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Mass spectrometry-based proteomic analysis of the DNA damage response

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1. ABSTRACT

In response to DNA damage, cells have evolved mechanisms to halt cell cycle progression, activate repair, or initiate apoptosis. These DNA damage response (DDR) pathways are critical for cellular survival in response to genomic insult, and play important roles in growth, development, and disease. Historically, mediators of DNA damage response signaling have been studied one or a few proteins at a time. Advances in mass spectrometry instrumentation and enrichment methods now allow for more global analysis of the DDR in cells and tissues. In this review we will discuss current methods in liquid chromatography tandem mass spectrometry (LC-MS/MS), enrichment strategies, and targeted analyses for the study of damage signaling. These methods have allowed a greater understanding of the DNA damage response and have highlighted the far-reaching effects of activation of damage-induced pathways.

2. INTRODUCTION

In order to maintain the integrity of the genome, cells have evolved complex mechanisms to deal with genomic insult to halt cell cycle progression allowing time to repair or bypass the damage, or, if the damage is too severe, to induce apoptotic programs (1–7). These mechanisms, collectively termed the DNA damage checkpoint, involve multiple signaling pathways and control of protein levels, localization, activity, and post-translational modification. Two PI3 kinase-like kinases, ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3 related (ATR) lie at the center of DNA damage checkpoint signaling (8–12). These kinases, through the effector kinases Chk1 and Chk2, serve to block cell cycle progression through modulation of Cdc25 phosphatase interaction with cyclin dependent kinases such as cdc2 (3, 13–25). Once activated, ATM/ATR and Chk1/Chk2 are also known to phosphorylate p53, affecting transcription of cell cycle regulating genes, again providing blockage of cell cycle progression (15, 16, 26–32). Canonical DNA damage checkpoint signaling at G1/S and G2/M are shown in Figure 1.

ATM and ATR dependent phosphorylation of Chk1 and Chk2 are critical regulatory mechanisms of these core DNA damage responses. ATM and ATR are themselves regulated by phosphorylation at multiple sites, most critically S1981 on ATM and S1989 on ATR (33–39). These sites, along with localization of the kinases at or near sites of damage, allow full activation of checkpoint signaling. ATM and ATR kinases phosphorylate target proteins such as the aforementioned Chk1, Chk2, and p53 on serine or threonine residues with a glutamine residue at the +1 position relative to the phosphorylation site (the S*Q/T*Q motif) (16, 40).

In recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become

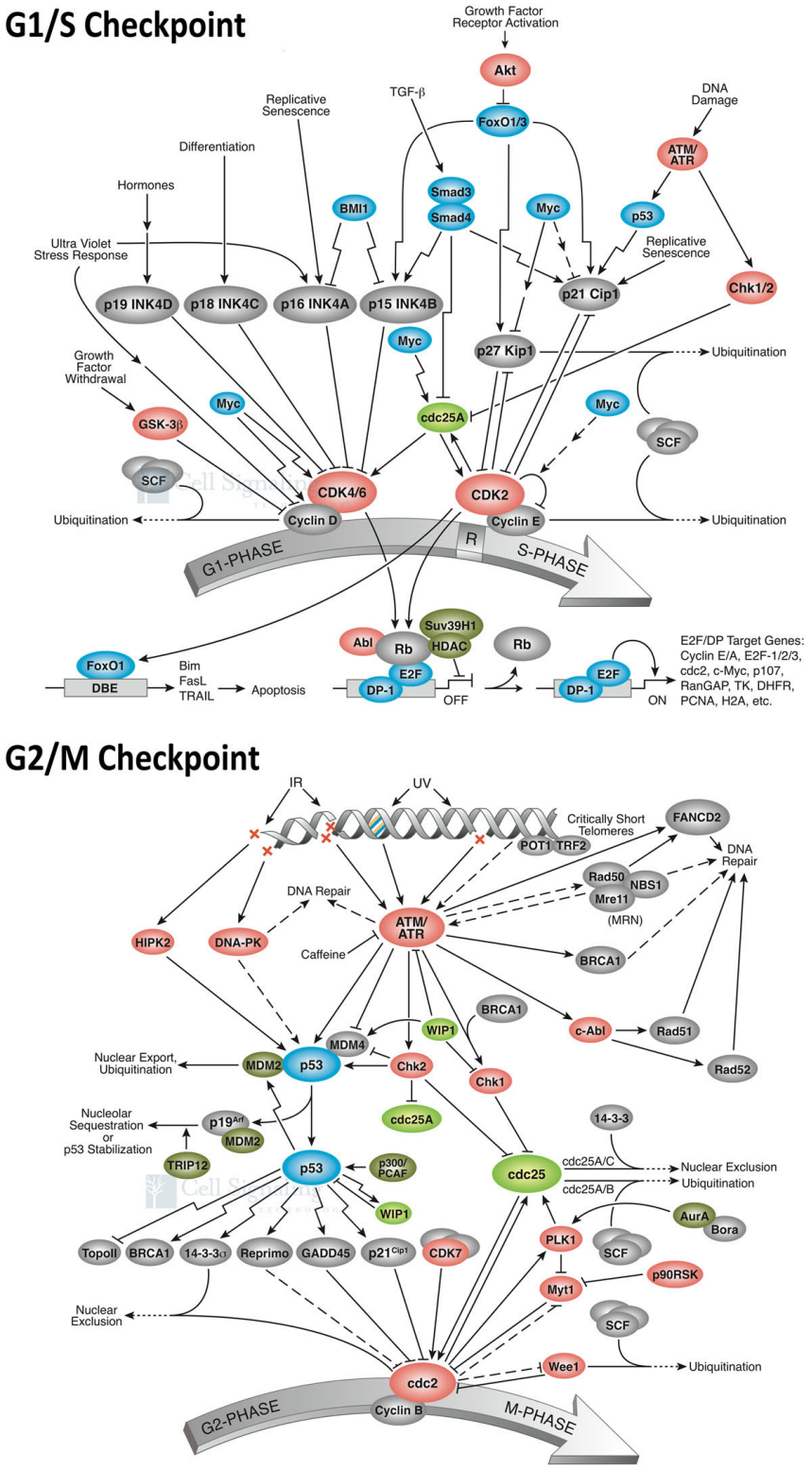

Figure 1. The G1/S and G2/M DNA damage checkpoints. The PI3K-like kinases ATM and ATR are critical regulators of DNA Damage Response signaling through Chk1 and Chk2 kinases as well as phosphorylating other substrates to promote cell cycle arrest, DNA repair, and apoptosis.

a widely used method to profile both protein levels and post translational modifications. In bottom-up or shotgun proteomics, samples are digested to peptides and purified. Peptides are separated over a reversed phase column, and as peptides elute from the column they are analyzed for their intact mass to charge ratio (m/z). Peptides are then selected for MS/MS analysis, involving fragmentation of the peptide and subsequent measurement of fragment or product ion m/z (41, 42). Peptides in the samples are identified by comparing the collected data to a species-specific database of in silico-digested proteins and scoring the resulting matches (43, 44). Relative abundance of the same peptide across multiple samples can be accurately measured, and peptides can be labeled with heavy isotopes or isobaric tags for sample multiplexing (45–58). Synthetic heavy isotope labeled peptides can also be added to samples prior to LC-MS/MS analysis for absolute quantification of peptide abundance (59, 60).

These methods allow simultaneous identification and quantification of thousands of proteins or sites in a single LC-MS/MS run. Advances in instrumentation and bioinformatic tools have allowed generation of datasets of ever increasing size and confidence in assignment of MS/MS spectra to peptide or protein sequences. Instruments can be programmed to make on the fly decisions as to which species to fragment for identification (data dependent analysis), or can be programmed to target specific peptides during the run (targeted, or data independent analysis) (61–63). In this review we will explore how these methods have been used to study the DNA damage response and checkpoint signaling, and future directions for mass spectrometry-based study of critical signaling components and entire networks.

3. PRINCIPLES OF MASS SPECTROMETRY

3.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Liquid chromatography tandem mass spectrometry is now widely used for the study of many different biological systems including development, signaling, and disease. LC-MS/MS can be performed top-down, profiling intact proteins and protein complexes, or bottom-up, profiling proteins that have been digested to peptides. In LC-MS/MS, analytes are first separated on a reversed phase column, in the case of bottom up peptide analysis usually packed with hydrophobic C18 resin. Peptides are eluted off the column using increasing concentrations of organic solvent such as acetonitrile. As peptides elute off the column, they are subjected to electrospray ionization (ESI) prior to MS/MS (64, 65).

In tandem mass spectrometry, the mass to charge ratios (m/z) of intact peptides (parent or MS1 ions) are first measured. In modern instruments, this measurement is exquisitely accurate, with mass errors commonly less than 10 parts per million (ppm), or less than 0.0.1 dalton for a peptide with m/z = 1000 dalton. Parent MS1 ions are then selected for MS/MS or MS2 analysis. Selected ions are fragmented into product or MS/MS ions, comprising breakdown products of the original MS1 parent ion, and the m/z of each of the product ions is again measured. Through this process, masses of both the intact peptide and many different fragments of that peptide are generated (66–68).

Once the m/z measurements of all selected parent and product ions have been collected, the mass spectrometry data can be matched to peptide identifications. The collected parent and product m/z values are compared to an *in silico* digested, species-specific protein database (43, 44). Identified peptides are scored for quality of the match between MS data and peptide sequence. In order to estimate the robustness of matches, the *in silico* digested database contains not only peptides corresponding to all proteins, but also reverse peptide sequences that do not exist in nature (a target-decoy strategy) (69–71). MS/MS spectra matching these reverse peptides are therefore known to be incorrect, and the dataset can be filtered based on scores of the reverse, incorrect assignments to a target false discovery rate, or FDR. Static cutoffs can be used for filtering data, or score filtering can be performed on each dataset as it is generated using linear discriminant algorithms (72–74).

When multiple samples are run in LC-MS/MS the relative abundance of each peptide across samples can be measured and compared. Quantification can be performed in a number of ways: Samples can be compared based on isotopic labeling of proteins prior to treatment and digestion using stable isotope labeling of amino acids in cell culture, or SILAC (51). Likewise, samples can also be labeled post-digestion and combined using reductive dimethylation (48). Isobaric tags such as iTRAQ or Tandem Mass Tags (TMT) can also be used for quantification (56–58, 75–77). These reagents are used to label peptides post-digestion and multiple samples (currently up to 11, with higher plex reagents on the horizon) can be combined. Each tag has a balancer moiety that gives the peptides the same m/z in MS1, but during MS/MS fragmentation the tag from each sample has a different mass, allowing quantification of the relative abundance of the peptide in each of the samples.

It is also possible to directly measure the relative abundance of each peptide without any labeling or special growth conditions. Software is available that allows alignment of LC-MS/MS data from multiple runs and extraction of peptide relative

chromatographic peak abundance across all samples (45, 46, 49, 50, 55, 78). These label free methods have the additional advantage of providing quantitative data even if a particular peptide was only MS/MS identified in a subset of the samples profiled, due to the accuracy of m/z measurement and quality of alignment software. Analytical and biological % CVs have been calculated for this type of quantitative analysis and are typically below 10% for analytical replicates (duplicate injections of the same sample) and below 20% for biological replicates (independent analysis of the same sample type) (79–82).

3.2. Total proteome profiling by LC-MS/MS

The previously described methods for performing LC-MS/MS analysis and quantification can be used to profile protein levels in cells and tissues. These total proteome profiling studies are performed by digesting proteins to peptides with trypsin, and subjecting them to LC-MS/MS with no further enrichment. Using these methods, thousands of proteins can be identified and quantified simultaneously. The relative abundance of each identified peptide can be measured and individual peptide data for each protein is combined to give protein-level quantitative changes. To increase the number of peptides and associated proteins identified in a given experiment, samples can be labeled, combined, and fractionated by ion exchange or reversed phase chromatography (74, 83–85). These fractions can then be run individually in LC-MS/MS, providing a simplified mixture of peptides in each fraction, thus allowing additional opportunities for peptide identification.

These total proteome profiling methods have been successfully applied to study of the DNA damage response across a wide range of cell types and damaging agents (86–90). Various cellular functions are affected at the protein level by DNA damage. These include proteins involved in DNA replication and repair, metabolism, cytoskeleton, NOTCH signaling, and chaperone proteins. Other studies have used subcellular fractionation to track changes in both protein abundance and localization in response to damage, for example changes between cytosolic and nuclear localization, or changes in the chromatin associated proteome (91–93). One study assessing changes in transcript and protein levels in response to alkylation damage of DNA in bacteria expanded the known repertoire of damage response proteins from four previously known proteins to dozens of proteins involved in diverse cellular functions such as DNA repair, transporters, chemotaxis, and flagellar synthesis (90). In some cases however, there are few protein-level changes during an acute DNA damage response, necessitating the use of methods that profile post-translational modification of proteins rather than protein levels.

4. ENRICHMENT STRATEGIES TO STUDY DNA DAMAGE SIGNALING

4.1. PTM/Motif antibodies and PTMScan

Antibodies for research applications are typically raised against a single peptide or protein injected into animals along with adjuvants. The antibodies selected and expanded by the immune response in the animal can then be harvested and purified. These traditional methods produce antibodies that are specific for the original sequence injected into the animal, whether an unmodified sequence on a protein or a site of post-translational modification, such as phosphorylation.

In order to generate antibodies that are more broadly reactive and thus of greater utility in enrichment of entire classes of peptides or proteins, methods have been developed using degenerate peptide libraries (Figure 2). To construct these libraries, each peptide in the library will contain the post-translationally modified residue of interest, or consensus kinase substrate motif. The amino acids flanking the modified residue(s) are then varied in the library. Instead of a single peptide being used as the antigen for antibody development, the entire library is used. This strategy produces antibodies that are exquisitely specific for the PTM or motif of interest but broadly reactive against many different primary amino acid sequences (94). PTM/Motif antibodies can be used for applications such as western blotting, immunoprecipitation/western, immunofluorescence, or flow cytometry, among others.

PTM/Motif antibodies can also be used to enrich peptides prior to LC-MS/MS analysis, known as the PTMScan method (Figure 3) (95). This enrichment allows the user to simplify the mixture of peptides delivered to the instrument, focusing the analysis on the peptides of interest. To perform a mass spectrometry experiment employing these antibodies, cells, tissues, serum, or other biological materials are harvested, reduced/alkylated, digested with trypsin (or other proteases) to peptides and purified over reversed phase columns. Peptides are then re-suspended in IP buffer, enriched with the appropriate PTM/Motif antibody, purified over reversed phase C18 tips (96), and run in LC-MS/MS. Database searching and score filtering match mass spectrometry data to peptide sequences, provide identification of thousands of post-translationally modified peptides in a single LC-MS/MS run. Quantitative analysis can be performed using label-free methods to measure relative abundance of each intact tryptic peptide in the MS1 channel, or using labeling methods such as SILAC, reductive dimethylation, or isobaric tagging methods such as TMT.

In the case of the DNA damage response, antibodies have been produced that enrich for the

Figure 2. PTM/Motif antibodies. Traditional antibody development uses a single peptide or protein as the antigen. To produce PTM/Motif antibodies, degenerate peptide libraries are created in which some residues are fixed and present in each peptide in the library while all other amino acids are varied. Antibodies can be generated against consensus kinase substrate motifs (middle panel) or PTMs (bottom panel). Antibodies generated in this manner are exquisitely specific for the targeted motif or PTM but broadly reactive against many primary amino acid sequences.

consensus ATM/ATR substrate motif, S*Q or T*Q. These ATM/ATR substrate motif antibodies have been successfully used to identify thousands of phosphorylation sites in cells and tissues treated with a variety of DNA damaging agents and inhibitor compounds (97–101). These studies have greatly expanded the known repertoire of proteins that participate in the DNA damage response, including proteins that participate in cell cycle progression, apoptosis, DNA replication and repair, transcriptional regulators, and components of the ubiquitin system, among others.

One study (99) identified over 900 S*Q/T*Q phosphorylation sites on 700 proteins induced by IR damage. A subset of these proteins, including LATS1, CSTF2, and DCK were functionally confirmed to be important regulators of damage signaling through siRNA of the identified proteins and screening for defects in H2AX phosphorylation, repair/recombination, and checkpoint activation. Another study (98) identified nearly 600 S*Q/T*Q sites on over 450 proteins in UV damaged cells. Damage responsive sites in this study were validated using western blotting and IP/western. The site-specific nature of LC-MS/MS data allowed confirmation of previously known sites, such as Ser343 of NBS1 (16), and provided the first localization of UV-inducible sites of phosphorylation of other DNA damage response proteins such as BAP1 Ser592 and Mre11 Ser676. Together, these studies have reinforced the notion that damage signaling goes far beyond the canonical ATM/R – Chk1/2 – Cdc25 – Cdk signaling pathways central to the damage checkpoint.

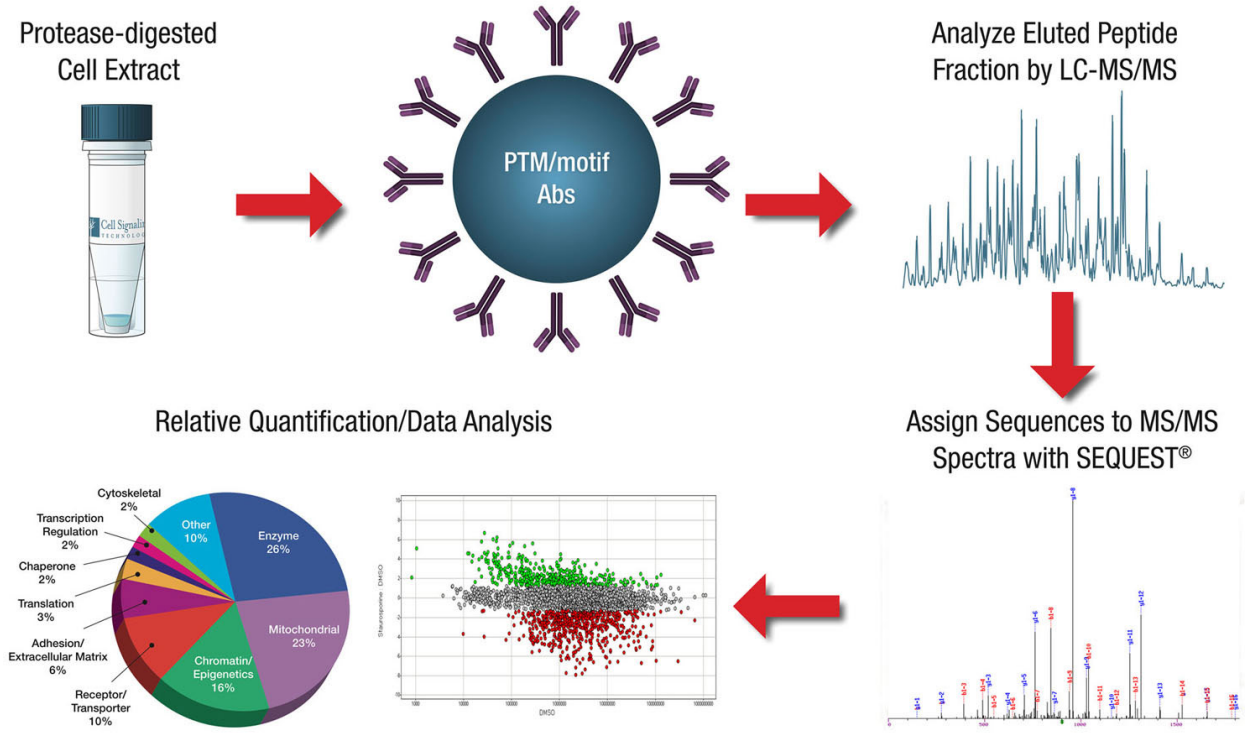

Figure 3. Antibody-based enrichment of peptides and LC-MS/MS analysis (PTMScan). Cells, tissues, serum, or other biological materials are digested to peptides and enriched with a PTM/Motif antibody or a multiplexed cocktail of site-specific antibodies. Enriched peptides are analyzed by LC-MS/MS to identify peptides and the relative abundance of identified peptides is then determined. These methods allow identification and quantification of thousands of peptides in a single LC-MS/MS run and comparison of quantitative changes across many runs.

Post-translational modifications beyond phosphorylation have also been implicated in the DDR, including acetylation and ubiquitination, such as the modification of the DNA replication protein PCNA or Fanconi's Anemia proteins in response to damage signals (102–106). To profile ubiquitination, an antibody was produced that recognizes the di-glycine remnant left behind after trypsin digestion at ubiquitinated lysine residues (107–109). This K-GG remnant antibody allows identification and quantification of thousands of sites of ubiquitin modification, and has been successfully used to profile changes in the ubiquitinome in response to DNA damage (93, 110). Similarly, antibodies have been generated that allow enrichment of peptides terminating in the caspase cleavage substrate motif (DEXD), to monitor apoptotic events regulated by DNA damage or other treatments (111–114).

Study of changes in ubiquitination and acetylation in response to UV or IR damage (110) yielded over 2,000 damage responsive ubiquitinated sites and over 200 acetylation sites. DDR proteins known to be ubiquitinated including CDC25, DDB2, and CDC6 were confirmed in the study. Novel sites on other known proteins such as PCNA were found, as well as identification of sites on proteins not previously known to be regulated by damage-induced ubiquitination including centromere proteins (CENPs) and spindle assembly proteins of the HAUS complex. Chemical inhibition of the Cullin-RING ubiquitin ligases (115) identified hundreds of damage-induced sites whose ubiquitination was mediated by these E3 ligase protein complexes. Changes in acetylation (both increased and decreased) were also observed for known DDR proteins such as RPA1, RAD50, NBS1, and Claspin. Together, these PTM/Motif antibodies have proven to be powerful tools for the study of cellular signaling networks, allowing identification of hundreds to thousands of new substrates and quantitative changes in specific post-translational modifications between treatment conditions.

4.2. Multiplexed pathway enrichment reagents (PTMScan Direct)

While PTM/Motif antibodies have shown great utility in the study of many diverse systems including the DNA damage response, enrichments that target any site sharing a common PTM or kinase substrate motif generate datasets with many new sites that may be poorly characterized. Often the goal of researchers and clinicians is two-fold: To discover novel signaling events regulated by their treatment or disease state, and to profile activation and inhibition of known signaling pathways in response to treatment/

disease. PTM/Motif antibodies can effectively solve the first goal, and to address the second need a novel strategy for multiplexed pathway enrichment has been employed.

Originally termed PTMScan Direct, this method uses multiplexed cocktails of site-specific antibodies to enrich for peptides containing critical modification sites on proteins that reside in the same signaling pathway. The method for this pathway-based enrichment is identical to that for PTM/Motif enrichment, with the only change being the antibodies used. By employing reagents that specifically target hundreds to thousands of critical signaling nodes from a single enrichment, whole signaling networks can be profiled from a single LC-MS/MS experiment (116, 117).

A collection of multiplexed antibody cocktails have been prepared to study diverse signaling pathways such as Akt/PI3K, Ser/Thr kinases, or a Multipathway reagent targeting key protein/sites across many different signaling areas. Of particular interest to the study of the DDR are a reagent that profiles DNA damage and cell cycle proteins, and one that targets apoptosis and autophagolytic pathways (117). To date, a number of studies have employed these reagents for analysis of cellular signaling. One such study proved the utility of the method in profiling DNA damage signaling in cells untreated or treated with UV damage of DNA. Activation of both ATM and ATR signaling pathways was observed upon UV damage, as well as activation of stress responsive MAP kinases JNK and p38, and the onset of apoptosis, measured by an increase in cleaved, activated forms of multiple caspases. Interestingly, although Chk1 activation was observed upon UV damage, ATR phosphorylation at the damage responsive Ser428 site was unchanged. This finding indicated that phosphorylation of Chk1 itself or another residue on ATR such as Thr1989 may be a better readout of UV damage-dependent checkpoint activation. These multiplexed reagents allow researchers to profile known components of the DNA damage response in cells and tissues, directly profiling the activation state and protein level of critical DDR regulators.

4.3. Other enrichment strategies for LC-MS/MS analysis

PTM/Motif antibodies and multiplexed pathway enrichment reagents are valuable tools for studying the DNA damage response. Other enrichment methods to profile phosphorylation changes are also commonly used. In lieu of an antibody-based enrichment, positively charged metal ions or metal oxides on beads can be used to globally enrich for phosphopeptides. (IMAC: Immobilized Metal Affinity Chromatography, MOAC: Metal Oxide Affinity Chromatography) (118–123). The positively charged metal ions or metal oxides interact with the negatively charged phosphates on peptides, allowing enrichment and identification of thousands to tens of thousands of phosphopeptides in a single experiment. IMAC or MOAC resins can also be used in combination with antibody-based enrichments prior to LC-MS/MS analysis (124, 125). By sequentially enriching with a phospho-motif antibody followed by a metal affinity resin, samples will be enriched for the specific motif of interest by the antibody while also having the high enrichment specificity (percentage of peptides identified containing a phosphorylated residue) characteristic of IMAC/MOAC.

As with total proteome analysis, samples can be labeled, combined, and fractionated prior to IMAC or MOAC enrichment to increase the number of phosphopeptides identified and quantified. IMAC or MOAC enrichments, run in parallel with total proteome analysis, have been performed on samples with and without DNA damage, and as with antibody-based studies, have again demonstrated the far-reaching effects of the DNA damage response beyond canonical ATM/ATR signaling pathways (86, 91, 110). In a study using both total proteome profiling and titanium dioxide enrichment of phosphopeptides to profile the response to alkylation damage of DNA (91) thousands of non-S*Q/T*Q sites were identified, including the first report of damage-inducibility of sites on DDR proteins such as Ser123 of the excision repair protein Rad23A, and decreased phosphorylation of Thr722 of the replication protein MCM3.

5. TARGETED METHODS FOR THE STUDY OF THE DNA DAMAGE RESPONSE

5.1. Multiple Reaction Monitoring (MRM) and related methods

In Section 4, antibody-based methods were discussed as powerful tools for dissecting the DDR in cells and tissues. Whether using PTM/Motif antibodies that enrich for peptides sharing common modifications or amino acid sequences, or using multiplexed cocktails of site and protein specific antibodies to enrich for peptides in the same signaling pathway, analysis is often performed in data-dependent mode. That is to say the samples are run in LC-MS/MS and the instrument makes on the fly decisions as to which peptides are identified in a given run. Data independent methods have been developed that allow the user to select proteins/sites to be analyzed either pre- or post-data acquisition and profile those endpoints across many samples (54, 126, 127).

A commonly used method to target specific peptides is multiple reaction monitoring (MRM). MRM involves selecting product ions from previously

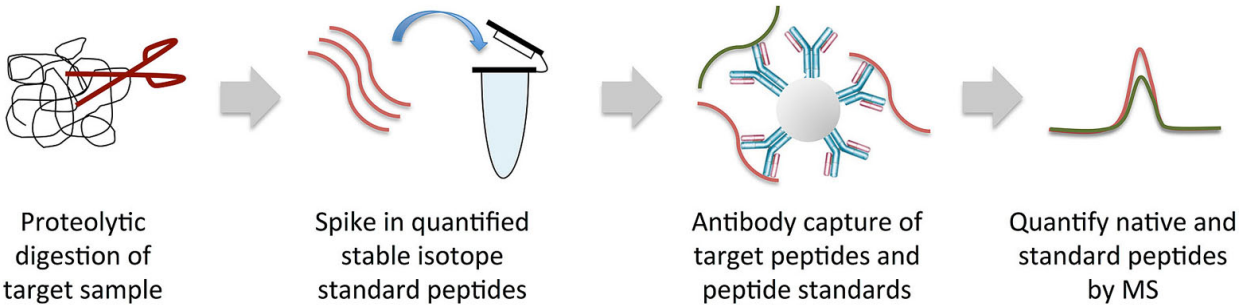

Figure 4. Targeted LC-MS/MS analysis workflow. For immuno-MRM/PRM analyses, endpoints are selected and synthetic heavy isotope labeled peptides are added to cells, tissues, or other biological materials to be profiled. Both endogenous and synthetic peptides are immunoaffinity purified and subjected to targeted MRM/PRM analysis.

collected MS/MS spectra to be targeted in the instrument. By limiting the instrument to selection of these ions, and including heavy isotope labeled synthetic peptides in each sample (stable isotope dilution, or SID), the amount of a given peptide across all samples profiled can be determined (128–130). A related method, termed parallel reaction monitoring (PRM) targets precursor peptides for analysis but instead of pre-selecting product ions to monitor, allows quantification based on any product ion generated by fragmentation of the parent ion (131, 132). Whether using MRM or PRM methods, the targeted nature of the assay allows higher throughput than traditional data dependent LC-MS/MS experiments and a fixed list of critical targets to profile.

In the case of the DNA damage response, as with many other cellular signaling systems, the most interesting candidate proteins/sites to target and quantify may be present in cells at low abundance or low phosphorylation site occupancy. In these cases, an MRM or PRM experiment with no enrichment will yield poor results, as the noise from all of the other, higher abundance peptides present will preclude accurate quantification. In order to overcome this limitation, some form of enrichment must be used to focus the analysis on the targets of interest. Metal affinity based enrichment can be used for this purpose, though the global nature of IMAC or MOAC resins as phosphopeptide enrichment tools can still lead to an overly complex mixture of peptides being delivered to the instrument. These methods also will not enrich for any protein or peptide that is not modified by phosphorylation. Antibody-based enrichment, for Immuno-MRM or -PRM (Figure 4) (133, 134), is therefore the best option to simplify the mixture and improve assay sensitivity. Antibodies against target proteins or sites can be purchased or produced and combined to create a reagent that will specifically enrich the desired peptides.

Both metal affinity and immunoaffinity-based strategies for targeted analysis have been successfully employed to profile DNA damage signaling (135, 136). DNA damage response proteins/sites were selected for analysis, and heavy isotope labeled synthetic peptides were produced and added to cellular peptides at known quantities. Cocktails of antibodies against known DNA damage response proteins were combined on beads to make a single reagent capable of simultaneously enriching multiple targets. This cocktail, or metal affinity IMAC beads were used to enrich for targeted proteins/sites, and both endogenous and synthetic peptides were immunoaffinity purified from the samples. This purified material was then run using targeted LC-MS/MS methods, providing sensitive, reproducible quantitative data to profile multiple, critical regulators of the DNA damage response.

A DNA damage response antibody cocktail containing 69 endpoints (DDR peptide targets) was used to profile human breast cancer tissue samples, and demonstrated variability in signaling between individuals when the tissues were IR damaged. The response of wild type and ataxia telangiectasia (ATM –/–) lymphoblast cell lines to IR and wild type lines to chemical inhibition of ATM was profiled, demonstrating differential phosphorylation of known ATM substrates such as NBS1 Ser343, BRCA1 Ser1524, and p53 Ser315. Interestingly, NBS1 Ser343 was still phosphorylated in ATM (–/–) or ATM inhibited cells, though at a slower rate than in wild type cells, suggesting compensatory mechanisms. Together, these data highlighted the utility of targeted methods for profiling the DDR to assess damage checkpoint activation and dynamics.

6. CONCLUSIONS

Traditional methods to study the DNA damage response have relied on systematic study of one or a few proteins/sites at a time, and the generation of reagents for each target to be studied. With advancements in mass spectrometry-based methods for proteomics, researchers are now able to generate quantitative data for thousands of endpoints

in a single experiment, giving a more global view of DNA damage signaling. Specialized antibodies against phosphorylation motifs or PTMs, multiplexed cocktails of antibodies against signaling pathway components, and targeted methods have all been successfully employed to create a much fuller picture of the responses to genomic insult. These methods will continue to be important tools in the study of the DNA damage response, with applications in cellular growth, development, and disease biology.

7. REFERENCES

1. R. T. Abraham: Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev*, 15(17), 2177–96 (2001) DOI: 10.1101/gad.914401

2. L. H. Hartwell and T. A. Weinert: Checkpoints: controls that ensure the order of cell cycle events. *Science*, 246(4930), 629–34 (1989) DOI: 10.1126/science.2683079

3. B. B. Zhou and S. J. Elledge: The DNA damage response: putting checkpoints in perspective. *Nature*, 408(6811), 433–9 (2000) DOI: 10.1038/35044005

4. S. Izumaru, N. Arima, Y. Toyozumi, S. Kato, M. Morimatsu and T. Nakashima: Down-regulation of p21Waf-1 protein facilitates IR- and UV-induced apoptosis in human squamous carcinoma cells. *Int J Oncol*, 24(5), 1245–55 (2004) DOI: 10.3892/ijo.24.5.1245

5. W. P. Roos and B. Kaina: DNA damage-induced cell death by apoptosis. *Trends Mol Med*, 12(9), 440–50 (2006) DOI: 10.1016/j.molmed.2006.07.007

6. W. P. Roos and B. Kaina: DNA damage-induced apoptosis: From specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett* (2012)

7. M. S. Sheikh, M. J. Antinore, Y. Huang and A. J. Fornace, Jr.: Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1. *Oncogene*, 17(20), 2555–63 (1998) DOI: 10.1038/sj.onc.1202292

8. R. T. Abraham: PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. *DNA Repair (Amst)*, 3(8–9), 883–7 (2004) DOI: 10.1016/j.dnarep.2004.04.002

9. M. F. Lavin and K. K. Khanna: ATM: the protein encoded by the gene mutated in the radiosensitive syndrome ataxia-telangiectasia. *Int J Radiat Biol*, 75(10), 1201–14 (1999) DOI: 10.1080/095530099139359

10. M. F. Lavin and Y. Shiloh: The genetic defect in ataxia-telangiectasia. *Annu Rev Immunol*, 15, 177–202 (1997) DOI: 10.1146/annurev.immunol.15.1.177

11. J. N. Sarkaria, E. C. Busby, R. S. Tibbetts, P. Roos, Y. Taya, L. M. Karnitz and R. T. Abraham: Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res*, 59(17), 4375–82 (1999)

12. W. A. Cliby, C. J. Roberts, K. A. Cimprich, C. M. Stringer, J. R. Lamb, S. L. Schreiber and S. H. Friend: Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *EMBO J*, 17(1), 159–69 (1998) DOI: 10.1093/emboj/17.1.159

13. Z. Guo, A. Kumagai, S. X. Wang and W. G. Dunphy: Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. *Genes Dev*, 14(21), 2745–56 (2000) DOI: 10.1101/gad.842500

14. T. P. Heffernan, D. A. Simpson, A. R. Frank, A. N. Heinloth, R. S. Paules, M. Cordeiro-Stone and W. K. Kaufmann: An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol*, 22(24), 8552–61 (2002) DOI: 10.1128/MCB.22.24.8552-8561.2002

15. M. B. Kastan and D. S. Lim: The many substrates and functions of ATM. *Nat Rev Mol Cell Biol*, 1(3), 179–86 (2000) DOI: 10.1038/35043058

16. S. T. Kim, D. S. Lim, C. E. Canman and M. B. Kastan: Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem*, 274(53), 37538–43 (1999) DOI: 10.1074/jbc.274.53.37538

17. Q. Liu, S. Guntuku, X. S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L. A. Donehower and S. J. Elledge: Chk1 is an essential kinase

that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev*, 14(12), 1448–59 (2000)

18. S. Matsuoka, G. Rotman, A. Ogawa, Y. Shiloh, K. Tamai and S. J. Elledge: Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*. *Proc Natl Acad Sci U S A*, 97(19), 10389–94 (2000)
DOI: 10.1073/pnas.190030497

19. K. Finn, N. F. Lowndes and M. Grenon: Eukaryotic DNA damage checkpoint activation in response to double-strand breaks. *Cell Mol Life Sci*, 69(9), 1447–73 (2011)
DOI: 10.1007/s00018-011-0875-3

20. H. Zhao and H. Piwnica-Worms: ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol*, 21(13), 4129–39 (2001)
DOI: 10.1128/MCB.21.13.4129-4139.2001

21. N. Mailand, J. Falck, C. Lukas, R. G. Syljuasen, M. Welcker, J. Bartek and J. Lukas: Rapid destruction of human Cdc25A in response to DNA damage. *Science*, 288(5470), 1425–9 (2000)
DOI: 10.1126/science.288.5470.1425

22. Y. Sanchez, C. Wong, R. S. Thoma, R. Richman, Z. Wu, H. Piwnica-Worms and S. J. Elledge: Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science*, 277(5331), 1497–501 (1997)
DOI: 10.1126/science.277.5331.1497

23. A. Ciccia and S. J. Elledge: The DNA damage response: making it safe to play with knives. *Mol Cell*, 40(2), 179–204 (2010)
DOI: 10.1016/j.molcel.2010.09.019

24. J. A. Wright, K. S. Keegan, D. R. Herendeen, N. J. Bentley, A. M. Carr, M. F. Hoekstra and P. Concannon: Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. *Proc Natl Acad Sci U S A*, 95(13), 7445–50 (1998)
DOI: 10.1073/pnas.95.13.7445

25. E. A. Nam and D. Cortez: ATR signalling: more than meeting at the fork. *Biochem J*, 436(3), 527–36 (2011)
DOI: 10.1042/BJ20102162

26. S. Banin, L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh and Y. Ziv: Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, 281(5383), 1674–7 (1998)
DOI: 10.1126/science.281.5383.1674

27. C. E. Canman, D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan and J. D. Siliciano: Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, 281(5383), 1677–9 (1998)
DOI: 10.1126/science.281.5383.1677

28. K. K. Khanna, K. E. Keating, S. Kozlov, S. Scott, M. Gatei, K. Hobson, Y. Taya, B. Gabrielli, D. Chan, S. P. Lees-Miller and M. F. Lavin: ATM associates with and phosphorylates p53: mapping the region of interaction. *Nat Genet*, 20(4), 398–400 (1998)
DOI: 10.1038/3882

29. R. S. Tibbetts, K. M. Brumbaugh, J. M. Williams, J. N. Sarkaria, W. A. Cliby, S. Y. Shieh, Y. Taya, C. Prives and R. T. Abraham: A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev*, 13(2), 152–7 (1999)
DOI: 10.1101/gad.13.2.152

30. S. Y. Shieh, J. Ahn, K. Tamai, Y. Taya and C. Prives: The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev*, 14(3), 289–300 (2000)

31. N. H. Chehab, A. Malikzay, M. Appel and T. D. Halazonetis: Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev*, 14(3), 278–88 (2000)

32. K. Tominaga, H. Morisaki, Y. Kaneko, A. Fujimoto, T. Tanaka, M. Ohtsubo, M. Hirai, H. Okayama, K. Ikeda and M. Nakanishi: Role of human Cds1 (Chk2) kinase in DNA damage checkpoint and its regulation by p53. *J Biol Chem*, 274(44), 31463–7 (1999)
DOI: 10.1074/jbc.274.44.31463

33. C. J. Bakkenist and M. B. Kastan: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*, 421(6922), 499–506 (2003)
DOI: 10.1038/nature01368

34. A. Dupre, L. Boyer-Chatenet and J. Gautier: Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex. *Nat Struct Mol Biol*, 13(5), 451–7 (2006) DOI: 10.1038/nsmb1090

35. J. H. Lee and T. T. Paull: Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. *Oncogene*, 26(56), 7741–8 (2007) DOI: 10.1038/sj.onc.1210872

36. S. Liu, B. Shiotani, M. Lahiri, A. Marechal, A. Tse, C. C. Leung, J. N. Glover, X. H. Yang and L. Zou: ATR autophosphorylation as a molecular switch for checkpoint activation. *Mol Cell*, 43(2), 192–202 (2011) DOI: 10.1016/j.molcel.2011.06.019

37. T. A. Mochan, M. Venere, R. A. DiTullio, Jr. and T. D. Halazonetis: 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res*, 63(24), 8586–91 (2003)

38. E. A. Nam, R. Zhao, G. G. Glick, C. E. Bansbach, D. B. Friedman and D. Cortez: Thr-1989 phosphorylation is a marker of active ataxia telangiectasia-mutated and Rad3-related (ATR) kinase. *J Biol Chem*, 286(33), 28707–14 (2011) DOI: 10.1074/jbc.M111.248914

39. S. Vidal-Eychenie, C. Decaillet, J. Basbous and A. Constantinou: DNA structure-specific priming of ATR activation by DNA-PKcs. *J Cell Biol*, 202(3), 421–9 (2013) DOI: 10.1083/jcb.201304139

40. T. O'Neill, A. J. Dwyer, Y. Ziv, D. W. Chan, S. P. Lees-Miller, R. H. Abraham, J. H. Lai, D. Hill, Y. Shiloh, L. C. Cantley and G. A. Rathbun: Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J Biol Chem*, 275(30), 22719–27 (2000) DOI: 10.1074/jbc.M001002200

41. J. Peng and S. P. Gygi: Proteomics: the move to mixtures. *J Mass Spectrom*, 36(10), 1083–91 (2001) DOI: 10.1002/jms.229

42. V. H. Wysocki, K. A. Resing, Q. Zhang and G. Cheng: Mass spectrometry of peptides and proteins. *Methods*, 35(3), 211–22 (2005) DOI: 10.1016/j.ymeth.2004.08.013

43. J. K. Eng, A. L. McCormack and J. R. Yates: An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom*, 5(11), 976–89 (1994) DOI: 10.1016/1044-0305(94)80016-2

44. D. N. Perkins, D. J. Pappin, D. M. Creasy and J. S. Cottrell: Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*, 20(18), 3551–67 (1999) DOI: 10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2

45. Y. Chen, F. Wang, F. Xu and T. Yang: Mass Spectrometry-Based Protein Quantification. *Adv Exp Med Biol*, 919, 255–279 (2016) DOI: 10.1007/978-3-319-41448-5_15

46. K. M. Coombs: Quantitative proteomics of complex mixtures. *Expert Rev Proteomics*, 8(5), 659–77 (2011) DOI: 10.1586/epr.11.55

47. S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*, 17(10), 994–9 (1999) DOI: 10.1038/13690

48. C. Ji, N. Guo and L. Li: Differential dimethyl labeling of N-termini of peptides after guanidination for proteome analysis. *J Proteome Res*, 4(6), 2099–108 (2005) DOI: 10.1021/pr050215d

49. J. Cox and M. Mann: MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*, 26(12), 1367–72 (2008) DOI: 10.1038/nbt.1511

50. W. M. Old, K. Meyer-Arendt, L. Aveline-Wolf, K. G. Pierce, A. Mendoza, J. R. Sevinsky, K. A. Resing and N. G. Ahn: Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics*, 4(10), 1487–502 (2005) DOI: 10.1074/mcp.M500084-MCP200

51. S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey and M. Mann: Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression

proteomics. *Mol Cell Proteomics*, 1(5), 376–86 (2002)
DOI: 10.1074/mcp.M200025-MCP200

52. A. Schmidt, J. Kellermann and F. Lottspeich: A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics*, 5(1), 4–15 (2005)
DOI: 10.1002/pmic.200400873

53. R. Shi, C. Kumar, A. Zougman, Y. Zhang, A. Podtelejnikov, J. Cox, J. R. Wisniewski and M. Mann: Analysis of the mouse liver proteome using advanced mass spectrometry. *J Proteome Res*, 6(8), 2963–72 (2007)
DOI: 10.1021/pr0605668

54. J. C. Silva, M. V. Gorenstein, G. Z. Li, J. P. Vissers and S. J. Geromanos: Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics*, 5(1), 144–56 (2006)
DOI: 10.1074/mcp.M500230-MCP200

55. M. C. Wiener, J. R. Sachs, E. G. Deyanova and N. A. Yates: Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. *Anal Chem*, 76(20), 6085–96 (2004)
DOI: 10.1021/ac0493875

56. P. L. Ross, Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson and D. J. Pappin: Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics*, 3(12), 1154–69 (2004)
DOI: 10.1074/mcp.M400129-MCP200

57. S. Wiese, K. A. Reidegeld, H. E. Meyer and B. Warscheid: Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics*, 7(3), 340–50 (2007)
DOI: 10.1002/pmic.200600422

58. A. Thompson, J. Schafer, K. Kuhn, S. Kienle, J. Schwarz, G. Schmidt, T. Neumann, R. Johnstone, A. K. Mohammed and C. Hamon: Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem*, 75(8), 1895–904 (2003)
DOI: 10.1021/ac0262560

59. S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner and S. P. Gygi: Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci U S A*, 100(12), 6940–5 (2003)
DOI: 10.1073/pnas.0832254100

60. D. S. Kirkpatrick, S. A. Gerber and S. P. Gygi: The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods*, 35(3), 265–73 (2005)
DOI: 10.1016/j.ymeth.2004.08.018

61. M. Bauer, E. Ahrne, A. P. Baron, T. Glatter, L. L. Fava, A. Santamaria, E. A. Nigg and A. Schmidt: Evaluation of data-dependent and -independent mass spectrometric workflows for sensitive quantification of proteins and phosphorylation sites. *J Proteome Res*, 13(12), 5973–88 (2014)
DOI: 10.1021/pr500860c

62. S. Eliuk and A. Makarov: Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annu Rev Anal Chem (Palo Alto Calif)*, 8, 61–80 (2015)
DOI: 10.1146/annurev-anchem-071114-040325

63. A. Hu, W. S. Noble and A. Wolf-Yadlin: Technical advances in proteomics: new developments in data-independent acquisition. *F1000Res*, 5 (2016)
DOI: 10.12688/f1000research.7042.1

64. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse: Electrospray ionization for mass spectrometry of large biomolecules. *Science*, 246(4926), 64–71 (1989)
DOI: 10.1126/science.2675315

65. M. Yamashita, Fenn, J.B.: Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem*, 88(20), 4451–4459 (1984)
DOI: 10.1021/j150664a002

66. Y. Zhang, B. R. Fonslow, B. Shan, M. C. Baek and J. R. Yates, 3rd: Protein analysis by shotgun/bottom-up proteomics. *Chem Rev*, 113(4), 2343–94 (2013)
DOI: 10.1021/cr3003533

67. Q. Hu, R. J. Noll, H. Li, A. Makarov, M. Hardman and R. Graham Cooks: The Orbitrap: a new mass spectrometer. *J Mass Spectrom*, 40(4), 430–43 (2005)
DOI: 10.1002/jms.856

68. J. V. Olsen, L. M. de Godoy, G. Li, B. Macek, P. Mortensen, R. Pesch, A. Makarov, O. Lange, S. Horning and M. Mann: Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics*, 4(12), 2010–21 (2005)
DOI: 10.1074/mcp.T500030-MCP200

69. H. Choi, D. Ghosh and A. I. Nesvizhskii: Statistical validation of peptide identifications in large-scale proteomics using the target-decoy database search strategy and flexible mixture modeling. *J Proteome Res*, 7(1), 286–92 (2008)
DOI: 10.1021/pr7006818

70. J. E. Elias and S. P. Gygi: Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods*, 4(3), 207–14 (2007)
DOI: 10.1038/nmeth1019

71. C. Y. Park, A. A. Klammer, L. Kall, M. J. MacCoss and W. S. Noble: Rapid and accurate peptide identification from tandem mass spectra. *J Proteome Res*, 7(7), 3022–7 (2008)
DOI: 10.1021/pr800127y

72. E. L. Huttlin, M. P. Jedrychowski, J. E. Elias, T. Goswami, R. Rad, S. A. Beausoleil, J. Villen, W. Haas, M. E. Sowa and S. P. Gygi: A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell*, 143(7), 1174–89 (2010)
DOI: 10.1016/j.cell.2010.12.001

73. D. H. Lundgren, H. Martinez, M. E. Wright and D. K. Han: Protein identification using Sorcerer 2 and SEQUEST. *Curr Protoc Bioinformatics*, Chapter 13, Unit 13 3 (2009)
DOI: 10.1002/0471250953.bi1303s28

74. J. Villen, S. A. Beausoleil, S. A. Gerber and S. P. Gygi: Large-scale phosphorylation analysis of mouse liver. *Proc Natl Acad Sci U S A*, 104(5), 1488–93 (2007)
DOI: 10.1073/pnas.0609836104

75. G. C. McAlister, E. L. Huttlin, W. Haas, L. Ting, M. P. Jedrychowski, J. C. Rogers, K. Kuhn, I. Pike, R. A. Grothe, J. D. Blethrow and S. P. Gygi: Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. *Anal Chem*, 84(17), 7469–78 (2012)
DOI: 10.1021/ac301572t

76. C. R. Braun, G. H. Bird, M. Wuhr, B. K. Erickson, R. Rad, L. D. Walensky, S. P. Gygi and W. Haas: Generation of multiple reporter ions from a single isobaric reagent increases multiplexing capacity for quantitative proteomics. *Anal Chem*, 87(19), 9855–63 (2015)
DOI: 10.1021/acs.analchem.5b02307

77. N. Rauniyar and J. R. Yates, 3rd: Isobaric labeling-based relative quantification in shotgun proteomics. *J Proteome Res*, 13(12), 5293–309 (2014)
DOI: 10.1021/pr500880b

78. J. Paardekooper Overman, J. S. Yi, M. Bonetti, M. Soulsby, C. Preisinger, M. P. Stokes, L. Hui, J. C. Silva, J. Overvoorde, P. Giansanti, A. J. Heck, M. I. Kontaridis, J. den Hertog and A. M. Bennett: PZR coordinates Shp2 Noonan and LEOPARD syndrome signaling in zebrafish and mice. *Mol Cell Biol*, 34(15), 2874–89 (2014)
DOI: 10.1128/MCB.00135-14

79. H. Weisser, S. Nahnsen, J. Grossmann, L. Nilse, A. Quandt, H. Brauer, M. Sturm, E. Kenar, O. Kohlbacher, R. Aebersold and L. Malmstrom: An automated pipeline for high-throughput label-free quantitative proteomics. *J Proteome Res*, 12(4), 1628–44 (2013)
DOI: 10.1021/pr300992u

80. H. Gu, J. M. Ren, X. Jia, T. Levy, K. Rikova, V. Yang, K. A. Lee, M. P. Stokes and J. C. Silva: Quantitative Profiling of Post-translational Modifications by Immunoaffinity Enrichment and LC-MS/MS in Cancer Serum without Immunodepletion. *Mol Cell Proteomics*, 15(2), 692–702 (2016)
DOI: 10.1074/mcp.O115.052266

81. A. Guo, H. Gu, J. Zhou, D. Mulhern, Y. Wang, K. A. Lee, V. Yang, M. Aguiar, J. Kornhauser, X. Jia, J. Ren, S. A. Beausoleil, J. C. Silva, V. Vemulapalli, M. T. Bedford and M. J. Comb: Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Mol Cell Proteomics*, 13(1), 372–87 (2014)
DOI: 10.1074/mcp.O113.027870

82. S. Gispert, N. Brehm, J. Weil, K. Seidel, U. Rub, B. Kern, M. Walter, J. Roeper and G. Auburger: Potentiation of neurotoxicity in double-mutant mice with Pink1 ablation and A53T-SNCA overexpression. *Hum Mol Genet*, 24(4), 1061–76 (2015)
DOI: 10.1093/hmg/ddu520

83. Y. Wang, F. Yang, M. A. Gritsenko, Y. Wang, T. Clauss, T. Liu, Y. Shen, M. E. Monroe, D. Lopez-Ferrer, T. Reno, R. J. Moore, R. L. Klemke, D. G. Camp, 2nd and R. D. Smith: Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics*, 11(10), 2019–26 (2011)
DOI: 10.1002/pmic.201000722

84. N. Dephoure, C. Zhou, J. Villen, S. A. Beausoleil, C. E. Bakalarski, S. J. Elledge and S. P. Gygi: A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci U S A*, 105(31), 10762–7 (2008)
DOI: 10.1073/pnas.0805139105

85. G. C. McAlister, D. P. Nusinow, M. P. Jedrychowski, M. Wuhr, E. L. Huttlin, B. K. Erickson, R. Rad, W. Haas and S. P. Gygi: MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal Chem*, 86(14), 7150–8 (2014)
DOI: 10.1021/ac502040v

86. A. Pines, C. D. Kelstrup, M. G. Vrouwe, J. C. Puigvert, D. Typas, B. Misovic, A. de Groot, L. von Stechow, B. van de Water, E. H. Danen, H. Vrieling, L. H. Mullenders and J. V. Olsen: Global phosphoproteome profiling reveals unanticipated networks responsive to cisplatin treatment of embryonic stem cells. *Mol Cell Biol*, 31(24), 4964–77 (2011)
DOI: 10.1128/MCB.05258-11

87. A. W. Truman, K. Kristjansdottir, D. Wolfgeher, N. Ricco, A. Mayampurath, S. L. Volchenboum, J. Clotet and S. J. Kron: Quantitative proteomics of the yeast Hsp70/Hsp90 interactomes during DNA damage reveal chaperone-dependent regulation of ribonucleotide reductase. *J Proteomics*, 112, 285–300 (2015)
DOI: 10.1016/j.jprot.2014.09.028

88. Q. Xiong, J. Feng, S. T. Li, G. Y. Zhang, Z. X. Qiao, Z. Chen, Y. Wu, Y. Lin, T. Li, F. Ge and J. D. Zhao: Integrated transcriptomic and proteomic analysis of the global response of Synechococcus to high light stress. *Mol Cell Proteomics*, 14(4), 1038–53 (2015)
DOI: 10.1074/mcp.M114.046003

89. H. G. Vieira, P. Grynberg, M. Bitar, F. Pires Sda, H. O. Hilario, A. M. Macedo, C. R. Machado, H. M. de Andrade and G. R. Franco: Proteomic analysis of Trypanosoma cruzi response to ionizing radiation stress. *PLoS One*, 9(5), e97526 (2014)
DOI: 10.1371/journal.pone.0097526

90. J. H. Baek, M. J. Han, S. Y. Lee and J. S. Yoo: Transcriptome and proteome analyses of adaptive responses to methyl methanesulfonate in Escherichia coli K-12 and ada mutant strains. *BMC Microbiol*, 9, 186 (2009)
DOI: 10.1186/1471-2180-9-186

91. A. Aslanian, J. R. Yates, 3rd and T. Hunter: Mass spectrometry-based quantification of the cellular response to methyl methanesulfonate treatment in human cells. *DNA Repair (Amst)*, 15, 29–38 (2014)
DOI: 10.1016/j.dnarep.2013.12.007

92. F. M. Boisvert, Y. W. Lam, D. Lamont and A. I. Lamond: A quantitative proteomics analysis of subcellular proteome localization and changes induced by DNA damage. *Mol Cell Proteomics*, 9(3), 457–70 (2010)
DOI: 10.1074/mcp.M900429-MCP200

93. S. Boeing, L. Williamson, V. Encheva, I. Gori, R. E. Saunders, R. Instrell, O. Aygun, M. Rodriguez-Martinez, J. C. Weems, G. P. Kelly, J. W. Conaway, R. C. Conaway, A. Stewart, M. Howell, A. P. Snijders and J. Q. Svejstrup: Multiomic Analysis of the UV-Induced DNA Damage Response. *Cell Rep* (2016)
DOI: 10.1016/j.celrep.2016.04.047

94. H. Zhang, X. Zha, Y. Tan, P. V. Hornbeck, A. J. Mastrangelo, D. R. Alessi, R. D. Polakiewicz and M. J. Comb: Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J Biol Chem*, 277(42), 39379–87 (2002)
DOI: 10.1074/jbc.M206399200

95. J. Rush, A. Moritz, K. A. Lee, A. Guo, V. L. Goss, E. J. Spek, H. Zhang, X. M. Zha, R. D. Polakiewicz and M. J. Comb: Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat Biotechnol*, 23(1), 94–101 (2005)
DOI: 10.1038/nbt1046

96. J. Rappsilber, Y. Ishihama and M. Mann: Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal Chem*, 75(3), 663–70 (2003)
DOI: 10.1021/ac026117i

97. N. A. Willis, C. Zhou, A. E. Elia, J. M. Murray, A. M. Carr, S. J. Elledge and N. Rhind: Identification of S-phase DNA damage-response targets in fission yeast reveals conservation of damage-response networks. *Proc Natl Acad Sci U S A*, 113(26), E3676–85 (2016)
DOI: 10.1073/pnas.1525620113

98. M. P. Stokes, J. Rush, J. Macneill, J. M. Ren, K. Sprott, J. Nardone, V. Yang, S. A. Beausoleil, S. P. Gygi, M. Livingstone, H. Zhang, R. D. Polakiewicz and M. J. Comb: Profiling of UV-induced ATM/ATR signaling pathways. *Proc Natl Acad Sci U S A*, 104(50), 19855–60 (2007)
DOI: 10.1073/pnas.0707579104

99. S. Matsuoka, B. A. Ballif, A. Smogorzewska, E. R. McDonald, 3rd, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi and S. J. Elledge: ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science*, 316(5828), 1160–6 (2007)
DOI: 10.1126/science.1140321

100. C. Zhou, A. E. Elia, M. L. Naylor, N. Dephoure, B. A. Ballif, G. Goel, Q. Xu, A. Ng, D. M. Chou, R. J. Xavier, S. P. Gygi and S. J. Elledge: Profiling DNA damage-induced phosphorylation in budding yeast reveals diverse signaling networks. *Proc Natl Acad Sci U S A*, 113(26), E3667–75 (2016)
DOI: 10.1073/pnas.1602827113

101. D. S. Kirkpatrick, D. J. Bustos, T. Dogan, J. Chan, L. Phu, A. Young, L. S. Friedman, M. Belvin, Q. Song, C. E. Bakalarski and K. P. Hoeflich: Phosphoproteomic characterization of DNA damage response in melanoma cells following MEK/PI3K dual inhibition. *Proc Natl Acad Sci U S A*, 110(48), 19426–31 (2013)
DOI: 10.1073/pnas.1309473110

102. P. L. Kannouche, J. Wing and A. R. Lehmann: Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol Cell*, 14(4), 491–500 (2004)
DOI: 10.1016/S1097-2765(04)00259-X

103. S. I. Ahmad, F. Hanaoka and S. H. Kirk: Molecular biology of Fanconi anaemia-an old problem, a new insight. *Bioessays*, 24(5), 439–48 (2002)
DOI: 10.1002/bies.10082

104. P. R. Andreassen, A. D. D'Andrea and T. Taniguchi: ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev*, 18(16), 1958–63 (2004)
DOI: 10.1101/gad.1196104

105. T. M. Thomson and M. Guerra-Rebollo: Ubiquitin and SUMO signalling in DNA repair. *Biochem Soc Trans*, 38(Pt 1), 116–31 (2010)
DOI: 10.1042/BST0380116

106. A. R. Lehmann: Ubiquitin-family modifications in the replication of DNA damage. *FEBS Lett*, 585(18), 2772–9 (2011)
DOI: 10.1016/j.febslet.2011.06.005

107. J. Peng, D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley and S. P. Gygi: A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol*, 21(8), 921–6 (2003)
DOI: 10.1038/nbt849

108. N. D. Udeshi, P. Mertins, T. Svinkina and S. A. Carr: Large-scale identification of ubiquitination sites by mass spectrometry. *Nat Protoc*, 8(10), 1950–60 (2013)
DOI: 10.1038/nprot.2013.120

109. K. A. Lee, L. P. Hammerle, P. S. Andrews, M. P. Stokes, T. Mustelin, J. C. Silva, R. A. Black and J. R. Doedens: Ubiquitin ligase substrate identification through quantitative proteomics at both the protein and peptide levels. *J Biol Chem*, 286(48), 41530–8 (2011)
DOI: 10.1074/jbc.M111.248856

110. A. E. Elia, A. P. Boardman, D. C. Wang, E. L. Huttlin, R. A. Everley, N. Dephoure, C. Zhou, I. Koren, S. P. Gygi and S. J. Elledge: Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response. *Mol Cell*, 59(5), 867–81 (2015)
DOI: 10.1016/j.molcel.2015.05.006

111. M. P. Stokes, Gu, H., Silva, J. C.: Antibody-Based Proteomic Analysis of Apoptosis Signaling. In: *Apoptosis Methods in*

Toxicology. Ed P. M. Muganda. Springer New York, New York, NY (2016)
DOI: 10.1007/978-1-4939-3588-8_9

112. V. C. Pham, R. Pitti, V. G. Anania, C. E. Bakalarski, D. Bustos, S. Jhunjhunwala, Q. T. Phung, K. Yu, W. F. Forrest, D. S. Kirkpatrick, A. Ashkenazi and J. R. Lill: Complementary proteomic tools for the dissection of apoptotic proteolysis events. *J Proteome Res*, 11(5), 2947–54 (2012)
DOI: 10.1021/pr300035k

113. U. Fischer, R. U. Janicke and K. Schulze-Osthoff: Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ*, 10(1), 76–100 (2003)
DOI: 10.1038/sj.cdd.4401160

114. R. V. Talanian, C. Quinlan, S. Trautz, M. C. Hackett, J. A. Mankovich, D. Banach, T. Ghayur, K. D. Brady and W. W. Wong: Substrate specificities of caspase family proteases. *J Biol Chem*, 272(15), 9677–82 (1997)
DOI: 10.1074/jbc.272.15.9677

115. M. D. Petroski and R. J. Deshaies: Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol*, 6(1), 9–20 (2005)
DOI: 10.1038/nrm1547

116. M. P. Stokes, C. L. Farnsworth, A. Moritz, J. C. Silva, X. Jia, K. A. Lee, A. Guo, R. D. Polakiewicz and M. J. Comb: PTMScan direct: identification and quantification of peptides from critical signaling proteins by immunoaffinity enrichment coupled with LC-MS/MS. *Mol Cell Proteomics*, 11(5), 187–201 (2012)
DOI: 10.1074/mcp.M111.015883

117. M. P. Stokes, J. C. Silva, X. Jia, K. A. Lee, R. D. Polakiewicz and M. J. Comb: Quantitative profiling of DNA damage and apoptotic pathways in UV damaged cells using PTMScan Direct. *Int J Mol Sci*, 14(1), 286–307 (2012)
DOI: 10.3390/ijms14010286

118. L. Beltran and P. R. Cutillas: Advances in phosphopeptide enrichment techniques for phosphoproteomics. *Amino Acids*, 43(3), 1009–24 (2012)
DOI: 10.1007/s00726-012-1288-9

119. S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt and F. M. White: Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat Biotechnol*, 20(3), 301–5 (2002)
DOI: 10.1038/nbt0302-301

120. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff and T. J. Jorgensen: Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics*, 4(7), 873–86 (2005)
DOI: 10.1074/mcp.T500007-MCP200

121. H. K. Kweon and K. Hakansson: Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal Chem*, 78(6), 1743–9 (2006)
DOI: 10.1021/ac0522355

122. S. A. Beausoleil, M. Jedrychowski, D. Schwartz, J. E. Elias, J. Villen, J. Li, M. A. Cohn, L. C. Cantley and S. P. Gygi: Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A*, 101(33), 12130–5 (2004)
DOI: 10.1073/pnas.0404720101

123. J. Fila and D. Honys: Enrichment techniques employed in phosphoproteomics. *Amino Acids*, 43(3), 1025–47 (2012)
DOI: 10.1007/s00726-011-1111-z

124. Y. Zhang, A. Wolf-Yadlin, P. L. Ross, D. J. Pappin, J. Rush, D. A. Lauffenburger and F. M. White: Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. *Mol Cell Proteomics*, 4(9), 1240–50 (2005)
DOI: 10.1074/mcp.M500089-MCP200

125. Y. Abe, M. Nagano, A. Tada, J. Adachi and T. Tomonaga: Deep Phosphotyrosine Proteomics by Optimization of Phosphotyrosine Enrichment and MS/MS Parameters. *J Proteome Res*, 16(2), 1077–1086 (2017)
DOI: 10.1021/acs.jproteome.6b00576

126. L. C. Gillet, P. Navarro, S. Tate, H. Rost, N. Selevsek, L. Reiter, R. Bonner and R. Aebersold: Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics*, 11(6), O111 016717 (2012)

127. B. K. Erickson, C. M. Rose, C. R. Braun, A. R. Erickson, J. Knott, G. C. McAlister, M. Wuhr, J. A. Paulo, R. A. Everley and S. P. Gygi: A Strategy to Combine Sample Multiplexing with Targeted Proteomics Assays for High-Throughput Protein Signature Characterization. *Mol Cell*, 65(2), 361–370 (2017)
DOI: 10.1016/j.molcel.2016.12.005

128. A. Wolf-Yadlin, S. Hautaniemi, D. A. Lauffenburger and F. M. White: Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc Natl Acad Sci U S A*, 104(14), 5860–5 (2007)
DOI: 10.1073/pnas.0608638104

129. N. R. Kitteringham, R. E. Jenkins, C. S. Lane, V. L. Elliott and B. K. Park: Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci*, 877(13), 1229–39 (2009)
DOI: 10.1016/j.jchromb.2008.11.013

130. D. C. Liebler and L. J. Zimmerman: Targeted quantitation of proteins by mass spectrometry. *Biochemistry*, 52(22), 3797–806 (2013)
DOI: 10.1021/bi400110b

131. A. C. Peterson, J. D. Russell, D. J. Bailey, M. S. Westphall and J. J. Coon: Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics*, 11(11), 1475–88 (2012)
DOI: 10.1074/mcp.O112.020131

132. S. Gallien, E. Duriez, C. Crone, M. Kellmann, T. Moehring and B. Domon: Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. *Mol Cell Proteomics*, 11(12), 1709–23 (2012)
DOI: 10.1074/mcp.O112.019802

133. N. L. Anderson, N. G. Anderson, L. R. Haines, D. B. Hardie, R. W. Olafson and T. W. Pearson: Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*, 3(2), 235–44 (2004)
DOI: 10.1021/pr034086h

134. J. R. Whiteaker, L. Zhao, S. E. Abbatiello, M. Burgess, E. Kuhn, C. Lin, M. E. Pope, M. Razavi, N. L. Anderson, T. W. Pearson, S. A. Carr and A. G. Paulovich: Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol Cell Proteomics*, 10(4), M110 005645 (2011)

135. J. R. Whiteaker, L. Zhao, P. Yan, R. G. Ivey, U. J. Voytovich, H. D. Moore, C. Lin and A. G. Paulovich: Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry Enables Multiplex, Quantitative Pharmacodynamic Studies of Phospho-Signaling. *Mol Cell Proteomics*, 14(8), 2261–73 (2015)
DOI: 10.1074/mcp.O115.050351

136. J. J. Kennedy, P. Yan, L. Zhao, R. G. Ivey, U. J. Voytovich, H. D. Moore, C. Lin, E. L. Pogosova-Agadjanyan, D. L. Stirewalt, K. W. Reding, J. R. Whiteaker and A. G. Paulovich: Immobilized Metal Affinity Chromatography Coupled to Multiple Reaction Monitoring Enables Reproducible Quantification of Phospho-signaling. *Mol Cell Proteomics*, 15(2), 726–39 (2016)
DOI: 10.1074/mcp.O115.054940

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