Lung Biology and Pathophysiology

Edited by Yutong Zhao

First edition published 2024

ISBN: 978-1-032-40902-3 (hbk)
ISBN: 978-1-032-40906-1 (pbk)
ISBN: 978-1-003-35524-3 (ebk)

8 Research Strategies for Investigating Protein Ubiquitination in Lung Diseases

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DOI: 10.1201/9781003355243-10

The funder for this chapter is The Ohio State University.

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

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8.1 INTRODUCTION

Ubiquitination is ubiquitin protein(s) or polyubiquitin chain(s) covalently conjugated to lysine (K) residues of target protein to regulate protein stability, cellular localization, and protein-protein interaction (1–7). Ubiquitination-dependent protein degradation has been well illustrated. Ubiquitination triggers protein degradation in proteasome or lysosome systems. Ubiquitin contains seven K residues (K6, K11, K27, K289, K33, K48, and K63). Polyubiquitin chains can be assembled using these K residues and the first methionine (Met1) residue (2–4, 7). In general, among these, K11-, K29- and K48-linked polyubiquitination is associated with proteasomal degradation, whereas mono- and K63-linked polyubiquitination regulates protein cellular translocation, protein-protein interaction, enzyme activity, and degradation in lysosomes (2–5, 8, 9). K63-linked polyubiquitination has been shown to increase protein stability possibly through counteracting its K48-linked polyubiquitination (10, 11).

A series of enzymatic reactions is necessary for ubiquitination. E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases work concertedly to transfer ubiquitin to the target proteins to yield mono- or polyubiquitination. E3 ligases selectively target substrates and transfer ubiquitin or ubiquitin chains to the substrates (12–16). Each E3 ligase targets multiple substrates to regulate cellular functions. Each protein may be targeted by different E3 ligases to form different types of ubiquitination, resulting in distinct functions. The association of E3 ligases and substrates is dependent on other Post-translational modifications (PTMs) of substrates (17). For instance, phosphorylation of substrates may increase E3 ligase association with substrates (18–23). Proteasomal degradation of the inhibitor of nuclear factor kappa B (I-κB) is mediated by the Skp1-cullin 1-F-box (SCF) β-Trcp E3 ligase in a phosphorylation-dependent manner (23). In response to inflammatory stimuli, phosphorylated I-κB binds to SCF β-Trcp E3 ligase and thereby is polyubiquitinated (23). Ubiquitination is a reversible process. Deubiquitinating enzymes (DUBs) remove mono-ubiquitin or polyubiquitin chains from substrates (24–27). The balance between the substrate association with its E3 ligase and DUB determines the outcome of the substrate. The shift of substrate binding to E3 ligase or DUB may be regulated by other induced PTMs such as phosphorylation.

Lung diseases impose a huge global health burden. Since 2020, nearly 7 million deaths have been caused by COVID-19 worldwide. Disfunction of lung cells, including epithelial, endothelial, and fibroblasts, contributes to the pathogenesis of lung diseases. Several E3 ligases have been identified to regulate lung inflammation, cell death, and differentiation through targeting transcriptional factors, plasma membrane receptors, signaling proteins, and mitochondrial proteins (18, 21, 28–43). Recent studies have demonstrated that DUBs counteract the effects of E3 ligases and modulate lung cell function in lung injury and fibrosis (21, 26, 44–50). Thus, a better understanding of how

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protein ubiquitination regulates physiological and pathological processes in lung cells is important to identify novel therapeutic strategies and targets for treating lung diseases. This chapter will focus on methodology and strategy of research in protein ubiquitination and deubiquitination in lung cells (Figure. 8.1).

8.2 DETERMINATION OF PROTEIN STABILITY IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Protein homeostasis is controlled by synthesis and degradation. Half-lives of proteins are highly variable, ranging from a few minutes to days in physiological condition. Cyclin D1 has a short half-life of around 30 minutes (51), whereas average half-lives of protein in HeLa cells were about 20 hours (52). To measure protein half-life, cycloheximide (CHX) chase assay is often performed. CHX is an inhibitor of protein biosynthesis because it inhibits translational elongation (53). Lung cells are treated with CHX (100–400 μg/ml) for different times (minutes to hours), and then cell lysates are analyzed by immunoblotting with a specific antibody against target protein. The concentration of CHX dependents on the cell types. To avoid the effect of cytotoxicity of CHX on protein degradation, cell viability needs to be monitored in the presence of CHX. Another traditional method to measure protein stability is the radioactive pulse-chase assay (54). Lung cells are incubated with ^{35}S-methionine for a short time to create the pulse of newly synthesized proteins. After designed period of time with "cold" media, cells are harvested and subjected to immunoprecipitation with an antibody against the target protein, followed by SDS-PAGE gel separation. Radioactive signals are detected by phosphoimager. The pulse-chase assay measures protein half-life without altering cellular activity; however, because of the use of radioactive materials, this method is not well used in the laboratory practice.

To determine if interest protein's stability is altered in the pathological conditions, such as infection, oxidative stress, and injury, first changes of protein levels are confirmed by immunoblotting analysis of interest protein after designed treatments, such as viral infection, endotoxin or cytokine treatments, or hypoxia exposure. In addition to protein stability, abnormal protein synthesis through transcription and translation machinery may alter protein levels in the pathological conditions. Thus, once changes of protein levels are confirmed by immunoblotting, mRNA levels of interest protein should be measured in the same condition by real-time polymerase chain reaction. In the case that changes in the mRNA levels are positively correlated with changes in protein levels, that suggests that protein level changes in a certain pathological condition are due to alteration of protein synthesis, not protein stability. If the protein level changes cannot be explained by changes in mRNA levels, it indicates that protein stability may be altered in the certain pathological condition. It is possible that under treatment, both synthesis and degradation of the interest protein are altered.

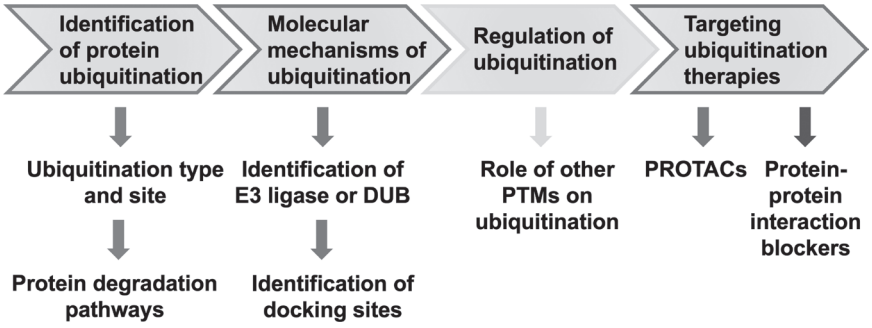

FIGURE 8.1 Research strategy to investigate protein ubiquitination.

8.3 DETERMINATION OF PROTEIN DEGRADATION PATHWAY

Once an interest protein's half-life or stability is confirmed in the physiological or pathological conditions, the next step is to investigate if the protein degradation is in the proteasome or lysosome system. The proteasome degrades damaged or unfolded proteins by proteolysis. The 20S proteasome contains β-subunits that have proteolytic active sites (55, 56). Specific inhibitors of the 20S proteasome, such as bortezomib and MG132, have been used to investigate if protein degrades in the proteasome (57). Lysosomes are membrane-bound organelles and degrade proteins in larger complexes, such as endosomes and mitochondria (58–60). Leupeptin, chloroquine, and bafilomycin A are often used to investigate if protein degrades in the lysosome (61, 62). To investigate which pathway regulates interest protein degradation in the physiological or pathological condition, lung cells are pretreated with inhibitors of the proteasome (MG132 or bortezomib) or lysosome (e.g., leupeptin) for 1 hour and then incubated with CHX (mimic physiological condition) or treatments (mimic pathological condition) for an additional designed period of time. If inhibitors of proteasome attenuate interest protein degradation, it suggests that interest protein degradation is by the proteasome system. The same adjustment is for using inhibitors of lysosome.

It has been shown that protein degradation in the proteasome is not totally dependent on ubiquitination. Misfolded and oxidized proteins may enter into the 20S proteasome and degrade (63). It is important to determine if the ubiquitination system regulates protein degradation. MLN7243 (TAK-243) is a selective E1-activating enzyme inhibitor (64) that inhibits the protein ubiquitination from the first step. Lung cells are treated with MLN7243 followed by CHX or pathological treatment. If protein degradation is prevented by MLN7243, it indicates that the interest protein degradation is regulated by the ubiquitination system.

8.4 DETERMINATION OF MONO- OR POLYUBIQUITINATION AND IDENTIFICATION OF UBIQUITIN SITES

After confirming the interest protein is degraded in the proteasome or lysosome, ubiquitination status of the interest protein needs to be determined. An in vivo ubiquitination assay can determine mono- or polyubiquitination of interest protein (21, 43, 65). Furthermore, different types of ubiquitination can be determined by using antibodies specific to K11-, K48-, or K63-linked polyubiquitin chains. Protein ubiquitination triggers protein degradation. In the pathological condition, loss of interest protein may reduce the overall ubiquitination degree of the substrate by in vivo ubiquitination assay, thus detecting the decreased ubiquitination cannot truly reflect the ubiquitination of the interest protein. To avoid this situation, cells are treated with the proteasome or lysosome inhibitor before pathological challenges. To increase the sensitivity of the assay, increases in ubiquitin levels in the cells can be achieved by overexpression of ubiquitin by plasmid or viral vector infection. After treatments, cells are collected in phosphate-buffered saline, and cell pellets are dissolved in 2% SDS-TBS buffer with deubiquitinating enzyme inhibitors (ubiquitin aldehyde and *N*-ethylmaleimide [NEM]), followed by sonication and boiling. These steps are to dissociate the interest protein from its binding or associated proteins. This is a critical step for the ubiquitination assay. Without separating the interest protein from its associated proteins, the detected ubiquitination bands may be due to ubiquitination of the associated proteins. After dissociation of the interest protein with its associated proteins, cell lysates are subjected to regulatory immunoprecipitation with an antibody against ubiquitin or the interest protein, followed by immunoblotting analysis with antibody against the interest protein or ubiquitin.

To further determine the types of ubiquitination, antibodies specific to K11-, K48-, or K63-linked polyubiquitin chains can be used for immunoprecipitation or immunoblotting analysis. Further, tagged ubiquitin K48 only, K63 only, K11 only, K48 deficient, K63 deficient, or K11 deficient plasmids can be used to transfect cells instead of pan-ubiquitin plasmid. Specific lysine-linked ubiquitination can be detected using these plasmids by in vivo ubiquitination assay.

K residues are common sites for accepting ubiquitin or polyubiquitin chains to the substrates. To initiate identifying the ubiquitin sites on interest proteins, computational methods may be applied to predict which K residues are potential ubiquitin-accepting sites. Computational machine learning methods have been well developed and employed for the ubiquitination site prediction based on protein sequences. However, researchers need to pay attention to the localization of the predicted K residues on the interest protein. For example, for the transmembrane protein, if the predicted K residues are on the extracellular membrane domain, the K sites do not need to be tested further. Experimental examination is needed to confirm whether the predicted K sites are ubiquitination sites. K residues are substituted with arginine (R) residues using site-directed mutagenesis to generate plasmid encoding K → R (K/R) mutants of the interest protein. The half-lives and degradation rates of wild type (Wt) and K/A mutants are compared by immunoblotting. The delay of degradation of K/R mutants suggests the K residue(s) are potential ubiquitination sites. To further validate if these K residues can be ubiquitinated or not, in vivo ubiquitination is used to measure the degree of ubiquitination of K/R mutants compared to Wt proteins. Multiple ubiquitination sites are possible within the interest proteins; thus several K residues need to be mutated. Proteomics have been employed to identify ubiquitination sites; however, it is challenging due to several factors, including small size of ubiquitination and low abundance of ubiquitinated proteins. Recently, improvement of tandem mass spectrometry (MS/MS) allows the detection of ubiquitination sites in enriched ubiquitinated proteins (66). Eventually, generation of K/R mutants and in vivo ubiquitination assay are needed to confirm the results from MS/MS.

8.5 IDENTIFICATION OF E3 LIGASES OR DUBs FOR THE INTEREST PROTEIN

Ubiquitination is regulated by a series of enzymatic reactions. E3 ligase is responsible for the ligation of ubiquitin or polyubiquitin chains to the substrate. There are major E3 ligase groups that have been well demonstrated, including RING-domain E3 ligases, HECT family E3 ligases, and RING-between-RING (RBR) E3 ligases (67–69). Like other PTMs, ubiquitination is negatively regulated by DUBs (70–72). There are an estimated 1000 E3 ligases and nearly 100 DUBs in human cells. The effects of E3 ligases and DUBs occurs in interaction with substrates. Identification of E3 ligase or DUB for the interest protein is important for further understanding the molecular regulation of substrate protein ubiquitination and deubiquitination. Enzyme screening library, protein interaction screening, and protein-protein interaction assays are used to identify E3 ligase or DUB for the interest protein.

Utilization of E3 ligase or DUB library assay is a common approach to identify E3 ligase or DUB in ubiquitination studies. A library that overexpresses or downregulates major E3 ligases or DUBs is conducted by plasmids, adenoviral or lentiviral vectors, small interfering (si)RNAs, or the CRISPR-cas9 system. The library is induced in the lung cells, and then the interest protein levels are examined by immunoblotting. If the interest protein levels are altered by the certain E3 ligase and DUB but the mRNA level of the interest protein is not affected, an E3 ligase or DUB is identified for the substrate.

Another common strategy to identify E3 ligase or DUB is based on unbiased protein-protein interaction assays. Endogenous or overexpressed interest protein as a bait can be co-immunoprecipitated (Co-IP) with its specific antibody. The precipitated complex is analyzed by mass spectrometry analysis (73, 74). The Co-IP–coupled MS provides a rapid, powerful, and unbiased screening to identifying the interest protein-associated partners including E3 ligases, DUBs, kinases, and other adaptors. Other methods including yeast two-hybridization (75, 76) and most recently developed proximity-based labeling techniques (BioID and TurboID) are often used for identifying ubiquitination-modifying enzymes (77–79).

Semi-unbiased strategies are be used to identify E3 ligase or DUB based on available knowledge about the interest protein. For instance, protein cellular localization is key information. If the interest protein is a membrane protein, the search for the ubiquitination modifying enzymes may be focused only on enzymes that have been known to be localized in the inner membrane. The sub-library for

overexpressing or knocking down E3 ligases or DUBs can be developed based on cellular localization and functions. Protein-protein interaction involves protein motifs or domains. For instance, if the interest protein contains a PY motif, it is possible that a E3 ligase with WW domain has a ability to target and induce the interest protein ubiquitination. Thus, E3 ligases containing WW domains (Nedd4, Nedd4L, WWP1, etc.) (80, 81) are priority candidates to be considered.

After identifying the potential E3 ligase or DUB for the interest protein, in vivo or in vitro ubiquitination assay is used to confirm the effect of identified E3 ligase or DUB on substrate ubiquitination. For in vivo cellular ubiquitination assay, E3 ligase or DUB will be overexpressed or downregulated in lung cells. Cell lysates are subjected to denatured IP and ubiquitination assay as described above. For in vitro ubiquitination assay, recombinant or purified E3 ligase or DUB protein are incubated with substrate protein along with ATP, ubiquitin, and E1 and E2 enzymes, followed by immunoblotting with a specific antibody against the substrate (18, 82). To confirm E3 ligase or DUB directly targets the substrate, the association of the enzymes and substrate are confirmed by co-IP and co-immunofluorescence staining in lung cells. Evaluation of co-localization is an important process. Co-IP/MS may detect the association of two proteins that are not localized in the same fractions of cells because cell lysates are mixtures of proteins from all the fractions.

8.6 IDENTIFICATION OF DOCKING SITE

To further investigate the molecular regulation of E3 ligase or DUB mediation of interest protein ubiquitination, it is necessary to identify the docking site on the interest protein for E3 ligase or DUB. This information may benefit design blocking small molecules or peptide to prevent or promote the interest protein degradation. If the protein-docking motif has been predicted by bioinformatic tools, the docking motif can be mutated or deleted with site-directed mutagenesis. For example, if the interest protein is the substrate of a WW domain-containing E3 ligase, it is highly possible that PY motif in the interest protein is the docking site for the E3 ligase. To confirm the hypothesis, a PY motif-deleted mutant can be generated. Protein-protein interaction, substrate ubiquitination, and stability are be performed to confirm if the PY motif (PPXY or LPXY) is critical for interacting with the identified WW domain-containing E3 ligase (83).

If there is no certain motif or domain predicted as the docking site, generation of a series of N-terminal or C-terminal deletion mutants of the substrate will be performed to identify docking site by protein-protein interaction assay, such as co-IP. It should be cautioned that the deletion mutants may affect substrate's cellular localization and conformational changes. All these changes may disrupt the interaction with E3 ligase or DUB. Thus, generation and use of mutant with precise mutation or deletion of key amino acids for protein-protein interaction assay can identify the docking site. Furthermore, the ubiquitination and stability of the mutant are examined to confirm that the key amino acids are critical for interaction with E3 ligase or DUB.

Similar to the strategy for identifying docking site in the substrate, it will be of interest to identify the domain on E3 ligase or DUB for interaction with the substrate. The F-box protein in the SCF E3 ligase complex plays a critical role in targeting the substrate. The C-terminal domain of the F-box protein contains a substrate binding site. Based on the structure of the C-terminal domain, F-box proteins are characterized as leucine-rich domain-containing proteins (FBXL family), WD domain-containing proteins (FBXW family), and "other" domain-containing proteins (FBXO family) (84, 85). A series deletion mutant of the C-terminal of the F-box protein can be generated and subjected to a protein-protein interaction assay to determine the binding site for the substrate.

8.7 DETERMINATION OF REGULATORY MECHANISMS

Protein ubiquitination is not constant in the cells, whereas its level changes in different cellular states of flux and responds to external stimuli. Cell cycle–related proteins, such as cyclins and cyclin-dependent kinases, may be ubiquitinated and degraded as the cell grows and divides. In

response to inflammatory stimuli, plasma receptors, such as interleukin-33R and lysophosphatidic acid R1, undergo ubiquitination and degradation in lung epithelial cells (18, 21). Ubiquitination is a regulatory process. Protein phosphorylation, acetylation, oxidization, or protein-protein interactions may influence ubiquitination (86), either promotion or reduction of ubiquitination through modulation of ubiquitination-related enzyme/substrate interaction, cellular localization, or ubiquitin conjugation. The chapter will introduce the research experiment design to investigate role of other PTMs and protein-protein interaction in ubiquitination and deubiquitination. Here, phosphorylation and acetylation are used as examples. First, phosphorylation or acetylation of substrate under physiological and pathological conditions should be examined by immunoblotting with specific phospho- or acetyl-antibodies, IP, or in vitro PTM assay. To investigate if the identified PTM regulates ubiquitination of the substrate, mutants of phosphorylation or acetylation sites are generated for investigating whether the site(s) are important for the substrate ubiquitination, degradation, or enzyme activity. Phosphorylation may enhance E3 ligase association with the substrate or reduce DUB/substrate interaction (21). To further understand the molecular mechanisms by which the PTMs influence ubiquitination, the effect of mutation on phosphorylation or acetylation sites on ubiquitination enzyme/substrate interaction and substrate's cellular localization should be considered. However, because both acetylation and ubiquitination use K residues, it is possible that the same K residue is responsible for both acetylation and ubiquitination. In this case, the K mutant may reduce both acetylation and ubiquitination. Thus, the conclusion from using K mutant needs be carefully considered. As discussed above, K residue is a common ubiquitin acceptor site. The ubiquitination site is not always exposed on the protein surface; this may prevent ubiquitination and degradation of the protein. During certain physiological and pathological changes, the K residue may be exposed to the protein surface because of changes in the substrate conformation. The protein structure conformation can be changed by PTMs including phosphorylation and protein-protein interaction. Substrate ubiquitination has been shown to be modulated by its association and disassociation with adapter proteins, such as 14–3–3 and calmodulin (87, 88). To investigate if protein ubiquitination is regulated by adapter protein interaction with a substrate, first modulate adapter protein levels by gene silencing or overexpression, and then determine substrate ubiquitination levels. If the binding site for adapter protein has been revealed, a mutant of the binding site can be generated and tested for ubiquitination.

In addition to the fact that PTMs of substrate regulate its ubiquitination, PTMs of E3 ligase or DUB may affect enzymatic activity or interaction with substrate (16, 19), therefore modulating substrate ubiquitination. Similar strategies as described above, such as mutation of phosphorylation or acetylation site, can be performed to investigate regulation of substrate ubiquitination.

8.8 DETERMINATION OF ROLE OF UBIQUITINATION IN LUNG CELLULAR FUNCTION AND DISEASES

Abnormal ubiquitination-dependent protein degradation has been shown to contribute to lung cellular disfunction and development of lung diseases (26, 89–91). Ubiquitination is a reversal and regulatory process (Figure 8.2). After confirming the interest protein is ubiquitinated, the next step is to investigate if the ubiquitination regulates protein-related cellular function. The mutant of ubiquitin acceptor site (K/R) or ubiquitination-related enzyme binding site is used to determine the substrate-related cellular functions, such as receptor-mediated signaling, cell proliferation, cytokine release, and cell death. The CRISPR-Cas9 system can be used to generate cell lines containing these mutants. As each E3 ligase or DUB targets and regulates multi-substrates, simple overexpression or knockdown of E3 ligase or DUB may result in unexpected and interest substrate-independent cellular response. The regulators, such as kinases or acetyltransferase, or adaptors (14–3–3 or calmodulin) may not only affect substrate ubiquitination but also ubiquitination-independent cellular functions. Thus, drawing a conclusion by modulation of E3 ligase, DUB, and regulators must be made carefully.

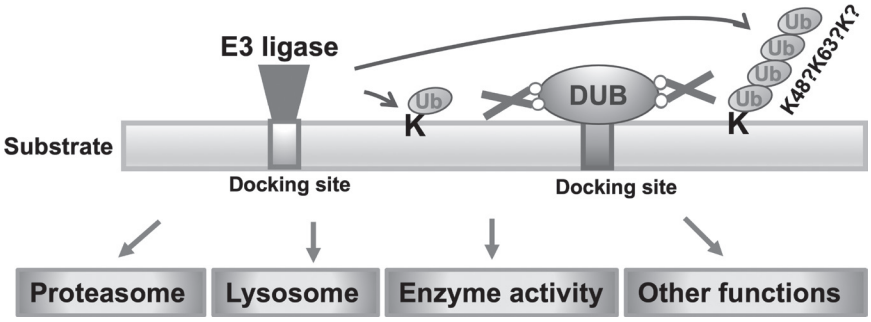

FIGURE 8.2 E3 ligase and DUB regulates protein ubiquitination.

To investigate the role of the ubiquitination of a substrate in the pathogenesis of lung diseases, the CRISPR-Cas9 system can be used to generate transgenic mice containing a mutant of ubiquitin acceptor site (K/R) or a ubiquitination-related enzyme binding site, followed by performing models of lung diseases. To precisely investigate the role of ubiquitination of interest protein in certain lung cell types during the development of lung diseases, cell type–specific transgenic mice may be generated.

8.9 UTILIZATION OF PROTEIN DEGRADATION PRINCIPLE TO DOWNREGULATE PROTEIN LEVELS BY PROTEOLYSIS TARGETING CHIMERAS

As discussed above, targeting a certain E3 ligase or DUB may affect multiple substrate-regulated cellular functions. Recently, a new biotechnology, proteolysis targeting chimeras (PROTACs), has been developed to target interest protein for its degradation based on modulation of protein ubiquitination (92–94). PROTAC technology does not need to identify specific E3 ligase for interest substrate, and it is suitable to degrade "undruggable proteins." Several PROTAC drugs have been tested in phase II clinical trials. The principle of PROTAC is to use an engineered small molecule comprising a E3 ligase binding site, a substrate binding site, and a linker between the two sites to recruit E3 ligase to substrate, therefore triggering substrate ubiquitination and proteasomal degradation. The substrate binding site can be designed based on known inhibitors of a substrate or a substrate's structure (Figure 8.3). The advantage of the design is not limited to a substrate's activation sites. Several endogenous E3 ligases, including von Hippel-Lindau (VHL) and cereblon (CRBN), have been widely used as baits to trap substrates (94). Small molecules binding to VHL or CRBN have been developed. Once the small molecule for substrate binding is revealed, the small molecule can be linked with a small molecule binding to E3 ligase with a linker. The new PROTACs can be used to test the substrate protein stability. However, the substrate may undergo various PTMs in the physiological and pathological conditions. The affinity of the small molecules binding to the substrate may vary; thus, it is causable that the PROTACs may have distinct efficiencies in different conditions.

Based on DUB-substrate or E3 ligase–substrate interaction, a docking site blocker can be designed and used to disrupt substrate interaction with DUB or E3 ligase to modulate its ubiquitination (95). For example, if the docking site for DUB is discovered, based on the docking site sequence and structure, a small molecule can be designed to prevent DUB interaction with the substrate. The small molecule will increase the protein ubiquitination. If the docking site for its specific E3 ligase is revealed, a small molecule can be used to reduce the interaction of E3 ligase, resulting in decreased ubiquitination.

FIGURE 8.3 PROTAC target substrate for its ubiquitination and degradation.

8.10 CONCLUSIONS

In this chapter, we have discussed the principle of protein ubiquitination, research strategies, experimental design, and PROTACs in investigating the role of protein ubiquitination in lung diseases. This is not a guideline, but it provides a thoughtful idea to identify protein ubiquitination, its underlying mechanisms, and its potential applications in understanding lung diseases. This chapter focuses on interest substrate ubiquitination and biological functions. In addition to investigating substrate ubiquitination, a growing number of studies have been focused on determining the regulation of E3 ligases or DUBs. Changes of E3 ligases or DUBs in lung cells have been shown to contribute to disease development. Understanding the regulation of these enzymes' transcription, protein stability, and activities in physiological and pathological conditions is important to develop new therapies to treat lung diseases and other disorders. In this chapter, we do not provide detailed experimental information because different cell types exhibit distinct responses to the same stimulation or inhibitor treatment. Researchers need to optimize experimental conditions based on their own cell culture systems and disease models. Rapid technological discoveries and improvement, including artificial intelligence in protein structure, drug design, and development, will lead to develop more efficient approaches to target protein ubiquitination-mediated cellular responses and therapies.

8.11 ACKNOWLEDGMENT

The author would like to thank Sarah J. Taleb for editing.

ABBREVIATIONS

CHX	cycloheximide
Co-IP	co-immunoprecipitate
CRBN	cereblon
DUB	deubiquitinating enzyme
MS/MS	tandem mass spectrometry
NEM	*N*-ethylmaleimide
PROTAC	proteolysis-targeting chimera
PTM	post-translational modification
RBR	RING-between-RING
VHL	von Hippel-Lindau
Wt	wild type

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