Enzyme-Substrate Relationships in the Ubiquitin System: Approaches for Identifying Substrates of Ubiquitin Ligases

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Abstract

Protein ubiquitylation is an important post-translational modification, regulating aspects of virtually every biochemical pathway in eukaryotic cells. Hundreds of enzymes participate in the conjugation and deconjugation of ubiquitin, as well as the recognition, signaling functions, and degradation of ubiquitylated proteins. Regulation of ubiquitylation is most commonly at the level of recognition of substrates by E3 ubiquitin ligases. Characterization of the network of E3-substrate relationships is a major goal and challenge in the field, as this it expected to yield fundamental biological insights and opportunities for drug development. There has been remarkable success in identifying substrates for some E3 ligases, in many instances using standard protein-protein interaction techniques (e.g., two-hybrid screens, co-immunoprecipitations paired with mass spectrometry). However, some E3s have remained refractory to characterization, while others have simply not yet been studied due to the sheer number and diversity of E3s. This review will discuss the range of tools and techniques that can be used for substrate profiling of E3 ligases.

Introduction

Ubiquitylation has many essential functions in cell cycle control, DNA damage responses, protein trafficking, protein turnover, the disposal of misfolded and damaged proteins, and many other biochemical pathways. The strict control of the substrate selection process is therefore imperative. A hierarchal system of enzymes, culminating in the recognition of substrates by E3 ligases, coordinates the conjugation of ubiquitin to substrates. The first step toward conjugation is ATP-dependent activation of ubiquitin by an E1 enzyme. The E1 first catalyzes the formation of a ubiquitin-adenylate intermediate, and then the active site cysteine of the E1 forms a thioester with the C-terminus of ubiquitin, with the release of AMP. Ubiquitin is then transferred from the E1 enzyme to the active-site cysteine of an E2 enzyme in a transthiolation reaction. E3 ligases bind ubiquitin-charged E2s and substrates and promote the transfer of ubiquitin from the E2 to a substrate lysine residue or a lysine of a previously conjugated ubiquitin molecule, forming an isopeptide bond linkage. In unusual cases, ubiquitin can be linked to the free N-terminal amino group of a substrate or of ubiquitin, or even to serine or threonine side chains (forming ester bonds). In humans, there are two E1 enzymes and 32 E2s that are known to facilitate ubiquitin conjugation [1], while *Saccharomyces cerevisiae* has a single E1 and 11 E2s [2]. There are over 600 E3s encoded in the human genome and at least 51 in *S. cerevisiae* [2], and these enzymes collectively

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coordinate the ubiquitylation of thousands of substrates. There are several types of E3s, varying substantially in their mode of ubiquitin transfer to substrates, as described below. E3s generally target more than one substrate and multiple E3s may target the same substrate.

There are two main mechanistic classes of E3s, RING (Really Interesting New Gene) and HECT (Homologous to the E6-AP Carboxyl Terminus) E3s, as well as a third class, RBR (RING-between-RING) E3s, which have characteristics of both RING and HECT E3s. The vast majority of E3s are RING E3s, with over 600 annotated in humans [3], while there are only 28 human HECT E3s [4] and 14 RBR E3s [5]. RING E3s range from 13 kDa to over 500 kDa and adopt a common structure through coordination of two zinc ions with 8 conserved Cys/His residues. The RING domain recruits the ubiquitin-E2 conjugate and a substrate recognition domain binds the substrate, allowing the transfer of ubiquitin to the substrate from the E2. U-box domain E3s (e.g. CHIP) are E3s that form a RING-like structure without the coordination of zinc ions [6]. RING E3s may function as a singular entity (e.g. MDM2) or as part of a modular complex.

The largest family of RING E3s, the Cullin-RING ubiquitin Ligases (CRLs), exist as multi-subunit complexes. One of six cullin proteins (CUL1, CUL2, CUL3, CUL4A/CUL4B, CUL5, CUL7) forms the scaffold for over 200 CRLs. SCF (SKP1-CUL1-F-box protein) and ECS (Elongin C–CUL2/5–SOCS box) complexes are the best-characterized CRLs [7]. For SCF ligases, the cullin, CUL1 (activated by NEDD8) binds the RING protein (RBX1 or RBX2), the RING protein recruits the E2, and the substrate receptor proteins (F-box proteins) recruits the substrate. SKP1 serves as an adaptor that connects the cullin to the F-box protein. An ECS ubiquitin ligase consists of a cullin (CUL2/5), a RING protein (RBX1 or RBX2) and a substrate receptor (e.g. VHL or SOCS box proteins), and adaptor proteins (e.g. Elongin B and Elongin C) that bridge the substrate receptor to the cullin protein [8]. The wide array of substrate receptors, which includes 69 different interchangeable F-box proteins, account for the extensive substrate profiles of CRL E3s. Subdivided by their protein interaction domains, there are three families of F-box proteins, FBXW, FBXL and FBXO. FBXL proteins contain leucine-rich repeats (LRRs); FBXW proteins have WD repeats; and FBXO proteins may or may not contain an additional motif [9]. Another type of CRL, the Anaphase Promoting Complex/Cyclosome (APC/C), is structurally very distinct from other CRLs and consists of 14 subunits, including multiple substrate adaptors. APC/C plays a critical role in mitosis, triggering the transition from metaphase to anaphase [10].

HECT E3s are structurally and mechanistically distinct from RING E3s in that they directly catalyze substrate ubiquitylation. This involves a second transthiolation reaction, with ubiquitin being transferred from the E2 to the catalytic cysteine of the HECT domain, before being transferred to substrate lysine residues. HECT E3s are relatively large monomeric E3 proteins (>90 kDa, up to ~500 kDa), with the HECT domain always located at the C-terminus. Outside of the HECT domain, these E3s are structurally diverse and the lack of easily identifiable substrate recognition domains for many HECT E3s (e.g. E6AP, HECTD1, HECTD2) has hindered progress in pairing substrates to enzymes. In some cases, there are obvious substrate recognition domains, such as the WW domains in the Rsp5/NEDD4 subfamily of HECT E3s, and these are in fact the most thoroughly characterized HECT E3s [11]. It should also be noted that while some HECT E3s have clearly defined motifs or

domains (e.g., RCC1 repeats in the HERC family of HECT E3s), in the majority of cases these have not been demonstrated to be direct substrate interaction motifs, even though they may be required for the function of the E3 [12].

RBR E3s contain a RING1 domain that, like a conventional RING E3, binds ubiquitin-charged E2s, however they also contain an active site cysteine (within the RING2 domain), that, like a HECT E3, forms a ubiquitin-E3 thioester intermediate [5]. There are 14 human RBR E3s [5] and two in *S. cerevisiae* [1]. PARKIN, associated with the neurological condition Parkinson's disease, is one of the best-studied RBR E3s [13]. Viruses and bacteria have exploited the ubiquitin system in many ways, including by the expression of viral and microbial ubiquitin ligases [14–16]. Some of these fall into recognizable classes of E3s (e.g., RING E3) and others are completely novel (e.g. IpaH family of Shigella ligases).

E3 substrates can be modified by a single ubiquitin at one or more sites (monoubiquitylated or multi-monoubiquitylated, respectively). More typically, a target is conjugated with chains of ubiquitin molecules at one or more sites (polyubiquitylation). Since polyubiquitin chain formation can occur through any one of the seven lysines on ubiquitin or the terminal amino group, polyubiquitin chains of different structural topology can be generated, which has important consequences for the target protein. For example, targets covalently conjugated with K48- linked chains are typically recognized by the proteasome and degraded [17]. Linear ubiquitin chains play an important role in innate and adaptive immune signaling [18], while those with K63-linked chains are involved in many different intracellular signaling events, including the internalization of plasma membrane proteins. Specific functions of most other chain types are unclear, however this is a very active area of research in the field and is reviewed elsewhere [19]. Although the molecular determinates for chain type formation are not well understood, for RING E3s it is generally the E2 that determines chain type, while determinants of chain type specificity of HECT E3-catalyzed reactions are a function of the HECT domain itself [20]. Most E3s result in the same type of ubiquitylation for all of their substrates. For example, substrates of RING-like E3 Rad5 typically have K63-linked chains while substrates of SCF ligases often display K48-linked chains. There are exceptions to this, generally as a result of an E3 interacting with multiple E2s that catalyze different types of chain linkages [21–25]. Chain type information can prove very useful for E3 substrate identification. For example, it may be possible to enrich for substrates by adding proteasome inhibitor to cells if K48 chains are typically generated by the E3. Alternatively, chain-specific antibodies or affinity matrices can be used to enrich for substrates with specific polyubiquitin linkages.

Substrate abundance and turnover rates vary substantially, which can make co-immunoprecipitation (Co-IP) strategies for identifying relevant E3s particularly challenging. This has fueled the use of proteasome inhibitors in conjunction with Co-IPs and the development of specialized tools, such as TUBES (Tandem Ubiquitin Binding Entities) [26], for the enrichment of ubiquitylated substrates. TUBES consist of artificial arrays of Ubiquitin Binding Associated (UBA) domains, which have a high avidity and affinity for specific polyubiquitin chain types. Such enrichment reagents were originally used to define the ubiquitinome, however they can be also be useful in a combinatorial approach for defining the substrates of a specific E3.

The class of E3, whether it is a monomeric or multimeric enzyme, and knowledge of previously characterized interacting proteins, functions, and intracellular localization are important considerations when choosing a suitable approach for pairing substrates to the E3 of interest. To date, protein-protein interaction techniques such as yeast two-hybrid and standard Co-IPs coupled with mass spectrometry have successfully identified many E3 substrates [27–30]. It is unlikely that any one technique can capture the full range of substrates for a particular E3 ligase, given a likely wide range of enzyme-substrate affinities and the potential complexity of regulatory mechanisms that govern enzyme-substrate interactions.

Other post-translational modifications can also influence ubiquitylation events. Neddylation of cullins activates the CRLs [31]. Phosphorylation can regulate ubiquitylation enzymes to influence E3-substrate interactions [32]. For example, phosphorylation can inhibit the E3 from interacting with its cognate E2 [33–35], which can profoundly affect the dynamics and specificity of the E3. The PINK1-induced phosphorylation of both ubiquitin and PARKIN is required for the complete activation of PARKIN [36, 37]. In addition, phosphorylation activates the HECT E3 ITCH [38]. Phosphorylation also directly influences the ubiquitylation of substrates; phosphorylation of C-Jun promotes its ubiquitylation [39], while phosphorylation results in the recognition of IκBα by the SCF$^{β\text{-}TrCP}$ E3 ligase and ultimately its degradation [40]. Proline hydroxylation of HIF1α, dependent on molecular oxygen concentration, stimulates the recognition of this substrate by the VHL E3 [41]. Considering this interplay of post-translational modifications, it is to be expected that some E3-substrate binding events might only be captured under certain experimental conditions. The following sections will focus on the techniques and resources for the identification of substrates of E3 ligases.

Pull-downs coupled with ubiquitylation reactions

Non-specifically bound proteins are the bane of co-immunoprecipitations and GST fusion protein pull-downs. In the case of E3s, substrates and non-specifically associated proteins can be potentially distinguished by essentially performing a ubiquitylation reaction following a pull-down; the assumption is that true substrates will be ubiquitylated, whereas non-specifically bound proteins will not be ubiquitylated. While this is not always a safe assumption, this approach has proven useful in some cases. For example, bacterially expressed glutathione sepharose- bound GST-Rsp5 (a yeast HECT E3) was mixed with ^{35}S-methionine-labeled yeast extracts and after pull-down and washing of the matrix, purified ubiquitin, E1, and E2 enzymes, and ATP were added directly to the beads [42]. It was predicted that Rsp5 substrates would be ubiquitylated by wild-type Rsp5, but not when pulled-down with the active-site cysteine-to-alanine mutant Rsp5 (C-A). SDS-PAGE and autoradiography was performed to identify bands that bound to both GST-Rsp5 and the C-A mutant, but were only ubiquitylated by WT GST-Rsp5. After identification of candidate substrate bands, the pull-down was repeated on a large scale (with unlabeled cell extract), and bands were excised from the gel and identified by protein sequencing. This approach identified Rpb1, the large subunit of RNA polymerase II, as an Rsp5 substrate [43, 44].

A similar approach was employed by Pagano and colleagues to identify targets of the human F-box protein SCF$^{\beta\text{-TrCP2}}$ [45]. HA-βTrCP2 was expressed in human 293T cells and following an anti-HA IP, all of the components necessary for a typical *in vitro* ubiquitylation reaction, including FLAG-ubiquitin, were mixed with the resin. An anti-FLAG IP was then performed to isolate ubiquitylated proteins, followed by LC-MS/MS. Claspin was identified as a new substrate by this approach. The specificity of the interaction between Claspin and SCF$^{\beta\text{-TrCP2}}$ was bolstered by the inability of 19 other F-box proteins to Co-IP with Claspin.

Screening protein arrays or protein expression libraries by *in vitro* ubiquitylation

Rotin and colleagues performed large-scale *in vitro* ubiquitylation assay on protein microarrays to identify new substrates of Rsp5 [46]. The protein microarrays were loaded with 4,000 GST and HIS- tagged *S. cerevisae* proteins and incubated with fluorescently-labeled ubiquitin along with E1, E2, Rsp5, and ATP. Ubiquitylation was allowed to proceed for 3 hours and was then detected with a fluorescent laser scanner. Candidates with the strongest signal relative to the positive control were deemed high confidence hits. This approach confirmed many previously identified substrates of Rsp5 and led to the identification of several new substrates, including Sna4 and Rcr1. Simply incubating the microarray slides with fluorescently-labeled Rsp5 protein also identified substrate proteins and interacting proteins, however this may not be a generally useful approach as the WW domains are protein-protein interaction domains that interact with small linear epitopes of substrates ("PY" motifs). Both data sets – direct Rsp5 interactors and protein ubiquitylated by Rsp5 - were enriched for known Rsp5-interacting proteins as well as proteins with PY motifs [11]. In a further variation of the approach, an *in vitro* assay for Rsp5 was developed using a bank of GST-tagged yeast proteins that were deemed potential substrates because they contained PY motifs (consensus sequence being PPxY) [47]. Fifty-eight PY-motif-containing yeast proteins were selected for screening, and a larger set of randomly chosen proteins were analyzed. To screen for potential Rsp5 substrates an *in vitro* ubiquitylation assay was performed using a GST-tagged set of candidate proteins and biotin-labeled ubiquitin. A mix of streptavidin and GST beads specifically isolated ubiquitylated substrates, which were detected by AlphaScreen™ Technology luminescence detection. The fluorescent detection of ubiquitylation increased the sensitivity of detection and offered a reduced workload and processing time compared to standard western blot detection. Nine positive hits were identified and most of these had PY motifs. Such an approach is obviously useful when substrates are predicted to share consensus E3 recognition domains, but is not useful when dealing with an E3 that does not contain previously characterized protein-protein interaction domains.

The principles described above have also been applied to find substrates of the APC/C, in an Extract-based Functional Assay (EFA) [48]. Here, a human protein microarray (Human ProtoArray microarrays; Invitrogen) of 8000 proteins was incubated with synchronized HeLa cell extracts. Since APC/C is activated at cell cycle transitions, extracts from two cycle stages were compared for ubiquitylation using an anti-ubiquitin antibody and fluorescent detection. As a preliminary experiment, extracts were mixed with the ^{35}S methionine-

labeled Securin, a known APC/C target, to verify the activity of APC/C in the extracts before proceeding with the screen. 132 candidates were identified and, at the time, there were 16 known substrates for APC/C, 11 of which were among the positive hits. Many of the putative substrates were associated with mitosis (based on GO terms) and were chosen for validation. Substrates were validated by incubating ^{35}S-labelled proteins with extracts containing active or inactive APC/C. Five of the six mitosis-associated candidates were shown to be substrates of APC/C. The protein microarray used in this study consisted of N-terminally GST-tagged proteins, however the nature of the tag and its location (N- or C-terminal) may be an important consideration in applying this to other E3s.

In 1997, Kirchner and colleagues developed the *in vitro* expression cloning (IVEC) technique [49] as a way of identifying the key players in the process of mitosis, and thereafter, the targets of the APC/C [50]. In this approach, pools of cDNAs (50–100 cDNAs per pool) were *in vitro* transcribed and translated in the presence of ^{35}S-labeled methionine. The pools of translated proteins were then either left untreated or a ubiquitylation reaction was initiated with the E3 and supporting E1 and E2 enzymes, ATP, and ubiquitin. The translation products were compared, with and without ubiquitylation components, to determine if any of the translated proteins from a given pool of cDNAs were substrates of the E3 (based on a molecular weight shift or degradation). If there was a positive hit with a pool, the pool was sorted and reanalyzed to identify the single cDNA that encodes the putative substrate. IVEC led to the discovery of Geminin as an APC/C substrate [51], which plays a critical role in cell cycle regulation. IVEC has been successfully used to identify other APC/C substrates as well, including Securin, Xkid, Tome-1, Sororin, and Mcph1-B [52–56], and has also been successfully applied for identifying substrates of kinases [49, 57], caspases [58], and p53 interacting proteins [59]. IVEC is likely to work well for E3-substrate interactions where smaller well-defined epitopes of substrates are recognized by the E3 (e.g. PY motifs recognized by NEDD4 family E3s, or D-box motifs by the APC/C). However, IVEC has its limitations since many of the translation products may come from incomplete cDNAs and translated proteins may therefore not be folded correctly and not be recognized by the E3. Another limitation is that it may not be possible to reconstitute regulated ubiquitylation (e.g., in response to substrate phosphorylation) in the in vitro translation system.

Cell-based Genome-Wide Screens

Advantages of cell-based screening approaches include the fact that 1) the results may have a greater chance of being biologically relevant and 2) that E3s that are difficult to express in an active form *in vitro* (e.g., multi-subunit E3s) may be more amenable to study in a cell-based system.

SCF ligases influence a wide range of cellular processes, mediated by the diverse array of F-box proteins. Toczyski and colleagues used a large-scale microscopy screening method in *Saccharomyces cerevisiae* to identify direct targets of Grr1, a F-box protein required for glucose sensing in yeast [60]. To develop the technique, a *grr1* deletion strain was constructed, in which *grr1* was replaced with Red Fluorescent Protein (RFP). The *grr1* (RFP-positive) deletion strain was crossed with a GFP-Open Reading Frame (ORF) fusion

library of 4000 yeast strains and mixed haploid populations of *grr1* (RFP-positive) and *GRR1* (RFP-negative) cells expressing an individual GFP-tagged protein were examined by immunofluorescence microscopy. An increase in median fluorescence intensity of the GFP signal in the *grr1* cells relative to the *GRR1* cells (as distinguished by the RFP signal) was indicative of that GFP fusion protein being a target of the Grr1-associated ligase. This large-scale screen was conducted in a 96-well plate format and over 100 putative targets were identified. After secondary screening, twenty-four of these proved to be indirect targets and were affected by *grr1* deletion at the mRNA level, while seven were stabilized by *grr1* deletion in a cycloheximide chase assay, confirming them to be direct ubiquitylation targets of Grr1. One of the main benefits of this technique is the precise comparative analysis of the wild-type (WT) and mutant strains.

Global Protein Stability (GPS) profiling was developed as a means of tracking the abundance of 8000 human proteins in response to environmental cues or cellular perturbations [61]. Dual fluorescent tags DsRed and GFP were transcribed from a single mRNA separated by an internal ribosome binding site (IRES), driven by a constitutive promoter which was integrated into the genome by retroviral transduction. GFP was fused to the ORF of interest and the IRIS allowed DsRed to serve as a control for protein expression, since alterations to protein stability due to interactions with an E3 ligase should only affect the levels of GFP. In addition, this ensured that transcriptional regulation does not affect protein levels, since both tags are transcribed from the same mRNA. Using GPS profiling and the human ORFeome library, the relative abundance of close to 8000 proteins were assessed at the single cell level. The total pool of cells, representing all 8000 ORFs, was subdivided into 7 populations using Fluorescence-activated cell sorting (FACS), according to the GFP/DsRed ratio, which indicated the relative stability of each protein. The DNA representing the ORFs from each subpopulation, as well as the total population, was then quantified by microarray hybridization. The relative enrichment of ORFs was assessed, and correspondingly, the levels of each protein in the library. To test the capabilities of GPS profiling, the proteasome inhibitor MG132 was added to cells. The effect was profound, with >80% of proteins showing increased stability. The subsequent validation of all 85 randomly chosen samples demonstrates the robustness of GPS analysis and the potential for this technique in detecting subtle cellular perturbations in response to expression or depletion of a particular E3 in the cell.

Elledge and colleagues used GPS for the identification of substrates for SCF ligases [62]. SCF ligases were inhibited by lentiviral delivery of dominant negative CUL1, the core component of SCF E3 ligases. Putative targets were those with increased ratio of GFP/DsRed when CUL1 was inhibited. The scope and sensitivity of GPS Profiling is exceptional: 73% of SCF substrates were identified and in addition, 31 of the 66 newly identified substrates were independently validated. Concerns for interference from N-terminal tags were addressed by testing ORF-HA fusions for 19 candidates; there was 89% correlation with the GFP-ORF results.

The technique was further improved upon with a more efficient lentivirus and an updated human ORFeome collection [63]. The updated ORFeome collection covered more ORFs and almost 13000 genes. The capacity of the GPS technique was once again interrogated, this

time by manipulating SCF ligases with the addition of MLN4924. MLN4924 is a NEDD8-activating enzyme inhibitor and inhibits the activity of all CRLs by preventing the activation of CUL1 and would be expected to increase the abundance of substrates. Indeed, half of the 190 known cullin-interacting proteins were identified in this screen. A quantitative proteomics approach was developed and used in parallel to the GPS profiling [63]. Quantitative ubiquitylation interrogation (QUAINT) analysis is essentially a variant of quantitative mass spectrometry. Ubiquitylated proteins were specifically co-immunoprecipitated using a diGly (diGlycine) antibody (discussed further below). Peptides that were enriched in response to MLN4924 treatment were identified using a SILAC (Stable Isotope Labeling with Amino acids in Cell culture)-based mass spectrometry approach. GPS and QUAINT appear to be good complementary techniques, since both identified known substrates that would have been missed with a single approach and putative substrates present in both data sets can be pursued with higher confidence.

Co-IP and Affinity Purification of E3s to identify substrates

Affinity purification coupled with mass spectrometry (AP/MS) is a widely used global approach for characterizing protein-protein interactions, in general. By expressing an affinity-tagged version of an E3 ligase in cells, substrates will often co-immunoprecipitate and can be identified by mass spectrometry. In the design of individual experiments, the size of the tag and its positioning at the N- or C-terminus of the protein may be important considerations, especially if functional domains are close to either end of the protein. Just as the design of AP experiments is essential to the generation of interpretable data, the ability to decipher genuine interactors from false positives is crucial to the success of AP/MS. Computational tools that can help here include HGSCore, CompPASS and SAINT [64–66]. CompPASS, for example, assigns a score to bait-prey pairs across multiple experiments by integrating the abundance, uniqueness and reproducibility of an interacting protein. In this manner, high-confidence candidate interacting proteins (HCIPs) are sorted from the common technical artifacts and false positives [67].

Dual affinity tags such as His6-FLAG allow for a two-step purification which can reduce background binding and, moreover, a His6 tag can tolerate denaturing conditions. Compared to His6-FLAG, a His6-biotin affinity purification allows for stringent conditions throughout the purification process due to the high affinity of biotin and streptavidin used for the IP [68]. Tagwerker *et al.*, used formaldehyde as a crosslinking reagent and His6-biotin-tagged Skp1 (adaptor component of SCF ligases) to identify substrates. In preparing the samples for MS, an on-bead tryptic digestion was used to circumvent the difficulty in removing the bound proteins from the beads.

Global approaches to map the ubiquitinome has been a major goal for the field and by using epitope-tagged ubiquitin, close to 1100 conjugates had been identified [69]. In 2011, Gygi, Harper and colleagues used a new approach called diGly proteomics to identify ~5000 ubiquitylated proteins in human cells [65]. In the process of preparing protein samples for diGly mass spectrometry analysis, the sample is trypsinized, which leaves a diglycine (diGly) remnant on the side chain of ubiquitylated lysine residues. DiGly remnant antibodies

were developed [65, 70] that can be used to immunoprecipitate these peptides, which are then identified by mass spectrometry.

The capacity of diGly proteomics for detecting subtle changes in ubiquitylation was tested in the initial study by Gygi and coworkers, by treating cells with proteasome inhibitor and/or MLN4924, compared to untreated cells. In the process, known and novel CRL substrates were identified and the specific detection of the ubiquitin-modified version of H2B validated this candidate as a CRL substrate. DiGly proteomics has since been used for the discovery of many substrates for E3s [71–74]. By using siRNAs to perturb E3 ligase activity and diGly proteomics, new substrates for HRD1 [72] and HUWE1 [74] have been identified and the Parkin interaction network was significantly expanded using a SILAC and diGly proteomics approach [71]. A new approach developed by the Gygi lab combines isobaric labeling of peptides and diGly proteomics [75]. By interrogating the PARKIN-PINK1 pathway, the authors successfully demonstrated the capacity of this technique for increased sensitivity and reproducibility in identifying candidate substrates for E3 ligases.

E6AP/UBE3A, a HECT E3, was one of the first E3 ligases to be characterized. E6AP cooperates with the HPV E6 protein in the degradation of p53 to promote HPV-associated cervical cancer [76, 77]. Mutations in the E6AP/UBE3A gene or alterations in expression are also the cause of Angelman syndrome, a severe neurologic disease [78]. Despite its clinical importance, few natural (E6-independent) substrates have been identified. A comprehensive approach to identify new substrates of E6AP compared the interactomes of the multiple isoforms of E6AP, both wild-type and a dominant negative (DN) mutant [79]. Based on previous work, it was hypothesized that a DN mutant may lead to accumulation of E6AP substrates in the cell [80]. Tagged proteins were inducibly-expressed in Flp-In T-REx 293 cells and affinity-purified. Mass spectrometry data was processed through CompPASS to identify high confidence candidate interactors. To assist the filtering process, NEDD4, another HECT E3, was also included in the experiment as a negative control. Despite no significant differences between isoforms of E6AP or between WT and the DN mutant, several interactors were identified. As confirmation, MAPK6, HIF1AN, and NEURL4 pulled down when E6AP was affinity-purified from cells. Moreover, these proteins were more readily detected in the DN mutant, however the physiological relevance and function of these interacting proteins remains unclear.

For a successful AP/MS-based substrate screen, it is extremely helpful when there are known substrates for the bait protein, and defined enzyme-substrate interaction domains. The tumor suppressor and F-box protein FBW7 is known to ubiquitylate phosphorylated substrates through a conserved Cdc4 phosphodegron (CPD) motif [81–84], which is essential for the binding of FBW7 to substrates [85]. To screen for new substrates, wild-type FBW7 and a mutant that does not bind known substrates were affinity-purified from human cells [86]. The MS data was filtered for proteins only present in WT and 72 proteins remained, including 5 known FBW7 targets. Analyzing the list of candidates using bioinformatics, it was noted that 26 of these proteins belonged to the same complex, which suggested that FBW7 could be pulling down indirect targets, as well as direct targets. A focus was placed on validating proteins with the highest spectral counts and determining the presence of the conserved phosphodegron (CPD) domain among candidates. This proved to

be an effective approach and MED13/13L were shown to be FBW7 targets, demonstrating a role for FBW7 in broadly regulating transcription.

In 2013, Harper and colleagues applied a Parallel Adaptor Capture (PAC) technique for the cross-comparison of AP/MS data from 19 FBXL proteins to systematically expand the interactome of FBXLs [87]. PAC is an approach to specifically enrich for F-box interacting proteins. Human HEK293 cells expressing HA-tagged F-box proteins were treated with either bortezomib (proteasome-inhibitor) or MLN4924 (CUL1 inhibitor) and HA-tagged proteins were precipitated in parallel and subjected to mass spectrometry (PAC proteomics). High Confidence Interacting Proteins (HCIPs) were identified using CompPASS analysis [67]. The approach proved extremely successful in identifying interactors. 80% of the candidates selected for validation by Co-IP/western blot were shown to interact with their cognate FBXL protein. In addition, almost 50% appeared to be substrates, with an increase in abundance in response to MLN4924 treatment. Interestingly, five of the candidates chosen for validation were sub-threshold HCIPs that appear to be substrates, which suggests that CompPASS filtering may have been too stringent and additional substrates could also have been missed. Similar PAC experiments were performed for 10 FBXLs in HCT116 cells (colon cancer cell line) and a significant overlap in targets identified in both cells types demonstrated the benefits of performing parallel experiments in different cell lines.

Bioinformatic analyses of E3-associated proteins can play a critical role in substrate verification. HERC2 is linked to a number of Autism-spectrum conditions and DNA damage repair pathways [88–90]. A small number of substrates have been characterized, such as NEURL4 and RNF8 [91, 92] but it has been notoriously difficult to study this very large E3 ligase and delineate its functions. Since the full-length protein is poorly expressed, Galligan et al created 6 constructs, each of about 1000 bp size, collectively spanning the full-length protein [93]. They hypothesized that substrates would co-fractionate with a specific fragment by binding to its corresponding interaction domain. By using HA-GFP as a control for non-specific bead binding and filtering for peptides that were present in one sample only, 239 putative interactors were identified. With such a large list of candidates, further *in silico* analysis was required. The Genomatrix Pathway system was applied to the data and it highlighted the abundance of proteins involved in intracellular vesicle-mediated organization and transport. Of the 40 HCIPs that were in this pathway, 27 of them were functionally linked. This approach proved successful and of the 21 potential interactors that were tested, 13 of them were shown to interact with endogenous HERC2.

Precise comparative analysis across many AP/MS experiments can eliminate background noise that is inherent to mass spectrometry data sets. A useful repository of common AP/MS contaminants has been compiled [94]. Moreover, a new interface, Spotlite provides similar scoring methods to analysis tools such as CompPASS but also integrates information on function, model organism phenotypes and human disease relevance [95]. With an interest in substrates of the E3 ligase Keap1, Major and colleagues reanalyzed their previously published Keap1 mass spectrometry using Spotlite. A library of 44 reference data sets from previous AP experiments, were comparatively analyzed to identify unique interactors. In this manner, 35 candidates were identified. As validation of their approach, eight of the

candidates co-immunoprecipitated with Keap1, and one of them, MCM3 has since been confirmed as a novel substrate [96].

Tandem ubiquitin-binding entities (TUBES) are tandem UBA domain repeats consisting of UBAs from UBQLN1 and HR23A [26, 97]. TUBES have become a widely used tool in molecular biology to trap ubiquitin-bound substrates and are commercially available. New substrates for the striated muscle-specific RING E3 MuRF1 were identified with the use of TUBES, in combination with two-dimensional differential in gel electrophoresis (2D-DIGE) and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry [98]. This technique centers on the comparative analysis of cells with differential (reduced or enhanced) E3 ligase activity. Either GFP alone or in combination with MuRF1 was overexpressed in primary cardiomyocytes and it was expected that known substrates as well as new candidates would be enriched for in response to MuRF1 overexpression. A pulldown with either TUBE or control beads was then performed. Two differentially-labeled experimental conditions were tested in a single 2D-DIGE gel. Gel1 compared the TUBE pull-down eluate for control (GFP only) versus the experimental cells (MuRF1). Gel 2 compared the supernatants following AP with either TUBE or control beads for experimental cells. Gel 3 compared the supernatants following AP with either TUBE or control beads for control cells. Protein spots displaying greater than 1.5 fold expression changes were deemed candidates in each individual gel and the list of candidates from all three gels were aligned to determine spots that were candidates by all three comparisons. The candidates across all multiple gels were selected for further analysis and removed from the 2D gels for protein identification by MALDI-TOF mass spectrometry. In this manner, Hspd1, Tpm1, and Atp5b were identified as new substrates of MuRF1.

Trypsin Resistant (TR)-TUBES have now been created, and may be more advantageous to use for mass spectrometry-based experiments, since they prevent the TUBES and ubiquitin itself from contributing to the overall peptides detected by mass spectrometry [99]. By co-expressing the TR-TUBES along with FBXO21, an uncharacterized F-box protein, candidate substrates were identified by diGly proteomics. Only two candidates were chosen for validation; these proteins had the highest spectral counts, and they were subsequently validated as substrates. With a focus on further optimizing the TUBES-type pull-down approach, Xu and colleagues systematically tested the binding capabilities of a number of different Ubiquitin Binding Domains (UBDs) [100]. By testing the binding capacity of five different UBAs, both alone and in combination, for ubiquitin, the authors designed Tandem hybrid UBDs (ThUBDs) that had unbiased binding to all seven types of polyubiquitin chains. Moreover, ThUBDs were more effective than naturally occurring UBDs at enriching for ubiquitylated proteins. Compared with HIS-MYC-Ub in yeast, ThUBDs purified more ubiquitylated proteins. In preparing the samples for mass spectrometry, the proteins were separated by high pH reversed-phase liquid chromatography (RP-LC), instead of SDS-PAGE. This greatly improved the yield of candidates, which compared favorably with previous studies, such as diGly proteomics, in mapping the global ubiquitinome. Other fusion protein approaches include the fusion to the proteasomal subunit S5A was developed to trap polyubiquitylated proteins [101]. In another approach, four tandem repeats of ubiquitin-associated domain from UBQLN1 were fused to a GST tag [102]. These

techniques, developed for enriching ubiquitylated proteins, may prove very useful as part of a combinatorial approach for E3-substrate pairing.

Substrate Traps

O'Connor *et al.*, developed a technique to covalently capture substrates of E3 ligases. Ubiquitin-activated Interaction Traps (UBAITs) are E3 ligase fusion proteins with ubiquitin, bridged by a flexible linker region, attached to the C-terminus of the E3 [103]. The C-terminus of ubiquitin remains free for activation by the E1 enzyme, forming a thioester-linked complex, which is then transferred to an E2 enzyme, as would be expected for any N-terminally-tagged ubiquitin (although in this case the N-terminal tag is an E3). For HECT E3s, the active site cysteine of the E3 can attack the UBAIT~E2 thioester bond in an intramolecular reaction, forming a thioester-linked "lariat" protein. If a substrate then interacts with the E3, even transiently, a lysine of the substrate might react with the thioester-linked lariat ubiquitin, which will result in formation of a stable amide bond-linked UBAIT-substrate complex (i.e., E3-linker-Ub-substrate), which can be purified via an N-terminal affinity tag on the UBAIT. The approach was also predicted to be applicable to non-HECT E3s, with the only difference being that the substrate lysine would react with the UBAIT when it is in a thioester linkage to the E2; the product would again be a stable amide bond-linked complex between the UBAIT and the substrate.

To validate the approach, N-terminally Tandem Affinity Purification (TAP)-tagged UBAITs were overexpressed in cells and affinity purified. Following SDS-PAGE, gel slices, excised from above the migration point of the UBAITs, were sent for mass spectrometry analysis. A UBAIT with the terminal glycine residues of ubiquitin mutated was used as a control for non-covalent interactors. The approach was validated in both yeast and human cells, for both HECT and RING E3s. Known substrates were trapped for the HECT E3s Rsp5 and Itch, as well as RING E3s Psh1 and RNF126. For yeast Rsp5, the UBAIT trapped 32% of known interactors, including regulators, substrates and adaptors. The presence of known adaptors and regulators as well as substrates likely reflects the ability of the UBAIT to serve as a covalent proximity-based protein interaction trap. The high ratio of known interactors for Rsp5 as well as for Psh1 highlights the specific and selective functionality of the UBAITs. Moreover, the UBAIT was also used to identify a new target for the RING E3 RNF168, a key component of the DNA damage response pathway. The technique has applications beyond E3s, as well, and can potentially be used to profile the interactome of almost any protein of interest, with the caveat that UBAITs must have the ubiquitin moiety at the C-terminal end, which may preclude its use with certain proteins [103].

The NEDDylator is another fusion-based strategy and takes advantage of the well-defined and short list of substrates for NEDD8 [104]. NEDD8 has its own E1 and a single E2, UBC12. By fusing UBC12 to the C-terminus of an E3 and co-expressing with tagged NEDD8, targets of the E3 will be NEDDylated, rather than ubiquitylated, and these targets can then be easily isolated by affinity purification (based on the tagged NEDD8) and identified by mass spectrometry. While natural NEDD8 substrates will also be affinity purified, the natural substrates are a short list of known proteins and these can be disregarded as candidate substrates of the NEDDylator. When tested with the RING E3

XIAP (or CIAP1), different orientations (N- versus C-terminal UBC12) were comparable *in vitro*, but C-terminal UBC12 was used in cells since N-terminal acetylation of UBC12 facilitated NEDDylation of certain NEDD8 substrates. Using Jurkat cells, a pull-down under denaturing conditions, and a SILAC-based MS strategy, known and unknown candidate substrates were isolated. The data was filtered for the enrichment of peptides with the NEDDylator compared to UBC12 alone. Since NEDD8 is not degraded by the proteasome, the NEDDylator may ensure stability of the conjugates compared to overexpression strategies that employ a tagged form of ubiquitin.

As an alternative to standard affinity purification, BioID was developed. This technique takes advantage of the biotinylation capabilities of the *E.coli* enzyme BirA. A mutant form of BirA promiscuously biotinylates proteins within a 10–20nm range [105]. By stably expressing the BirA-POI (Protein Of Interest) fusion protein in mammalian cells, it is expected that substrates and other binding partners can be biotinylated and subsequently identified by a streptavidin pull-down and mass spectrometry [106]. This technique was successfully applied to identify substrates of the F-box protein SCF$^{\beta\text{-TrCP1/2}}$ [107].

The Toczyski lab developed the Ubiquitin Ligase Substrate Trapping technique to identify substrates of SCF-F-box ligases in yeast [108]. The UBA domains of Dsk2 or Rad23 (ubiquitin receptor proteins that typically mediate the transfer of ubiquitylated proteins to the proteasome) were fused, via a 3X FLAG linker, to eight different F-box proteins. The UBA-tagged F-box substrate receptors allowed for anti-FLAG co-immunoprecipitation of the polyubiquitylated forms of the substrates with the UBA-F-box proteins. This was done in cells expressing His6-tagged ubiquitin so that a secondary Ni-NTA purification of ubiquitylated proteins could be performed from the anti-FLAG immunoprecipitate. Artifacts of overexpression were avoided by expressing the UBA-F-box proteins from an endogenous promoter, where the bait was the only copy of the F-box protein in the cell. Both known and novel substrates of the F-box E3s were identified. An important factor for the success of this technique was the ability to compare the proteins identified with the different F-box traps, and a 250-fold enrichment was chosen as the cutoff point in order to filter the data for unique peptides among the different F-box traps. The Toczyski lab has expanded this technique for use in mammalian cells [109], using stable cell lines expressing doxycycline-inducible His6-tagged ubiquitin and the E3-3XFLAG-Rad23B fusion proteins.

Concluding Remarks

There are clearly many options for profiling the substrate specificity of E3, and the characteristics and preexisting knowledge of any given E3 will allow some degree of prioritization of approaches. Additional recent perspectives on these issues are also available [110, 111]. The development of approaches that take unique advantage of ubiquitin and Ubl biochemistry (e.g., UBAITs, Neddylators, Ubiquitin Ligase Substrate Traps) may improve the depth of coverage and help identify less abundant substrates for E3 ligases. The direct tagging of endogenous proteins for Co-IP approaches using CRISPR/Cas9 techniques may also help eliminate artifacts of overexpression in human cell systems, and CRISPR/Cas9 approaches will also allow the investigator to infer ligase substrates by proteomic analyses in a clean deletion background. The importance of modern bioinformatics and statistical tools

in analyzing output of many of the approaches described here should not be underemphasized. For example, the RBR E3 ARIH1 was recently discovered to interact with CRLs by Co-IP experiments, however retrospective analysis of older CRL interactome studies had previously demonstrated this connection [112]. Therefore, improved bioinformatics tools and analysis of multiple datasets obtained with a variety of approaches can be useful for refining lists of candidate substrates and guiding further experimentation.

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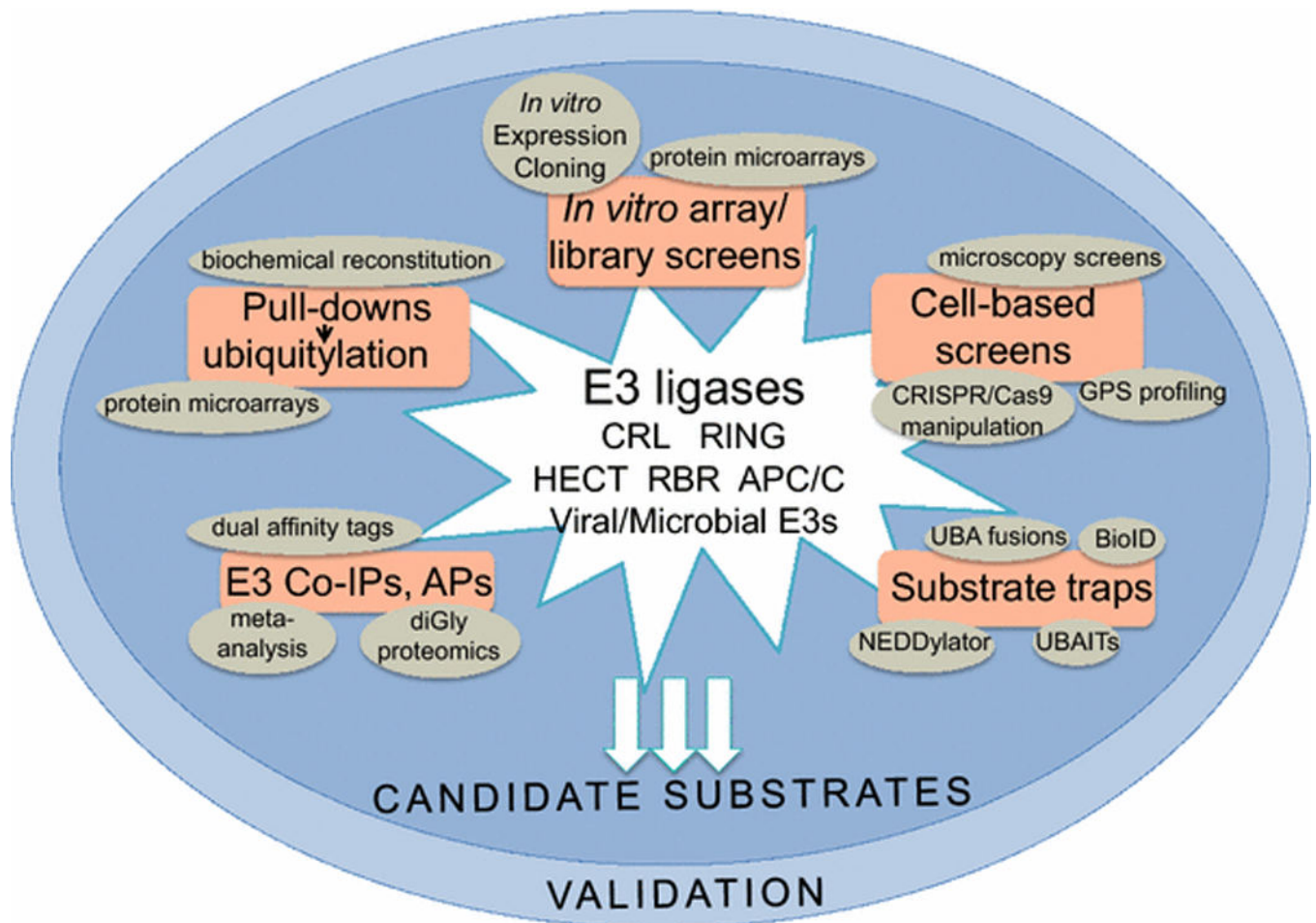
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Figure. Methods to Identify Substrates of E3 Ligase

Classes of E3s are indicated in the center, and techniques and approaches for the identification of substrates are categorized as follows (orange boxes): Pull-downs coupled with *in vitro* ubiquitylation reactions; Cell-based screens; Co-immunoprecipitations (Co-IPs) or affinity purification (AP) of the E3; Substrate traps. Examples of techniques covered under these subcategories are circled.