Breaking the limits of artificial ubiquitination

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Neurodegenerative diseases such as Parkinson, Alzheimer's, and Huntington are proteopathic disorders characterized by the accumulation of intracellular insoluble aggregates of misfolded proteins and are one of the major medical concerns of our modern society. Particularly, Parkinson disease is a synucleinopathy caused by the cytosolic aggregation of α -Synuclein into Lewy bodies (1). Although its biological function remains obscure, numerous lines of evidence clearly point at α -Synuclein oligomers or aggregates as the causative agent of Parkinson disease (1). However, despite intense research, the detailed etiology of Parkinson disease remains elusive. Apart from missense point mutations in the α -Synuclein gene (which account for familial cases of early onset Parkinson disease), increased intracellular expression levels

and posttranslational modifications of α -Synuclein have been implicated in the formation of Lewy bodies. Currently, known modifications of α -Synuclein include proteolytic truncation, oxidation, nitration, phosphorylation, and ubiquitination (1). The key to understanding proteopathic diseases such as Parkinson disease rests on elucidating the role of these modifications and how they influence the properties of the nascent α -Synuclein polypeptide. In their current research article, Haj-Yahya et al. report on an important milestone in the synthesis of selectively di- and tetra-ubiquitinated (K48) α -Synuclein, which can be used to study the biophysical and biochemical consequences of α -Synuclein ubiquitination (2). In addition to offering valuable insights into molecular biology of synucleinopathies, their findings

also open up new horizons in the research on ubiquitination—a crucial posttranslational protein modification involved in quintessential aspects of cell biology (3).

Ubiquitin is a small protein of 76 amino acids and is highly conserved among all eukaryotic organisms. Ubiquitin functions as a posttranslational protein modifier that is covalently attached to lysine residues on the target substrates by its C-terminal glycine (4). Protein targets become ubiquitinated by an enzymatic transfer reaction, which involves three types of enzymes: E1, the ubiquitin-activating enzyme; E2, the ubiquitin-conjugating enzyme; and an E3 ubiquitin ligase. The concerted action of this enzymatic triad is opposed by deubiquitinating enzymes, which remove ubiquitin moieties from modified target proteins, thus contributing to highly dynamic changes in the ubiquitination status of the substrate proteins. Interestingly, the human genome encodes >600 E3 ubiquitin ligases and ~100 deubiquitinases, clearly illustrating the complexity of the intracellular machinery involved in producing ubiquitin signals (5, 6). Importantly, ubiquitin itself contains seven lysine residues enabling assembly of seven homotypic types of polymeric ubiquitin chains. Notably, assembly of ubiquitin chains can also be achieved by head-to-tail fusion of ubiquitin moieties, in that C-terminal glycine of ubiquitin is conjugated to N-terminal amino group of the next ubiquitin molecule, giving rise to linear ubiquitin chains (7). The structural, biophysical, and signaling properties of these distinct types of ubiquitin chains are determined by the specific type of linkage between single ubiquitin molecules within the polymeric chain, which can be recognized by numerous ubiquitin binding domains of a diverse range of effector proteins (7).

Despite the remarkable progress reached over the past decades of studying ubiquitination, further advances are severely hampered by our inability to obtain workable amounts

Fig. 1. Summary of the impact of diverse forms of protein ubiquitination on the biophysical and biochemical properties of α -Synuclein.

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of selectively ubiquitinated protein species. Available enzymatic methods generally do not grant sufficient specificity of introduced modifications, resulting in complex mixtures of products of unsatisfactory yield. From this perspective, synthetic and semisynthetic methods of protein chemistry proved unparalleled in delivering ubiquitinated protein species of strictly defined molecular identity. Currently, applied chemical methods for protein ubiquitination predominantly rely on native chemical ligation inspired by intein-based protein splicing, which allows traceless coupling of chemically synthesized ubiquitinated peptides with the expressed C-terminal protein fragments (8). Furthermore, the methodology for generation of isopeptide bonds can be accomplished by either (i) GOPAL (genetically encoded orthogonal protection and activated ligation), with its sophisticated amine protection/deprotection steps (9); or (ii) utilization of thiolysine, a lysine derivative that can be applied in a manner reminiscent of native chemical ligation (10). The recent progress in genetically encoded incorporation of thiolysine into proteins by using the expanded genetic code makes the latter method highly attractive (11). Spectacular results obtained thus far by protein chemistry involve monoubiquitinated histone H2B (8) and α -Synuclein (12), free di-ubiquitin conjugates of all possible linkage types (13), or tetra-ubiquitin chains conjugated to a short peptide (14), and all these synthetic molecules offered invaluable insights into the broad spectrum of the structural, biophysical, or biochemical aspects of protein ubiquitination. However, no successful synthesis of a full-length, native protein selectively modified by a polyubiquitin chain has been reported. The work of Haj-Yahya and colleagues provides an example of successful synthesis of substantial amounts of a protein modification by a longer chain of four conjugated ubiquitin molecules.

The synthetic route used by Haj-Yahya et al. relied on native chemical ligation and thiolysine-based coupling chemistry. This multistep procedure enabled a traceless attachment of a K48-linked di- or tetra-ubiquitin chain to lysine K12 of α -Synuclein, a residue previously shown to be ubiquitinated in isolates from Lewy bodies. The homogenous semisynthetic di- and tetra-ubiquitin conjugates of α -Synuclein, along with their mono- and nonubiquitinated counterparts, enabled a precise and direct comparison of the impact of ubiquitin chain length on the biophysical and biochemical properties of α -Synuclein (Fig. 1). Relevant to

the etiology of synucleinopathies, Haj-Yahya et al. evaluated the influence of ubiquitination on the aggregation tendency of α -Synuclein and its suitability as a substrate of C-terminal phosphorylation and proteasomal degradation (Fig. 1). Importantly, tetra-ubiquitinated α -Synuclein tended to form amorphous aggregates rather than fibrillar ones, suggesting that cells may deploy ubiquitination as means

Haj-Yahya et al. report on an important milestone in the synthesis of selectively di- and tetra-ubiquitinated (K48) α -Synuclein.

to dissolve the aggregates within the Lewy bodies. Furthermore, the investigation of the interplay between ubiquitination and phosphorylation and their impact on the solubility of α -Synuclein yielded interesting insights into the cross-talk of these two pivotal post-translational protein modifications. Ubiquitinated α -Synuclein was resistant to serine phosphorylation by polo-like kinase 3 (PLK3) but was efficiently phosphorylated by tyrosine kinase Syk. Interestingly, Y125 phosphorylated and tetra-ubiquitinated α -Synuclein formed precipitate more readily than the di-ubiquitinated form, illustrating the differential impact of ubiquitin chains of varying length on biophysical properties of the modified protein. In addition, analysis of the proteasomal degradation kinetics of mono- vs. polyubiquitinated α -Synuclein species, assayed under biochemical conditions closely mimicking intracellular environment,

adds valuable insight regarding the suggested restriction of deubiquitinase activity by long ubiquitin chains (15), because the monoubiquitinated α -Synuclein was subjected to deubiquitination and remained stable, whereas its di- and tetra-ubiquitinated counterparts were efficiently degraded. This addresses an important issue of ubiquitin chain editing: a highly dynamic interplay between the synthesis and degradation of ubiquitin chains, which precisely tailors the ubiquitin signal to the changing needs of the cell.

Despite its key importance to the research in the ubiquitination field, it should be mentioned that the elegant semisynthetic strategy followed by Haj-Yahya et al. is not universally applicable. The use of thiolysine residues for isopeptide chemical ligation requires a subsequent desulphurization step that is incompatible with proteins containing endogenous cysteine residues. Moreover, harsh chemical treatments at certain synthesis steps may be deleterious to proteins with a complex tertiary structure. Fortunately, α -Synuclein, containing no cysteine and with intrinsically disordered structure, provides no such constraints. It can also be expected that further advances in protein chemistry may alleviate these limitations of the current technology.

In summary, the progress in semisynthetic production of ubiquitin conjugates reported by Haj-Yahya et al. (2) opens completely new and long-awaited perspectives in the research on posttranslational modifications of proteins. The advancements made in this study increase our understanding of the pathophysiology of synucleinopathies and may prove to be indispensable for the dissection of the complexity of ubiquitin signals in the future.

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