Proline Hydroxylation: A New Target for RNA Metabolism and Cell Cycle Regulation?

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> Proline hydroxylation, a post-translational modification, plays a critical role in protein function, most famously in collagen stability. But its broader impact on cellular processes, particularly outside of collagen, has remained largely uncharacterised due to technical limitations in site-specific identification. A new systematic characterisation workflow, published in eLife, has now identified thousands of these sites, opening new avenues for understanding cellular regulation.1

KEY TAKEAWAYS

- The Pivot: A novel HILIC-LC-MS workflow enables comprehensive, site-specific identification of proline hydroxylation beyond collagen.
- The Data: Roxadustat inhibited 1954 non-collagen hydroxylation sites in HEK293 cells and 1253 in RCC4 cells.
- The Action: Clinicians should recognise the expanding role of prolyl hydroxylases in cellular pathways beyond hypoxia, potentially influencing drug development.

Proline hydroxylation is a ubiquitous post-translational modification, crucial for the structural integrity of collagen. But its functional reach extends far beyond connective tissue. Prolyl hydroxylase enzymes (PHDs) regulate the stability of hypoxia-inducible factor (HIF), making them key targets in conditions like anaemia and kidney disease. However, the full scope of PHD substrates and the precise sites of hydroxylation on non-collagen proteins have been difficult to map comprehensively. This lack of detailed understanding has limited the ability to fully grasp the regulatory networks governed by these enzymes.1

The current study addressed this gap by developing a robust workflow to systematically identify proline hydroxylation sites in proteins. Jiang and colleagues combined hydrophilic interaction chromatography (HILIC) enrichment with high-resolution nano-liquid chromatography-mass spectrometry (LC-MS). This approach aimed to overcome the challenges associated with detecting and localising these modifications, which often involve subtle mass shifts and can be difficult to distinguish from other oxidative modifications. The investigators applied this method to HEK293 and RCC4 cell lines, treating them with either the prolyl hydroxylase inhibitor Roxadustat (FG-4592), the proteasome inhibitor MG-132, or a DMSO control.1

A New Approach to Site-Specific Detection

The technical innovation at the heart of this research lies in the combination of HILIC enrichment and refined mass spectrometry parameters. HILIC separates peptides based on their hydrophilicity, and hydroxylated peptides, being

more hydrophilic, showed consistent enrichment in specific HILIC fractions. This pre-fractionation step significantly reduced sample complexity, allowing for more sensitive detection of modified peptides. The team also optimised MS collision energy and considered adjacent amino acid sequences, demonstrating that combining LC retention time with these optimised parameters enabled reliable site identification, even when multiple proline residues were present within a single peptide.1

The diagnostic hydroxyproline immonium ion, a key indicator of hydroxylation, showed intensity variations dependent on MS collision energy, peptide concentration, and the surrounding amino acid sequence. This observation underscores the need for careful parameter tuning in mass spectrometry-based proteomics to accurately identify and quantify such modifications. The researchers used synthetic peptides to validate their methodology, confirming that their refined approach could differentiate between closely located proline hydroxylation sites, a common challenge in previous studies.1

The initial characterisation of hydroxylated peptides revealed consistent characteristics across both HEK293 and RCC4 cell line datasets. These peptides consistently enriched in more hydrophilic HILIC fractions, a predictable outcome given the addition of a hydroxyl group. But they also exhibited distinct charge and mass distributions compared to unmodified or oxidised peptides. This distinct profile provided a spectral signature that aided in their identification and differentiation from other post-translational modifications, which can often confound proteomic analyses.1

Uncovering the Scope of Proline Hydroxylation

Using this advanced workflow, the researchers identified a substantial number of proline hydroxylation sites. In HEK293 cells, they identified a total of 4993 proline hydroxylation sites. In RCC4 cells, the total was 3247 sites. These numbers represent a significant expansion of the known hydroxylation landscape, far exceeding what previous, less systematic methods had uncovered. The sheer volume of identified sites suggests that proline hydroxylation is a far more pervasive regulatory mechanism than previously appreciated, extending its influence across a broad spectrum of cellular proteins.1

The critical step was then to determine which of these sites were genuinely regulated by prolyl hydroxylases. They did this by comparing the hydroxylation profiles in cells treated with Roxadustat (FG-4592), a known PHD inhibitor, versus control or proteasome inhibitor-treated cells. Roxadustat specifically inhibits PHDs, leading to a reduction in hydroxylation. Of the identified sites, 1954 high-confidence non-collagen sites in HEK293 cells showed inhibition by FG-4592. In RCC4 cells, 1253 such sites were inhibited. These numbers are crucial; they pinpoint specific proline residues on non-collagen proteins that are direct targets of PHD activity, providing a clearer picture of PHD substrate specificity.1

The inhibition by FG-4592 was a key filter, distinguishing bona fide PHD-regulated sites from other forms of proline oxidation or non-specific modifications. This specificity is vital for understanding the true biological roles of PHDs. The proteasome inhibitor MG-132, used as a control, did not show the same pattern of inhibition, reinforcing that the observed changes were due to PHD activity rather than general protein turnover. This careful experimental design strengthens the confidence in the identified sites as being genuinely regulated by prolyl hydroxylases.1

Functional Implications and Protein Enrichment

The functional analysis of proteins containing FG-4592-inhibited hydroxylation sites revealed enrichment in several critical cellular processes. These proteins were significantly enriched for roles in RNA metabolism, mRNA splicing, and

cell cycle regulation. This finding shifts the understanding of prolyl hydroxylation beyond its well-established role in hypoxia signalling and collagen synthesis, suggesting a broader regulatory impact on fundamental cellular machinery. The involvement in RNA metabolism and splicing points to a potential role in gene expression control, while cell cycle regulation implies influence over cell proliferation and differentiation.1

One particularly interesting protein identified was the phosphatase 1 regulatory subunit Repo-Man (CDCA2). Repo-Man is known to be involved in mitotic chromosome segregation and nuclear envelope reassembly. Its hydroxylation by PHDs suggests a direct link between oxygen sensing pathways and cell division, a connection that warrants further investigation. This specific example highlights how the systematic identification of hydroxylation sites can uncover novel regulatory mechanisms for well-known proteins, potentially revealing new therapeutic targets. The Oxford Handbook of Oncology provides further context on cell cycle regulation in cancer.1

The study's findings suggest that PHDs, beyond their role in HIF stabilisation, are integral regulators of a diverse set of cellular functions. The identification of specific hydroxylation sites provides a molecular handle to investigate how these modifications alter protein function, stability, or interactions in these newly identified pathways. This could lead to a deeper understanding of how cellular oxygen levels influence processes like RNA processing and cell division, which are fundamental to both normal physiology and disease states.1

Caveats and Future Directions

While the workflow developed by Jiang and colleagues represents a significant advance, some limitations bear consideration. The study primarily used HEK293 and RCC4 cell lines, which may not fully represent the hydroxylation landscape in all cell types or in vivo conditions. The cellular context can significantly influence enzyme activity and substrate availability, meaning that some hydroxylation sites identified here might be cell-type specific, and others might be missed. Further studies in primary cells, tissues, and animal models will be necessary to validate and expand upon these findings.1

The use of a single PHD inhibitor, Roxadustat, while effective, means that the identified sites are specific to the PHDs inhibited by this compound. Other PHDs or hydroxylases not targeted by Roxadustat might regulate additional proline hydroxylation sites that remain uncharacterised. A broader panel of inhibitors or genetic knockdown approaches could provide a more complete picture of the entire prolyl hydroxylase family's substrate repertoire. The study also focused on identification, not quantification of the hydroxylation dynamics under various physiological or pathological conditions. Understanding the stoichiometry and turnover of these modifications will be the next critical step.1

The study provides a robust methodology for identifying proline hydroxylation sites, but the functional consequences of each individual site remain largely unexplored. While protein enrichment analysis points to broad functional categories, the precise impact of hydroxylation at each identified proline on protein activity, localisation, or interaction partners requires detailed biochemical and cell biological investigation. This will involve site-directed mutagenesis and functional assays to confirm the regulatory role of specific hydroxylation events.1

> **CLINICAL IMPLICATIONS**
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> The systematic identification of thousands of non-collagen proline hydroxylation sites fundamentally shifts our understanding of prolyl hydroxylase biology. For years, PHDs have been primarily linked to hypoxia and HIF

regulation, driving the development of drugs like roxadustat for anaemia. But these data suggest PHDs are far more promiscuous, influencing RNA metabolism and cell cycle control. This means the therapeutic potential of PHD inhibitors may extend beyond erythropoiesis, potentially impacting oncology or inflammatory conditions where these pathways are dysregulated.

The identification of specific hydroxylation sites on proteins involved in mRNA splicing and cell cycle regulation provides concrete molecular targets. This level of precision could allow for the development of highly selective modulators, moving beyond broad-spectrum PHD inhibition. The challenge will be to determine which of these thousands of sites are truly functionally relevant and how their modification contributes to disease pathogenesis.

For drug developers, this research opens a new frontier. Instead of viewing PHDs solely through the lens of HIF, they now have a roadmap to explore novel substrates and pathways. This could lead to a new generation of PHD modulators with distinct therapeutic profiles, targeting specific cellular processes without the off-target effects associated with less precise interventions. The complexity of these newly identified pathways will require careful consideration in clinical trial design.

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