Biocompare

The Buyer's Guide for Life Scientists

Discovery and Characterization of Post-Translational Modifications

Sponsored by

Cytoskeleton, Inc.

Contents

Simple Technology, Powerful Results

Signal-Seeker™ PTM Toolkits

Signal-Seeker™ Detection Kits

Simple

- Comprehensive kit minimizes optimization time, while maximizing PTM detection
- Analyse with standard western immunoblotting

Powerful

- Unparalleled sensitivity and precision to identify low abundance modified proteins
- Study multiple PTMs using the same system

Essential Kit Components:

- IP Affinity Beads
- IP control Beads
- Universal Lysis System
- Key Inhibitors
- Wash Reagents
- Elution Reagents
- Spin Columns
- Protein Quantitation System
- Chemiluminescence

The Protein Experts
Cytoskeleton, Inc.

Ordering information
for USA and Canada:
Online - cytoskeleton.com
Phone - 303.322.2254
Fax - 303.322.2257

PTMs: Functional Regulators of Disease

Misregulation of key proteoforms occurs in numerous diseases; as a result, many PTMs are being investigated as targets for therapeutic intervention.

Angelo DePalma

Post-translational modification (PTM) includes more than 200 diverse, covalent alterations that alter a protein's behavior. Phosphorylation, glycosylation, and acetylation are the most common PTMs, resulting from the enzymatic addition of phosphate, glycans, and acetyl groups, respectively. PTMs may also involve much larger additions to the protein backbone. For example, ubiquitination tacks on the regulatory protein ubiquitin, and SUMOylation joins the protein of interest with one of several small ubiquitin-like modifier proteins.

Phosphorylation, the most intensely studied PTM, plays critical roles in cellular homeostasis and often serves as a type of on–off switch for active proteins. Acetylation is another PTM with profound effects on such cellular processes as cell cycle, apoptosis, DNA repair, and signal transduction.

PTMs in cancer

Cancer is a process through which cells are reprogrammed, through several layers of regulation, to fulfill the tumor's need to migrate and proliferate.[1] Recent research has revealed significant layers of regulation controlling cell signaling and metabolic pathways to which PTMs contribute. Moreover, the crosstalk between and among PTMs, as well as deregulation of PTM–PTM interactions, has been implicated in the pathogenesis of several diseases, including many cancers.[2]

Over- or under-expression of acetylation, glycosylation, phosphorylation, methylation, neddylation, prenylation, SUMOylation, and ubiquitination occurs in numerous proteins associated with cancer, including GPCR, p53, DAPK1, NF-kB, and others.[3]

Understanding the roles of PTMs in cancer therefore affords researchers and clinicians novel therapeutic intervention points, and potential biomarkers for diagnosing cancer, monitoring its progression, and assessing treatment efficacy.

The tumor suppressor protein PTEN, a phosphatidylinositol and tensin homolog, regulates cellular adhesion, migration, proliferation, growth, and survival. PTEN's tumor suppression arises from its inhibition of the PI3K/Akt signaling pathway integral for cell survival and growth.[4] A wealth of information in fact reveals that common PTEN PTMs (phosphorylation, ubiquitination, SUMOylation, acetylation, and oxidation) dynamically alter the protein's stability, activity, localization, and inter-protein interactions. Moreover, defective post-translational regulation of PTEN, which occurs more frequently in cancerous vs. normal cells, leads to loss of PTEN activity and is one of the most common mutations in human cancers.[5]

Similarly, the transcription factor p53 regulates the expression of up to 3,000 genes involved in apoptosis, senescence, cell cycle arrest, DNA repair, apoptosis, tumor microenvironment, autophagy, and tumor invasion and metastasis.[6,7] p53 regulation occurs through as many as 50 PTMs, among which ubiquitination, phosphorylation, and acetylation appear to be the most significant (Figure 1).[8,9]

The Wnt/β-catenin signaling pathway regulates cellular proliferation, differentiation, and migration during embryonic development and participates in adult cell homeostasis. This pathway is regulated through PTMs, and its dysregulation has been implicated in several illnesses, including degenerative diseases and cancer.[10]

As with most proteins, Wnt signaling and its significant molecular actors are regulated by multiple PTMs, with individual PTMs capable of both

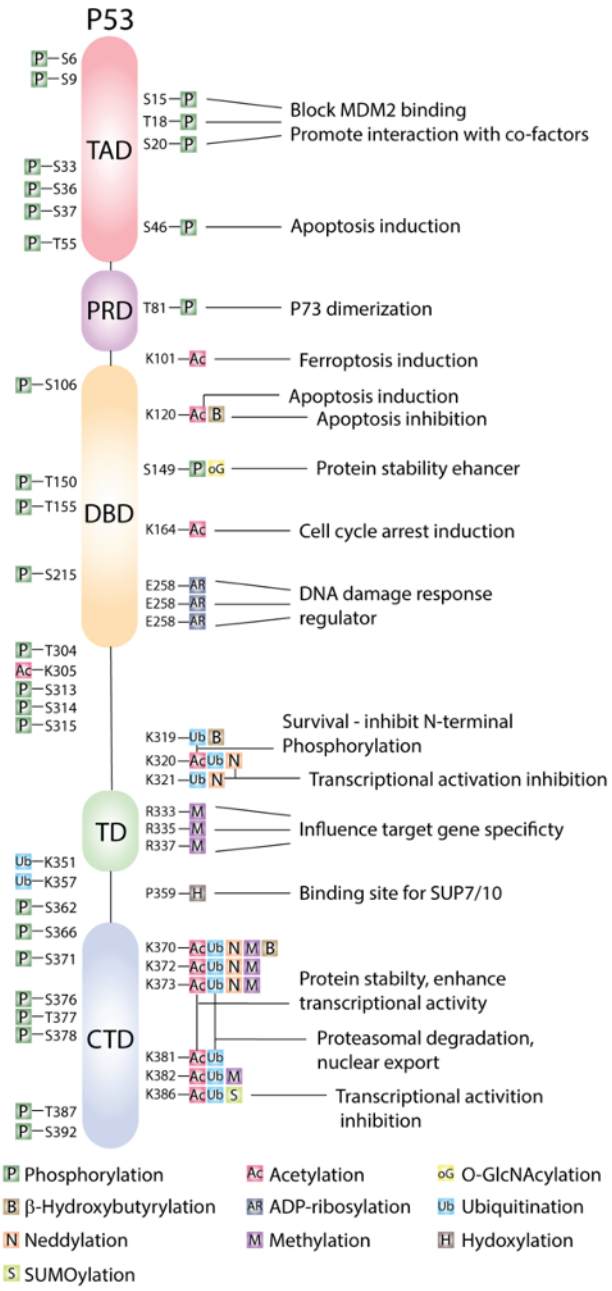

Figure 1. Schematic of P53 post-translational modifications

enhancing and inhibiting activity, depending on the amino acid residue on which it acts. In addition, the influence of PTMs may be cooperative or mutually exclusive. Understanding these complex, diverse, and interoperating regulatory processes, with the

goal of treating and diagnosing human cancers, requires sensitive analytical methods and reagents, a topic we will cover extensively in this eBook.

PTMs in cardiovascular disease

The pathophysiology of heart disease often involves the death or dysfunction of cardiomyocytes, whose contractile abilities depend on the proper functioning of ion channels and pumps, cytoskeletal proteins, and receptors. Many of the relevant proteins are regulated through PTMs, which rapidly but profoundly affect the activity of affected proteins and hence the cellular responses.[11]

For example, the trimeric troponin complex, which is controlled by calcium, regulates sarcomere contraction. One component of the complex, cardiac troponin I (cTnI), serves as a biomarker of heart disease due to its degradation and appearance in the blood. Moreover, phosphorylation of cTnI is altered in human heart disease.[12] Studies have uncovered other PTMs on cTnI, including acetylation, oxidation, cleavage,[13] and methylation, with implications for diagnosis and treatment of cardiomyopathies, such as myocardial infarction and heart failure.[14]

PTMs in stem cell research

Pluripotent stem cells (PSCs), which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), maintain their primitive differentiation status through the action of transcription factors that activate pluripotency-promoting genes and concomitantly suppress differentiation-promoting genes. The expression levels and transactivation capabilities of these transcription factors are regulated by PTMs.[15]

The pluripotent transcription factors Oct4, Sox2, and Nanog regulate transcription through complex mechanisms whereby each transcription factor functions independently but is also capable of auto-inhibition.[16] All three transcription factors are in turn regulated through PTMs.

Oct4 expression level and transcriptional activity are regulated by ubiquitination, SUMOylation, and phosphorylation. For example, ubiquitinated Oct4 decreases levels of that transcription factor and induces ESC differentiation.[17] Similarly, Nanog undergoes phosphorylation on multiple serine residues, which reduces its activity and enhances ESC differentiation,[18] while Sox2 expression in ESCs is regulated through competition between methylation and phosphorylation.[19]

PTMs and pharmaceutical development

PTMs influence cellular processes (and hence the etiologies of numerous diseases) through their modulation of a protein's physical and chemical properties, folding, conformation, stability, and activity—all of which must exist within narrowly defined states for normal functioning. The number of sites where PTMs may occur, and the diversity of possible post-translational changes, transforms a proteome originating from about 21,000 genes into millions of proteins with unique functions and activities.[20,21]

Our understanding of the role of PTMs in disease is still very much focused on the local effects of PTMs, their proximity to pharmaceutical binding sites, and their involvement in protein–protein interactions.[22] Those limitations, however, have not stopped drug developers from designing both protein and small-molecule drugs that enhance or disrupt the activity of PTMs or the proteins carrying them. Many

PTM-disrupting or -activating drugs belonging to several classes have been approved, and many more are in development (Table 1).

Future perspectives

PTMs govern a broad swath of cellular regulatory events, many of which are implicated in disease. This brief introduction provides a small sample of the significance of PTMs in this context and hints at possible future directions for the development of drugs and diagnostics based on PTM analysis. There is every reason to expect that as the roles of PTMs in disease become better understood, new small-molecule and biologic drugs will emerge that are safer and more effective than the examples we have provided. That understanding, however, depends on the availability of reagents and analytics that are up to the task of unraveling the complex and often interrelated activities of PTMs in both healthy and diseased cells.

Table 1: PTM Therapeutics

	PTM Target	Description	Ref
Approved Drugs			
Kinase targeted cancer therapies	Phosphorylation	49 approved PKIs	brimr.org/PKI/PKIs.htm reviewed in 23
HDAC inhibitors	Acetylation	5 approved HDAC inhibitors. (e.g. Belinostat–peripheral T-cell lymphoma)	reviewed in 24
PARP inhibitors	PARylation	4 approved PARP inhibitors (e.g. Olaparib–ovarian cancer)	reviewed in 25
Proteasome inhibitors	Ubiquitination	3 approved proteasome inhibitors (e.g. Ixazomib–multiple myeloma)	reviewed in 26
Clinical Drugs and Testing			
HDAC inhibitors	Acetylation	15 HDAC inhibitor drugs in phase 1-III (e.g. EX-527–Huntington disease)	reviewed in 24
Search: Phosphorylation	Phosphorylation	487 clinical trials	clinicaltrials.gov
Search: Acetylation	Acetylation	137 clinical trials	clinicaltrials.gov
Search: Methylation Protein	Methylation (Proteins)	157 clinical trials	clinicaltrials.gov
Search: Glycosylation	Glycosylation	50 clinical trials	clinicaltrials.gov
Search: Ubiquitination	Ubiquitination	59 clinical trials	clinicaltrials.gov
MLN4924	NEDDylation	30 clinical trials	reviewed in 27
Search: Prenylation	Prenylation	5 clinical trials	clinicaltrials.gov
Preclinical (Newer Drug Targets)			
SUMO E1, E2, or SENP targeting drugs	SUMOylation	19 drugs in development that target SUMOylation regulatory enzymes	reviewed in 28
DUB inhibitors	Ubiquitination	27 DUB inhibitors	reviewed in 29
PROTAC	Ubiquitin E3 ligase	Chimeric drugs utilizing E3 recognition	reviewed in 30
Glycosylation chemical inhibitors	Glycosylation	Several classes of chemical inhibitors	reviewed in 31

About the author

Angelo DePalma earned his Ph.D. in organic chemistry from Stony Brook University and was previously senior scientist at Schering-Plough. He has written extensively on biotechnology, biomanufacturing, medical devices, pharmaceutical commerce, laboratory instrumentation, and advanced materials.

References

1. Martin-Bernabe A. et al. 2017. The importance of post-translational modifications in systems biology approaches to identify therapeutic targets in cancer metabolism. Curr. Opin. Struct. Biol. 3, 161–169.
2. Zheng W. et al. 2019. Crosstalk of intracellular post-translational modifications in cancer. Arch. Biochem. Biophys. 676, 108-138.
3. Sharma B.S. et al. 2019. Post-translational Modifications (PTMs), from a Cancer Perspective: An Overview. Oncogene. 2. 10.35702/onc.10012.
4. Song M.S. et al. 2012. The functions and regulation of the PTEN tumor suppressor. Nat. Rev. Mol. Cell Biol. 13, 283–296.
5. Chalhoub N. and Baker S.J. 2009. PTEN and the P13-Kinase Pathway in Cancer. Annu Rev Pathol. 4, 127–150.
6. Bieging K.T. et al. 2014. Unravelling mechanisms of p53-mediated tumour suppression. Nat. Rev. Cancer. 14, 359–370.
7. Kruiswijk F. et al. 2015. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat. Rev. Mol. Cell Biol. 16, 393–405.
8. Kruse J.P. and Gu W. 2009. Modes of p53 regulation. Cell. 137, 609–622.
9. Bode A.M. and Dong Z. 2004. Post-translational modification of p53 in tumorigenesis. Nat. Rev. Cancer. 4, 793–805
10. Polakis P. 2000. Wnt signaling and cancer. Genes Dev. 14, 1837–1851
11. Liddy K. et al. 2013. Functional decorations: post-translational modifications and heart disease delineated by targeted proteomics. Genome Med. 5, 20.
12. Wijnker P.J.M. et al. 2014. Troponin I phosphorylation in human myocardium in health and disease. Neth. Heart J.22, 463–469.
13. Zabrouskov V. et al. 2008. Unraveling molecular complexity of phosphorylated human cardiac troponin I by top down electron capture dissociation/electron transfer dissociation mass spectrometry. Mol. Cell Proteomics. 7, 1838–1849.
14. Onwuli D.O. et al. 2019. The inhibitory subunit of cardiac troponin (cTnl) is modified by arginine methylation in the human heart. Int J Cardiol. 282, 76–80.
15. de los Angeles A. et al. 2015. Hallmarks of pluripotency. Nature. 525, 469–478.
16. Pan G. et al. 2006. A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J. 20, 1730–1732.
17. Xu H.M. et al. 2004. Wwp2, an E3 ubiquitin ligase that targets transcription factor Oct-4 for ubiquitination. J. Biol. Chem. 279, 23,495–23,503.
18. Kim S.H. et al. 2014. ERK1 phosphorylates Nanog to regulate protein stability and stem cell self-renewal. Stem Cell Res. 13, 1–11.
19. Fang L. et al. 2014. A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. Mol Cell. 255, 537–551.
20. van Kasteren S.I. et al. 2007. Expanding the diversity of chemical protein modification allows post-translational mimicry. Nature. 446(7139).1105–1109.
21. Lee T.Y. et al. 2006. dbPTM: an information repository of protein post-translational modification. Nucleic Acids Res. 34(Database issue), D622–D627.

22. Min-Gang S. et al. 2017. Investigation and identification of functional post-translational modification sites associated with drug binding and protein-protein interactions. BMC Syst Biol. 11 (Suppl7), 132.

23. Bhullar K.S. et al. 2018. Kinase-targeted cancer therapies: progress, challenges and future directions. Mol Cancer. 17(1):48.

24. Eckschlager T. et al. 2017. Histone deacetylase inhibitors as anticancer drugs. Int J Mol Sci. 18(7).

25. Veneris J.T. 2019. Choosing wisely: Selecting PARP inhibitor combinations to promote anti-tumor immune responses beyond BRCA mutations. Gynecol Oncol.

26. Gupta N. et al. 2019. Clinical pharmacology of Ixazomib: The first oral proteasome inhibitor. Clin Pharmacokinet. 58(4):431–49.

27. Zhou L. et al. 2019. Neddylation: a novel modulator of the tumor microenvironment. Mol Cancer. 18(1):77.

28. Yang Y. et al. 2018. Small-molecule inhibitors targeting protein SUMOylation as novel anticancer compounds. Mol Pharmacol. 94(2):885–94.

29. Poondla N. et al. 2019. Deubiquitinating enzymes as cancer biomarkers: new therapeutic opportunities? BMB Rep. 52(3):181–9.

30. Zou Y., Ma D., Wang Y. 2019. The PROTAC technology in drug development. Cell Biochem Funct. 37(1):21–30.

31. Esko J.D., Bertozzi C., Schnaar R.L. 2015. Chemical tools for inhibiting glycosylation. Essentials of Glycobiology. Cold Spring Harbor (NY). 701–12.

Proteoform Discovery and Validation Techniques for the Molecular Biologist

Identification of key technical issues that hinder PTM proteoform investigation and useful biological techniques utilized to overcome them.

Kim Middleton and Henrick Horita

Post-translational modifications (PTMs) are enzymatically regulated, covalent modifications that change a protein's structure. These alterations to functional groups on a protein can affect its stability, spatial localization, and binding partners, ultimately regulating its function.[1] Any given protein is likely to be modified by a number of different PTMs, and each specific PTM primes the protein to perform a unique and dynamic function in the cell. Therefore, it is reasonable to hypothesize that PTMs are an omnipresent mechanism regulating many diverse functions of any given protein.

In support of this hypothesis, PTMs such as ubiquitination, glycosylation, acetylation, SUMO-ylation, and phosphorylation have been shown to regulate nearly every biological process through control of signal transduction, protein turnover, protein recognition, and localization.[2,3] These PTMs and others vastly increase proteomic complexity by orders of magnitude because each post-translationally modified protein represents a unique protein-form (proteoform).[4-6] Importantly, characterization of specific proteoforms has resulted in great insight into the target protein's function (See the last article in this eBook, PTM Functional Characterization and Beyond, page 26, for more information), and a significant number are being actively pursued as promising therapeutic targets or biomarkers (See the previous article in this eBook, PTMs: Functional Regulators of Disease, page 4, for further insight). For example, a particularly promising therapeutic targets the p53 proteoform MDM2 ubiquitinated p53, which has been shown to be deregulated in a wide variety of cancers.[7]

Unraveling mechanistic aspects of PTM proteoforms via molecular biology approaches is technically very challenging. With the exception of phosphoproteins, histones, and several medically relevant proteins such as p53 and Tau, there are few proteoform-specific antibodies in the PTM toolbox, necessitating the use of more indirect analytical techniques. The remainder of this article addresses key technical issues associated with PTM proteoforms as well as useful discovery and validation techniques utilized to overcome them.

Technical issues when studying PTMs

- Low stoichiometry
  - Proteoforms tend to be sub-stoichiometric; any one proteoform accounts for less than 1–5% of a protein's total population at any given time, making them difficult to detect
- Highly dynamic
  - Proteoforms are dynamic, often appearing transiently in response to a specific signal
- Cell or tissue specificity
  - Proteoforms can be cell or tissue type specific
- Sample preparation: Gain or loss of PTMs
  - PTM profiles can be positively and negatively altered during sample processing
- Utilizing proper controls
  - Key controls are critical for interpreting results

The preceding list comprises some of the major technical issues that make detecting PTM proteoforms challenging (see page 26 for specific examples). The following are commonly used techniques that molecular biologists use to investigate novel proteoforms of their target protein.

Enrichment of PTM proteoforms

Enrichment of PTM proteoforms is necessary due to stoichiometry issues[8-10] and is often achieved by immunoprecipitation (IP) techniques. Enrichment with IP utilizes antibodies, protein-binding domains, metal ion affinity, and other specific binding molecules that are attached to a solid support matrix (such as agarose resin). These affinity matrices bind to the target protein or PTM, while non-targeted proteins in the complex lysate are not captured and removed through wash steps. The enriched PTM proteoforms are then detached and isolated from the support matrix using a concentrated volume of elution buffer. The isolated population is analyzed in downstream applications like western blotting or mass spectrometry to determine if a target protein is post-translationally modified.

Two approaches are possible for endogenous PTM proteoform enrichment; one approach uses an affinity matrix that recognizes the target protein, while the other uses an affinity matrix specific for the PTM of interest. An affinity matrix against the target protein of interest may immunoprecipitate potentially all proteoforms of that specific protein, while IP with a PTM-specific affinity matrix may immunoprecipitate nearly all proteoforms modified by that PTM.

Western blotting analysis

Western blotting is a standard molecular biology approach used to identify a specific protein of interest from a complex protein mixture or lysate. In the case of PTM proteoform analysis, the enriched sample is mixed with SDS, separated to specific regions in an acrylamide gel based on size (SDS-PAGE), transferred to a blotting membrane, and specifically identified by antibody recognition.

Figure 1A shows an example where ubiquitinated EGFR was enriched with an ubiquitin affinity reagent and visualized with an EGFR antibody. The western blot results show unambiguous identification of ubiquitinated EGFR. These data are supported by the loss in the ubiquitinated EGFR band when the deubiquitinase inhibitor NEM was removed from

the lysis buffer, thus allowing deubiquitination to occur (Figure 1A). The reciprocal experiment was performed using an EGFR antibody for IP enrichment. The enriched proteins were then visualized by probing with a pan-ubiquitin antibody. Figure 1B shows minimal ubiquitinated EGFR was detected with this approach. One possible explanation for this stark difference could be due to the bound-ubiquitin protein blocking the EGFR antibody recognition site (Figure 1C and D). Previous research has shown that some PTM modifications may block the antibody-binding site on a target protein and prevent interaction with the antibody—thus producing a false negative result.[11]

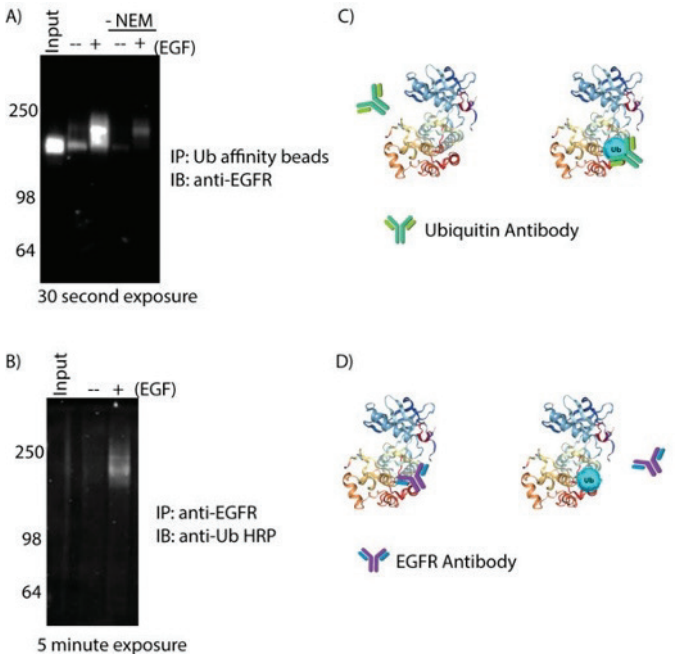

Figure 1. Detection of EGFR ubiquitination. Serum-restricted A431 cells were either unstimulated (–) or stimulated with EGF (+) for 15 minutes prior to lysis with BlastR lysis buffer with or without NEM. WCL was analyzed for EGFR levels (Input). (A) Lysates were incubated with UBA01 ubiquitin affinity beads and analyzed for ubiquitinated EGFR with an anti-EGFR antibody. (B) Lysates were incubated with anti-EGFR antibody and protein-G beads. Captured proteins were analyzed for ubiquitinated EGFR with an anti-Ub-HRP conjugated antibody.

Mass spectrometry analysis

Mass spectrometry (MS) is an analytical method that can identify a PTM proteoform based on its mass-to-charge ratio. Two distinct MS approaches are used to investigate PTM proteoforms, bottom-up and top-down analysis (which are described in more detail in Mass Spectrometry and Post-Translational Modifications, page 22). Benefits of mass spectrometry are its potentially unbiased approach, PTM site specificity, and independence from antibodies for detection. Technical challenges when utilizing MS include protein abundance bias,[12] method sensitivity[13] and methodological expertise, such as sample preparation, digestion strategies, fractionation approaches, and other considerations.[14] Investigating PTM proteoforms with MS requires both a comprehensive understanding of the biological model and technical expertise with the instrumentation/analysis; thus, we have found that successful PTM proteoform investigation with this method requires strong collaboration between molecular biologists and MS experts.

Overexpression and mutagenesis

Overexpression is a well-established system where a plasmid of a tagged version of a target protein is transfected into cells, which usually results in high expression levels. The expressed protein normally contains a tag that is recognized by a well-characterized antibody (e.g., His). The increased expression and optimized enrichment antibody improves the chance of identifying the PTM proteoform and is commonly analyzed by western blot. The overexpression system is particularly important when a proteoform is challenging to study endogenously because it allows investigators to control expression, localization, and other factors

through mutagenesis approaches. Importantly, mutagenesis approaches enable site-specific PTM investigation and characterization. Due to the overexpressed nature, any critical identification should be validated by additional methods, and the final article in this eBook, page 26, provides examples where overexpression studies were performed during PTM proteoform characterization.

In vitro biochemical assay

Biochemical assays utilize purified or *in vitro* translated versions of a target protein to determine if it can be modified by a specific PTM. The purified protein is added to a test tube with specific enzymes (e.g., E1, E2, E3 ubiquitin ligase) and the appropriate substrate (e.g., ubiquitin), co-factors, and energy sources. After incubation, the sample is then analyzed by western blot analysis. It is important to note that *in vitro* biochemical analysis is not available for all types of PTMs; however, it is routinely performed to investigate phosphorylation,[15] ubiquitination,[16] SUMOylation,[17] and other PTM modifications. A limiting step in performing *in vitro* biochemical assays is obtaining purified versions of the target protein and modifying enzymes.[18]

Proximity ligation assay

Proximity ligation assay (PLA) is a novel immunoassay technology that can be used to study protein interactions and PTMs. PLA is unique in its ability to identify proteoforms in fixed tissues and cells.[19] The principle of PLA-PTM works by utilizing two antibodies; one targets a PTM of interest while the other binds a specific protein of interest.[20] The initial steps are similar to standard immunofluorescence staining where the primary antibodies bind the epitopes of interest, and the secondary antibodies recognize their respective primary antibodies. The difference with PLA is that these secondary antibodies have short DNA strands covalently attached to them (these antibody–DNA complexes are called PLA probes). If the two PLA probes are in close enough proximity, presumably because the two antibodies are bound to a proteoform, they will form circularized DNA. PCR amplification of circularized DNA is performed, and fluorescently labeled complementary DNA probes are added for visualization. Due to the significant DNA amplification, which can be up to several hundred-fold, the fluorescent signal from very few molecules will be visible by microscopy.

Specialized techniques

An emerging technology that will have profound effects on the PTM field is genetic code expansion. This technology allows researchers to specifically add a PTM of interest, homogenously onto a target protein without a requirement for the specific modifying enzyme.[21] Having homogenous purified versions of the PTM proteoform will be critical for structural studies, *in vitro* functional studies, and others. There are numerous other techniques available that are outside the scope of this article, such as NMR spectroscopy for site specificity and structural insight of proteoforms,[22] thermal dissociation assays for detection of enzymatic PTM removal,[23] and Nanopore technology for label-free PTM detection.[24] Additionally, for each specific PTM there are highly specialized methods that have been developed. For example, investigators are using Hotspot Thermal Profiling of native proteins in live cells to identify which site-specific phosphorylation events alter protein stability.[25] Other investigators are utilizing novel ubiquitin clipping mechanisms to gain insight into polyubiquitin chains and architecture.[26] Furthermore, novel approaches have been developed to quantitate kinetic parameters of

Table 1. Pros and cons of PTM enrichment and detection strategies		
Enrichment Strategies		
PTM Antibody Immunoprecipitation	**Protein Antibody Immunoprecipitation**	**PTM and Protein Overexpression**
Pros: • May be able to study endogenous PTM changes • Kits are available: minimal IP optimization • No PTM interference with PTM affinity reagents • No specialized tools required • No specialized expertise required • Familiarity with target protein antibody for western application	**Pros**: • Familiarity with target protein antibody • May be able to study endogenous PTM changes • No specialized tools required • No specialized expertise required	**Pros:** • Useful when target specific antibody is not available • Confirm site specific modification • Useful if modification is undetectable by protein specific IP • Increase chance of capturing overexpressed protein (expression and tagging) • May not require as much starting material as the target protein should be highly expressed
Cons: • Identification of PTM IP antibody, bead conjugation, IP optimization (if kits are not available) • False negatives are possible if lower abundance PRM-modified proteins are not captured • No site specificity	**Cons:** • Must validate target protein antibody works in IP applications • PTM modification may block binding site of antibody against target protein; thus, false negatives are possible • Heavy and light chain contamination, unless additional bead conjugation steps are performed • Requires optimization of IP methodology • Must identify PTM antibody for western blotting • No site specificity	**Cons:** • Requires plasmids for overexpression of tagged target protein and tagged PTM • Requires optimization for transfection and expression and/or producing stable overexpression cell lines • Adding tags to proteins may alter their function relative to the endogenous target protein • Not physioligical; thus, may produce false positive data • Added time and expenses due to transfection • Requires optimization of IP methodology
Detection Strategies		
Western Blotting	**Mass Spectrometry**	***In Vitro* Biochemical Analysis**
Pros: • Familiarity with target protein anitbody for western application • Familiarity with western methodology • No specialized tools required • No specialized expertise required	**Pros:** • Broad spectrum analysis of proteins modified by PTM • Broad spectrum analysis of PTMs that modify target protein • Unbiased approach to identify novel PTM modified proteins • Identify site specific modifications • Cores may be available to perform mass spectrometry analysis (for a fee)	**Pros:** • Determine if target protein is modified by specific PTM • Useful tool to identify regulatory enzymes for PTM modivication of specific POI • Good validation tool to confirm PTM modification of target POI
Cons: • No site specificity • Western protocol may alter some modification like oxidation • False negatives are possible if lower abundance PTM-modified proteins are not captured	**Cons:** • Requires mass spectrometer • Requires mass spectrometry expertise to run samples and analyze large data sets • Significant mass spectrometry and sample preparation optimization • False negatives are possible due to mass spectrometry limitations and bias	**Cons:** • Proteins, enzymes, and substrates are not a physiologic concentrations, thus, false positive results may be possible • Requires obtaining purified or translated POI as well as enzymes required for specific PTM modification • May not be representative of what occurs in cells and animals

regulatory enzymes such as lysine acetyl transferases using reverse-phase HPLC.[27]

Conclusions

Due to a PTM proteoform's biological nature, (i.e., post-translationally modified proteins are present at low stoichiometric levels), identification of a specific proteoform often goes undetected via standard protein detection methods like western blotting and immunofluorescence staining. Table 1 highlights the pros and cons of several enrichment and detection approaches that may be used for preliminary investigation. The PTM-specific IP approach is particularly beneficial, as kits are available in this format that allow investigators to bypass extensive optimization issues. A recent review identified Signal-Seeker kits as a novel tool to investigate PTMs of target proteins.[28] More information is provided in the next article, Signal-Seeker PTM Discovery Toolkits: Utilities and Applications, page 17. Better tools and approaches to efficiently detect these important modifications will undoubtedly facilitate the PTM proteoform discovery and validation process and, when used in combination with other PTM investigation tools, will allow for successful functional characterization of a PTM proteoform.

About the authors

Kim Middleton, Ph.D., and Henrick Horita, Ph.D., are scientists at Cytoskeleton in Denver, Colorado.

References

1. Buuh ZY, Lyu Z, Wang RE. Interrogating the Roles of Post-Translational Modifications of Non-Histone Proteins. Journal of Medicinal Chemistry. 2017.
2. Marko-Varga G, Fehniger TE. Proteomics and disease—the challenges for technology and discovery. J Proteome Res. 2004;3(2):167–178.
3. Seo J, Lee KJ. Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J Biochem Mol Biol. 2004;37(1):35–44.
4. Jensen ON. Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. Curr Opin Chem Biol. 2004;8(1):33–41.
5. Archakov A, Lisitsa A, Ponomarenko E, Zgoda V. Recent advances in proteomic profiling of human blood: clinical scope. Expert Rev Proteomics. 2015;12(2):111–113.
6. Regnier FE, Kim J. Proteins and Proteoforms: New Separation Challenges. Anal Chem. 2018;90(1):361–373.
7. Sanz G, Singh M, Peuget S, Selivanova G. Inhibition of p53 inhibitors: progress, challenges and perspectives. J Mol Cell Biol. 2019;11(7):586–599.
8. Johnson H, Eyers CE. Analysis of post-translational modifications by LC-MS/MS. Methods Mol Biol. 2010;658:93–108.
9. Wu R, Haas W, Dephoure N, Huttlin EL, Zhai B, Sowa ME, et al. A large-scale method to measure absolute protein phosphorylation stoichiometries. Nat Methods. 2011;8(8):677–683.
10. Ordureau A, Munch C, Harper JW. Quantifying ubiquitin signaling. Mol Cell. 2015;58(4):660–676.
11. Fuchs SM, Strahl BD. Antibody recognition of histone post-translational modifications: emerging issues and future prospects. Epigenomics. 2011;3(3):247–249.
12. Nordon IM, Brar R, Hinchliffe RJ, Cockerill G, Thompson MM. Proteomics and pitfalls in the search for potential biomarkers of abdominal aortic aneurysms. Vascular. 2010;18(5):264–268.
13. Toby TK, Fornelli L, Kelleher NL. Progress in Top-Down Proteomics and the Analysis of Proteoforms. Annu Rev Anal Chem (Palo Alto Calif). 2016;9(1):499–519.
14. Feist PE, Sun L, Liu X, Dovichi NJ, Hummon AB. Bottom-up proteomic analysis of single HCT 116 colon carcinoma multicellular spheroids. Rapid Commun Mass Spectrom. 2015;29(7):654–658.

15. Glickman JF. Assay Development for Protein Kinase Enzymes. In: Sittampalam GS, Coussens NP, Brimacombe K, Grossman A, Arkin M, Auld D, et al., editors. Assay Guidance Manual. Bethesda (MD)2004.

16. Lim SO, Li CW, Xia W, Cha JH, Chan LC, Wu Y, et al. Deubiquitination and Stabilization of PD-L1 by CSN5. Cancer Cell. 2016.

17. Sarge KD. Analysis of Protein Sumoylation. Curr Protoc Protein Sci. 2016;83:14 8 1–8.

18. Brockly F, Piechaczyk M, Bossis G. Production and Purification of Recombinant SUMOylated Proteins Using Engineered Bacteria. Methods Mol Biol. 2016;1475:55–65.

19. Leuchowius KJ, Weibrecht I, Soderberg O. In situ proximity ligation assay for microscopy and flow cytometry. Curr Protoc Cytom. 2011;Chapter 9:Unit 9 36.

20. Elfineh L, Classon C, Asplund A, Pettersson U, Kamali-Moghaddam M, Lind SB. Tyrosine phosphorylation profiling via in situ proximity ligation assay. BMC Cancer. 2014;14:435.

21. Chen H, Venkat S, McGuire P, Gan Q, Fan C. Recent Development of Genetic Code Expansion for Posttranslational Modification Studies. Molecules. 2018;23(7).

22. Kumar A, Narayanan V, Sekhar A. Characterizing Post-Translational Modifications and Their Effects on Protein Conformation Using NMR Spectroscopy. Biochemistry. 2019.

23. Eskonen V, Tong-Ochoa N, Valtonen S, Kopra K, Harma H. Thermal Dissociation Assay for Time-Resolved Fluorescence Detection of Protein Post-Translational Modifications. ACS Omega. 2019;4(15):16501–16507.

24. Restrepo-Perez L, Wong CH, Maglia G, Dekker C, Joo C. Label-Free Detection of Post-translational Modifications with a Nanopore. Nano Lett. 2019;19(11):7957–7964.

25. Huang JX, Lee G, Cavanaugh KE, Chang JW, Gardel ML, Moellering RE. High throughput discovery of functional protein modifications by Hotspot Thermal Profiling. Nat Methods. 2019;16(9):894–901.

26. Swatek KN, Usher JL, Kueck AF, Gladkova C, Mevissen TET, Pruneda JN, et al. Insights into ubiquitin chain architecture using Ub-clipping. Nature. 2019;572(7770):533–537.

27. Njeri CW, Ononye OE, Balakrishnan L. Quantification of *In Vitro* Protein Lysine Acetylation by Reversed Phase HPLC. Methods Mol Biol. 2019;1983:49–56.

28. Antfolk D, Antila C, Kemppainen K, Landor SK, Sahlgren C. Decoding the PTM-switchboard of Notch. Biochim Biophys Acta Mol Cell Res. 2019:118507.

Signal-Seeker PTM Discovery Toolkits: Utilities and Applications

Novel tools drive advances in PTM proteoform discovery of target proteins in several biological processes, such as viral pathogenicity, immune checkpoints, and molecular signaling pathways.

Henrick Horita, Andy Law, and Kim Middleton

Dysregulation of post-translational modifications (PTMs) have been implicated in the progression of diseases like cancer, metabolic diseases, neurologic diseases, and others.[1-4] Therapies such as tyrosine kinase inhibitors specifically target PTM regulatory mechanisms. Emerging therapeutics that target the acetylation (Ac), SUMOylation 2/3 (SUMO 2/3), and ubiquitination (Ub) pathways are in development and demonstrate the relevance of PTMs as critical physiological and pathological regulators (Go back to the first article in this eBook, PTMs: Functional Regulators of Disease, page 4, for more information).

The characterization of proteins such as p53, epidermal growth factor receptor (EGFR), and histones has unveiled the essential role that PTMs play in regulating a protein's function to effectively orchestrate cellular events.[5-7] PTM regulation is not restricted to this small subset of proteins; on the contrary, evidence from proteomic analyses suggests greater than 70% of proteins are phosphorylated or ubiquitinated at some point during their "life cycle."[8]

Identification of a novel PTM for a target protein, defining its physiologic role, and studying its potential cross-talk with other PTMs is still a challenging process. The previous article in this eBook, Proteoform Discovery and Validation Techniques for the Molecular Biologist, page 10, highlights tools and approaches for investigating PTMs and provides tips and suggestions for the molecular biologist to utilize when performing initial discovery studies. Here we provide additional insight into Signal-Seeker kits, a PTM discovery tool that enables investigators to rapidly identify if their protein of interest is endogenously modified by one or more of these critical PTMs in their biological system.

Importantly, every Signal-Seeker kit is a comprehensive immunoprecipitation (IP) system that utilizes optimized reagents including a universal lysis system, validated IP and elution buffers, and finely tuned affinity matrices to simplify and accelerate the discovery process. Figure 1 highlights the general workflow for utilizing this system. Below we describe three applications where investigators utilized these toolkits to determine if their target protein was post-translationally modified.

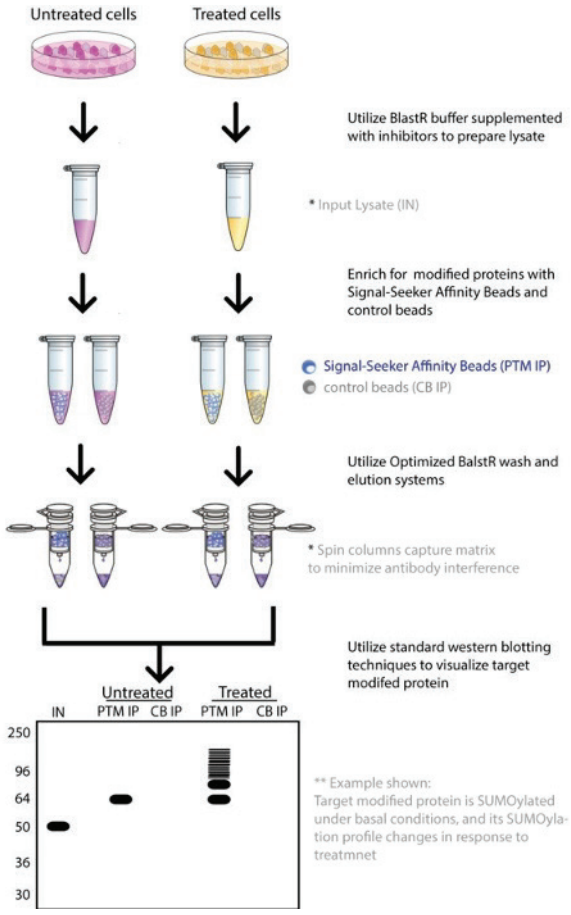

Figure 1. Workflow of Signal-Seeker PTM identification kits. Diagram depicting steps performed in order to obtain PTM profiles for a target protein.

SUMOylation of viral protein APH-2

Human T lymphotropic virus type 2 (HTLV-2) is an asymptomatic virus that shares similar genomic organization to the HTLV-1 virus, which has been linked to several diseases including cancer.[9] Both viruses have antisense proteins, but while the HTLV-1 antisense protein HBZ plays a significant role in virus-induced pathogenicity such as cell transformation,[10] the HTLV-2 antisense protein APH-2 does not play a role in cell proliferation or transformation. Interestingly, several studies have shown that the APH-2 protein is highly unstable with a half-life of 20–30 minutes, which may explain its minimal role in HTLV-2 infection.[11,12] Comparative studies were performed to decipher critical and functional differences between the two antisense proteins to better understand their roles in viral pathogenicity.

There is a growing body of knowledge that suggests that viruses hijack the cellular SUMOylation machinery to aid in propagation.[13,14] Since SUMOylation can affect protein stability, Dubuisson et al. sought to decipher if this PTM altered APH-2 instability. A critical first step was the discovery that APH-2 is endogenously modified by SUMO 2/3, which was determined using the Signal-Seeker toolkit.[15] Additional molecular studies determined that SUMO modification localized APH2 to PML nuclear bodies for degradation. Interestingly, alteration of SUMOylation had little effect on HBZ expression. This study highlights the importance of PTMs like SUMOylation in virus regulation and supports ongoing strategies to utilize the SUMO molecular machinery to potentially combat viral infection.[15]

Mono-ubiquitination of immune checkpoint protein PD-L1

Cancer cells have evolved mechanisms to suppress the host's immune system through cell surface expression of checkpoint inhibitors, like the programmed cell death ligand 1 (PD-L1) protein, as a key mechanism for cancer progression.[16] PD-L1 is overexpressed in many different cancers[17] and has garnered significant attention as a key target for anticancer therapies. Several antibody-based drugs targeting the PD-L1/PD-1 axis have been FDA approved[18]; still, ongoing research aims to determine why only some PD-L1–positive tumors respond to treatment.[19] A better understanding

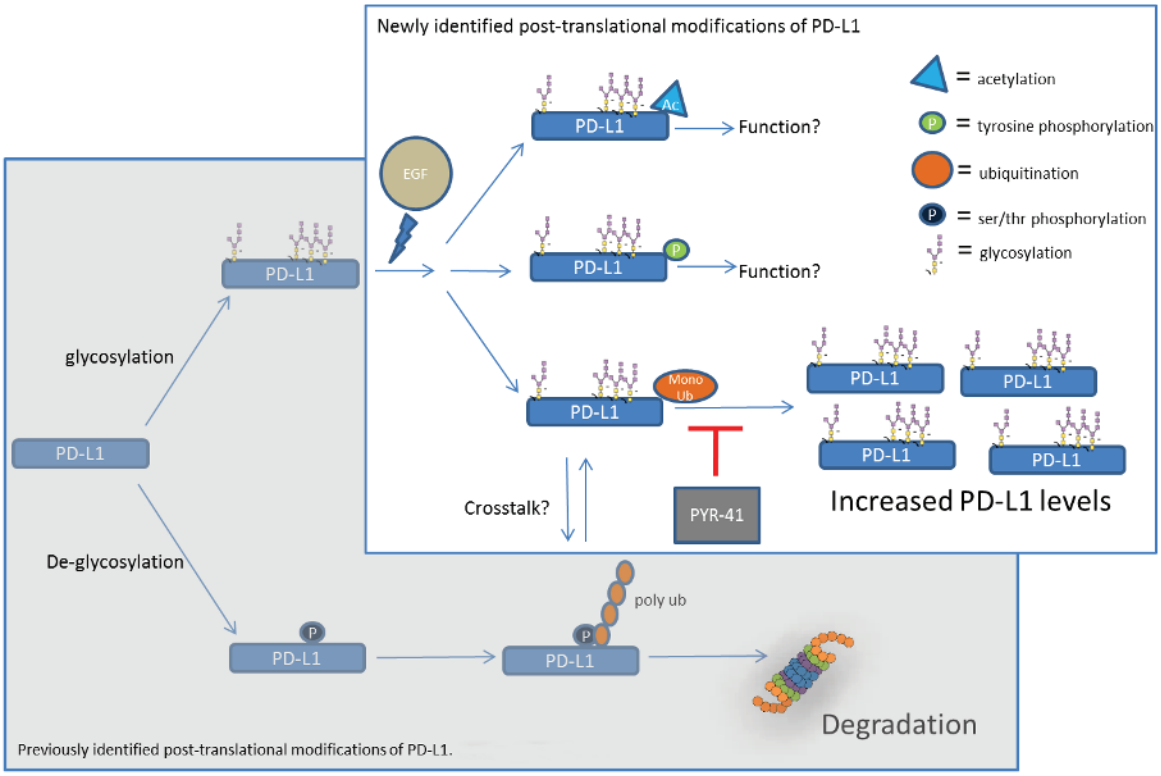

Figure 2. Schematic of PD-L1 PTMs. Figure adapted from Horita et al. 2017. Neoplasia.21 Model: Profile of PD-L1 post-translational modifications and their roles in regulating PD-L1 protein levels.

of the mechanisms regulating PD-L1 may result in more efficacious therapeutics.

While the transcriptional regulatory mechanisms of PD-L1 expression have been reported previously,[20] it was difficult to find reports regarding PTM of this protein. Using the Signal-Seeker toolkits, the authors examined PD-L1's levels of tyrosine phosphorylation, ubiquitination, acetylation, and SUMOylation in A431 cells treated with epidermal growth factor (EGF).[21] This study led to the novel identification of PD-L1–modified tyrosine phosphorylation, acetylation, and mono- and multi-ubiquitination (Figure 2).

The investigators utilized these toolkits to examine the functional role of PD-L1 ubiquitination and determined, through temporal studies, that mono- and multi-ubiquitination preceded an EGF-stimulated increase in total PD-L1 protein expression.[21] Pharmacological inhibition of EGFR activation further demonstrated that mono- and multi-ubiquitination of PD-L1 relies on EGFR activation. Importantly, inhibition of ubiquitin E1 activating enzyme blocked EGF-induced increases in total PD-L1 protein, revealing a potential mechanistic role for mono-ubiquitinated PD-L1 in the regulation of total PD-L1 protein levels. Ultimately, these studies suggest that regulatory PTM mechanisms of PD-L1 may be important for regulating its expression and function for immune homeostasis.

PTM characterization of the EGFR signaling pathway

The third example highlights the use of Signal-Seeker toolkits to examine the PTM profile changes in the well-studied EGFR–rat sarcoma (Ras)–c-Fos axis in response to EGFR stimulation. This pathway was selected for several reasons: (i) the level of endogenous, non-EGF stimulated target proteins spans a range from abundant to low level expression (EGFR > Ras > c-Fos), which gave some indication of the dynamic range of the toolkits; (ii) selected protein targets represent transmembrane (EGFR), cytoplasmic/membrane bound (Ras), and nuclear (c-Fos) proteins, which gave an indication of the efficiency of the toolkits to detect protein targets from a comprehensive range of cellular compartments; and (iii) multiple reports of PTM proteoforms for this set of proteins are available in the literature, which gave an indication of the reliability of the toolkits.[22]

While tyrosine phosphorylation (pY) of this pathway is well characterized, investigation of other PTMs like Ac, SUMO 2/3, and Ub in the same biological system using a single lysis system has not been previously reported. Eleven of the possible 12 proteoforms were identified using the Signal-Seeker system, which correlates with all 10 of the previously identified proteoforms, while also identifying an unreported proteoform, acetylated c-Fos.[22]

The Signal-Seeker toolkits enabled investigation of the PTM status of proteins in various cellular compartments that ranged from low to high abundance. The dynamic and endogenous levels of these PTMs were investigated in a single lysis system, providing insight into potential crosstalk between these four PTMs in response to physiologic stimulants like EGF. Collectively, the data suggests that these toolkits provide a simple approach for effective investigation of established and novel PTMs for any target protein.

Conclusions

Often, it is only a very limited pool of researchers that have studied any given target protein and therefore have the expertise and insight to know what experimental system, conditions, and timelines are necessary to study their target protein. A set of validated tools that empower these researchers to effectively determine if their protein is modified in their specific experimental model, without the need to develop specialized methods, should greatly facilitate PTM discovery. We highlighted three examples where Signal-Seeker tools identified endogenous PTMs of the target proteins. While Signal-Seeker toolkits were developed based on conventional IP principles, they are far from a standard IP reagent. The comprehensive, highly refined, and optimized nature of the toolkits, akin to QIAGEN MAXI prep kits for plasmid purification, make them simple yet powerful PTM discovery tools that are perfect for non-PTM experts.

About the authors

Henrick Horita, Ph.D., Andy Law, M.Sc., and Kim Middleton, Ph.D., are scientists at Cytoskeleton Inc. in Denver, Colorado.

References

1. Wu Z, Huang R, Yuan L. Crosstalk of intracellular post-translational modifications in cancer. Arch Biochem Biophys. 2019;676:108138.

2. Marcelli S, Corbo M, Iannuzzi F, Negri L, Blandini F, Nistico R, et al. The Involvement of Post-Translational Modifications in Alzheimer's Disease. Curr Alzheimer Res. 2018;15(4):313–335.

3. Yan K, Wang K, Li P. The role of post-translational modifications in cardiac hypertrophy. J Cell Mol Med. 2019;23(6):3795–3807.

4. Gao J, Shao K, Chen X, Li Z, Liu Z, Yu Z, et al. The involvement of post-translational modifications in cardiovascular pathologies: Focus on SUMOylation, neddylation, succinylation, and prenylation. J Mol Cell Cardiol. 2019;138:49–58.

5. Jenuwein T, Allis CD. Translating the histone code. Science. 2001;293(5532):1074–1080.

6. Liu Y, Tavana O, Gu W. p53 modifications: exquisite decorations of the powerful guardian. J Mol Cell Biol. 2019;11(7):564–577.

7. Nguyen LK, Kolch W, Kholodenko BN. When ubiquitination meets phosphorylation: a systems biology perspective of EGFR/MAPK signalling. Cell Commun Signal. 2013;11:52.

8. Mertins P, Qiao JW, Patel J, Udeshi ND, Clauser KR, Mani DR, et al. Integrated proteomic analysis of post-translational modifications by serial enrichment. Nat Methods. 2013;10(7):634–637.

9. Xie L, Green PL. Envelope is a major viral determinant of the distinct *in vitro* cellular transformation tropism of human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2. J Virol. 2005;79(23):14536–14545.

10. Mesnard JM, Barbeau B, Cesaire R, Peloponese JM. Roles of HTLV-1 basic Zip Factor (HBZ) in Viral Chronicity and Leukemic Transformation. Potential New Therapeutic Approaches to Prevent and Treat HTLV-1-Related Diseases. Viruses. 2015;7(12):6490–6505.

11. Halin M, Douceron E, Clerc I, Journo C, Ko NL, Landry S, et al. Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. Blood. 2009;114(12):2427–2438.

12. Panfil AR, Dissinger NJ, Howard CM, Murphy BM, Landes K, Fernandez SA, et al. Functional Comparison of HBZ and the Related APH-2 Protein Provides Insight into Human T-Cell Leukemia Virus Type 1 Pathogenesis. J Virol. 2016;90(7):3760–3772.

13. Lowrey AJ, Cramblet W, Bentz GL. Viral manipulation of the cellular sumoylation machinery. Cell Commun Signal. 2017;15(1):27.

14. Everett RD, Boutell C, Hale BG. Interplay between viruses and host sumoylation pathways. Nat Rev Microbiol. 2013;11(6):400–411.

15. Dubuisson L, Lormieres F, Fochi S, Turpin J, Pasquier A, Douceron E, et al. Stability of HTLV-2 antisense protein is controlled by PML nuclear bodies in a SUMO-dependent manner. Oncogene. 2018;37(21):2806–2816.

16. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450–461.

17. Ritprajak P, Azuma M. Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma. Oral Oncol. 2015;51(3):221–228.

18. Li Y, Li F, Jiang F, Lv X, Zhang R, Lu A, et al. A Mini-Review for Cancer Immunotherapy: Molecular Understanding of PD-1/PD-L1 Pathway & Translational Blockade of Immune Checkpoints. Int J Mol Sci. 2016;17(7).

19. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. Clin Cancer Res. 2013;19(5):1021–1034.

20. Chen J, Jiang CC, Jin L, Zhang XD. Regulation of PD-L1: a novel role of pro-survival signalling in cancer. Ann Oncol. 2016;27(3):409–416.

21. Horita H, Law A, Hong S, Middleton K. Identifying Regulatory Posttranslational Modifications of PD-L1: A Focus on Monoubiquitinaton. Neoplasia. 2017;19(4):346–353.

22. Horita H, Law A, Hong S, Middleton K. A simple toolset to identify endogenous post-translational modifications for a target protein: a snapshot of the EGFR signaling pathway. Biosci Rep. 2017.

MS and Post-Translational Modifications

An essential tool for the growth in proteoform identification and its evolution towards more effective PTM investigation.

Angelo DePalma

There are more than 200 known post-translational modifications (PTMs), as mentioned in the first article in this eBook, PTMs: Functional Regulators of Disease, page 4, which provides a detailed explanation of PTMs. As a result of these diverse chemical modifications, more than one million unique protein isoforms (proteoforms) arise from the human coding genome consisting of just around 21,000 genes (Figure 1). PTMs are valued as biomarkers and are useful in diagnosing and monitoring diseases as well as treatments, while their underlying processes have been drug discovery targets. Realizing those opportunities depends on the availability of robust, reliable analytic tools, and mass spectrometry (MS) is an emerging technology for this purpose.

Because PTMs affect the molecular weight of both intact proteins and protein fragments, MS allows the identification of specific PTMs on specific amino acid residues. MS interfaces nicely with centrifugation, electrophoresis, liquid chromatography, and many other front-end separation/purification techniques, so MS analysis is suited to many different sample types. A typical MS proteomic experiment will therefore seek PTM identification and location, as well as primary structural data.

Enrichment

Recent studies have uncovered more than 20,000 phosphorylation sites, 19,000 ubiquitination sites, and 3,600 acetylation sites on proteins commonly found in human cell lines.[1] Given the molecular diversity of a typical proteomic sample, the presence of multiple PTM-isoforms for each protein, and the presence of background contaminants,[2] most samples must undergo enrichment to improve MS detection. For example, phosphorylated peptides may be enriched with immobilized metal affinity chromatography,[3] while acetylated or ubiquitinated peptides may be enriched through antibodies.[4,5] Other peptide fractionation strategies include cation exchange, hydrophilic interaction liquid chromatography, stable-isotope labeling, and serial enrichment.[1] All of these methods share the goal

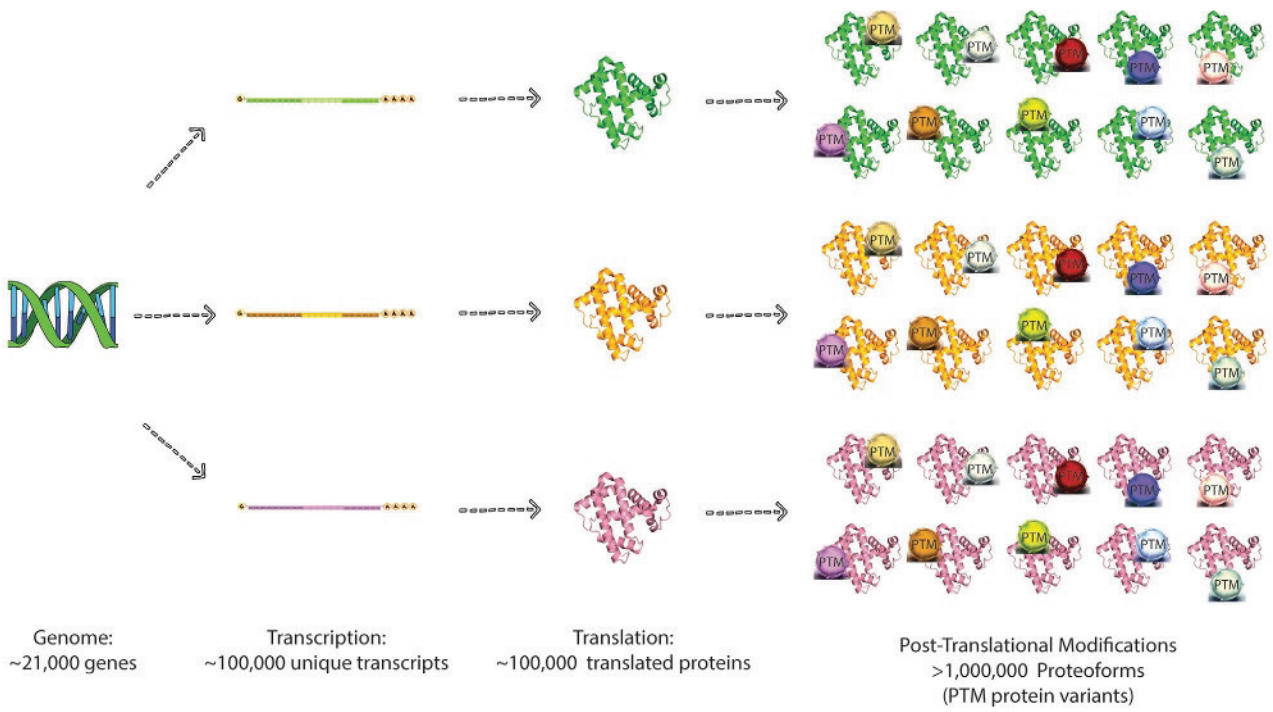

Figure 1. Expansion of genes to protein proteoforms with PTMs

of increasing the concentration or accessibility of target post-translationally modified proteins in the presence of interfering species.

Top-down vs. bottom-up proteomics

MS-based analysis of PTMs employs two major methods: top-down and bottom-up proteomics.

Conventionally, bottom-up proteomics digests proteins with proteolytic enzymes, separates them using chromatography or electrophoresis, and analyzes them by MS. Top-down methods use intact protein ions, often generated by electrospray ionization, which undergo gas-phase fragmentation.[6] Some experts also refer to methods that purify intact proteins first, followed by digestion and MS analysis, as top-down.[7] This definition emphasizes the purification step at the expense of mass spectrometry.

Depending on when the purification steps, if any, occur, methods utilizing proteolytic digestion may contain thousands or even tens of thousands of fragments from many different proteins, or the signature pieces of just a few proteins. For this reason, many proteomics samples undergo two-dimensional purification, followed by peptide mass fingerprinting and tandem MS (MS–MS).

Strengths and weaknesses

Bottom-up proteomics is relatively inefficient in terms of protein-sequence coverage, as only a small, variable fraction of the peptide fragments from any one protein is recoverable. This means that a significant quantity of PTM information is lost.[8]

Since the top-down approach operates on intact proteins, structural characteristics that are lost in bottom-up MS are retained. In addition, as the

"starting material" is intact protein, all fragmented species are relevant, and detection is possible for all PTMs. Eliminating the protein digestion step—essentially allowing the MS instrument to do the heavy lifting—also saves significant time.

Despite its advantages in some areas, the top-down approach is not a panacea. The technique will need to undergo additional improvement and refinement before it may be considered a robust approach to proteomics.

Intact proteins are, for one, more difficult to work with than peptides. Proteins tend to be less soluble than peptides, a factor affecting their suitability to purification by liquid chromatography. Large proteins, particularly membrane proteins, require detergents for solubilization. Common detergents, like sodium dodecyl sulfate (SDS), are incompatible with ESI, the ionization method of choice for very large, labile molecules.

Sensitivity and detection limits of MS on proteins are lower, generally, than for peptides, and, while less complex operationally, the throughput of top-down is significantly lower than for bottom-up methods. Hence the top-down approach is used mostly for single, isolated proteins, protein mixtures of fairly low complexity, or proteins smaller than about 50 kDa.[6]

Future directions

Top-down proteomics is improving, however. Enhancements to enrichment/prefractionation methodology, MS instrumentation, and dissociation methods for intact-protein ions have made top-down MS analysis more accessible, particularly in translational medicine.[8] For example, the top-down approach known as "spectrometric immunoassay" uses antibody-derived microcolumns to isolate intact proteins of interest as a front-end to MALDI-TOF MS analysis.[9] Techniques such as these tend to automate top-down workflows, and will continue to support its adoption as an alternative to the bottom-up approach.

Investigators are also looking into middle-up and middle-down proteomics for unequivocal characterization of PTMs. Middle-up techniques break up the protein of interest by either stopping proteolytic digestion before it is complete, by chemically cleaving disulfide bonds, or through cyanogen bromide digestion of C-terminal methionine residues.[10] Similarly, middle-down proteomics cleaves proteins into large fragments, but instead of direct MS analysis, gas-phase dissociation is performed first.[11]

MS has been instrumental in fueling growth in the PTM field, evident by the expansive number of proteoforms identified. However, functional characterization of the majority of these proteoforms is still unknown. Several different MS-based approaches aim to quantify PTM site stoichiometry as a means to prioritize investigation of abundant proteoforms.[12] Molecular biology tools and approaches may be useful to characterize promising proteoform targets. Likely, a combination of these approaches will be utilized to effectively characterize the next generation of proteoform biomarkers and therapeutic targets.

About the author

Angelo DePalma earned his Ph.D. in organic chemistry from Stony Brook University and was previously senior scientist at Schering-Plough. He has written extensively on biotechnology, biomanufacturing, medical devices, pharmaceutical commerce, laboratory instrumentation, and advanced materials.

References

1. Mertins P. et al. 2013. Integrated proteomic analysis of post-translational modifications by serial enrichment. Nat Methods. 10, 634–637.
2. Dattatreya Mellacheruvu D. et al. 2013. The CRAPome: a contaminant repository for aAffinity purification mass spectrometry data. Nat Methods. 10(8), 730–736.
3. Ficarro S.B. et al. 2009. Magnetic bead processor for rapid evaluation and optimization of parameters for phosphopeptide enrichment. Anal. Chem. 81,4566–4575.
4. Kim S.C. et al. 2006. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol. Cell. 23, 607–618.
5. Xu G., Paige J.S., Jaffrey S.R. 2010. Global analysis of lysine uiquitination by ubiquitin remnant immunoaffinity profiling. Nat. Biotechnol. 28, 868–873.
6. Proteomics: Top-down or Bottom Up. 2016. News Medical Lifeciences. Jan 12. https://www.news-medical.net/whitepaper/20160112/Proteomics-top-down-or-bottom-up.aspx
7. Wehr, T. 2006. Top-down versus bottom-up approaches in proteomics. LCGC. 24. 1004-1010.
8. Toby TK et al. 2016. Progress in top-down proteomics and the analysis of proteoforms. Annu Rev Anal Chem. 9(1), 499–519.
9. Trencheska O., Nelson R.W., Nedelkov, D. 2016. Mass spectrometric immunoassays for discovery, screening, and quantification of clinically relevant proteoforms. Bioanalysis. 8(15),1623–1633.
10. Zhang, Z., Pan, H., Chen, X. 2009. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom. Rev. 28, 147–176.
11. Fornelli L. et al. 2014. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap Fourier transform mass spectrometry. Anal. Chem. 86, 3005–3012.
12. Prus G et al. 2019. Analysis and Interpretation of Protein Post-Translational Modification Site Stoichiometry. Trends Biochem Sci. Nov; 44(11):943–960.

PTM Functional Characterization and Beyond

Characterization of Ras and Actin proteoforms using a multitude of approaches uncovered novel target-protein regulation that produced profound physiological and biological consequences.

Henrick Horita and Kim Middleton

Post-translational modifications (PTMs) are dynamic and often reversible alterations to a protein's structure. These discrete structural changes have profound regulatory effects on the protein's stability, binding partner interactions, and functions.[1-3] With the advancement in PTM identification, championed by mass spectrometry (MS) as described in the previous article in this eBook (MS and Post-Translational Modifications, page 22) and the computational biology field[4-6] scientists now estimate that millions of PTM protein-forms (proteoforms) exist in mammalian cells.[7-9]

Clearly PTMs of proteins are prevalent biological processes, but how do we unravel whether a specific proteoform has an essential biological function? Currently, only a very small fraction of proteoforms have been validated via molecular biology approaches and even fewer have been functionally characterized. When identifying key targets from omics data, validation is a critical step. The second article in this eBook (Proteoform Discovery and Validation Techniques for the Molecular Biologist, page 10) highlights effective approaches to validate whether or not a proteoform exists in an investigator's specific biological model.

Once a promising target has been validated the next step is to determine its functional role in a biological system or disease model. Functional characterization should be performed utilizing a combination of *in vitro* biochemical assays, molecular biology techniques, and animal-model approaches. Below we describe two examples where biologically important proteoforms were characterized and highlight essential tools, models, and approaches.

Ubiquitinated Ras isoforms

The Ras GTPase plays an important role in multiple signal transduction pathways involved in normal cell growth and differentiation as well as several forms of cancer.[10,11] The three isoforms of Ras—H-Ras, N-Ras, and K-Ras—were identified over 30 years ago for their oncogenic activation in human tumors.[11] Aberrant Ras signaling has been identified in more than 30%

of all human cancers with the most common being lung, colon, and pancreatic cancers.[10,12] In particular, KRas has been identified as the most important Ras protein in cancer research and, as such, correlated with over 21% of human cancers.[13] Despite extensive research on these proteins, no effective Ras inhibitor has been identified, earning KRas the reputation of an undruggable protein.

Ras proteins undergo several post-translational modifications, including proteolytic cleavage by RCE1, farnesylation, carboxymethylation by ICMT, and palmitoylation.[14] A seminal study by Jura et al. discovered that HRas protein was mono- and di-ubiquitinated to regulate its localization.[15] These studies were done primarily with overexpression of both Ras and ubiquitin proteins in a heterologous CHO-K1 cell line.[15] Utilizing these molecular biology tools allowed them to determine that HRas and NRas were ubiquitin substrates while KRas was not. Furthermore, they performed mutagenesis techniques and determined that the region of HRas that was ubiquitinated exists in the hypervariable region of the protein, which is distinct from KRas. These studies clearly showed the strength in utilizing molecular overexpression approaches to characterize the ubiquitinated HRas proteoform.

In contrast, a recent study utilizing HEK293T cells determined that KRas can be mono-ubiquitinated, and this modification altered KRas GTP loading and enhanced its affinity for specific downstream effectors.[16] The investigators hypothesized that a possible explanation for these opposing findings is that CHO-K1 cells may not have the E3 ligase ubiquitin machinery necessary for KRas ubiquitination, and highlights the importance of utilizing multiple biological models during functional characterization. KRas ubiquitination occurred on amino acid K147 as determined by western blotting and mass spectrometry.[16] Functional studies on GTP loading and downstream effectors were performed with *in vitro* biochemical assays and co-immunoprecipitation approaches.

Another interesting result from the aforementioned study in CHO-K1 cells found that HRas di-ubiquitination (di-Ub) was important for HRas activation, but was unchanged in response to upstream EGF regulation.[15] In contrast, a more recent study investigating endogenous Ras di-Ub in response to EGF was examined using the Signal-Seeker tools.[17] This study reported robust, dynamic changes in di-ubiquitinated Ras in response to EGF stimulation (Figure 1). It is likely that the overexpression system performed in CHO-K1

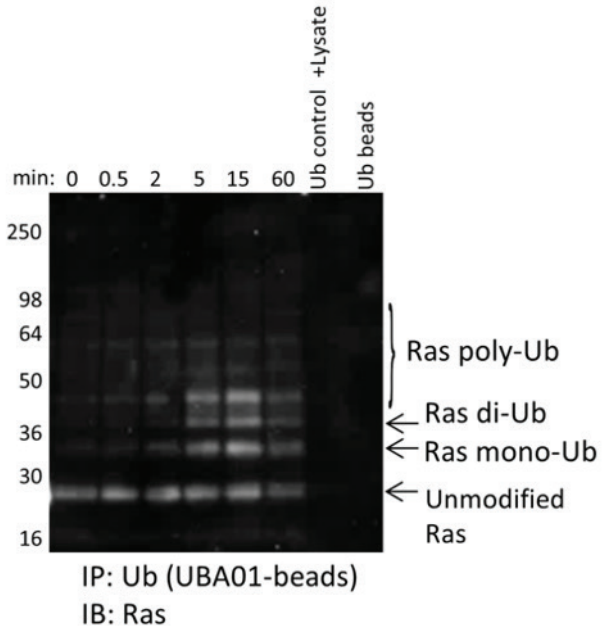

Figure 1. Endogenous ubiquitination of Ras. Figure adapted from Horita et al. 2017. Biosci Rep.[17] Serum-restricted A431 cells were stimulated with EGF for the given time period. Unstimulated and EGF-treated A431 lysates were incubated with ubiquitin-affinity beads (UBA01) to immunoprecipitate ubiquitinated proteins or ubiquitin control beads (CUB02). Samples were separated by SDS-PAGE and analyzed by western immunoblotting using a pan Ras antibody to identify ubiquitinated pan Ras. Representative western blots from n≥3 independent experiments are shown.

cells masked physiologic changes in di-Ub Ras, a result postulated by the authors of the original study. Molecular overexpression approaches are indispensable PTM investigation tools, as they enable in-depth investigation on site specificity and isoform specificity as highlighted above; however, we recommend that physiological and biological studies should be validated via endogenous PTM detection approaches. While these studies have conflicting isoform results, they collectively support the original finding that Ras ubiquitination is a biologically significant proteoform; in support of this, a recent study identified the protein LZTR1 as a functional regulator of KRas ubiquitination, and this novel mechanism may contribute to tyrosine kinase inhibitor (TKI) drug resistance.[18]

Methionine oxidation of actin

Actin is a well-characterized, abundant, and essential cytoskeletal protein. Its dynamic properties allow it to shift between monomeric (G-actin) and polymeric (F-actin) states, which is vital for many cellular processes. Actin's dynamicity and function is regulated by many internal and external cues that are facilitated by actin binding proteins (ABPs), signal transducers, and others. Additionally, several studies now indicate that actin itself is highly modified by post-translational modifications (PTMs). Furthermore, intensive studies of specific actin PTMs have detailed their effect on actin dynamics, ABP interactions, and actin-dependent physiology.[19,20]

A seminal study by the Terman group uncovered a role for the enzyme MICAL (molecule interacting with CasL) in mediating oxidation of Met44 and Met47 of actin, which they identified with mass spectrometry.[21] Functional studies, deduced primarily with elegant *in vitro* biochemical assays, showed that actin polymerization dynamics was significantly augmented due to oxidation by the redox protein MICAL1. They confirmed these findings with mutagenesis M44L or M47L studies and found that M44 was essential for MICAL1 regulation of actin polymerization. When oxidized, Met44 becomes negatively charged and interferes with actin monomer–monomer interactions, thus promoting F-actin severing and depolymerization. Importantly, the group performed studies with an *in vivo Drosophila* model and found that MICAL1 overexpression had profound consequences, producing deformed bristle formation that was rescued with M44L actin mutants.[21,22]

The robust results produced in this seminal study were reproduced by several different groups, and these models and approaches were subsequently utilized to identify the counterpart to MICAL1, which is the SelR/MsrB family of methionine sulfoxide reductase enzymes. Two groups independently identified SelR (MsrB) as the enzyme responsible for reduction of Met44 and Met47 and restoration of normal actin dynamics.[23,24] Collectively, the findings identify a reversible and regulatory PTM of actin that controls its dynamics and cytoskeletal organization (Figure 2).

Since MICALs appear to be expressed ubiquitously and the Met44 and Met47 residues of actin are highly conserved, it is likely that this mechanism of redox regulation may play a prominent role in modulating actin function in all tissue and cell types. A recent study identified MICAL-2 as a regulator of nuclear G-actin levels, subsequent MRTF-A/SRF transcriptional regulation, and physiological regulation of heart development.[25] These studies were performed with a combination of *in vitro* biochemical assays, overexpression models, and *in vivo* zebrafish studies. Another study investigated the effects that growth factors and chemo-repellents have on MICAL-oxidation regulation of actin and

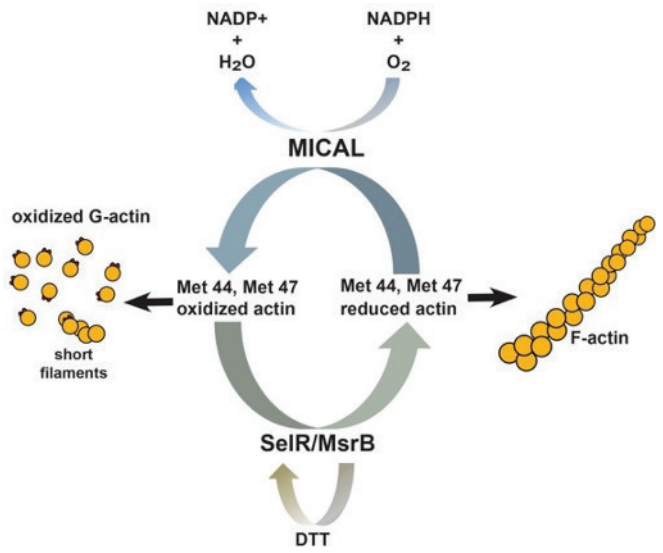

Figure 2. Actin Met44 and Met47 physiological redox system

found paradoxically that effects of the chemo-repellent were amplified by growth factor signaling, which had profound effects on physiological axon guidance regulation, as well as pathological tumor progression and response to treatment.[26] In totality, these studies identified an actin proteoform with diverse biological functions.

Conclusions

The examples above identify two critical proteoforms of very distinct proteins, which were found to be important for their respective protein's function and when altered, produced profound physiological and pathological consequences. In both cases it took a combination of *in vitro* biochemical assays, mass spectrometry, overexpression/mutagenesis, endogenous physiologic models, and *in vivo* models to effectively characterize these proteoforms. The example with Ras ubiquitination highlights potential pitfalls that arise when utilizing a single system with overexpression approaches. While overexpression systems allow investigators to hone in on specific questions regarding form and function of a proteoform, it can mask physiologic mechanisms. Conversely, physiological studies investigating endogenous proteoforms can often provide great correlative data, but gain/loss of function experiments using molecular mutagenesis approaches are useful tools to prove causation and are critical when investigating site specificity for a particular PTM. Ultimately, it is essential to reinforce the idea that utilizing multiple approaches for functional characterization is the best path forward as exemplified with actin oxidation.

Scientist's understanding of a few PTMs on a select number of target proteins has led to several therapeutic drug targets; still, there are hundreds of thousands of proteoforms waiting to be investigated with in-depth functional studies. The approaches and tools to perform proper biological and physiological validation and characterization are growing, which will simplify the task while providing more accurate and reproducible results that elucidate the importance of these PTMs and proteoforms.

About the authors

Henrick Horita, Ph.D., and Kim Middleton, Ph.D., are scientists at Cytoskeleton Inc. in Denver, Colorado.

References

1. Seo J, Lee KJ. Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J Biochem Mol Biol. 2004;37(1):35–44.
2. Bah A, Forman-Kay JD. Modulation of Intrinsically Disordered Protein Function by Post-translational Modifications. J Biol Chem. 2016;291(13):6696–6705.
3. Buuh ZY, Lyu Z, Wang RE. Interrogating the Roles of Post-Translational Modifications of Non-Histone Proteins. Journal of medicinal chemistry. 2017.

4. Beltrao P, Albanese V, Kenner LR, Swaney DL, Burlingame A, Villen J, et al. Systematic functional prioritization of protein posttranslational modifications. Cell. 2012;150(2):413–425.
5. Li S, Iakoucheva LM, Mooney SD, Radivojac P. Loss of post-translational modification sites in disease. Pac Symp Biocomput. 2010:337–347.
6. Nickchi P, Jafari M, Kalantari S. PEIMAN 1.0: Post-translational modification Enrichment, Integration and Matching ANalysis. Database (Oxford). 2015;2015:bav037.
7. Jensen ON. Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. Curr Opin Chem Biol. 2004;8(1):33–41.
8. Regnier FE, Kim J. Proteins and Proteoforms: New Separation Challenges. Anal Chem. 2018;90(1):361–373.
9. van Kasteren SI, Kramer HB, Jensen HH, Campbell SJ, Kirkpatrick J, Oldham NJ, et al. Expanding the diversity of chemical protein modification allows post-translational mimicry. Nature. 2007;446(7139):1105–1109.
10. Wang W, Fang G, Rudolph J. Ras inhibition via direct Ras binding—is there a path forward? Bioorg Med Chem Lett. 2012;22(18):5766–5776.
11. Castellano E, Santos E. Functional specificity of ras isoforms: so similar but so different. Genes Cancer. 2011;2(3):216–231.
12. Burns MC, Sun Q, Daniels RN, Camper D, Kennedy JP, Phan J, et al. Approach for targeting Ras with small molecules that activate SOS-mediated nucleotide exchange. Proc Natl Acad Sci U S A. 2014;111(9):3401–3406.
13. Baines AT, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. Future Med Chem. 2011;3(14):1787–1808.
14. Gysin S, Salt M, Young A, McCormick F. Therapeutic strategies for targeting ras proteins. Genes Cancer. 2011;2(3):359–372.
15. Jura N, Scotto-Lavino E, Sobczyk A, Bar-Sagi D. Differential modification of Ras proteins by ubiquitination. Mol Cell. 2006;21(5):679–687.
16. Sasaki AT, Carracedo A, Locasale JW, Anastasiou D, Takeuchi K, Kahoud ER, et al. Ubiquitination of K-Ras enhances activation and facilitates binding to select downstream effectors. Sci Signal. 2011;4(163):ra13.
17. Horita H, Law A, Hong S, Middleton K. A simple toolset to identify endogenous post-translational modifications for a target protein: a snapshot of the EGFR signaling pathway. Biosci Rep. 2017.
18. Bigenzahn JW, Collu GM, Kartnig F, Pieraks M, Vladimer GI, Heinz LX, et al. LZTR1 is a regulator of RAS ubiquitination and signaling. Science. 2018;362(6419):1171–1177.
19. Varland S, Vandekerckhove J, Drazic A. Actin Post-translational Modifications: The Cinderella of Cytoskeletal Control. Trends Biochem Sci. 2019.
20. Terman JR, Kashina A. Post-translational modification and regulation of actin. Current opinion in cell biology. 2013;25(1):30–38.
21. Hung RJ, Pak CW, Terman JR. Direct redox regulation of F-actin assembly and disassembly by Mical. Science. 2011;334(6063):1710–1713.
22. Hung RJ, Yazdani U, Yoon J, Wu H, Yang T, Gupta N, et al. Mical links semaphorins to F-actin disassembly. Nature. 2010;463(7282):823–827.
23. Hung RJ, Spaeth CS, Yesilyurt HG, Terman JR. SelR reverses Mical-mediated oxidation of actin to regulate F-actin dynamics. Nat Cell Biol. 2013;15(12):1445–1454.
24. Lee BC, Peterfi Z, Hoffmann FW, Moore RE, Kaya A, Avanesov A, et al. MsrB1 and MICALs regulate actin assembly and macrophage function via reversible stereoselective methionine oxidation. Mol Cell. 2013;51(3):397–404.
25. Lundquist MR, Storaska AJ, Liu TC, Larsen SD, Evans T, Neubig RR, et al. Redox modification of nuclear actin by MICAL-2 regulates SRF signaling. Cell. 2014;156(3):563–576.
26. Yoon J, Kim SB, Ahmed G, Shay JW, Terman JR. Amplification of F-Actin Disassembly and Cellular Repulsion by Growth Factor Signaling. Dev Cell. 2017;42(2):117–129.

Keep It Simple

Signal-Seeker™ PTM Toolkits

Signal-Seeker™ Detection Kits

Description	Amount	Item #
Signal-Seeker™ Acetyl-Lysine Detection Kit	30 assays	BK163
Signal-Seeker™ Ubiquitination Detection Kit	30 assays	BK161
Signal-Seeker™ SUMO 1 Detection Kit	30 assays	BK165
Signal-Seeker™ SUMO 2/3 Detection Kit	30 assays	BK162
Signal-Seeker™ Phosphotyrosine Detection Kit	30 assays	BK160

Perfect Toolkits for non-PTM Experts

- Comprehensive PTM detection kits to simplify investigation for non-PTM experts
- Simple analysis with standard western immunoblotting
- Investigate the role of known PTMs in your system
- Detect endogenous levels (vs. transfected amounts) of modified proteins
- Use different kits to build a temporal protein regulation profile

The Protein Experts
Cytoskeleton, Inc.

Ordering information
for USA and Canada:
Online - cytoskeleton.com
Phone - 303.322.2254
Fax - 303.322.2257