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Invited Mini Review

Deubiquitinase dynamics: methodologies for understanding substrate interactions

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Deubiquitinases (DUBs) are essential regulators of protein homeostasis that influence cellular signaling, protein stability, and degradation by removing ubiquitin chains from substrate proteins. Understanding DUB–substrate interactions is critical to elucidate their functional roles and therapeutic potential. This review highlights key methodologies to investigate DUB activity and substrate interactions, including biochemical assays, fluorescence-based approaches, and *in vitro* deubiquitination assays. Biochemical methods, such as those measuring protein degradation rates, ubiquitination dynamics, and protein–protein interactions, provide valuable insights into DUB function and specificity. Fluorescence-based techniques that include photoconvertible reporters, fluorescent timers, and FRET enable the real-time monitoring of DUB dynamics and substrate turnover in live cells. Furthermore, *in vitro* deubiquitination assays provide direct mechanistic insights into DUB activity on target substrates. While each method provides unique insights, they also present challenges, like limited specificity or sensitivity, technical difficulties, or insufficient physiological relevance. Integrating complementary approaches can enhance accuracy and provide deeper insights into DUB–substrate interactions, facilitating the development of DUB-targeted therapeutic strategies. [BMB Reports 2025; 58(5): 191-202]

INTRODUCTION

In the late 1970s, the discovery of ubiquitin-mediated protein degradation fundamentally transformed our understanding of cellular protein regulation (1, 2). This breakthrough, later recognized by the 2004 Nobel Prize in Chemistry, revealed that protein degradation is an exquisitely regulated process that is central to cellular homeostasis. The ubiquitin–proteasome system (UPS) has since emerged as a sophisticated regulatory network that orchestrates protein stability, localization, and activity through post-translational modifications (PTMs) (3-6). We now understand that protein degradation mechanisms should not be regarded as merely a 'garbage disposal' system for malfunctioning or redundant proteins, but rather constitute a highly specific and actively regulated process that ensures timely control through precise substrate selection.

UPS regulates various cellular processes by regulating protein stability, localization, and activity, ensuring the delicate balance required for protein homeostasis (7-9). This process relies on the dynamic interplay of ubiquitinating and deubiquitinating enzymes (10). Deubiquitinases (DUBs) serve as master regulators by catalyzing the removal of ubiquitin modifications from substrate proteins, thereby controlling their cellular fate. Beyond their role in preventing proteasomal degradation, DUBs maintain cellular ubiquitin pools, and influence diverse biological processes that include protein degradation via the proteasome (11), endocytosis (12-15), DNA repair mechanisms (16-18), and kinase activation (19-22). This regulatory reach positions DUBs as critical determinants of cellular homeostasis, with their dysfunction implicated in various pathological conditions, such as cancer (23-26), inflammatory disorders (25, 27), and neurodegenerative diseases like Parkinson's disease (28, 29).

The therapeutic potential of targeting DUBs has attracted significant attention, driven by their druggable active sites, and their dysregulation in various diseases (30, 31). Determining the specific DUBs involved in particular diseases is a critical step toward modulating their activity for disease intervention (24, 29, 32). Despite advances in proteomics and molecular biology, systematic methodologies to elucidate DUB–substrate interactions remain complex, and insufficiently explored. Key obstacles include distinguishing direct from indirect substrates, limited throughput of existing methods, and capture of the dynamic nature of DUB activity in cellular contexts. Emerging technologies, such as proximity labeling, photoconvertible fluorescent protein tagging, and advanced mass spectrometry (MS), have begun to address these limitations, and offer new insights into DUB–substrate networks.

This review examines cutting-edge experimental strategies to identify and characterize DUB–substrate interactions, encompassing both the identification of DUB targets, and the regulatory DUBs for individual substrates. By synthesizing current methodologies and emerging technologies, we provide a frame-

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work to advance DUB biology, and accelerate the development of DUB-targeted therapeutics.

DEUBIQUITINASE (DUB)

Ubiquitination: precision modulation of cellular function

The ubiquitin system that represents a fundamental molecular mechanism of cellular regulation is characterized by specificity and versatility (4, 33). This PTM enables cells to modify protein function, relocate proteins, and interact with cellular machinery through two primary mechanisms of mono-ubiquitination, and poly-ubiquitination.

Mono-ubiquitination targets histones, thus serves as a critical epigenetic regulatory mechanism (34). By attaching a single ubiquitin molecule, cells alter chromatin structure, modulate gene expression, and respond to cellular stress without changing genetic sequence. This modification acts as a dynamic molecular switch that facilitates rapid transcriptional responses, enables DNA damage repair (35), and supports chromosomal dynamics (36). Poly-ubiquitination of non-histone substrates functions as a complex signaling platform extending beyond protein degradation (37). Diverse ubiquitin chain configurations allow cells to coordinate immune responses (38), regulate cell cycle progression (39), and control programmed cell death (40). Such poly-ubiquitin chains recruit cellular machinery, modulate protein interactions, and signal complex cellular responses.

The enzymatic cascade involves a hierarchical system of E1, E2, and E3 enzymes, with E3 ligases providing critical substrate specificity (41). This selective mechanism enables cells to respond to environmental changes, maintain protein homeostasis, and preserve cellular adaptive potential (42).

Molecular mechanisms of DUB activity

DUBs catalyze the hydrolysis of isopeptide bonds that link ubiquitin to the target protein, which is central to their biological function. Their active sites, which consist of critical amino acids that allow for substrate specificity and catalytic efficiency, facilitate this enzymatic activity. The primary biochemical mechanism involves the nucleophilic attack by a water molecule or a thiol group on the carbonyl carbon of the isopeptide bond, leading to bond cleavage and the release of free ubiquitin (43).

Based on their catalytic mechanisms, the DUBs can be broadly categorized into three main types: cysteine proteases, metalloproteases, and serine proteases (44). Cysteine proteases, which include the largest family of DUBs (USPs), contain a catalytic cysteine residue that performs a critical role in the hydrolysis reaction. The cysteine residue forms a covalent intermediate with the ubiquitin moiety, which is subsequently attacked by water, resulting in the cleavage of the isopeptide bond. This mechanism allows DUBs to specifically recognize and cleave polyubiquitin chains, particularly those linked through lysine 48 (K48) residues, which typically target proteins for degradation (43).

In addition to their catalytic activity, DUBs engage in protein–protein interactions that modulate their activity and substrate specificity. For example, the binding of DUBs to ubiquitinated substrates often involves additional domains that enhance their specificity and affinity, such as ubiquitin-binding motifs (45). This specificity is paramount, as it ensures that DUBs selectively deubiquitinate target substrates, and thereby regulate their stability, localization, and activity within the cell. Moreover, certain DUBs are known to interact with co-factors or PTMs that can further influence their activity; for example, the inactivation of DUBs due to phosphorylation or other modifications can mediate a rapid response to cellular stress signals, which highlights the dynamic regulatory mechanisms at play (46).

Functional diversity of DUBs

Based on their structural characteristics and mechanisms of action, DUBs are classified into seven distinct families: Ubiquitin-specific Proteases (USPs), the first class and the largest family of DUBs, are primarily known for their ability to cleave ubiquitin from K48-linked polyubiquitin chains, which commonly target proteins for degradation. USPs are critical to regulate protein stability and cellular signaling pathways. Ubiquitin C–Terminal Hydrolases (UCHs), the second class, specialize in removing single ubiquitin molecules from substrates, thereby helping maintain the pool of free ubiquitin; this activity plays a vital role in the dynamic control of ubiquitination. Ovarian Tumor Proteases (OTUs), the third class, are characterized by unique catalytic properties and substrate specificity. Such OTUs often deubiquitinate K63-linked polyubiquitin chains, which are involved in signaling processes, rather than targeting proteins for proteasomal degradation. Machado–Joseph Disease Proteases (MJDs), the fourth class, are involved in processing both ubiquitin and non-ubiquitin substrates, and MJDs are recognized for their role in neurodegenerative conditions, emphasizing their significance in disease contexts. Jab1/Mov34/Mpr1 (JAMM) Metalloproteases, the fifth class, require metal ions for their activity, and are important in the regulation of various cellular processes that include immune responses and protein homeostasis. While the JAMM family consists solely of zinc metalloproteases, the other six families are cysteine proteases. Motif Interacting with Ubiquitin (MIU)-containing Novel DUB Family (MINDY) Proteases, the sixth class, are characterized by the presence of MIU domains (47). These domains allow MINDY proteases to specifically interact with ubiquitin and influence various cellular functions (43). Zinc finger-containing ubiquitin peptidase 1 (ZUP1), the seventh class, exhibits specificity for Lys63-linked chains conferred by multiple ubiquitin-binding domains, and is associated with genome integrity pathways. The human genome contains only one representative of the ZUP1 family (48).

Pathophysiological implications of DUBs

DUBs have been implicated in various diseases, reinforcing their importance in pathophysiology. For example, USP7 has been associated with cancer progression through its regulation of key tumor suppressors and oncogenes, leading to the promotion of malignancy (49). Further, OTUD5 is known to facilitate bladder cancer progression by modulating the mTOR signaling pathway, thereby influencing cell growth and survival (50). The role of DUBs in neurodegenerative diseases is also notable; aberrant regulation of UCH−L1 has been linked to Parkinson's disease, highlighting the potential of targeting these enzymes for therapeutic intervention (28). These examples illustrate how specific DUBs can alter cellular pathways, and ultimately contribute to the onset and progression of various diseases.

METHODOLOGIES TO IDENTIFY DUB–SUBSTRATE DYNAMICS

Biochemical assays: protein degradation rate

The initial step to experimentally validate DUBs that stabilize specific substrates is to determine whether there are resulting changes in the degradation rate of the target substrate protein after inducing changes in DUB activity. By altering the DUB protein levels or using inhibitors, researchers can induce changes in DUB activity. To effectively evaluate protein degradation, researchers need to master various methodologies that facilitate these observations. Understanding the appropriate techniques is vital to accurately interpret the results and confirm the role of DUBs in substrate stabilization.

A direct and traditional approach to studying protein degradation is through the labeling of nascent proteins with radioactive amino acids, a technique known as the *isotopic pulse–chase method*. In this method, during the *pulse* phase, cells are first exposed to radiolabeled amino acids to label newly synthesized proteins. Then during the *chase* phase, the labeled precursor is replaced with an excess of unlabeled amino acids to halt further labeling. The fate of the labeled proteins over time can then be monitored to assess their stability, modification, or degradation by following analysis, such as immunoprecipitation and SDS−PAGE (Fig. 1A). However, the handling of radioactive isotopes requires strict safety protocols, and may limit the applicability of the method in labs without appropriate facilities (51).

Another technique, *stable isotope labeling with amino acids in cell culture (SILAC)*, offers a non-radioactive alternative to study protein degradation by labeling nascent proteins. This method employs stable isotopes, such as [13C6] arginine, during cell culture to incorporate labels into newly synthesized proteins. Advanced MS is then used to quantify the degradation of labeled proteins, which provides a reliable means to measure individual protein degradation rates (Fig. 1B). However, SILAC requires specialized MS equipment, which not all laboratories may have available (52).

While isotopic pulse–chase methods and SILAC are powerful techniques, they come with certain limitations. The methods introduce slight alterations to the chemical properties of the amino acid residues; these may lead to improper folding of the protein, resulting in anomalous detection in subsequent assays such as SDS−PAGE and antibody-based quantification. Another challenge is the inaccuracies that the potential for labeled amino acids in degraded proteins to be reused for the synthesis of new proteins could introduce (52). Independent validation using complementary approaches is essential to address the limitations of direct protein labeling methods.

A widely utilized alternative is *Cycloheximide (CHX) chase immunoblotting*; this allows researchers to accurately quantify the half-life of specific proteins within cells without direct labeling. In this approach, CHX is added to cell culture media to halt protein synthesis by inhibiting the elongation step of protein translation. Following this inhibition, total protein is harvested in a time-dependent manner. Immunoblotting is then used on these samples to quantify the protein of interest, allowing precise measurement of its degradation rate over time (Fig. 1C) (53). The primary advantage of this approach is that the target protein is not chemically modified, thus its structure and function are preserved. Moreover, the subsequent immunoblotting analysis allows for detection regardless of the composition of the protein extraction buffer; this feature is particularly beneficial when the target protein may be misfolded or aggregated. However, this method is limited when the target protein exhibits a long half-life, as inhibition of the overall protein synthesis can rapidly diminish cell viability over time. Therefore, it is not recommended to perform CHX chase experiments for more than 24 hours. If extended chase times are necessary, more accurate assessments can be facilitated by increasing the concentration of CHX, while reducing treatment duration. Given that the dosing of CHX can influence the degradation rate, experiments need always to incorporate appropriate controls to ensure reliable comparisons.

Biochemical assays: ubiquitination assay

Once changes in protein degradation rates are observed, the next step is to confirm whether the ubiquitination of the substrate has been altered in response to DUB activity. Two commonly used methods to assess ubiquitination are Nickel Pull-Down assays, and Activity-based Probe labeling.

Nickel pull-down assays provide a method to visualize the ubiquitination of substrate proteins via Western blotting, which typically employs anti-substrate antibodies. In these assays, the substrate, DUB, and His-tagged ubiquitin are overexpressed in cells. Ni–NTA agarose, a nickel-charged affinity resin that selectively binds His-tagged proteins with high specificity, is used to subsequently isolate the ubiquitinated proteins. The strong affinity between the histidine residues in the His-tag of ubiquitin and the immobilized nickel ions facilitate the selective capture, while non-tagged proteins pass through the resin matrix. The ubiquitinated substrates are then detected as high-molecular-weight bands on the immunoblot, which indicate

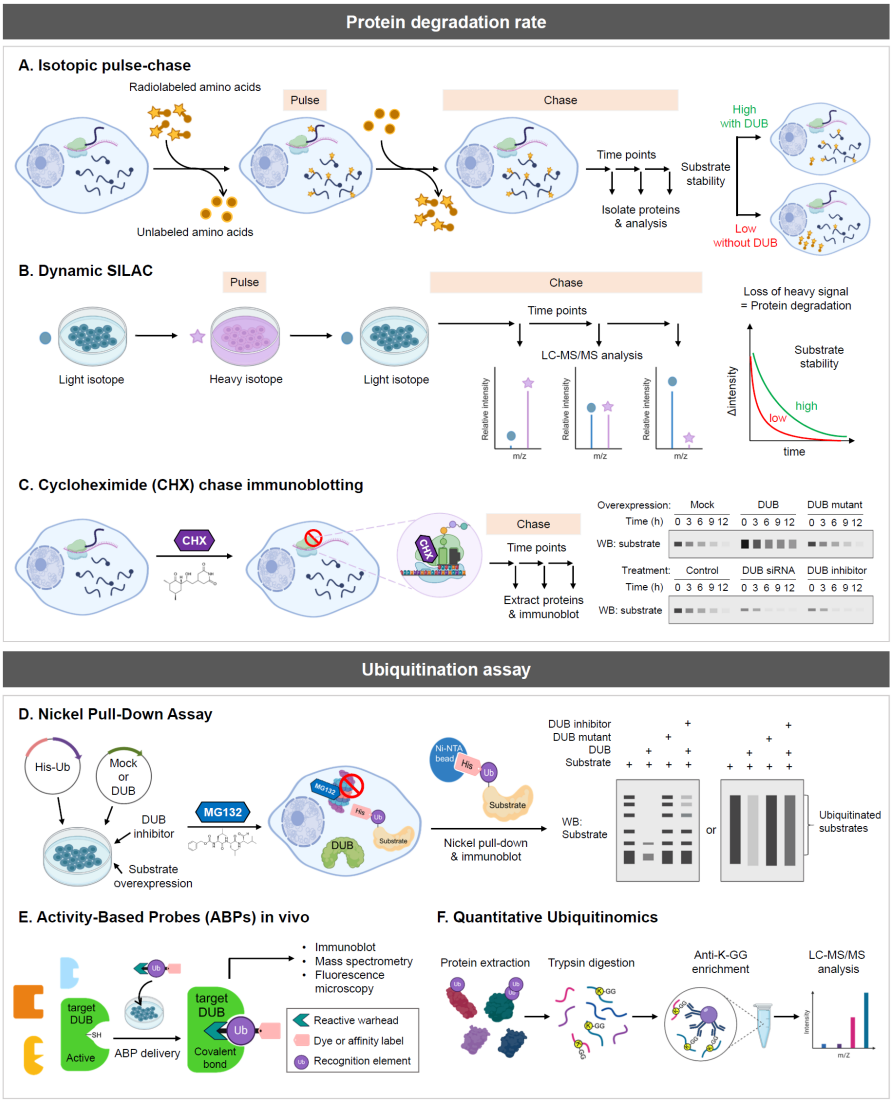

Fig. 1. Diagrams of biochemical assays to reveal protein degradation rate and ubiquitination. (A) The isotopic pulse-chase method labels proteins with metabolically radiolabeled amino acids to measure degradation rates. (B) Dynamic SILAC incorporates stable isotopes during synthesis to assess protein stability via MS analysis. (C) Cycloheximide chase assay monitors protein decay by inhibiting translation and comparing degradation rates with a control group by immunoblotting. (D) Nickel pull-down assay captures His-tagged ubiquitinated proteins for downstream ubiquitination analysis. Bands larger than native substrates in a ladder-like or continuous pattern indicate ubiquitinated substrates. (E) Activity-based probes covalently bind to active DUBs, enabling detection via SDS-PAGE or MS. (F) MS-based quantitative ubiquitinomics identifies and quantifies ubiquitination sites and levels using immunoaffinity purification of di-glycine (K-GG) peptides.

their ubiquitination (Fig. 1D). Due to the strong affinity between His-tags and nickel ions, this method is simple, efficient, and highly specific. It is adaptable for various ubiquitination studies by incorporating different tags. However, careful consideration should be given to potential overexpression artifacts, the requirement for His-tagged proteins, the risk of non-specific binding, and the sensitivity to buffer conditions inherent to in pull-down assays (54).

Activity-based probes (ABPs), which are derived from ubiquitin or ubiquitin-like molecules, contain reactive warheads that covalently bind to the active site residues of target enzymes, such as cysteine in DUBs. These probes label active DUBs in cells or tissue samples, to allow DUBs to be detected through techniques like Western blotting or MS (Fig. 1E). In deubiquitination assays, ABPs shift labeled DUBs to a higher molecular weight on SDS−PAGE, with band intensity corre-

lating to DUB activity, enabling both qualitative and quantitative analysis. ABPs can also be used with fluorophores for live-cell imaging to study DUB localization and dynamics. In contrast to Nickel pull-down ubiquitination assays, ABP-based assays utilize untransfected whole-cell lysates, making them suited to high-throughput screening. For *in vivo* applications, ABPs have been delivered into cells using pore-forming toxins, electroporation, or are optimized with cell-penetrating peptides (CPPs) to enhance membrane translocation (54). However, ABPs present several limitations. The reactive nature of some ABPs can cause instability or non-specific labeling, complicating data interpretation. Critically, ABPs do not directly identify DUB substrates; instead, by using probes that mimic specific ubiquitin chain linkages, they offer indirect insights into substrate specificity. While useful for studying enzyme families and structural features, ABPs cannot replace complementary methods, such as proteomics or biochemistry approaches, that are essential to identify actual endogenous DUB substrates (55).

MS-based techniques are critical for the quantification and characterization of ubiquitinated proteins. The most widely used method for mapping ubiquitination sites involves the *immunoaffinity purification of di-glycine (K−GG) peptides*. During tryptic digestion of ubiquitinated proteins, ubiquitin-modified lysines leave behind a K−GG remnant; this serves as a marker for the presence of ubiquitination. Enrichment of these ubiquitinated peptides is typically achieved through immunoaffinity methods, followed by quantitative MS techniques that compare ubiquitination levels across samples (Fig. 1F) (56). The main advantages of MS-based ubiquitinomics lie in its high sensitivity and ability to provide site-specific information on ubiquitination. This approach can identify thousands of ubiquitination sites from a small amount of starting material (e.g., 1 mg) (57). Furthermore, it is compatible with quantitative proteomics techniques, such as SILAC and tandem mass tags (TMT) (58), which enhance its capacity for quantitative analysis. Despite its potential, the low stoichiometry of ubiquitinated species in cells presents a challenge for MS-based quantification, which thus requires efficient enrichment methods to enhance detection sensitivity. Additionally, DUB activity during sample preparation can lead to deubiquitination, which potentially affects the accuracy of ubiquitin site identification. Optimized protocols are therefore needed to preserve ubiquitination during the process.

Biochemical assays: protein–protein interaction

We have described experimental approaches to determine whether the activity of a DUB alters its target substrate protein levels. However, these approaches offer only indirect evidence, and represent merely an initial step toward elucidating DUB–substrate interactions. To gain deeper insights, it is crucial to determine whether the DUB and substrate function as physical binding partners.

One of the most commonly employed techniques to investigate protein–protein interactions is *co-immunoprecipitation (Co-IP)*. This method uses specific antibodies to capture the DUB of interest from cell lysates, followed by identification of associated binding partners with methods such as immunoblotting and MS. Co-IP is a powerful method to directly identify binding partners, and is a standard approach in nearly all studies investigating protein–protein interactions. It provides valuable insights into the physiological interactions between proteins under native or near-native conditions. However, the specificity of Co-IP is highly dependent on antibody quality; suboptimal antibodies may result in cross-reactivity or non-specific binding, leading to issues with reliability. To circumvent this problem, proteins with tags, such as HA, FLAG, or Myc, are often expressed in cells, enabling the use of validated antibodies for Co-IP (59). However, overexpressing tagged proteins can lead to artifacts, including the formation of non-physiological complexes that may not accurately represent endogenous protein interactions. The protein–protein interactions that are weak, transient, or involve low-abundance proteins may escape detection. Additionally, some buffer conditions, including salt or detergent concentrations, can disrupt native protein conformations and potentially alter interaction strength or stability (60). Therefore, even though Co-IP provides strong evidence, it is crucial to critically interpret the results, and validate them with complementary experiments to ensure accurate and reliable findings.

While *Pull-down assays* are similar to Co-IP, they allow for the identification of protein–protein interactions without the use of antibodies. In this method, the target protein attached to a fusion tag, like His or GST, is expressed in cells and incubated with lysates to facilitate binding with potential interaction partners. The tagged protein complex is precipitated using specific beads, such as nickel or glutathione beads, then analyzed through SDS−PAGE and immunoblotting. Although this approach provides a direct method to study protein interactions, overexpression or nonspecific binding may make it prone to artifacts, and potentially compromise the interpretation of results (61).

Proximity labeling is a valuable method to study protein–protein interactions in living cells that offers insights into both stable and transient interactions within the native cellular environment. Techniques such as BioID and APEX provide spatial and temporal resolution by labeling proteins that lie in close proximity to the target protein. BioID utilizes a mutant biotin ligase to biotinylate nearby proteins, while APEX employs engineered ascorbate peroxidase to generate biotin–phenoxyl radicals for labeling (62). Turbo ID, an improved version of BioID, has achieved a significant advance by reducing labeling time from 18 hours to less than 10 minutes (63). These methods are particularly advantageous for capturing weak or transient interactions, and work with both soluble and insoluble proteins with minimal disruption. A major limitation of proximity labeling is that the close proximity of two proteins does not necessarily indicate direct physical interaction. Proteins can be brought into close proximity by other binding partners

or physiological conditions, even in the absence of direct interaction. Furthermore, a larger labeling radius increases the likelihood of labeling unrelated proteins that happen to be spatially near the target, potentially leading to false positives. Despite these limitations, proximity labeling remains a valuable tool, as it enables the detection of transient interactions that are often missed by conventional methods.

Yeast Two-Hybrid (Y2H), a widely utilized method to detect protein–protein interactions, capitalizes on the modular architecture of eukaryotic transcription factors. In this system, the DNA-binding domain (BD) and activation domain (AD) of a transcription factor are separated, with the *bait* protein fused to the BD, and the *prey* protein fused to the AD. Interaction between the bait and prey proteins brings the BD and AD together, thereby activating the reporter gene and producing a measurable signal. Y2H offers several advantages, including its ability to screen large libraries and detect weak or transient interactions in living cells. This high-throughput, cost-effective method has been pivotal to identify novel protein interactions. However, the technique has limitations. Overexpression artifacts may result in false positives and negatives. Further, requiring interactions in the yeast nucleus might not reflect native cellular conditions, while the technique is less effective for membrane-bound or transcriptionally inactive proteins (64).

Fluorescence-based methods: visualization of DUB or substrate dynamics

Fluorescence-based techniques are powerful tools to study DUB–substrate interactions, offering real-time insights into protein stability and turnover within cellular contexts. These methods utilize fluorescent markers and dyes to visualize and quantify protein degradation, which enable simultaneous observation of both the degradation rate and protein–protein interactions between the DUB and its substrate. Fluorescence-based approaches provide a versatile platform to study the dynamic relationships of proteins within living cells, and offer key advantages in terms of sensitivity and temporal resolution.

The simplest approach involves fusing enhanced green fluorescent protein (EGFP) or other fluorescent proteins to either the DUB or substrate, which enables the tracking of their co-localization and interactions within living cells. This method allows for the visualization of dynamic changes in protein quantities and co-localization over time. For example, CHX chase can also be achieved with fluorescence signals. Tagging specific substrates with fluorescent markers allows, after CHX treatment with fluorescence microscopy or flow cytometry, their stability and degradation to be monitored under various DUB conditions (65, 66). Global Protein Stability Profiling (GPSP) is a high-throughput method to measure protein turnover in live cells on a proteome-wide scale. The technique uses a reporter construct encoding both sole DsRed and an EGFP–protein fusion in 1:1 ratio. The EGFP/DsRed fluorescence ratio reflects protein stability, which allows efficient tracking of protein turnover over time. This technique thus offers real-time insights into protein dynamics within cells (67).

However, caution is warranted when employing fluorescent protein tagging, as it can significantly impact protein stability and native function. For example, large molecular complexes, such as ribosomal subunits, may experience disrupted assembly when tagged with GFP, reducing their stability due to interference with proper subunit formation. Similarly, when fused with fluorescent tags, proteins localized to specific subcellular organelles may fail to reach their target compartments, compromising functionality. Additionally, GFP tagging has been shown to interfere with protein biogenesis. For example, EGFP, either expressed alone or as part of a fusion protein, can inhibit polyubiquitination and disrupt multiple cell signaling pathways (68). While fluorescent protein tagging remains a highly effective and convenient tool, such side effects can lead to misinterpretation of experimental results. Therefore, relying solely on fluorescence-based experiments without further validation is risky and should be avoided.

Fluorescence-based methods: protein turnover rate

The *bleach–chase method* tracks protein degradation rate by using light pulses to irreversibly convert fluorescent proteins into non-fluorescent forms. The method employs libraries of cell clones expressing endogenous yellow fluorescent protein (YFP)-tagged proteins. The bleaching step generates two populations, fluorescent and non-fluorescent, with the decay of non-fluorescent proteins reflecting degradation events. This method estimates degradation rates using relative fluorescence intensities between non-fluorescent and fluorescent groups in control (Fig. 2A). This approach limits real-time measurement within a single cell, while being highly dependent on the accuracy of control data (69).

Photoconvertible Fluorescent Proteins (PCFPs), such as Dendra2, can be switched from green to red fluorescence upon exposure to specific light (Fig. 2B). This allows the degradation of the photoconverted red population to be tracked over time, measuring protein turnover rate (70-72).

Tandem fluorescent protein timers (tFTs), composed of mCherry and Superfolder GFP (sfGFP), are effective tools to study protein turnover in living cells. When mCherry–sfGFP is tagged to a specific substrate, the construct initially fluoresces green, after synthesis and gradually shifts to red over time, providing a visual timeline of the turnover dynamics of the substrate (Fig. 2C) (73).

Fluorescence-based methods: protein–protein interaction

Förster Resonance Energy Transfer (FRET) is a highly sensitive and versatile technique to study protein–protein interactions in living cells, and offers nanometer-scale spatial resolution. FRET occurs when two fluorophores, a donor and an acceptor, are within 1-10 nm of each other, enabling non-radiative energy transfer from the excited donor to the acceptor (Fig. 2D) (74). This proximity-based mechanism makes FRET particularly effective for detecting transient or weak interactions that are

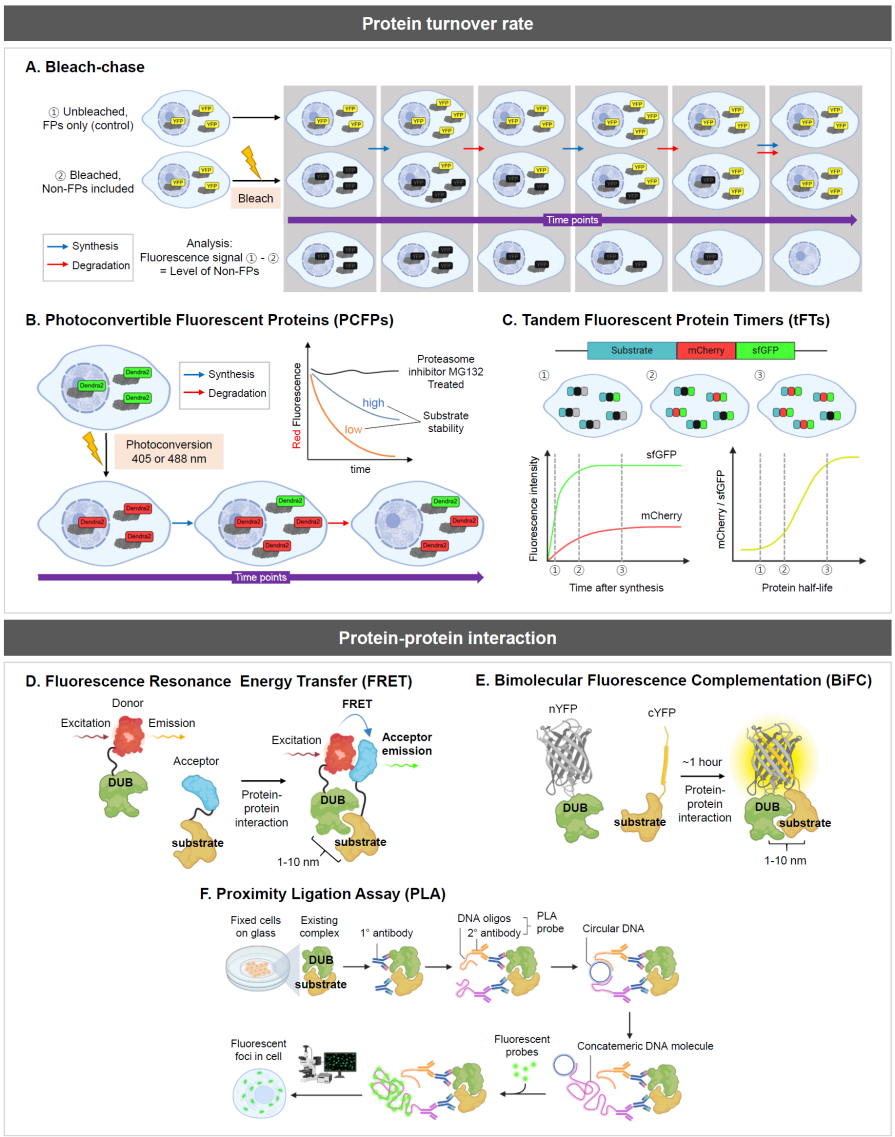

Fig. 2. Diagrams of fluorescence-based methods to investigate DUB-substrate dynamics. (A) The Bleach-Chase method involves photobleaching FP-tagged proteins to measure their degradation. (B) Photoconvertible Fluorescent Proteins, such as Dendra2, allow the photoconversion of FP-tagged proteins to track protein degradation over time. (C) Tandem Fluorescent Protein Timers, like GFP to mCherry, exhibit time-dependent fluorescence shifts to measure protein turnover. (D) Fluorescence Resonance Energy Transfer (FRET) enables the detection of protein-protein interactions through energy transfer between fluorophores. (E) Bimolecular Fluorescence Complementation (BiFC) restores fluorescence when two non-fluorescent FP fragments reassemble, revealing protein-protein interactions. (F) Proximity Ligation Assay (PLA) generates a fluorescent signal when antibodies with oligonucleotides are in close proximity (below 40 nm), enabling the detection of protein-protein interactions.

challenging to capture with conventional methods. The technique supports real-time monitoring of PPIs in live-cell contexts and provides quantitative insights into interaction strength, kinetics, and protein conformational changes. These characteristics make FRET effective to investigate the UPS including DUBs, which have thus contributed to recent advances in the field (75). Despite its advantages, FRET requires careful controls to minimize spectral bleed-through. Moreover, fluorescent protein tags must be judiciously selected to avoid perturbing protein function or localization.

Bimolecular Fluorescence Complementation (BiFC) is another powerful technique to investigate protein–protein interactions in living cells. This method involves fusing two non-fluorescent fragments of a fluorescent protein, such as YFP or Venus,

to the proteins of interest. Upon interaction, the fragments of the split fluorescent protein reassemble, restoring fluorescence, which can be detected (Fig. 2E). BiFC enables the real-time, *in vivo* monitoring of protein interactions with high spatial resolution of 1-10 nm, making it especially useful to detect weak or transient interactions, even at low endogenous expression levels. Additionally, it provides insights into the subcellular localization of interactions. The intensity of the fluorescence signal reflects the strength of the interaction, offering quantitative information as well. However, its limitations include a relatively slow maturation time (~1 hour), and the potential for non-specific interactions at high expression levels. Furthermore, the irreversible nature of the fluorescence complex can stabilize weak interactions, leading to potential overestimation of their strength (76).

Proximity Ligation Assay (PLA) is a highly sensitive and specific method that is used to detect and visualize protein–protein interactions *in situ* within fixed cells or tissues. This technique utilizes antibody pairs that are conjugated to DNA oligonucleotides, which bind to target proteins. When the proteins are in close proximity (within 40 nm), the DNA strands are ligated, generating an amplifiable signal. This signal is then amplified through rolling circle amplification, producing detectable fluorescent spots under a microscope (Fig. 2F) (77). PLA offers several advantages that include the ability to detect interactions of endogenous proteins at low expression levels, and provide spatial information on transient or weak interactions. Importantly, unlike other techniques, PLA does not require tagging the target proteins, thus avoiding potential artifacts. However, PLA necessitates the use of antibodies and probes in permeabilized cells, meaning that it is not applicable to live-cell imaging. As with other imaging-based techniques, PLA may suffer from false positives, due to the detection of proteins that are in proximity, but not directly interacting (78).

In vitro deubiquitination assays

In vitro deubiquitination assays provide clear and quantifiable results, offering an advantage over cell-based methods. These assays typically utilize purified recombinant DUBs, although recombinant proteins produced in *E. coli* may lack the essential PTMs required for optimal DUB activity. To address this limitation, immunoprecipitated DUBs from cell lysates can be used to provide a more accurate representation of native DUB activity. A standard approach to assess DUB function begins with assays using recombinant DUBs, followed by testing with immunoprecipitated DUBs or mammalian cell-based systems to assess intracellular deubiquitination activity, thus confirming the physiological relevance of the findings (54).

Various *in vitro* deubiquitination assays are commonly employed. Ubiquitin chain cleavage assays, which use purified ubiquitin chains as substrates, detect DUB activity through the appearance of mono-ubiquitin bands (47, 79-81). Fluorescent substrate assays, such as those utilizing Ub—AMC probes, allow the real-time monitoring of DUB activity by measuring the increase in fluorescence as ubiquitin is cleaved (82, 83). Activity-based probe labeling, such as with HA–ubiquitin vinyl sulfone (HA—UbVS), covalently labels active DUBs and can be detected via immunoblotting (54, 84, 85).

While these assays offer several advantages—including quantifiable results, high-throughput screening capabilities for DUB inhibitors, and the ability to study DUB activity without interference from cellular context—they also face limitations. The most significant limitation is that *in vitro* assays often fail to replicate the complexity of the physiological environment; this makes it essential to optimize conditions like pH, temperature, and cofactors for each specific DUB. Therefore, it is premature to dismiss cellular findings on the grounds that *in vitro* assays fail to show the same results. Moreover, some assays may fail to detect weak or transient deubiquitination events. To confirm the specificity of the results, it is critical to include control groups, such as no-enzyme and catalytically inactive DUB mutants.

CONCLUSIONS

DUBs are fundamental regulators of protein homeostasis that orchestrate cellular processes ranging from protein turnover to signal transduction. The methodological toolkit to study DUB biology has expanded significantly, encompassing quantitative biochemical assays, advanced fluorescence-based approaches, and detailed molecular studies (Table 1). Each method offers unique advantages: for example, biochemical assays quantify degradation rates and ubiquitination dynamics, fluorescence-based techniques enable the real-time monitoring of DUB activity in live cells, while *in vitro* assays provide direct insights into substrate-specific activity. Together, these complementary strategies have illuminated the pivotal roles of DUBs in maintaining cellular homeostasis, and revealed their dysfunction in various pathological conditions.

Recent technological advances have revolutionized our understanding of DUB biology. Single-cell proteomics has unveiled previously unappreciated heterogeneity in DUB activity across cellular populations (86), while structural studies have provided insights into substrate recognition and catalysis at atomic-resolution scale (87, 88). The integration of artificial intelligence with multiomics datasets has accelerated the mapping of DUB–substrate networks, and enabled the prediction of regulatory mechanisms (89, 90).

Several critical questions remain at the forefront of DUB research. The expanding complexity of PTMs beyond ubiquitination requires further exploration, particularly in the context of SUMOylation (91) and other ubiquitin-like modifiers (92). Additionally, the identification and validation of physiologically relevant substrates across diverse cellular states remain significant challenges that necessitate innovative methodological approaches.

The therapeutic targeting of DUBs has emerged as a promising frontier in drug development, particularly for diseases that are

Table 1. Overview of methodologies to study DUB-substrate interaction

Category	Purpose	Methodology	Strengths	Limitations	Key principle	References
Biochemical assays	Measure protein degradation rate	Isotopic Pulse-Chase	Minimal disturbance to cell physiology	Requires radioactive isotopes	Radiolabeling nascent proteins and track decay	(51)
		Dynamic Stable Isotope Labeling with Amino Acids in Cell Culture (Dynamic SILAC)	Non-radioactive, high accuracy	Requires specialized MS equipment	Stable isotope labeling during protein synthesis	(52, 94)
		Cycloheximide (CHX) Chase Immunoblotting	Simple, cost-effective. Observe native proteins.	Alters cell metabolism dramatically and decrease cell viability	Monitor decay by blocking translation	(53)
	Ubiquitination assay	Nickel Pull-Down Assay	Simple enrichment for ubiquitin-modified proteins	Limited specificity	Affinity capture of His-tagged ubiquitinated proteins	(54, 95)
		Activity-Based Probes (ABPs) *in vivo*	High specificity for active DUBs	Cannot directly detect substrates	Covalent binding of probes to active DUBs	(54, 55)
		Quantitative Ubiquitinomics	High-throughput, unbiased	High cost, requires specialized MS equipment	MS-based detection of ubiquitinated proteins	(56-58)
	Protein-protein interaction	Co-Immunoprecipitation (Co-IP)	Widely accessible, simple, cost-effective	Requires validated antibodies, low sensitivity, non-specific binding	Antibody-based capture of interacting proteins. MS analysis can be followed.	(59, 60)
		Pull-Down Assay	Widely accessible, cost-effective, antibody-free	overexpression artifacts, non-specific binding	Affinity purification of interacting proteins. MS analysis can be followed.	(61)
		Proximity Labeling (BioID/APEX)	Dynamic interactions can be detected	Off-target labeling, lack of direct interaction evidence	Enzyme-mediated biotinylation of nearby proteins	(62, 63)
		Yeast Two-Hybrid (Y2H)	High sensitivity, high-throughput screening	Target proteins must present interaction in yeast nucleus	Reporter gene activation from direct bait-prey interaction	(64)
Fluorescence-based methods	Visualize DUB or substrate dynamics	Fluorescent Protein (FP) Fusion Reporters	Real-time monitoring in live cells, wide applicability (e.g., CHX, MG132)	Artifacts from fluorescent tags	Tracking FP-tagged target proteins with fluorescent microscopy	(65, 67)
	Measure protein degradation rate	Bleach-Chase	Spatially resolved dynamics	Accuracy depends on control group, requires advanced microscopy	Photobleaching irreversibly FP-tagged proteins	(69)
		Photoconvertible Fluorescent Proteins (PCFPs)	Tracking is available in single cell, High temporal control	Requires advanced microscopy	Photoconversion marks FP-tagged proteins for tracking over time (e.g., Dendra2)	(70, 72)
	Measure protein turnover rate	Tandem Fluorescent Protein Timers (tFTs)	Visual timeline of both synthesis and degradation	Artifacts from fluorescent tags	Time-dependent fluorescence shifts (e.g., GFP to mCherry)	(73)
	Protein-protein interaction	Fluorescence Resonance Energy Transfer (FRET)	Detects proximity in 1-10 nm	Limited dynamic range, artifacts from tags	Energy transfer between fluorophores	(74, 75)
		Bimolecular Fluorescence Complementation (BiFC)	Allow multicolor analysis, interaction strength can be detected	Irreversible binding may hinder dynamics, slow fluorescence maturation, artifacts from tags	Fluorescence restore when two non-fluorescent FP fragments reassemble	(76)
		Proximity Ligation Assay (PLA)	High sensitivity and specificity, no overexpression or tagging artifacts	Requires validated antibodies and probes, fixed cells only, false positives from random proximity	Fluorescent signal is generated when antibodies with oligonucleotides are in close proximity below 40 nm	(77, 78)
In vitro assays	Measure DUB activity on ubiquitin chains	Ubiquitin Chain Cleavage Assay	Mechanistic insights into DUB specificity	May lack physiological relevance	Incubate DUBs with ubiquitinated substrates	(47, 79-81)
		Fluorescent Substrate Assay	High sensitivity	May lack physiological relevance	Monitor fluorescence changes upon cleavage	(82, 83)
	Measure DUB enzyme activity	Activity-Based Probes (ABPs) *in vitro*	Direct assessment of enzymatic activity	Cannot determine substrate specificity	Probe covalently binds to active enzyme	(84, 85)

characterized by aberrant protein homeostasis. As they advance through preclinical and clinical development, small-molecule inhibitors and activators of DUB activity offer potential precision medicine approaches to treat cancer, neurodegenerative diseases, and inflammatory conditions (30, 32, 82, 83, 90, 93). In future, the convergence of technological innovation with mechanistic insights promises to accelerate our fundamental understanding of DUB biology, as well as the development of DUB-targeted therapeutics. These advances are anticipated to ultimately transform our ability to treat diseases through precise modulation of the ubiquitin system.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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