an example whereby post-translational modifications regulate signalling fidelity, modifications within a specific sequence of amino acids results in the formation of recognition motifs, which subsequently recruit interaction partners through specific protein domains. This thereby allows for ordered assembly and disassembly of protein interactions in a dynamic regulated

manner (Yaffe 2002; Scott and Pawson 2009). Therefore, global analysis of protein modification is critical to our understanding of the cellular response to changes in intra- or extra-cellular environment.

Mass spectrometric analysis of modified proteins, and peptides, is an essential tool for both discovery and initial characterisation. However, global analysis of post-translational modifications is a challenge due to their low stoichiometric abundance. Therefore, analyses typically require enrichment strategies prior to the identification and quantification (also see recent reviews by Jensen 2006; Witze et al. 2007). Antibody-mediated affinity purification of target proteins and modified peptides (Rush et al. 2005; Moritz et al. 2010; Zhang et al. 2005) has permitted global analysis of phosphorylation, acetylation and ubiquitination (Choudhary et al. 2009; Peng et al. 2003; Xu et al. 2010; Wagner et al. 2011; Kim et al. 2011). The basis for these enrichment strategies is the development of an antibody recognising a specifically modified peptide for capture and analysis. This approach has been used to identify and quantify global changes in protein acetylation (Choudhary et al. 2009), serine/threonine phosphorylation (Matsuoka et al. 2007; Moritz et al. 2010), tyrosine phosphorylation (Rush et al. 2005; Rikova et al. 2007) as well as protein ubiquitination (Xu et al. 2010; Wagner et al. 2011; Kim et al. 2011). These studies have proved very important to the general understanding of protein modifications and cellular organisation.

Protein stability and ubiquitination

There has been considerable interest in determining protein stability, and several MS-based workflows have been established to monitor global changes. Importantly, these workflows each provide a unique and complementary analytical tool. Classically, protein stability would be determined by pulse-labelling experiments where the incorporation of S-35 methionine would be monitored for a protein of interest. With the advent of stable isotope labelling for quantitative proteomics (Ong et al. 2002; Oda et al. 1999) it has now become feasible to determine protein stability on a global level. Importantly, this approach also permits analysis of protein synthesis where the incorporation of heavy-labelled amino acids specifically marks the newly synthesised protein (Selbach et al. 2008; Schwanhausser et al. 2011). Interestingly, analyses of protein turnover have recently shown a high correlation between translation efficiency and protein abundance, whereas the regulation of gene expression was found to have less impact (Maier et al. 2011; Schwanhausser et al. 2011). Integration of such data with a description of protein complex stoichiometry (Kuhner et al. 2009) further suggested that proteins in complexes with stable subunit stoichiometry had a lower half-life variation (Maier et al. 2011).

One of the best-described modifications regulating protein stability is ubiquitination, where one effect is that targeted proteins are subsequently degraded. Therefore, much interest exists in identifying ubiquitinated proteins. To address this, MS has proved an essential role where earlier experiments have been using tagged versions of ubiquitin or ubiquitin-recognising domains to enrich for modified proteins (Peng et al. 2003; Danielsen et al. 2011; Xu et al. 2009; Akimov et al. 2011). However, until recently, it was challenging to specifically identify the modified peptides and therefore determine the site of ubiquitination. In a recent series of papers (Xu et al. 2010; Wagner et al. 2011; Kim et al. 2011), newly developed antibodies specifically recognising the characteristic di-Gly modified lysine (existing as a result from digestion of ubiquitinated proteins with trypsin) have successfully been used to identify thousands of modified peptides. Notably, through these large-scale studies it became clear that protein ubiquitination is a widespread modification affecting several cellular processes from vesicle trafficking, tyrosine kinase signalling and immune signalling. Furthermore, several ubiquitinated proteins serve as hubs in protein interaction networks underscoring the impact of this modification in regulating cellular information processes. Curiously, Kim et al. (2011) noted that following proteasomal inhibition, the ubiquitinated portion of many proteins increased in the absence of overt alterations in total protein levels, indicating a paradox between protein ubiquitination and proteasomal degradation that requires further interrogation.

Protein phosphorylation, identification and quantification

Protein phosphorylation impacts multiple aspects of cellular function through its ability to control protein–protein interactions, localisation, activity and degradation. Protein phosphorylation arises as a result of the transfer of a phosphate group from ATP onto the hydroxyl amino acids serine, threonine and tyrosine. While protein kinases add the phosphate group, protein phosphatases remove the added phosphate group. Deregulated kinases (and phosphatases) have been extensively linked to the development of diseases such as cancer, which has resulted in recent efforts to develop specific inhibitors.

Mass spectrometry has had a significant impact on the understanding of protein phosphorylation through its ability to specifically determine the position of modification and to quantify the amount of a given phosphorylation site. Today, several complementary approaches exist whereby phosphorylated proteins and peptides can be enriched.

Some of the widely used approaches include immobilised metal affinity purification (IMAC), titanium dioxide (TiO_2) and phosphoamidate chemistry (PAC) (Tao et al. 2005; Ficarro et al. 2002; Pinkse et al. 2004). Recently, additional protocols have emerged including the combination of IMAC and TiO_2 (Thingholm et al. 2008), IMAC and phosphate precipitation (Zhang et al. 2007) as well as combinations of peptide fractionation and phospho enrichment, such as strong cation exchange (SCX), hydrophilic liquid chromatography (HILIC), electrostatic repulsion–hydrophilic interaction chromatography (ERLIC) and phospho-enrichment by IMAC or TiO_2 (Choi et al. 2008; Villén and Gygi 2008; Albuquerque et al. 2008; Bennetzen et al. 2010). With these workflows proteome-wide and quantitative analysis have been undertaken of tyrosine kinase signalling (Olsen et al. 2006), DNA damage response (Bennetzen et al. 2010), nuclear phosphorylations (Beausoleil et al. 2004) and cell-cycle progression (Dephoure et al. 2008; Olsen et al. 2010). In addition, novel insight has been provided for the phosphorylation-dependent regulation of embryonic stem cells (Prokhorova et al. 2009; Van hoof et al. 2009) and tissue-specific signalling in vivo (Huttlin et al. 2010). In addition, using global phospho-proteomics analysis, Bodenmiller et al. (2010) recently identified widespread changes in the phospho-proteomic composition following systematic disruption of yeast kinases. This study further underlines the interconnected structure of kinase signalling and underscores the necessity of global analysis in kinase signalling studies. Importantly, while each of the above-mentioned approaches has been successfully employed in global phospho-proteomics analysis, they recover complementary parts of the phosphoproteome (Bodenmiller et al. 2007), suggesting that complementary strategies are necessary for complete mapping.

In addition to these approaches, tyrosine-phosphorylated proteins have been more specifically interrogated through the use of specific phospho-tyrosine-recognising antibodies and recognition domains (Pandey et al. 2000; Blagoev et al. 2003, 2004; Kratchmarova et al. 2005). Moreover, phospho-tyrosine-recognising antibodies were recently used to specifically enrich tyrosine-phosphorylated peptides, thereby increasing the number of phosphorylation sites identified (Rush et al. 2005). This approach has proven particular important to our understanding of phospho-tyrosine-dependent signalling (Rikova et al. 2007; Guo et al. 2008), since this modification is particularly low abundant (approximately 1–2 %, Hunter and Sefton 1980; Olsen et al. 2006). These workflows have permitted novel insight into signalling aberrations in cancer cells (Huang et al. 2007; Rikova et al. 2007; Guo et al. 2008), and tyrosine kinase signalling (Blagoev et al. 2004; Kratchmarova et al. 2005; Jorgensen et al. 2009; Rubbi et al. 2011). In contrast to phospho-tyrosine-recognising antibodies, motif-based affinity purification of phospho-serine and phospho-threonine is restricted by the high sequence specificity imposed by several of these kinases. Although this could be viewed as a disadvantage, the distinct benefit is that specific signals now can be directly interrogated. For example, DNA damage signalling and Akt signalling were recently analysed by antibodies specifically recognising either ATM/ATR or Akt consensus sites (Matsuoka et al. 2007; Stokes et al. 2007; Moritz et al. 2010). In addition to increasing our understanding of signalling dynamics, several of these studies have provided the foundation for evolutionary analysis, which have brought novel aspects to our interpretation of the mechanisms that regulate signalling fidelity (Tan et al. 2009; Levy et al. 2010; Gnad et al. 2010; Beltrao et al. 2009).

Defining functional relationships within signalling networks: kinases and substrates

The technological advancement in phospho-peptide purification in combination with increased sensitivity and speed of current mass spectrometers has markedly increased the number of identified phosphorylation sites, leading to a widening in our knowledge gap of kinase–substrate relationships. Since analysis of individual kinase–substrate relationships is labour intensive, there is a growing need for techniques associating individual kinases with cognate substrates. In a recent series of papers, a combination of genetically engineered cells and specific kinase-inhibitors has been used to identify kinase substrates of mTORC1, Aurora kinase A, ERK2 and CDK1 (Yu et al. 2011; Hsu et al. 2011; Koch et al. 2011; Holt et al. 2009; Carlson et al. 2011). For example, mTORC1 targets were recently identified by systematic phospho-proteomic analysis of cells treated with inhibitors and activators of mTORC1. Fibroblasts deleted in genes responsible for tuberous sclerosis (TSC1 and 2) have high levels of mTORC1 activity and thus direct mTORC1 targets would be expected to decrease in abundance following inhibitor treatment. Importantly, by integrating the effects from several inhibitors, off-target and feedback-regulated phosphorylations were excluded thereby creating a high confidence data set. Using a similar strategy, in vivo substrates of cdk1 and Aurora kinase A were recently identified in yeast. Here, the endogenous kinase was substituted for an analogue sensitive version, permitting a highly specific inhibition by the addition of 1NM-PP1 (Bishop et al. 2000; Holt et al. 2009; Koch et al. 2011). The sequences of regulated substrates overlapped with the known consensus site for cdk1, highlighting that specific in vivo experiments can also be used to derive information regarding kinase specificity. Furthermore, most of the regulated sites were high confidence endogenous targets.

Although this suggests that most kinase–substrate relationships readily could be identified through similar approaches, selective high affinity inhibitors only exist for a small subset of kinases. Furthermore, small molecule inhibitors frequently exhibit 'off target' effects, which can skew the analysis. To overcome this, putative kinase–substrate relationships have been filtered by combining information of their substrate specificity (Mok et al. 2010; Miller et al. 2008) with interaction data (Linding et al. 2007). The basis for this approach is the notion that kinases are more likely to phosphorylate a given substrate connected through protein networks. In fact, calculations suggest that up to 70 % of the specificity of kinase–substrate recognition is dependent on interactions (Linding et al. 2007). To further pinpoint relevant kinase–substrate relationships, it would be beneficial to have simultaneous and kinome-wide measurements of kinase activities. By including this additional layer of information, the dynamics of the kinase activities could be related to the kinetics of substrate phosphorylation. Unfortunately, kinome-wide analysis of the specific activity of individual kinases is still not feasible by conventional technologies such as affinity capture coupled with activity measurements. However, proof of principle experiments have indicated that kinase activities can be measured using peptides matching the sequence specificity of individual kinases as a substrate. Phosphorylation of the peptide can then be quantified by MS (Cutillas et al. 2006; Yu et al. 2009; Kubota et al. 2009). Here, the specific kinase activity can be determined in a single kinase–substrate reaction or multiplexed for up to 90 substrates in parallel. As a consequence of the experimental setup, the phosphorylation of individual peptides cannot yet be ascribed to a single kinase and therefore the combined effect on multiple peptides must be used as a proxy for kinase activity. Importantly, once this assay has been benchmarked, it holds great promise as a high throughput assay to identify targets of inhibitors and to globally determine the activation state of the cellular kinome.

As an alternative approach to identify targets of inhibitors, proteomics has also been used to determine substrate specificities through competition experiments. Using immobilised promiscuous small molecule inhibitors, ATP or nucleotide acyl phosphates, kinases and other purine-binding cellular proteins can be isolated and identified by MS (Bantscheff et al. 2007; Daub et al. 2008; Oppermann et al. 2009; Patricelli et al. 2007, 2011). By including small molecule inhibitors, competition between the inhibitor and the affinity matrix can be determined on a kinome-wide basis. Using this approach, several known and novel drug–kinase relationships have been identified. Importantly, in contrast to methods using recombinant kinases, the relative cellular expression level of kinases, some with overlapping drug-specificities, can be taken into account. This is important, as it will impact the cellular response to drug–treatment in vivo. In addition, this methodology is not restricted by prior notions of relevant kinase–drug interactions and as such novel relationships may be identified. Although the throughput and scale of the proteomics approach is currently lagging behind alternative methodologies using recombinant kinases (Fedorov et al. 2007; Karaman et al. 2008), the advantages justify the approach as a parallel bioassay.

Stoichiometry of protein modification: importance and challenges

As enrichment methods for protein phosphorylation, in general, relies on specific capture of phosphorylated peptides and thereby discards non-phosphorylated peptides, the stoichiometry of individual phosphorylation events is rarely determined. Therefore, one critical assumption is that the composition of the proteome remains unaltered between the experimental conditions and that the identified change in protein phosphorylation is a direct consequence of altered kinase and phosphatase activities. Critically, determining the phosphorylation stoichiometry will provide valuable insight into the putative effect of the phosphorylation. For example, a threefold increase in the level of phosphorylation could be the result of a 1–3 or 10–30 % increase in protein phosphorylation stoichiometry (Fig. 3). While an increase of 1–3 % might be sufficient for activating enzymatic activity, it would be unlikely to have a significant inhibitory effect. Targeted approaches have recently been applied to accurately determine phosphorylation stoichiometry using a combination of label-free and standard peptides (Domanski et al. 2010; Jin et al. 2010; Johnson et al. 2009). As an example of label-free analysis, Steen et al. calculated the 'flyability' of the peptides and used this to determine phosphorylation stoichiometry. This approach was subsequently expanded to rigorously determine the phosphorylation stoichiometry of the anaphase-promoting complex in mitotic cells (Steen et al. 2005, 2008). Recently, analyses have included parallel quantitative analysis of the proteome and phospho-proteome in order to determine the phospho-site stoichiometry. For example, phospho-proteomic analysis of wild type and yeast deleted for components in the mating pathway, Fus3 or Ste7, was normalised to the relative protein abundance. This showed that 25 % of seemingly regulated phospho-peptides were a consequence of differences in protein abundance (Wu et al. 2011a). Interestingly, by determining the relative levels of phospho-peptides as well as protein levels, Olsen et al. (2010) calculated phosphorylation site occupancy during mitosis and observed a significant increase in site occupancy compared to asynchronous cells.

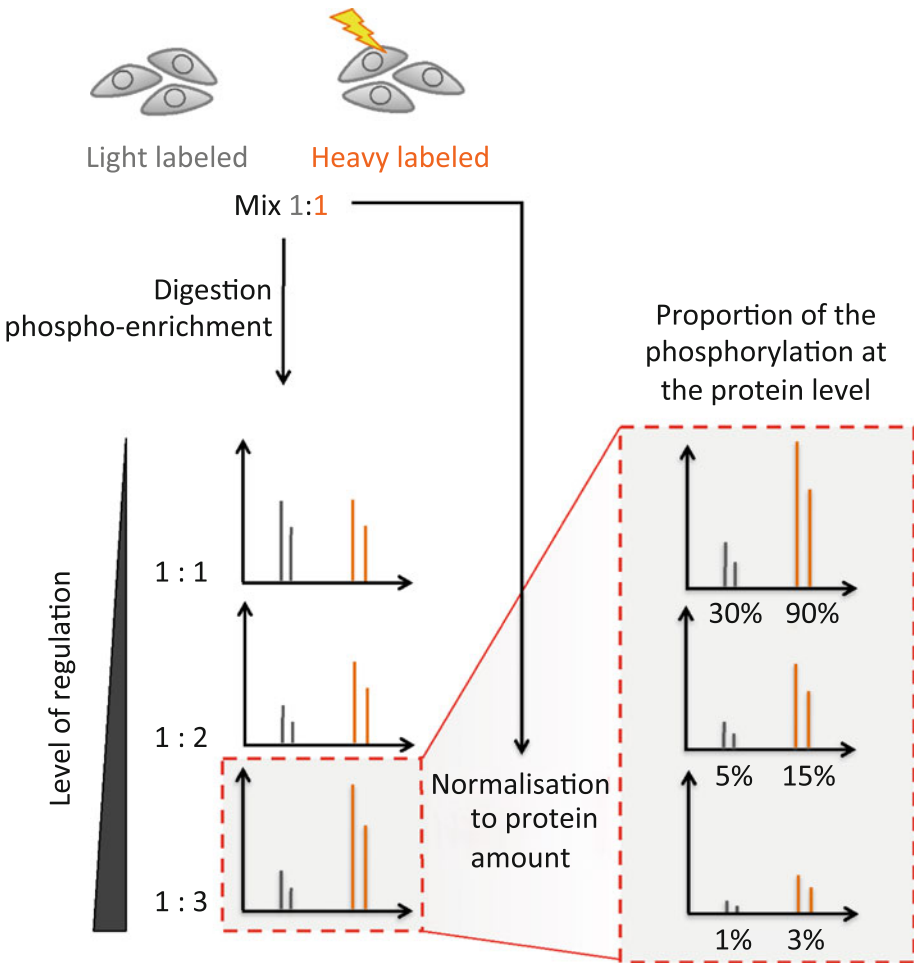

Fig. 3 Determination of phosphorylation stoichiometry. Analysis of phosphorylation stoichiometry is critical in order to obtain further biological insight. Comparison of the level of post-translational modification between differently labelled samples results in the identification of regulated proteins (*left*), whereas normalisation to the protein amount is necessary to obtain the stoichiometry of the signal (*right*). The relative abundance of a modified peptide may lead to a different hypothesis of the cellular function once the modified site is normalised to the protein abundance

Phosphorylation stoichiometry can also be determined by combining phosphatase treatment with quantification. Here, one half of the sample is phosphatase treated, thereby removing all phosphorylated forms, and is subsequently compared to the control treated sample. Analysis of these samples will therefore provide information of peptide phosphorylation stoichiometry by quantitatively comparing the relative levels of the non-modified peptides (Johnson et al. 2009; Wu et al. 2011b). Although this type of workflow provides a straightforward analysis of global protein phosphorylation stoichiometry, the exact position of the phosphorylation within peptides with more than one phospho-acceptor site cannot be determined. However, Wu et al. used this approach to conduct a comparative analysis of yeast phosphorylation stoichiometry and showed that phosphorylation sites containing acidic (casein kinase-II) like motifs were overall of higher stoichiometry, whereas proline-directed phosphorylation events were of low stoichiometry (Wu et al. 2011b). Furthermore, in agreement with previous publications (Tan et al. 2009; Holt et al. 2009), most of the phosphorylation sites were in disordered regions. Interestingly, the authors noticed that phosphorylation sites in ordered regions were of low stoichiometry and that they were overall more conserved than sites in disordered regions. With these findings in mind, it would be interesting to revisit some of the earlier global phosphorylation analysis of various cancerous cells and tumour biopsies. While most of the findings in these reports were based on the relative abundance of phosphorylation between samples, determination of the stoichiometric abundance of targets in control and tumour samples might provide further valuable insight.

Perspective

To fully utilise the continuous identification of genomic aberrations in disease, it is crucial to establish the link from genotype to phenotype. Recent developments in mass spectrometric platforms and sample preparations make it feasible to study the impact of genetic aberrations on the cellular proteome. To further interrogate these effects, it is important to determine the effects on protein expression, signalling complexes and post-translational modifications. However, it is also critical to continuously evaluate the systems that are being interrogated. For example, MS-

based analysis of cellular signalling is typically carried out by comparing control treated cells to cells that have been perturbed by addition of ligands, inhibitors or other treatments. These types of analysis have provided much valued insight. However, the cellular environment comprises multiple cell types, extracellular matrix components and other factors not easily recapitulated in a petri dish. Therefore, one outstanding question is how best to determine the impact of these environmental factors on the cellular proteome. For example, to analyse the cell-specific signals from co-culture experiments and subsequently determine their effect on cellular behaviour, tools are required to de-convolute data to establish the cellular origin of identified peptides. To enable mass spectrometric analysis of cell-specific signalling between co-cultured cells, stable isotopic labelling (Ong et al. 2002; Oda et al. 1999) can be used as a lineage tracer as well as for relative quantification. This approach has recently been used to identify contact-initiated signalling in a cell-specific manner (Jorgensen et al. 2009) as well as to identify cell-specific synthesis of proteins from pathogens (Rechavi et al. 2010). Therefore, by further development of technology and workflows, MS-based analysis will continue to play a role in understanding basic biological and disease-causing mechanisms.

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