Molecular Cell
Perspective

The Colossus of Ubiquitylation: Decrypting a Cellular Code

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http://dx.doi.org/10.1016/j.molcel.2013.01.028

Ubiquitylation is an essential posttranslational modification that can regulate the stability, activity, and localization of thousands of proteins. The reversible attachment of ubiquitin as well as interpretation of the ubiquitin signal depends on dynamic protein networks that are challenging to analyze. In this perspective, we discuss tools of the trade that have recently been developed to dissect mechanisms of ubiquitin-dependent signaling, thereby revealing the critical features of an important cellular code.

Introduction

Codes enable communication between two parties that are not in direct contact. Humans have used codes as different as hieroglyphics, alphabets, and quipus to share information among their peers, while often concealing the same information from others. If the rules that define these codes are unknown, deciphering encoded documents can be a daunting task that often relies on the invention of new decryption technology such as the Colossus, an early programmable computer used to break military codes.

Analogous to human societies, cells communicate by encoding, interpreting, and erasing information. A particularly intriguing system is centered on the posttranslational modification of cellular factors with ubiquitin, a conserved protein that can be covalently attached to substrates as a single moiety or a polymeric chain (Komander and Rape, 2012). Ubiquitin chains can adopt distinct topologies depending on whether they are linked through ubiquitin's N terminus or one of its seven Lys residues (homogenous chains), different Lys residues at successive chain positions (mixed chains), or forks that are characterized by one moiety being decorated with at least two ubiquitin molecules (branched chains). These ubiquitylation marks can trigger unique outcomes and, hence, may form the basis of a code: monoubiquitylation changes interaction or localization patterns (Hicke and Riezman, 1996), chains connected through K48 of ubiquitin trigger degradation by the 26S proteasome (Chau et al., 1989), and K63-linked chains regulate assembly of oligomeric complexes (Deng et al., 2000; Spence et al., 2000). However, the functions of atypical chains linked through M1, K6, K11, K27, K29, or K33 are only beginning to emerge, and roles of mixed or branched chains are unknown. How much information can be encoded with ubiquitin, therefore, remains to be established.

Ubiquitylation depends on an enzymatic cascade that, in human cells, consists of two E1 enzymes, ~40 E2s and ~600 E3s, with the latter being tasked to recruit specific substrates (Deshaies and Joazeiro, 2009; Schulman and Harper, 2009; Ye and Rape, 2009). The E3s fall into three classes referred to as HECT, RING, or RING-between-RING (RBR) enzymes (Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009; Wenzel et al., 2011). HECT- and RBR-E3s possess an active site cysteine that is charged with ubiquitin before this ubiquitin is used to modify a substrate. By contrast, RING-E3s promote the direct transfer of ubiquitin from the active site of an E2 to a substrate lysine, and they rely on their E2 enzymes to endow them with linkage specificity (Komander and Rape, 2012). While the general architecture of this cascade has long been established, many E3-substrates, physiological E2-E3 pairs, and mechanisms of E3 regulation remain to be discovered, leaving a gap in our understanding of how the ubiquitin code is established.

In human cells, ubiquitylation marks are recognized by ~20 ubiquitin-binding domains (UBDs) that are present in ~200 proteins (Husnjak and Dikic, 2012). UBDs can preferentially bind to a particular chain type, thereby coupling a linkage-specific modification to a downstream signaling event. Linkage preference can also be observed for deubiquitinases (DUBs), which cleave ubiquitin off substrates to terminate or edit ubiquitin-dependent signaling (Sato et al., 2008). As with E3s, relatively few UBDs and DUBs have been matched with their substrates in cells, leaving us with an incomplete knowledge of how the ubiquitin code is interpreted or erased.

As ubiquitylation depends on a complex interplay between enzymes, substrates, and effectors, the rules guiding ubiquitin-dependent signaling are only beginning to be understood. During recent years, several technologies have been developed that have addressed key challenges inherent to the study of such a transient and tightly regulated modification. These new strategies build upon and complement traditional genetic and biochemical approaches that have laid a strong foundation for understanding principles of ubiquitylation. Here, we discuss the new tools of the trade, focusing on areas that witnessed the most progress: identification of ubiquitylation enzymes, substrate discovery, analysis of mechanisms underlying ubiquitylation, and determination of functional consequences of ubiquitylation for cellular regulation.

Discovery of Important Ubiquitylation Enzymes

Many traditionally studied ubiquitylation enzymes were identified in genetic screens of model systems, such as *S. cerevisiae or D. melanogaster*, or found to be mutated in human diseases. Evidence for roles in ubiquitylation was then provided by biochemical reconstitution. For example, subunits of the SCF

(Skp, Cullin, F-box) complex and APC/C (anaphase-promoting complex/cyclosome) were isolated in screens for regulators of cell division in yeast and later found to constitute multimeric E3s that are required for cell division in all eukaryotes (Peters, 2006; Petroski and Deshaies, 2005). Conversely, mutations in the genes encoding *BRCA1* or *FBXW7* were associated with an increased risk of developing breast or colon cancer, thereby drawing interest to the corresponding E3s (Lipkowitz and Weissman, 2011).

The advent of siRNA (small interfering RNA) technology has allowed the straightforward identification of ubiquitylation enzymes that play important roles in human cells. Whole genome siRNA screens pointed to E3s that control the cellular defense against viral infection (Mercer et al., 2012) or autophagy (Orvedahl et al., 2011). Providing a more focused approach, many laboratories have developed siRNA libraries that selectively target ubiquitylation enzymes, allowing them to employ complex assay systems and analysis methods. This strategy was first implemented for human DUBs, revealing roles for these enzymes in transforming growth factor β (TGF-β) signaling, DNA repair, and splicing (Dupont et al., 2009; Nijman et al., 2005; Song et al., 2010). In a similar manner, siRNA libraries that selectively target E3s have been successful in discovering enzymes that regulate DNA repair (Bekker-Jensen et al., 2010; Gudjonsson et al., 2012; Nakada et al., 2010), vesicular transport (Jin et al., 2012), and mRNA degradation (Cano et al., 2012).

Similar to other genetic approaches, the success of siRNA screens is hampered if a substrate is regulated by partially redundant ubiquitylation enzymes. For example, four different E3s have been proposed to control the abundance of cyclin D1 (Kanie et al., 2012). Moreover, if an E3 is present in excess of its critical substrate, the partial depletion achieved by most screening conditions might not suffice to produce a phenotype. In both instances, the likelihood of identifying ubiquitylation enzymes can be increased by sensitizing cells by altering the abundance or activity of other components of the signaling pathway in question. These approaches are similar to synthetic lethality-driven gene discovery (Kaelin, 2005) and have revealed roles for ubiquitylation enzymes in cell division or survival (Kessler et al., 2012; Luo et al., 2009; Stegmeier et al., 2007). Alternatively, additional information, such as expression profiles, can be integrated into the screening platform to generate libraries that target fewer enzymes but allow a more careful optimization of depletion efficiency. This strategy formed the foundation of identifying a Cul3 ubiquitin ligase that controls vesicle trafficking (Jin et al., 2012), and it pointed to the function of the E3s RNF43 and Znrf3 in triggering the removal of Wnt receptors from the plasma membrane (Hao et al., 2012; Koo et al., 2012).

The power of focused siRNA screens for the identification of critical ubiquitylation enzymes is illustrated by work in Wnt signaling, a pathway whose frequent misregulation in cancer has created interest for new drug targets. Novel small molecule inhibitors of Wnt signaling were found to act by stabilizing a key component of this pathway, the destruction complex subunit axin, by virtue of their inhibitory effect on the poly-ADP-ribosylation enzyme tankyrase (Huang et al., 2009). Subsequent focused siRNA screens identified RNF146 as a poly-ADP-ribosylation-dependent E3 that targets axin for degradation (Callow et al., 2011; Zhang et al., 2011). This example underscores that a combinatorial approach relying on genetics, biochemistry, and chemical biology continues to be a promising route to identify important roles for ubiquitylation enzymes.

As expected for central components of signaling networks, many E3s and DUBs form multimeric complexes with distinct catalytic and regulatory subunits. As screens often fail to identify all components of multimeric enzymes, it can be prudent to search for functional interacting partners of a candidate enzyme. Affinity purification and mass spectrometry provide a rapid means to defining the composition of ubiquitylation complexes, as recently seen with subunits of the APC/C or cofactors of Cul5 that are hijacked by a pathogenic virus (Hubner et al., 2010; Hutchins et al., 2010; Jäger et al., 2012). Another mass spectrometry approach, CompPASS, can detect high-confidence interactors, even if they are expressed at low levels (Sowa et al., 2009). In this strategy, multiple affinity purifications are performed under identical experimental conditions and using the same epitope tags and cell types (Figure 1). By employing a statistical filter, CompPASS then extracts high-confidence interactors from this mass spectrometric data set. CompPASS was first used to pinpoint binding partners of human DUBs or regulators of autophagy and ER-associated degradation, thereby generating high-quality interaction maps for ubiquitylation enzyme families (Behrends et al., 2010; Christianson et al., 2012; Sowa et al., 2009). When combined with small-molecule inhibition or siRNA-mediated depletion, CompPASS can reveal dynamic changes in interaction networks (Bennett et al., 2010), and it can also be used to isolate specific E3 substrates (Lee et al., 2011).

Identification of Specific Ubiquitylation Substrates

The role of ubiquitylation in signaling can only be understood if the physiological targets of critical E3s are known, yet substrate identification remains a major bottleneck in the field. Many E3s bind their substrates transiently, which complicates affinity purification approaches that gain specificity through comparably lengthy incubation and wash steps. Indeed, a kinetic analysis of SCF-dependent ubiquitylation revealed that even ideal substrates dissociate from the SCF within seconds (Pierce et al., 2009). Moreover, cellular decision makers are often present at low levels, which increases the likelihood that these potential E3 substrates will be missed in mass spectrometry. This situation is aggravated if binding an E3 leads to degradation, a common outcome of ubiquitylation.

One of the earliest approaches to isolate E3 targets, in vitro expression cloning (IVEC), addressed the issue of low substrate levels in cells (Ayad et al., 2005). In this procedure, pools of radiolabeled proteins are generated in vitro, incubated with extracts or E3s, and analyzed by autoradiography (Figure 2A). As modern cDNA (complementary DNA) libraries are based on gene collections, such as the ORFeome (set of open reading frames) (Lamesch et al., 2007), IVEC screens can rapidly interrogate the whole genome. In lieu of radiolabeled substrates, arrays with recombinant proteins immobilized on glass surfaces can be used to detect ubiquitylation events catalyzed by an E3 or extract (Merbl and Kirschner, 2009; Persaud et al., 2009). IVEC and its related strategies are particularly successful if substrates or degron motifs are known for an E3, as this information

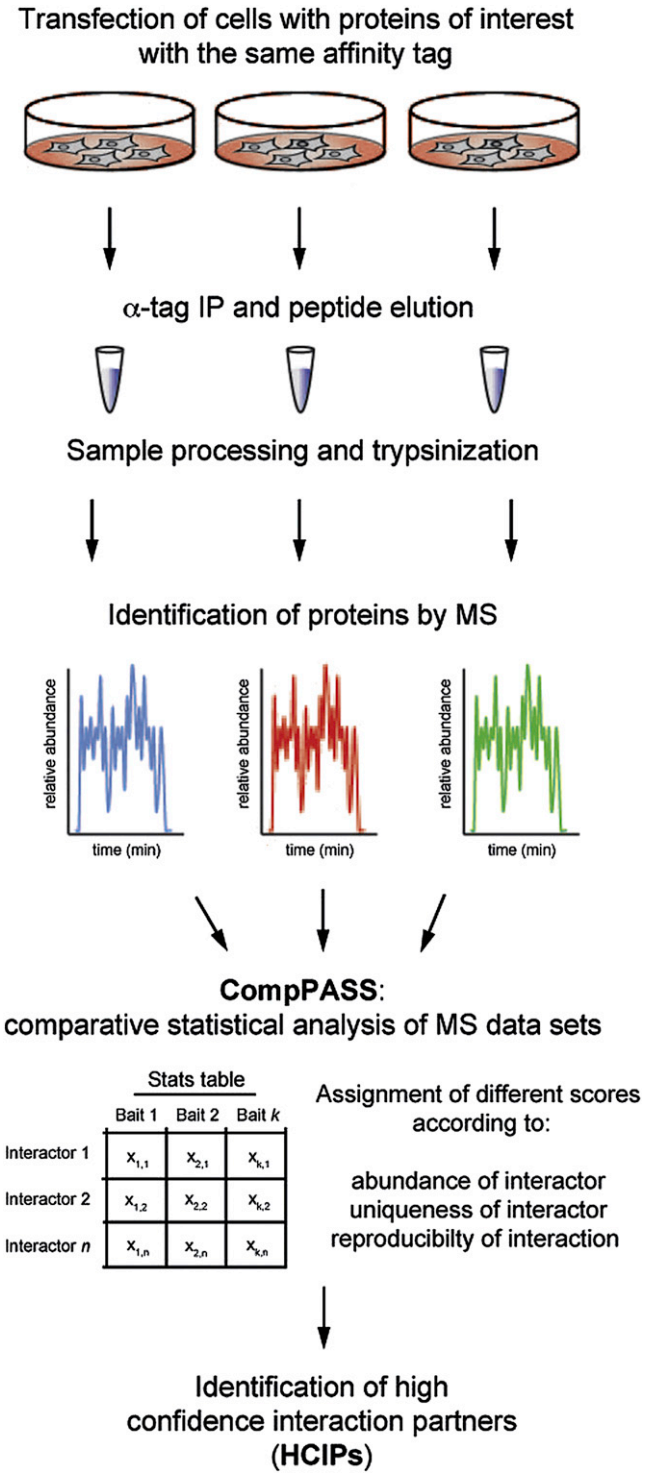

Figure 1. Characterization of Ubiquitylation Enzyme Composition by CompPASS Mass Spectrometry

CompPASS utilizes a statistical filter to identify high-confidence interactors from mass spectrometry data sets that were generated from purifications of multiple bait proteins prepared under identical experimental conditions.

facilitates the development of efficient assays that increase the likelihood of isolating substrates (McGarry and Kirschner, 1998; Song and Rape, 2010; Zou et al., 1999). However, in vitro or bacterial expression systems can be limited in their capacity to produce properly folded proteins, a shortcoming that potentially hampers the recognition of candidate substrates by their cognate E3s.

Addressing these problems, global protein stability (GPS) profiling allows for the identification of ubiquitylation substrates in cells, as demonstrated for E3s of the cullin-RING ligase family (Emanuele et al., 2011; Yen and Elledge, 2008; Yen et al., 2008). In current GPS libraries, ~13,000 human genes are cloned as fusions with GFP (green fluorescent protein) into vectors that also express RFP (red fluorescent protein) from internal ribosomal entry sites (Figure 2B). The bicistronic reporters are packaged into viruses, which are used to transduce populations of cells that differ in the activity of an E3. The cells are then sorted into bins dependent on the ratio of GFP-tagged substrate versus the RFP control; proteins that are turned over depending on a specific E3 display a characteristic enrichment in bins with higher GFP/RFP-ratio if this E3 is inhibited. While potent in identifying proteolytic substrates, GPS does not yet allow identification of nonproteolytic ubiquitylation events. The GFP tag might also interfere with substrate recognition by the E3, as is frequently observed for substrates of the APC/C.

While IVEC and GPS rely on heterologous protein expression, mass spectrometry enables substrate discovery by identifying endogenous ubiquitylation sites. This methodology is based on the observation that the ubiquitylation of a substrate lysine ablates its recognition by trypsin, a protease that is employed to generate peptides for mass spectrometry. Instead of the substrate lysine, trypsin cleaves the attached ubiquitin after its residue R74, resulting in peptides with a Gly-Gly (GG) remnant of ~140 Da. Thus, the identity of ubiquitylated proteins and actual modification sites can be discovered by interrogating mass spectrometric data sets for peptides that contain a GG remnant. Following groundbreaking work in yeast (Peng et al., 2003), global ubiquitylation site analyses have reported thousands of modification events in eukaryotes (Beltrao et al., 2012; Emanuele et al., 2011; Kim et al., 2011; Tagwerker et al., 2006; Wagner et al., 2011).

To facilitate identification of GG remnant peptides, either ubiquitylated proteins or peptides can be enriched from lysates prior to their analysis by mass spectrometry. This can be achieved by purifying conjugates on high-affinity tandem ubiquitin-binding entities (Hjerpe et al., 2009) or under denaturing conditions from cells that express His- or His/biotin-tagged ubiquitin (Peng et al., 2003; Tagwerker et al., 2006). While the latter approach has the advantage of blocking deubiquitylation during the purification procedure, overexpressed ubiquitin can lead to the modification of nonphysiological substrates. This pitfall has recently been documented for the ubiquitin-like modifier Nedd8, which, if expressed at higher levels, can be activated by the ubiquitin-activating enzyme E1 and used to modify proteins that are usually modified with ubiquitin (Hjerpe et al., 2012). Rather than proteins, ubiquitylated peptides can be enriched after trypsin digestion of reaction mixtures by employing antibodies that recognize the GG remnant with little specificity

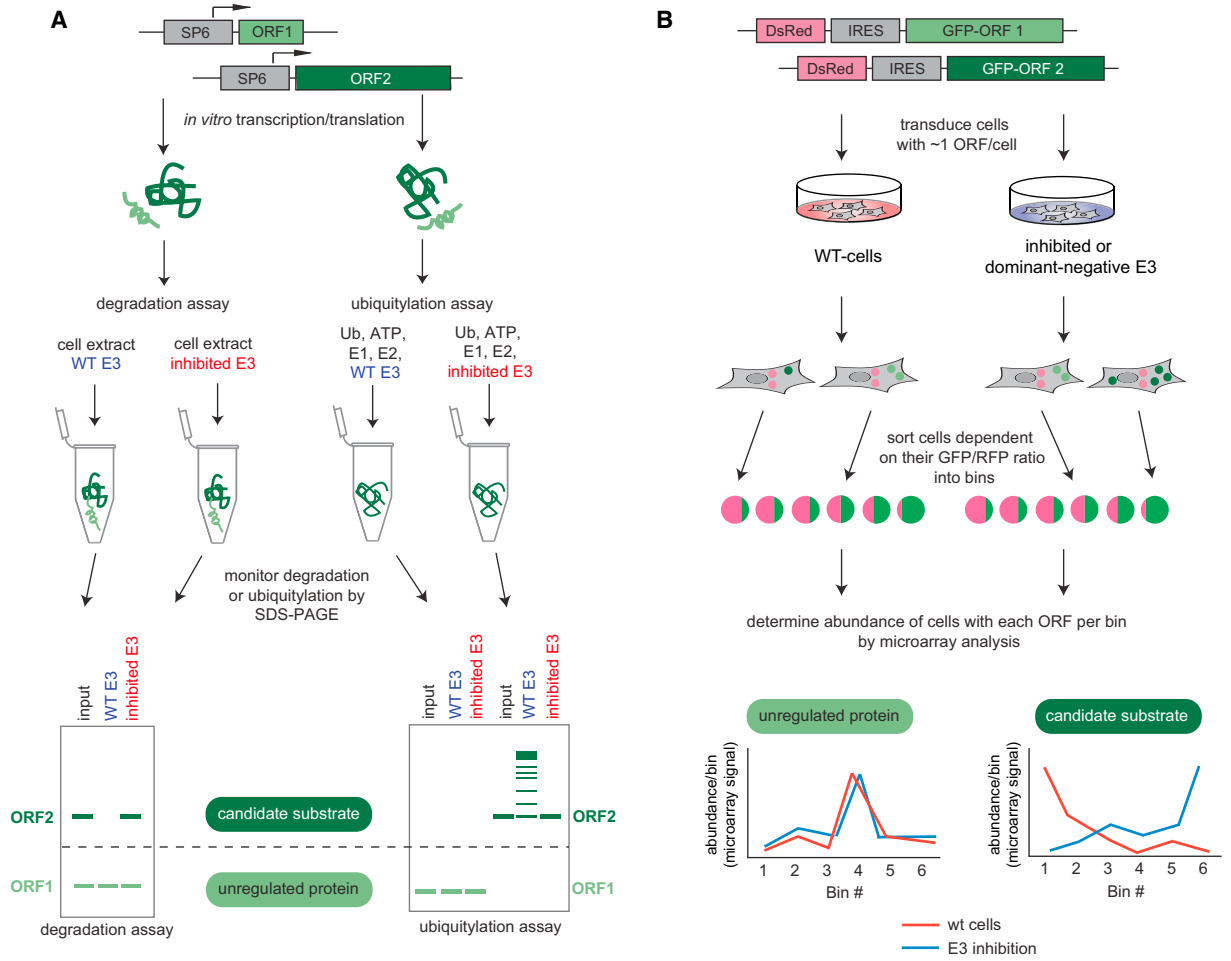

Figure 2. Identification of E3 Substrates

(A) In vitro expression cloning. In this technique, proteins are synthesized in vitro and analyzed in pools for degradation in extracts containing active E3s or for ubiquitylation by purified enzymes. Controls using inactive E3s are essential for ensuring specificity.

(B) Global protein stability (GPS) profiling. GPS relies on fluorescence sorting of transduced cells depending on the ratio between control RFP and the candidate, a GFP-tagged ORF. After sorting into bins, cells are identified by microarray analysis using primers against the ORF. Inhibition of an E3 responsible for degradation leads to sorting of cells expressing the GFP-tagged candidate into bins with higher GFP/RFP ratio.

for the sequence surrounding the ubiquitylated lysine (Figure 3) (Xu et al., 2010).

If inhibition of a particular E3 is combined with stable isotope labeling by amino acids in culture (SILAC), a reduction in the levels of GG peptides can rapidly point to specific substrates of this enzyme. Work using GG peptide antibodies along with MLN4942, a compound that interferes with neddylation and inhibits cullin-RING ligases, identified hundreds of potential substrates for this class of E3s (Emanuele et al., 2011; Kim et al., 2011). Alternatively, substrates can first be enriched in affinity purifications of E3s performed under nonstringent conditions. Incubation of these E3 preparations with ubiquitylation cocktails should then lead to modification of substrates, but not nonspecific interactors. In this case, it is an increase in the abundance of GG remnant peptides that points to specific substrates (Dorrello et al., 2006; Guardavaccaro et al., 2008).

As E3s have been reported to control a large number of proteins, it is a major improvement of the described approaches that they can point to many substrates at a time. This allows researchers to gain insight into global effects of E3 function rather than focusing on the consequences of modifying a single substrate. However, as is the case for most screening strategies, it is critical to follow up the initial discovery of candidate substrates with an in-depth analysis of the ubiquitylation events. Experiments that reveal the modification of endogenous proteins and dependency on candidate E3s or substrate degron motifs provide the most compelling evidence for a physiologically relevant event.

Determining Functional Consequences of Ubiquitylation

Although ubiquitylation has often been equated with degradation, it is now established that ubiquitin modifications trigger a variety of distinct outcomes. The crystal structures of ubiquitin molecules connected through different linkages provide a visual reminder of the diversity embedded in this modification (Komander and Rape, 2012). Distinct crystal and NMR (nuclear

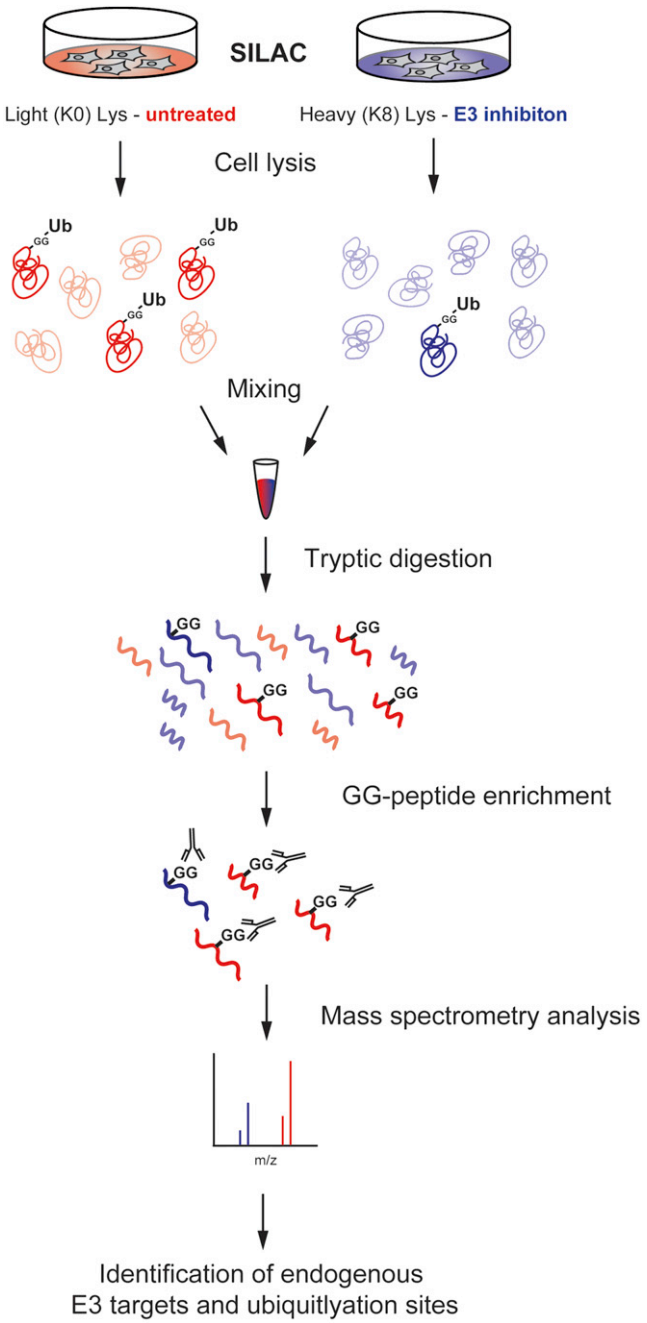

Figure 3. Identification of Ubiquitylation Sites Following Enrichment of Modified Peptides by GG-Peptide Antibodies
Following tryptic digestion of lysates, peptides containing the GG remnant are affinity purified using GG-peptide antibodies. This enrichment protocol significantly improves the detection of ubiquitylated peptides to determine the identity of modified proteins.

magnetic resonance) structures obtained from polymers connected by the same linkage suggested flexibility in ubiquitin chain conformations (Eddins et al., 2007; Lai et al., 2012; Varadan et al., 2004). Indeed, when K48- or K63-linked ubiquitin dimers were analyzed by single-molecule fluorescence energy transfer, they were found to adopt multiple conformations that are in a dynamic equilibrium with each other (Ye et al., 2012). Binding partners can selectively interact with a subset of these conformations, suggesting that altering the conformational equilibrium of ubiquitin chains can affect ubiquitin-dependent signaling.

This result underscores that ubiquitylation has to be analyzed within its biological context, yet it can be difficult to obtain sufficient material to study the function of modified proteins. Overcoming this obstacle, chemical biology has been used to generate defined conjugates that can be analyzed for activity or binding partners. For example, synthesis of monoubiquitylated histones provided evidence for structural and functional changes brought about by this modification (Fierz et al., 2011; Kim et al., 2009; McGinty et al., 2008), while production of K29-linked ubiquitin dimers identified a deubiquitinase with the respective linkage preference (Virdee et al., 2010). However, purified systems cannot recapitulate all functions of ubiquitylation, as seen with K63-linked chains. These linkages effectively target proteins to purified proteasomes in vitro, yet are unlikely to be a frequent degradation signal in cells (Kirkpatrick et al., 2006; Xu et al., 2009b).

To better recapitulate physiological conditions, ubiquitin-dependent signaling can be reconstituted in cell extracts. Extracts can be depleted of effectors or supplemented with ubiquitin mutants that lack acceptor sites for chain formation, and consequences on signaling can be determined quantitatively. Extract-based experiments recently uncovered functions of K63-linked chains in NF-κB transcription factor activation or the cellular response to viral infection (Deng et al., 2000; Hou et al., 2011), and they led to the discovery of K11-linked chains as key mediators of mitotic degradation (Jin et al., 2008). It is important to note that experiments using ubiquitin mutants have to be interpreted carefully, as Lys residues can fulfill functions other than being a conjugation site. Indeed, K6 is a critical residue of the ubiquitin surface that is recognized by the E2 Ube2S during K11-linked chain formation (Wickliffe et al., 2011).

As neither purified systems nor extracts fully recapitulate reactions that rely on proper localization of ubiquitylation enzymes or substrates, detecting conjugates in cells and interfering with their formation is paramount to deciphering the ubiquitin code. Providing a technological breakthrough, antibodies that specifically recognize K48- or K63-linkages were engineered and used to analyze ubiquitin chain editing, a process in which a K63-linked chain is removed to terminate nonproteolytic signaling and then replaced by a K48-linked chain to target the signaling molecule for degradation (Newton et al., 2008). K48- and K63-linkage-specific antibodies have now been widely employed and have, for example, underscored the importance of K63-linked chains in DNA repair (Doil et al., 2009; Stewart et al., 2009). In addition, antibodies against K11- and M1-linked chains confirmed critical functions of these chain types in mitotic control and NF-κB signaling, respectively (Matsumoto et al., 2012, 2010; Tokunaga et al., 2009). If a specific E3 accounts for most of a particular chain type in cells, as is the case for the APC/C and its role in driving K11-linked chain formation, linkage-specific antibodies can be used in combination with siRNA

depletion to connect a particular linkage with a specific enzyme (Matsumoto et al., 2010; Wickliffe et al., 2011).

Although antibodies can provide key snapshots of ubiquitin-dependent signaling, they do not allow researchers to analyze ubiquitylation in real time. For M1- and K63-linked chains, such data can be obtained with genetically encoded reporters that combine a fluorescent protein, such as GFP, with linkage-specific ubiquitin-binding domains (Sims et al., 2012; van Wijk et al., 2012). These ubiquitin sensors can detect conjugates that accumulate at particular locations such as on mitochondria destined for mitophagy or on DNA that is undergoing repair. However, current sensors are limited in their ability to monitor the ubiquitylation of specific substrates. Sensors can also affect ubiquitin-dependent signaling if they compete with a ubiquitin-binding protein for access to the conjugate. This property can be exploited to collect evidence for a certain linkage in a signaling pathway, yet it should be remembered as a potential caveat when analyzing ubiquitin-driven signaling events.

While detecting linkage formation at a particular location can provide data that are consistent with a function of this modification, it does not probe it directly. To obtain such evidence, cells expressing mutant forms of ubiquitin can be investigated for defects in signaling. These experiments are complicated by ubiquitin being expressed from four loci: two genes encode ubiquitin as a fusion with ribosomal proteins, whereas two others encode cassettes that express multiple copies of ubiquitin fused to each other (Pickart, 2001). Groundbreaking studies in yeast showed that it is possible to replace all copies of wild-type ubiquitin with mutants, asserting the importance of ubiquitin's hydrophobic patch and providing evidence for K63-linked chains in nonproteolytic regulation (Sloper-Mould et al., 2001; Spence et al., 2000). Similar experiments can now be conducted in mammalian cells using siRNA-dependent depletion of all genes encoding ubiquitin combined with expression of the two ribosomal proteins fused to mutant ubiquitin (Xu et al., 2009a). In the ideal case, studies that are consistent with the function of a chain type should be complemented by experiments that change the linkage specificity of the E3. An improved understanding of the mechanisms underlying ubquitylation, as obtained by techniques discussed below, might pave the way for such E3 reprogramming.

Dissecting the Mechanism of Ubiquitylation

Dissecting biochemical mechanisms of ubiquitylation has long been at the heart of this field, and insights gained from these studies greatly improved our understanding of how ubiquitylation controls cell behavior. Crystallography and NMR illustrated the architecture of E3s and DUBs, but they have also been powerful in elucidating catalytic mechanisms for these enzymes. Structural tours-de-force recently revealed the effects of neddylation on the flexibility of the RING subunit in cullin-RING ligases, the mechanism of RING-dependent ubiquitylation, or dramatic rearrangements occurring during the E1 activation cycle (Duda et al., 2008; Olsen et al., 2010; Plechanovová et al., 2012). Paired with electron microscopy, such studies can visualize allosteric changes in large E3s, as seen for the APC/C and its regulation by the spindle checkpoint (Chao et al., 2012; Herzog et al., 2009; Schreiber et al., 2011).

Due to the short-lived nature of many interactions in the ubiquitylation cascade, complexes between substrates, enzymes and effectors often resist their crystallographic analysis. To fill this gap, NMR chemical shift perturbation analysis has been used to monitor transient, yet specific, protein interactions of ubiquitylation enzymes. Combined with bioinformatic docking and mutagenesis, this strategy can help illuminate enzymatic transition states, such as those used by E2s during linkage formation. Chemical shift perturbation analyses were critical tools in the discovery of substrate-assisted catalysis, a mechanism that can determine the linkage specificity of chain formation, and they pointed to pivotal interactions of the RING domain with ubiquitin-loaded E2 (Hamilton et al., 2001; Pruneda et al., 2012; Saha et al., 2011; Wickliffe et al., 2011).

Insights gleaned from structural analyses of ubiquitylation enzymes are most powerful if combined with biochemical reconstitution of the modification reaction, an approach that has been facilitated by an advance in purification systems that allowed the generation of recombinant 1.5 MDa APC/C or the proteasomal lid (Lander et al., 2012; Schreiber et al., 2011; Uzunova et al., 2012). In vitro experiments enable stringent testing of structural models by mutational analyses, and they can also be used to study the kinetics of ubiquitylation in a rigorous manner. Indeed, time-resolved analyses that used fluorescence energy transfer reporters to dissect interactions of the SCF with E2s or substrates underscored the high dynamics of protein interactions driving ubiquitylation reactions (Kleiger et al., 2009; Pierce et al., 2009; Saha and Deshaies, 2008).

It is important to bear in mind that the biochemical analysis of RING-E3s requires knowledge of their physiological E2s, which determine the linkage-specificity of the modification (Komander and Rape, 2012). This cautionary point was illustrated by the recent discovery of physiological E2s for the APC/C. Only after yeast Ubc4/Ubc1 or human Ube2C/Ube2S was used in in vitro reactions, was the separation of chain initiation and elongation or the linkage specificity of human APC/C discovered (Jin et al., 2008; Rodrigo-Brenni and Morgan, 2007; Williamson et al., 2009).

Ultimately, understanding mechanisms of ubiquitylation should allow isolation of small molecules that target important enzymes. Such compounds can deepen our understanding of ubiquitin-dependent signaling, but they might also be of therapeutic benefit. The paradigm that E3s can be regulated by low molecular weight compounds was established in plants, where hormones bind E3s of the SCF family and increase their affinity for substrates (Tan et al., 2007). Recently, screens for antagonists of ubiquitylation led to the discovery of proTAME, a molecule that inhibits the APC/C and was used to confirm the role of this E3 in disassembling the spindle checkpoint (Zeng et al., 2010), and inhibitors of the E2 Cdc34 that underscored the importance of donor-ubiquitin binding for catalysis (Ceccarelli et al., 2011). Notably, the inhibitors of Cdc34 as well as molecules blocking SCF^{Cdc4} engage allosteric sites that are not obvious prior to drug binding (Ceccarelli et al., 2011; Orlicky et al., 2010). These findings raise the possibility that more compounds targeting ubiquitylation enzymes that will help illuminate biochemical mechanisms and cell biological functions of critical ubiquitylation enzymes could be discovered.

New Methods of Targeting the Ubiquitin System?

As this perspective hopefully conveyed, understanding principles of ubiquitin-dependent signaling requires the integration of multiple methods, and gaps in our understanding are especially evident when different strategies yield inconsistent results. For example, in vitro studies suggested that the protein Cand1 (cullin-associated and neddylation-dissociated 1) inhibits SCF-dependent ubiquitylation, yet in vivo assays found it to promote degradation by the same E3 (Chuang et al., 2004; Goldenberg et al., 2004). This paradox was resolved by novel kinetic approaches that identified Cand1 as an exchange factor that allows new substrate adaptors to bind the SCF (Deshaies, personal communication).

The development of new technologies should, therefore, continue to inform our understanding of ubiquitylation. Such innovation could provide insight into questions of ubiquitin-dependent signaling that remain incompletely understood: do all chain types encode unique information, or does another reason account for the existence of multiple chain topologies? To this end, we need to develop more efficient means to identify E3s with novel linkage specificity or reprogram known E3s to synthesize new chain types. Complex issues arise for mixed or branched chains: can we determine the architecture of such chains and connect it with a particular function? Moreover, is it possible to probe the ubiquitylation of specific substrates in cells? This question becomes more urgent as more proteins are identified that are only targeted at particular locations or when in complex with specific binding partners (Williamson et al., 2011). While linkage-specific antibodies or sensors monitor global ubiquitylation events, other strategies might be required to measure substrate-specific ubiquitylation in cells.

Finally, the dynamic architecture of ubiquitin-dependent signaling networks is only beginning to be understood. We know comparably little about physiological E2-E3 pairs, collaborations between E3s, or opposition of E3s though specific DUBs, yet such functional interactions likely play important roles in determining the output of ubiquitin-dependent signaling in cells. To gain insight into the systems biology of ubiquitylation it might require a combination of interaction studies as pioneered with CompPASS, large-scale substrate identification as with ubiquitylation site analyses, discovery of functional interaction as revealed by siRNA screens, and computational modeling of signaling networks. Such studies will likely be informative with respect to understanding consequences of aberrant ubiquitylation for the pathology of diseases and identifying ubiquitylation events that should be targeted for therapeutic benefit.

It is the complexity of ubiquitylation—many chain types, hundreds of enzymes, and thousands of substrates—that is exploited by the cell to regulate essential signaling pathways. The same complexity poses a daunting challenge to those trying to uncover the rules that govern ubiquitin-dependent signaling. As described in this perspective, it might be an integrated approach that is built on the continuous development of new technologies—collaboration and innovation—that could ultimately enable us to decipher this fascinating cellular code.

ACKNOWLEDGMENTS

The authors apologize to all whose work could not be included due to space limitations. We thank Julia Schaletzky for critically reading the manuscript and all members of our laboratory for stimulating discussions. We are grateful to Ning Zheng, Ray Deshaies, and David Komander for sharing results prior to publication. Research in our laboratory is funded by grants from the National Institutes of Health, the California Institute for Regenerative Medicine, and the Korean Institute of Science and Technology.

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