POST-TRANSLATIONAL MODIFICATIONS

A shift for the *O*-GlcNAc paradigm

Many nuclear and cytoplasmic proteins are post-translationally modified by both *O*-GlcNAc and phosphate, but determining whether a single copy of a protein bears multiple modifications remains a challenge. A new analytic approach reveals a surprising correlation between phosphorylation and the *O*-GlcNAc modification.

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Since its discovery less than 30 years ago[1], *O*-GlcNAc glycosylation has emerged as a ubiquitous post-translational modification[2]. Nearly 1,000 transcription factors, translational regulators, cytoskeletal components and other nucleocytoplasmic proteins have been found to bear this simple glycan, which consists of a single *N*-acetylglucosamine (GlcNAc) residue attached in a β linkage to a serine or threonine side chain. Like phosphorylation, *O*-GlcNAc glycosylation is a dynamic modification, and all metazoans harbor genes encoding at least one O-GlcNAc transferase (OGT) that adds *O*-GlcNAc and one O-GlcNAcase (OGA) that removes it. Although historically overlooked, *O*-GlcNAc has clear physiological significance: it has been shown that OGT is required for embryogenesis in mice[3], whereas OGT overexpression leads to insulin resistance and hyperleptinemia[4]. Through these and other organismal studies[5], *O*-GlcNAc glycosylation is now coming into view as an essential regulatory modification. The current challenges are to discover the protein functions that are altered by *O*-GlcNAc modification and reveal the molecular mechanisms by which *O*-GlcNAc imparts these changes. Although some studies have documented the functional significance of *O*-GlcNAc modification at specific sites (see, for example, ref. 6), the competing and interrelated effects of multiple post-translational modifications often complicate the interpretation of data. In this issue, Rexach *et al.* report a simple yet innovative mass-tagging approach for quantifying *O*-GlcNAc modification levels and use this technique to characterize simultaneously the *O*-GlcNAc glycosylation states and phosphorylation states of intact proteins[7]. When compared to existing methods, their technique has the distinct advantage of providing information about the coexistence of multiple modifications on a single protein.

Proteins modified with *O*-GlcNAc are also known to be subject to phosphorylation. Indeed, these two post-translational modifications are often found at the same residues, leading to the idea that they might bear a reciprocal relationship[8]. This proposed relationship was termed the "yin-yang hypothesis" in reference to the Taoist principle that seemingly opposing forces in nature in fact complement one another. Further support for the yin-yang hypothesis came from the observation that OGT associates with a

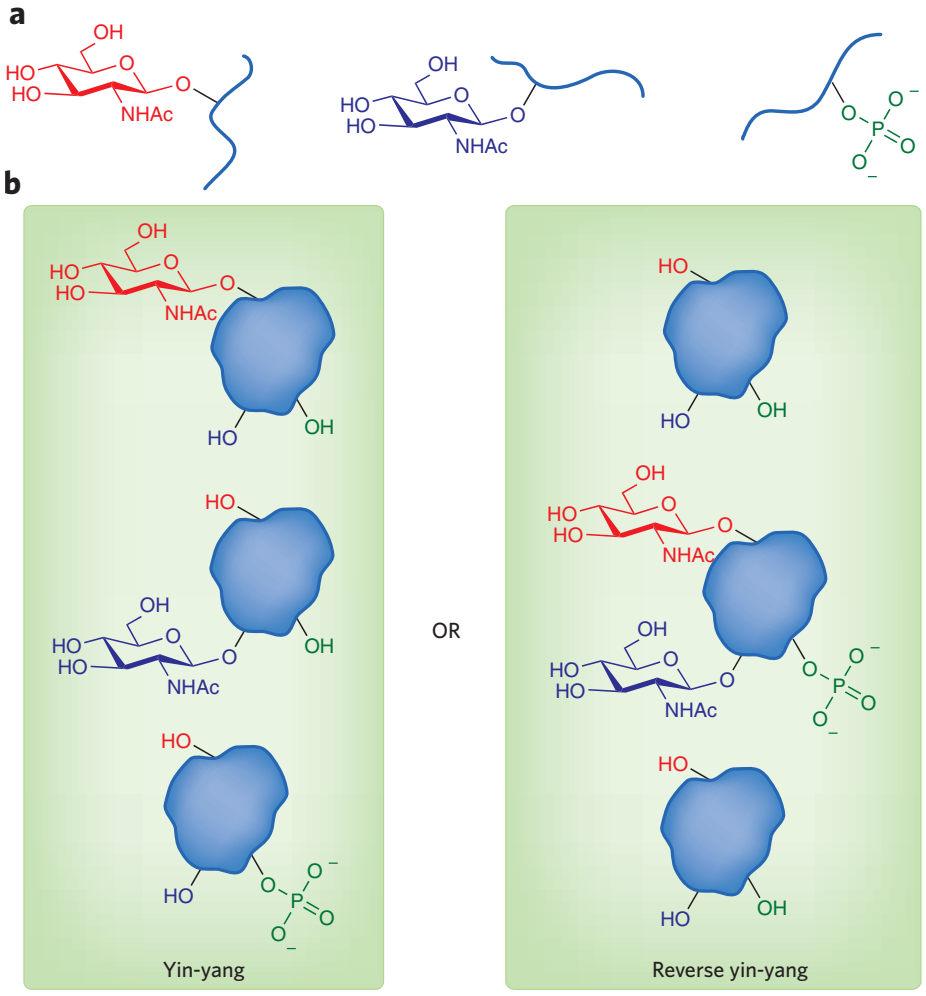

Figure 1 | Mapping multiple post-translational modifications. (**a**) Typical methods to map post-translational modifications rely on proteolytic digestion of proteins, thereby losing information about which modifications reside on the same molecule. (**b**) A new mass-tagging approach can be used to quantify and determine the stoichiometry of *O*-GlcNAc modification. Furthermore, this technique reveals new information about the interplay between *O*-GlcNAc glycosylation and phosphorylation. In at least one case, *O*-GlcNAc modification is associated with an increased probability of phosphorylation of the same molecule, a surprising reverse of the predicted yin-yang relationship.

phosphatase, forming a complex that has both dephosphorylating and *O*-GlcNAc glycosylating activity[9]. But more recent evidence suggests that *O*-GlcNAc glycosylation is not simply a placeholder for phosphorylation and that the relationship between these two modifications is much more complex. Because most post-translational modification mapping techniques use proteolytic digests that decouple modification sites (**Fig. 1a**), it remains difficult to decipher the relationship between post-translational modifications on individual copies of a protein. Does the presence of *O*-GlcNAc at one site affect the probability of phosphorylation elsewhere on the same protein? Do multiple *O*-GlcNAc modifications have a more potent effect than one? New top-down mass spectrometry approaches promise a route to answering these questions, but these methods require highly specialized equipment and laborious analysis. In addition, the notorious lability of the *O*-GlcNAc modification poses added challenges for detection and quantification via mass spectrometry[10].

In contrast, the mass-tagging approach reported by Hsieh-Wilson and co-workers[7] offers an easy way to quantify *O*-GlcNAc modification levels. In their two-step derivatization process, they first use a mutant galactosyltransferase to add a ketone 'handle' to all *O*-GlcNAc residues. In the second step, a chemoselective reaction is used to derivatize each ketone with a large polyethylene glycol (PEG) tag. The defined masses of these PEG tags predictably affect proteins' mobility in SDS-PAGE. Quantitative immunoblotting using an antibody against the protein of interest enables the authors to detect distinct bands corresponding to unmodified protein and to proteins with one or more PEG tags, an analysis that is visually similar to traditional immunoblot characterization of poly-ubiquitinated proteins. Because the authors simultaneously detect unglycosylated and glycosylated proteins, they obtain quantitative information about glycosylation levels, a key difference between this technique and methods that rely on affinity purification of glycosylated proteins. In addition to glycosylation levels, the immunoblot analysis also reveals glycosylation stoichiometry: in the case of the highly glycosylated nucleoporin Nup62, they detect molecules bearing up to ten *O*-GlcNAc residues. Perhaps the most intriguing application of this technique is its use to scrutinize the relationship between *O*-GlcNAc and phosphorylation. The authors examined methyl CpG binding protein 2 (MeCP2), a protein involved in gene silencing. Phosphorylation of MeCP2 at Ser80 is critical to its ability to associate with chromatin and regulate gene expression[11]. Using the mass-tagging approach in combination with antibodies that detect total MeCP2 and MeCP2 phosphorylated at Ser80, Rexach *et al.* showed that phosphorylation at Ser80 is correlated with increased levels of glycosylation on the same protein—the opposite of the predicted yin-yang relationship (**Fig. 1b**). These results emphasize the fact that features of distinct subpopulations of molecules can be obscured by global analysis.

Because of this technique's ease of implementation and the fact that it can be used on virtually any kind of sample, we can expect to see future studies using mass tagging to quantify *O*-GlcNAc glycosylation levels in physiologically relevant specimens. Indeed, the authors use their technique to show that the extent of CREB glycosylation in the rat brain is remarkably similar in multiple animals, suggesting that this modification is under tight regulatory control. Several lines of evidence point to disregulation of *O*-GlcNAc in diabetes and other metabolic diseases, so it will be of compelling interest to investigate how *O*-GlcNAc glycosylation levels and stoichiometry change in animal models or even patient samples. The major limitation of the mass-tagging approach is that it does not provide information about sites of glycosylation; thus, a way to link this technique with mass spectrometry–based site mapping would be extremely powerful. Finally, although the current report focuses exclusively on *O*-GlcNAc glycosylation, one can imagine using analogous strategies to add mass tags to other post-translational modifications, perhaps simultaneously quantifying multiple modifications.

Ten years ago, the report of the first draft of the human genome focused attention on the surprisingly small number of human genes, leading to the prediction that much of the diversity of protein function must arise from differential post-translational modification. The past decade has seen a massive effort to inventory where and when these modifications occur. The complementary information provided by the new mass-tagging technique demonstrates exciting progress toward properly cataloging protein modifications, developing a mechanistic picture of the underlying processes and—the ultimate goal—understanding how the combinatorial complexity of post-translational possibilities translates into human physiology. ■

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Competing financial interests

The author declares no competing financial interests.