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The new landscape of protein ubiquitination

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Abstract

Recently developed technologies allow proteome-wide quantification of ubiquitination sites and provide avenues for identifying targets of ubiquitin ligases.

Many properties of proteins, such as their stability, localization, binding partners, and their activity, can be regulated by ubiquitination, a post-translational modification (PTM) involving the transfer of ubiquitin, a 76-amino acid protein, to lysine residues[1]. Ubiquitin is targeted to specific proteins by E3 ligases, each of which catalyzes the transfer of ubiquitin to specific protein targets. With over 500 E3 ligases encoded in the human genome[2], the complexity of the cellular machinery devoted to ubiquitination rivals phosphorylation, which is regulated by a comparably large network of kinases and phosphates. However, the number of known ubiquitination sites is markedly fewer than that of phosphorylation sites, largely due to the lack of technical approaches to profile the sites of ubiquitination across the proteome. Three recently published papers in *Molecular Cell*[3], *Molecular Cellular Proteomics*[4], and *Cell*[5] have largely remedied this, providing a comprehensive view of the ubiquitinome and revealing functional consequences of ubiquitination for a large subset of the proteome.

Ubiquitination is an umbrella term for several different types of ubiquitin modifications, each of which can have different effects on target proteins. The most well known ubiquitin modification is the polyubiquitin chain that targets proteins for degradation by the proteasome. The chain begins with a ubiquitin that is conjugated via its C-terminus to the ε-amine of a lysine residue in a target protein. Subsequent lysines in the chain are conjugated to a lysine residue in the previous ubiquitin, typically through Lys48, thereby creating a signal for proteasomal degradation[6]. However, polyubiquitin chains that are linked through other lysines may have proteasome-independent functions, such as altering subcellular localization or protein interactions[7]. An additional form of ubiquitination is monoubiquitination, in which only one ubiquitin is attached to a target protein. Monoubiquitination can alter subcellular localization. These different types of ubiquitin modifications account for the diverse effects of ubiquitination on protein function.

Kim *et al.*,[3] Wagner *et al.*,[4] and Emanuele *et al.*[5] used ubiquitin remnant profiling, a mass spectrometry-based proteomic approach to identify ubiquitinated proteins and the modified lysine residues[8]. Unlike proteomic methodologies used to profile other PTMs, such as phosphorylation and acetylation, which use modification-specific antibodies, ubiquitin remnant profiling uses antibodies that recognize a protein modification that is only

generated after a ubiquitinated protein is digested with trypsin (Fig 1a). Because the C-terminal residues of ubiquitin are Arg-Gly-Gly, cleavage of ubiquitin after arginine leaves a Gly-Gly (diglycine) remnant attached to the modified lysine residues in proteins. Antibodies specific for diglycine-modified lysines do not precipitate free ubiquitin, but only precipitate ubiquitin remnant-containing peptides from tryptic digests, allowing them to be analyzed by mass spectrometry[8].

Wagner *et al.*[4] and Kim *et al.*[3] used ubiquitin remnant profiling of human cells to generate the largest dataset of ubiquitination sites. Using powerful mass spectrometry instrumentation, these investigators identified ~11,000[4] and ~19,000[3] ubiquitination sites. Consistent with previous studies, these datasets reveal that a significant number of proteins are ubiquitinated on multiple lysine residues, and that the ubiquitination status of each lysine residue can be independently regulated[3].

A striking finding was that the majority of ubiquitination in cells occurs on newly synthesized proteins[3]. Earlier investigations revealed that a considerable portion of the proteasome's attention is directed towards newly synthesized proteins, which are particularly susceptible to aggregation and misfolding[9]. Consistent with this idea, Kim *et al.* found that the level of ubiquitination at over 70% of ubiquitinated sites significantly dropped following protein synthesis inhibition[3]. These findings indicate that catalogs of ubiquitination sites may contain large numbers of these "quality control" ubiquitination events, rather than "regulatory" ubiquitination events that have roles in cellular signaling pathways (Fig 1b). Therefore, datasets of ubiquitination sites need to be viewed cautiously because they may contain sites that are irrelevant to signaling pathways. Since these sites will be used to inform mutagenesis experiments to establish the function of ubiquitination, the presence of extraneous sites can be problematic. Therefore, it will be important to consider including protein synthesis inhibitors to reduce the level of quality control sites in future proteomics experiments. Alternatively, screens for ubiquitination sites that are induced in a signal-dependent manner are likely to yield regulatory ubiquitination events. Carefully annotated databases will be needed to help researchers assess whether a ubiquitination event is part of cellular housekeeping or is potentially used for regulating protein function.

As Kim *et al.*[3] point out, the ubiquitination sites that occur on nascent misfolded proteins may actually be valuable biomarkers to identify cellular states in which protein synthesis or protein folding has been disrupted. Protein misfolding and aggregation contribute to the pathogenesis of several diseases, such as Alzheimer's and Parkinson's disease. Additionally, several therapeutic strategies, such as chaperone inhibitors, aim to disrupt protein folding[10]. The quality control ubiquitination sites identified by Kim *et al.* may serve as a useful pool of biomarkers to establish the fidelity of protein folding in different disease states or in response to drug treatment.

A major challenge is establishing the functional consequence of ubiquitination on a target protein. In many cases, ubiquitination leads to protein degradation, which in principle can be established by detecting increases in protein levels after proteasome inhibition. However, these experiments can be inconclusive since ubiquitination may target only the protein in a

specific subcompartment of the cell, which may not result in a demonstrable change in overall protein levels. Both Kim *et al.* and Wagner *et al.* provide considerable assistance with this problem by characterizing how the level of ubiquitination at each lysine is influenced by proteasome inhibitors. In many cases ubiquitination levels of specific lysines increased, demonstrating that ubiquitination of these sites leads to proteasomal degradation. Surprisingly, for some ubiquitination sites, treatment with proteasome inhibitors reduced ubiquitin levels. Ubiquitination of these lysines is probably not linked to proteasomal degradation, but is likely to have alternate functions. Since these sites are presumably subjected to physiological cycles of ubiquitination and deubiquitination, ubiquitination levels drop when the released ubiquitin is sequestered in proteins that would otherwise have been degraded by the proteasome. By determining whether the ubiquitination level increases upon proteasome inhibition, the ubiquitination site can be classified as having a proteasome-dependent or independent role.

Kim *et al.* and Emanuele *et al.* both use ubiquitin remnant profiling to identify targets of E3 ligases. E3 ligases constitute a valuable set of potential drug targets, although determining which ones are suitable depends on identifying the specific proteins that they ubiquitinate. Both groups used MLN4924, an inhibitor of cullin-RING ubiquitin ligases, to identify several hundred target proteins, and the corresponding ubiquitination sites. Emanuele *et al.* also used a previously described cellular method for global protein stability (GPS) profiling[11], which relies on a library of dual-fluorescent reporters expressing DsRed and each of over 15,000 open reading frames (ORFs) tagged with EGFP. The ratio of EGFP to DsRed is used as an indicator of the half-life of the ORF in the cell. Cells that exhibit an increase in the EGFP/DsRed ratio after MLN4924 treatment express ORFs that are stabilized by cullin-RING ubiquitin ligase inhibition. Although the GPS and ubiquitin remnant profiling data sets exhibited considerable overlap, unique proteins were found by both methods. In part, this may be because GPS only detects proteins that undergo changes in ubiquitin modifications that affect protein stability. Ubiquitin remnant profiling identifies ubiquitination events regardless of whether they are linked to proteasome-dependent degradation. On the other hand, ubiquitin remnant profiling may be less likely to detect targets which are expressed at low levels in cells.

It will be important to extend ubiquitin remnant profiling to enable selective profiling of ubiquitination events that are due to specific chain linkages or to monoubiquitination. Ubiquitin remnant profiling can be performed on protein samples that are enriched for specific chain linkages using chain linkage-specific antibodies[12]. Alternatively, monoubiquitinated proteins can be prepared for ubiquitin remnant profiling by immunodepleting polyubiquitinated proteins using antibodies specific for polyubiquitin. These ubiquitin modification-specific analyses will provide insight into the roles of these modifications in ubiquitination signaling.

In addition to providing a powerful resource, these studies demonstrate the ability of ubiquitin remnant profiling to match specific targets to E3 ligases, and to identify ubiquitination events that are regulated by potential therapeutic compounds. General application of this approach to other E3 ligases will undoubtedly uncover novel E3 ligase-regulated pathways and reveal E3 ligases that target disease-relevant proteins.

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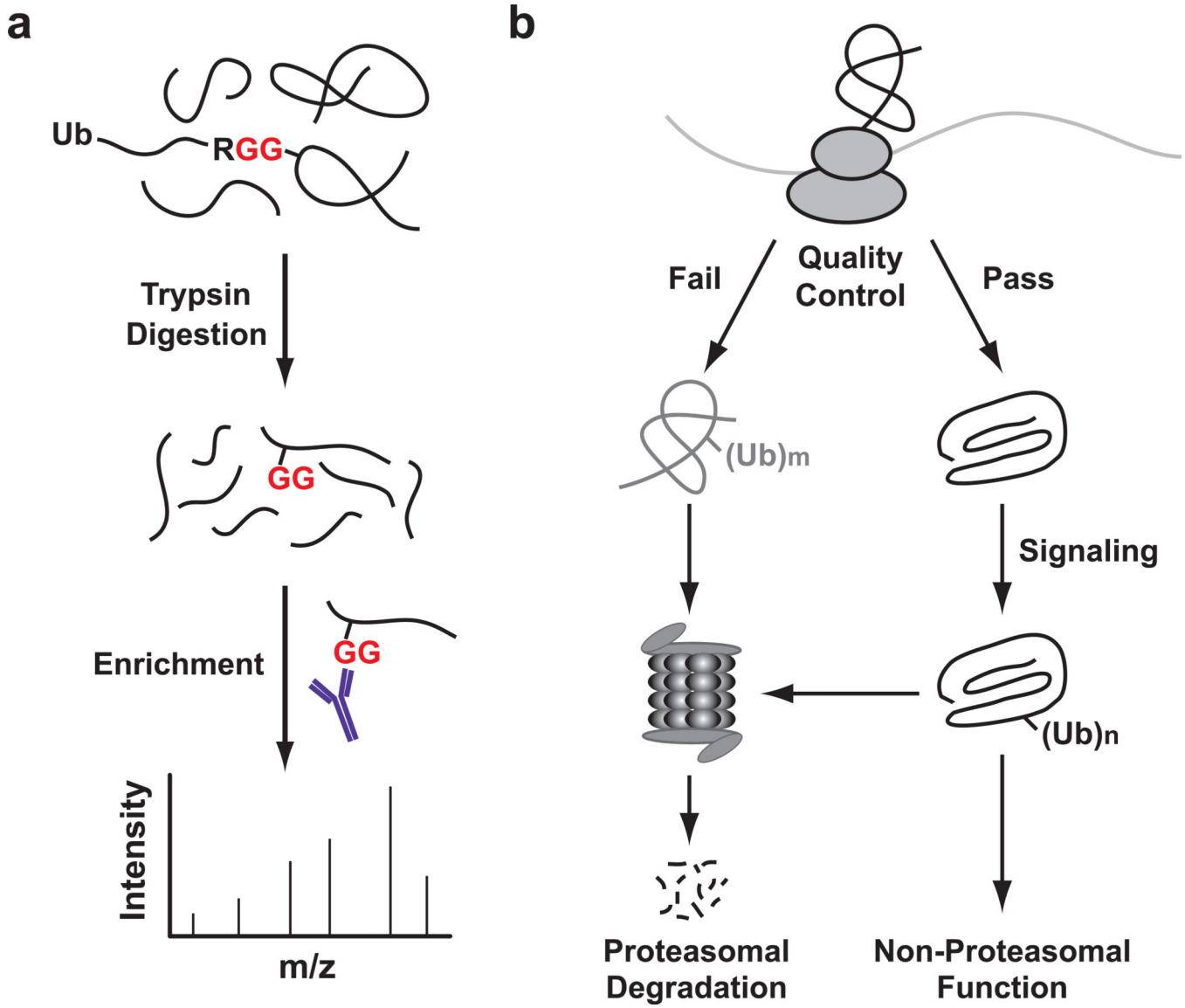

Figure 1. Ubiquitin remnant profiling for proteomic identification of ubiquitination sites

(**a**) In ubiquitin remnant profiling, ubiquitin remnants are revealed after digestion of lysates with trypsin. Trypsin cleaves ubiquitin after an arginine residue near its C-terminus, leaving a ubiquitin-derived diglycine remnant on lysine residues in target proteins. Ubiquitin-remnant containing peptides are purified with a diglycyl-lysine-specific antibody. Mass spectrometry is used to sequence these peptides and identify the modified lysine residue. (**b**) Two classes of ubiquitination. A major role for ubiquitination is to target newly synthesized proteins, which are highly susceptible to misfolding and aggregation. This "quality control" ubiquitination mechanism ensures that misfolded proteins are subjected to proteasomal degradation. In response to signaling or other pathways, properly folded proteins may become subjected to "regulatory" ubiquitination, which can target a protein for proteasomal degradation, or otherwise influence protein function.