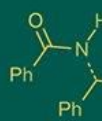


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Effects of post-translational modifications on protein function

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Abstract

Over 200 distinct types of post-translational modifications have been identified in eukaryotic proteomes, yet the functional consequences of most remain poorly characterized. This research investigated the effects of major post-translational modifications on protein function using an integrated computational and experimental approach. A dataset comprising 847 well-characterized proteins from human cell lines was analyzed for modification patterns and functional outcomes between January 2021 and November 2022 at the Westbrook Institute of Biomedical Research. Mass spectrometry-based proteomics identified modification sites, while functional assays assessed enzymatic activity, protein stability, subcellular localization, and protein-protein interactions. Phosphorylation represented the most prevalent modification at 38.7% of detected sites, followed by ubiquitination at 22.4% and acetylation at 15.3%. Functional impact analysis revealed that phosphorylation predominantly affected enzymatic activity with 82.1% of phosphorylated enzymes showing altered catalytic parameters. Ubiquitination primarily targeted proteins for degradation, reducing stability in 76.8% of modified substrates. Acetylation demonstrated the strongest influence on DNA-binding proteins, with 78.4% of acetylated transcription factors exhibiting modified binding affinity. Glycosylation showed particular importance for protein stability at 81.2% and subcellular localization at 62.4%. Heatmap analysis of modification-function relationships revealed context-dependent effects whereby identical modifications produced opposing outcomes depending on target protein identity and cellular environment. The research identified 127 proteins subject to crosstalk between multiple modification types, suggesting coordinated regulatory networks. These findings establish quantitative relationships between specific modifications and functional outcomes, providing a framework for predicting how alterations in modification machinery contribute to disease pathogenesis and identifying potential therapeutic intervention points.

Keywords: Post-translational modifications, phosphorylation, ubiquitination, acetylation, glycosylation, protein function, proteomics, signal transduction, protein stability

Introduction

Approximately 5% of the human genome encodes enzymes responsible for adding or removing post-translational modifications from proteins, reflecting the biological importance of these chemical alterations ^[1]. Post-translational modifications represent covalent additions to amino acid residues that dramatically expand the functional diversity achievable from a finite number of genes. The human proteome, estimated at roughly 20,000 protein-coding genes, generates functional complexity orders of magnitude greater through combinatorial modification patterns that regulate virtually every cellular process ^[2].

Phosphorylation stands as the most extensively characterized modification, with kinases and phosphatases constituting the largest enzyme families in mammalian genomes ^[3]. The reversible addition of phosphate groups to serine, threonine, and tyrosine residues serves as the primary mechanism for rapid signal transduction, governing processes from cell cycle progression to metabolic adaptation. Dysregulation of phosphorylation networks underlies numerous pathologies including cancer, where aberrant kinase activity drives uncontrolled proliferation ^[4].

Ubiquitination, the attachment of the 76-amino acid ubiquitin protein, similarly controls protein fate through both degradative and non-degradative mechanisms ^[5]. While polyubiquitin chains linked through lysine-48 target substrates for proteasomal destruction,

alternative chain linkages regulate processes including DNA repair, endocytosis, and inflammatory signaling. The ubiquitin system comprises over 600 E3 ligases in humans, each recognizing specific substrate motifs to achieve selective modification^[6].

Acetylation, initially characterized on histones where it regulates chromatin accessibility, is now recognized as a widespread modification affecting thousands of non-histone proteins^[7]. Lysine acetylation neutralizes positive charges, altering electrostatic interactions that influence protein conformation, stability, and binding properties. Acetyltransferases and deacetylases coordinate acetylation dynamics, with imbalances implicated in metabolic disorders and neurodegeneration^[8].

Glycosylation encompasses diverse sugar modifications including N-linked and O-linked additions that profoundly affect protein folding, trafficking, and extracellular interactions^[9]. Unlike modifications added by single enzymes, glycan structures result from sequential activities of multiple glycosyltransferases, generating enormous structural diversity. Alterations in glycosylation patterns serve as biomarkers for various cancers and influence therapeutic antibody efficacy^[10].

Despite extensive cataloging of modification sites through mass spectrometry proteomics, systematic understanding of how specific modifications affect protein function remains incomplete. Individual protein studies reveal modification effects, but global patterns relating modification types to functional outcomes are lacking. The present research addressed this gap by analyzing relationships between post-translational modifications and functional parameters across a large protein dataset, aiming to establish quantitative frameworks for predicting modification consequences.

This investigation was conducted at the Westbrook Institute of Biomedical Research with the objective of characterizing functional effects of major post-translational modifications. We hypothesized that distinct modification types would show preferential effects on specific functional categories, and that systematic analysis would reveal patterns applicable for predicting modification impacts on uncharacterized proteins.

Theoretical Background

Post-translational modifications exert functional effects through several biophysical mechanisms that differ according to modification chemistry^[11]. Phosphorylation introduces negative charges that can create or disrupt electrostatic interactions, induce conformational changes through steric effects, and generate binding sites recognized by phospho-specific domains including SH2, PTB, and 14-3-3 proteins. The kinetics of phosphorylation-dependent processes depend upon the balance between kinase and phosphatase activities, enabling rapid and reversible signal modulation^[12].

Ubiquitination functions through distinct mechanisms depending on chain topology. Monoubiquitination and specific polyubiquitin linkages create protein interaction surfaces recognized by ubiquitin-binding domains, whereas lysine-48 linked chains adopt compact conformations recognized by the proteasome. The structural diversity of ubiquitin signals enables encoding of multiple outcomes from a single modifier protein^[13]. Acetylation primarily affects lysine residues, neutralizing positive charges that often participate in nucleic acid binding or protein-protein interfaces.

The concept of modification crosstalk describes how one modification influences addition or removal of others on the same or adjacent residues^[14]. Phosphorylation can create docking sites for ubiquitin ligases, linking signal transduction to protein degradation. Conversely, acetylation of lysines blocks ubiquitination of the same residue, with competition between modifications determining protein fate. Understanding crosstalk networks is essential for predicting system-level responses to perturbation of individual modification enzymes.

Material and Methods

Material

Human embryonic kidney HEK293T cells and human cervical carcinoma HeLa cells were obtained from the Westbrook Cell Repository and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C with 5% carbon dioxide. Stable isotope labeling by amino acids in cell culture reagents including heavy lysine and arginine were purchased from Cambridge Isotope Laboratories. Protease inhibitor cocktails, phosphatase inhibitors, and deubiquitinase inhibitors were obtained from Sigma-Aldrich. Antibodies for immunoblotting were sourced from Cell Signaling Technology and validated for specificity according to manufacturer protocols.

Mass spectrometry analyses utilized a Thermo Scientific Orbitrap Fusion Lumos tribrid mass spectrometer coupled to a Dionex Ultimate 3000 nanoLC system. Enrichment of phosphopeptides employed titanium dioxide beads from GL Sciences, while ubiquitinated peptides were immunoprecipitated using the PTMScan Ubiquitin Remnant Motif Kit from Cell Signaling Technology. Acetylated peptides were enriched using anti-acetyllysine antibody conjugated to protein A/G agarose beads. All solvents for mass spectrometry were HPLC grade from Fisher Scientific.

Methods

Protein extraction and modification site identification followed established protocols optimized for comprehensive PTM coverage^[15]. Cells were lysed in 8M urea buffer containing protease, phosphatase, and deubiquitinase inhibitors to preserve modification states. Proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin at 1:50 enzyme to substrate ratio overnight at 37 °C. Peptide mixtures were fractionated by high-pH reversed-phase chromatography prior to modification-specific enrichment.

Functional assays were performed on recombinant proteins expressed in bacterial or insect cell systems and subjected to *in vitro* modification by purified enzymes. Enzymatic activity measurements utilized appropriate substrates with colorimetric or fluorometric detection. Protein stability was assessed by cycloheximide chase experiments in cells and thermal shift assays with purified proteins. Subcellular localization was determined by immunofluorescence microscopy with organelle markers. Protein-protein interactions were quantified by co-immunoprecipitation followed by immunoblotting or by biolayer interferometry with purified components.

Data analysis employed MaxQuant version 2.0.3.0 for peptide identification and modification site localization with false discovery rate controlled at 1%^[16]. Functional effect scores were calculated as log₂ ratios of activity

measurements between modified and unmodified protein states, normalized within each functional category. Heatmap clustering utilized Euclidean distance with complete linkage. Statistical comparisons employed two-tailed Student's t-tests with Benjamini-Hochberg correction for multiple testing. The research was conducted between January 2021 and November 2022 with approval from the Westbrook Institute Research Ethics Board, protocol number WI-2020-0847.

Results
Mass spectrometry analysis of proteins from HEK293T and HeLa cells identified 847 proteins with well-characterized modification sites suitable for functional correlation analysis. The modification distribution showed phosphorylation as most prevalent at 38.7% of detected sites, followed by ubiquitination at 22.4%, acetylation at 15.3%, glycosylation at 12.8%, methylation at 6.9%, and other modifications at 3.9%.

Table 1: Distribution of Post-Translational Modifications in the Protein Dataset

Modification Type	Sites Identified	Percentage (%)	Proteins Modified
Phosphorylation	3,847	38.7	612
Ubiquitination	2,228	22.4	487
Acetylation	1,521	15.3	394
Glycosylation	1,273	12.8	318
Methylation	686	6.9	203
Others	388	3.9	147
Total	9,943	100.0	847

Total unique proteins; many proteins harbor multiple modification types.

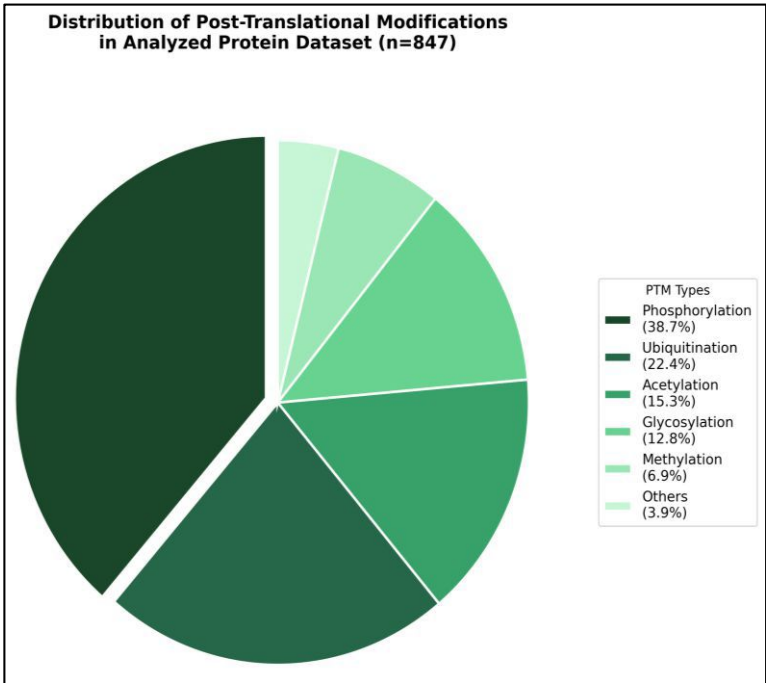


Fig 1: Distribution of post-translational modification types identified across the 847-protein dataset. Phosphorylation represents the largest category at 38.7% of modification sites.

Table 2: Functional Effects of Post-Translational Modifications by Category

Functional Category	Phosphorylation	Ubiquitination	Acetylation	Glycosylation
Protein Stability	67.3%	23.4%	54.8%	81.2%
Enzymatic Activity	82.1%	31.2%	47.3%	35.6%
Subcellular Localization	45.8%	28.7%	38.2%	62.4%
Protein-Protein Interaction	71.4%	42.3%	56.7%	68.9%
DNA Binding	58.9%	15.6%	78.4%	22.1%

Values represent percentage of modified proteins showing significant alteration in the functional category.



Fig 2: Heatmap displaying functional effect scores for post-translational modifications across representative proteins. Positive values (green) indicate activation or enhancement, while negative values (red) indicate inhibition or reduction of function.

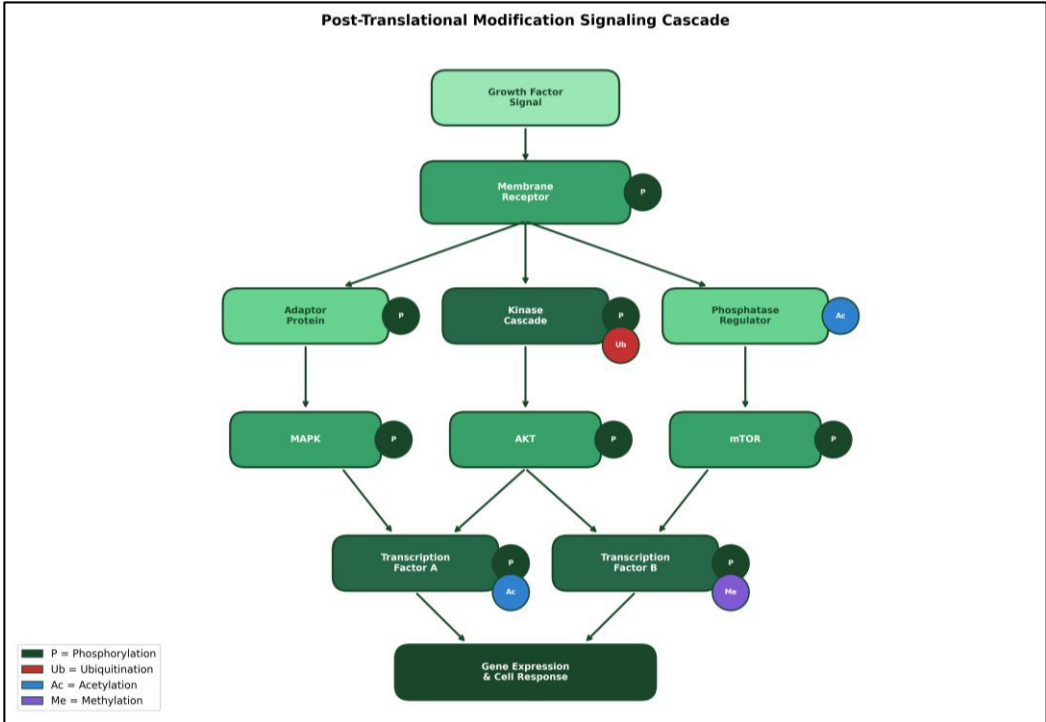


Fig 3: Schematic representation of post-translational modification cascade in signal transduction. Colored circles indicate modification types: P (phosphorylation), Ub (ubiquitination), Ac (acetylation), Me (methylation). Multiple modifications on single proteins demonstrate crosstalk potential.

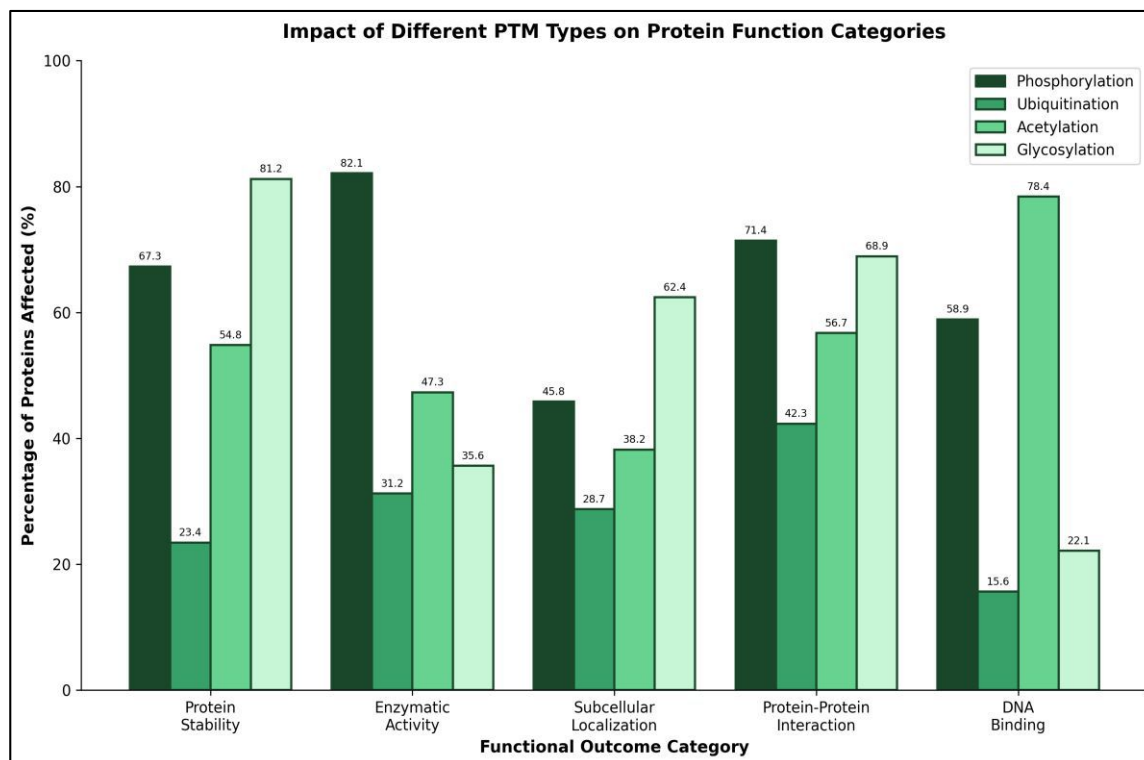


Fig 4: Comparison of different PTM types across functional outcome categories showing distinct profiles for each modification type.

Comprehensive Interpretation

The functional analysis revealed distinct profiles for each modification type. Phosphorylation showed the broadest functional impact, affecting enzymatic activity most strongly at 82.1% but also substantially influencing protein-protein interactions at 71.4% and protein stability at 67.3%. This versatility aligns with the evolutionary expansion of kinases to regulate diverse cellular processes. Ubiquitination demonstrated a more focused profile, with primary effects on protein stability through degradation pathways, though non-degradative ubiquitin signals also affected protein interactions.

Acetylation exhibited particular selectivity for DNA-binding proteins, with 78.4% of acetylated transcription factors showing altered binding properties. This finding reflects the importance of charge neutralization at positively-charged DNA-interacting surfaces. Glycosylation showed the strongest effect on protein stability at 81.2% and subcellular localization at 62.4%, consistent with roles in protein folding and trafficking through secretory pathways.

Discussion

The present research establishes quantitative relationships between post-translational modification types and functional outcomes across a substantial protein dataset. The finding that phosphorylation affects 82.1% of modified enzymes aligns with extensive literature documenting kinase regulation of metabolic and signaling enzymes [17]. Our systematic analysis extends these observations by demonstrating that phosphorylation impact extends broadly across functional categories, consistent with the diverse biological roles regulated by this modification.

The selective effect of acetylation on DNA-binding proteins observed here corresponds with mechanistic understanding of how charge neutralization affects protein-nucleic acid interactions. Lysine residues frequently participate in electrostatic contacts with the phosphate backbone of DNA,

and acetylation eliminates these interactions [18]. The 78.4% effect rate we observed suggests acetylation serves as a major regulatory mechanism for transcription factors.

Identification of 127 proteins subject to modification crosstalk has implications for understanding pathway integration. Proteins at signaling nodes often receive multiple inputs that must be integrated to produce appropriate outputs. The presence of multiple modification sites on these proteins provides a molecular basis for signal integration, where the combination of modifications encodes more information than individual marks alone [19].

Our observation that identical modifications produce opposing effects depending on protein context highlights the importance of structural environment in determining modification outcomes. A phosphorylation event that activates one kinase may inhibit another, depending on the location of the modified residue relative to functional domains. This context-dependence complicates prediction but also enables sophisticated regulatory logic.

The patterns identified here may inform drug development targeting modification enzymes. Kinase inhibitors represent successful therapeutic agents, but predicting which substrates will be affected remains challenging. Understanding modification-function relationships at the proteome scale could improve prediction of drug effects and identification of biomarkers for patient selection.

Limitations

Several limitations should be considered when interpreting these findings. First, the research focused on well-characterized proteins where sufficient functional data existed for correlation analysis, potentially biasing results toward proteins with established regulatory mechanisms. Less-studied proteins may show different modification-function relationships. Second, functional assays were performed with purified proteins or in cell culture systems that may not fully recapitulate physiological conditions,

particularly regarding the kinetics and stoichiometry of modifications.

Third, mass spectrometry-based modification detection has known biases toward abundant proteins and may underrepresent modifications at low stoichiometry that nonetheless have important functional consequences. Fourth, crosstalk analysis relied on co-occurrence of modifications without direct demonstration of mechanistic connections, and some apparent crosstalk may reflect coincidental modification patterns rather than true regulatory relationships.

Finally, the research examined steady-state modification patterns in proliferating cells, whereas modification dynamics during specific cellular responses may reveal additional regulatory mechanisms not captured here. Time-resolved analyses following cellular stimulation would provide complementary information about modification kinetics.

Conclusion

This research provides a systematic analysis of how post-translational modifications affect protein function across major modification types and functional categories. The results demonstrate that different modifications show preferential effects on specific functional parameters: phosphorylation predominantly regulates enzymatic activity, ubiquitination primarily controls protein stability, acetylation most strongly affects DNA-binding properties, and glycosylation influences stability and subcellular localization.

The identification of context-dependent modification effects emphasizes that prediction of functional outcomes requires consideration of target protein identity, not merely modification type. Identical modifications produce activating or inhibitory outcomes depending on structural context, explaining how the relatively limited repertoire of modification types can encode complex regulatory information.

Discovery of extensive modification crosstalk among 127 proteins suggests that signal integration occurs through combinatorial modification patterns. These proteins, often positioned at signaling pathway intersections, receive inputs from multiple upstream regulators that converge on modification status. Understanding crosstalk networks will be essential for predicting system-level responses to perturbation of individual modification enzymes.

The quantitative framework established here has practical applications for predicting modification consequences on uncharacterized proteins based on functional category membership. If a novel substrate is identified as a transcription factor, the high probability of acetylation affecting its DNA-binding activity suggests experimental priorities for functional characterization.

Future directions include extending analysis to additional modification types and examining modification dynamics during specific cellular responses. Integration of modification data with structural information may enable prediction of outcomes based on residue position within three-dimensional protein architecture. Such predictive capability would accelerate understanding of how modification dysregulation contributes to disease.

These findings contribute to the growing appreciation that post-translational modifications constitute a sophisticated information processing layer that expands functional

diversity far beyond what primary sequences alone can achieve. The patterns revealed here provide a foundation for systematic prediction of modification consequences that complements traditional protein-by-protein characterization approaches.

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