Mukesh Nandave
Priti Jain *Editors*

PROTAC-Mediated Protein Degradation: A Paradigm Shift in Cancer Therapeutics

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Foreword

In the ever-evolving landscape of cancer therapeutics, innovation is paramount. The quest for more effective treatments has led to groundbreaking discoveries, and among them, PROteolysis-TArgeting Chimera- or PROTAC-mediated protein degradation represents frontier science. As we delve into the pages of *PROTAC-mediated Protein Degradation: A Paradigm Shift in Cancer Therapeutics*, edited by Dr. Mukesh Nandave and Dr. Priti Jain, we embark on a journey through the forefront of molecular medicine.

The ubiquitin proteasome system, a cornerstone of cellular regulation, has long fascinated scientists with its intricate machinery and profound implications for health and disease. This book begins by laying the foundation of our understanding and tracing the historical milestones and revolutionary breakthroughs that have shaped our perception of this system.

At the heart of this paradigm shift lies the principle of PROTACs—a transformative approach that harnesses the cell's own machinery to selectively degrade disease-causing proteins. With chapters dedicated to elucidating the principles, mechanisms, and structural considerations of PROTACs, the reader is equipped with a comprehensive understanding of this innovative strategy.

As with any groundbreaking technology, there are inherent advantages and challenges. This book meticulously examines these, offering insights into the novel technologies driving PROTAC design and the future trends shaping its development. Moreover, it navigates the complex landscape of global regulatory requirements and clinical development, illuminating the path from bench to bedside.

One of the most promising aspects of PROTACs is their potential for tissue-specific targeting, offering a tailored approach to cancer therapy. From the toxicological aspects to the management of specific malignancies such as prostate cancer, breast cancer, and hematological malignancies, each chapter delves into the intricacies of applying PROTACs in clinical practice.

Furthermore, this volume explores the synergies between PROTACs and emerging technologies like artificial intelligence and machine learning, offering a glimpse into the future of precision medicine. Whether in the treatment of glioma or cardiovascular diseases, the potential of PROTACs to reshape therapeutic landscapes knows no bounds.

In closing, "PROTAC-mediated protein degradation" transcends the boundaries of traditional oncology, offering a holistic perspective that encompasses not only the

molecular intricacies but also the clinical implications and future directions of this transformative technology. It is my sincere belief that this book will serve as a cornerstone for researchers, clinicians, and students alike, driving us closer to the ultimate goal of conquering cancer.

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Preface

In the ongoing quest to develop more effective treatments for cancer, researchers are exploring innovative approaches that target the molecular mechanisms underlying the disease. One such promising avenue is the development of proteolysis-targeting chimeras or PROTACs. These molecules represent a novel class of therapeutic agents with the potential to revolutionize cancer therapy by exploiting the body's own protein degradation machinery to eliminate disease-causing proteins.

Though, traditional approaches like monoclonal antibodies and small molecule inhibitors have shown significant efficacy, they often encounter challenges such as drug resistance and limited selectivity. PROTACs tend to overcome these obstacles by harnessing the cell's natural protein degradation pathway to degrade target proteins, thereby achieving more potent and selective inhibition.

In the context of cancer therapy, PROTACs hold tremendous promise for targeting oncogenic drivers and overcoming drug resistance. PROTACs have the potential to disrupt multiple signaling pathways simultaneously, thereby exerting potent antitumor effects. Furthermore, the ability to target specific proteins within complex signaling networks offers the possibility of personalized treatment strategies tailored to the molecular profile of individual tumors.

Despite these exciting prospects, challenges remain in the development and optimization of PROTAC-based therapies. These include issues related to selectivity, pharmacokinetics, and off-target effects, which must be carefully addressed to ensure the safety and efficacy of PROTACs in clinical settings. Additionally, the identification of suitable E3 ligases and the design of optimal ligands pose significant hurdles in the rational design of PROTACs.

This book entitled *PROTAC-Mediated Protein Degradation: A Paradigm Shift in Cancer Therapeutics* is published with an intention to provide in-depth concepts of PROTACs and their role in the treatment of cancer. This book comprises 17 chapters presented logically to understand PROTACs from the fundamental to the latest development. This book begins with ubiquitin-proteasome system, its history and development, and principles of PROTACs. Further, it proceeds with the chemistry of PROTACs and the designing aspects for novel compounds. This book then progresses with technological aspects and global regulatory requirements for PROTACs and then discusses the pharmacological aspects covering the role in prostrate cancer, breast cancer, lymphocytic leukemia, and glioma. The delivery systems of PROTACs and exploration of AI/ML have also been dealt with in this book.

In conclusion, PROTACs represent a promising new approach in cancer therapy that capitalizes on the cell's natural protein degradation machinery to eliminate disease-causing proteins. The potential of PROTACs to overcome limitations associated with traditional inhibitors and to target previously undruggable proteins makes them an exciting area of research with the potential to significantly impact the treatment of cancer and other diseases.

Overall, our book aims to be a comprehensive and authoritative resource, catering to both experts in the field and those seeking to gain a deeper understanding of PROTAC technology and its transformative potential in cancer therapeutics.

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New Delhi, Delhi, India Priti Jain

Contents

1 Understanding the Ubiquitin Proteasome System: History and Revolution 1
Abdul Qadir, Amit Kumar, Riya Nagpal, Aqsa Khan, Abhishek Wahi, and Priti Jain

2 PROTACs: Principles and Mechanisms 21
Ranbir Singh Saluja, Madhura P. Vaidya, and Prashant S. Kharkar

3 Structural Considerations and Chemistry of PROTACs 39
Abhishek Wahi, Hemant R. Jadhav, Shikha Thakur, Sushma Dev, Priyanka Mohanty, and Priti Jain

4 Advantages and Disadvantages of PROTACs 67
Rohini Pujari, Shvetank Bhatt, Urvashi Soni, Shreya Sharma, and Shubham Patil

5 Novel Technologies in PROTAC Design 89
Keerti Jain, Sukuru Chinna Reddy, Sreevardhan Moode, Piyush Mehra, Sofiya Tarannum, Manisha Patel, Vineet Kumar Jain, and Harvinder Popli

6 Future of Trends in the Design and Development of PROTAC 117
Popat Kumbhar, Kaustubh Kolekar, Vikas Kamble, Chukwuebuka Emmanuel Umeyor, John Disouza, and Vandana B. Patravale

7 Global Regulatory Requirements Applicable for PROTACs 135
Rohini Pujari, Urvashi Soni, and Ashwini Gawade

8 Tissue-Specific Targeting Strategies with PROTAC Technology 153
Ruchi Jakhmola Mani and Deepshikha Pande Katare

9 Clinical Development of PROTACs 169
Advait Dubey, Kavita Pal, and Vikram Gota

10 Toxicological Aspects of PROTACs 193
Rohini Pujari and Urvashi Soni

11 PROTACs in the Management of Prostate Cancer 209
Deepa S. Mandlik and Satish K. Mandlik

12 Exploring the Role of PROTACs for the Treatment of Breast Cancer 239
Arzoo Pannu, Ramesh K. Goyal, and Shikha Goswami

13 Role of PROTACs in Hematological Malignancies 279
Ashif Iqubal, Kashif Iqubal, Sanjeev Yadav, V. A. Arun, and Kundan Mishra

14 Artificial Intelligence and Machine Learning for Exploring PROTAC in Underutilized Cells 297
Ruchi Tandon and Parveen Kumar

15 PROTACs in Treatment of Glioma 327
Sachin Kumar and Vikas Maharshi

16 Proteolysis Targeting Chimera (PROTACs): An Attractive Technology in CVD Therapeutics—Opportunities and Challenges . . . 347
Dhruv Sanjay Jaya Gupta, Nidhi Khedpande, and Kalyani Barve

17 Delivery Systems: Miniaturised PROTAC, Nano PROTAC, and Aptamer-Based RNA PROTAC 367
Manish P. Patel, Hard K. Kalyani, Kashyap M. Patel, Bharat R. Patel, Dipti H. Patel, and Jayvadan K. Patel

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Abbreviations

Ab-TAC	Antibody-based PROTAC
AD	Alzheimer's disease
ADAs	Anti-drug antibodies
ADME	Absorption, distribution, metabolism, excretion
AE	Adverse effects
AhR	Aryl hydrocarbon receptor
AI	Artificial Intelligence
AKT	Protein kinase B
ALCOA	Attributable, Legible, Contemporaneous, Original, and Accurate
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
ANN	Artificial neural networks
AP	Affinity purification
APC	Anaphase-promoting complex
APC	Aptamer-PROTAC conjugates
API	Active pharmaceutical ingredient
APOA2	Apolipoprotein A-II precursor
AR	Androgen receptor
ARCC-4	Androgen receptor PROTAC degrader-4
ARL	Androgen receptor ligand
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
BCL	B-cell lymphoma
BCL-2	B-cell lymphoma-2
BCL-XL	B-cell lymphoma extra large
BCM	Binary classification model
BET	Bromodomain and extra-terminal domain
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRCA	BReast CAncer gene
BRD	Bromodomain protein
BRD4	Bromodomain containing protein 4

BTK	Bruton's tyrosine kinase
BUB1	Budding uninhibited by benzimidazole 1
CADD	Computer-aided drug design
CALR	Calreticulin gene
CAMs	Cell adhesion molecules
CAR-T	Chimeric antigen receptor T-cell therapy
CBR	Clinical benefit rate
CCR5	C-Chemokine receptor-5
CDK	Cyclic-dependent kinase
CDK4/6	Cyclin-dependent kinase(s) 4/6
CDK6	Cyclin-dependent kinase 6
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
CETSA	Cellular thermal shift assays
CHK	Checkpoint kinases
cIAP1	Cellular inhibitor of apoptosis protein 1
CLIPTAC	Click-formed proteolysis-targeting chimera
CMA	Chaperone-mediated autophagy
CMC	Chemistry manufacturing and controls
CNN	Convolutional neural networks
CNS	Central nervous system
COX-1/2	Cyclooxygenase ½
CRABP1	Cellular retinoic acid-binding protein 1
CRBN	Cereblon
CRFs	Case report forms
CRISPR	Clustered regularly interspaced short palindromic repeats
CRL	Cullin-Ring ligase
CRP	C-reactive protein
CRPC	Castration-resistant prostate cancer
CSRs	Clinical study reports
CTA	Clinical Trial Application
CUL2	Cullin-2
CVD	Cardiovascular disease
Cys	Cysteine
DAPK1	Protein kinase 1
DBPs	DNA-binding proteins
DDIs	Drug–drug interactions
DGM	Deep generative model
DL	Deep learning
DNA	Deoxyribonucleic acid
DSG	Di(N-succinimidyl) glutarate
DSS	Di(N-succinimidyl) suberate
DUBs	Deubiquitylating enzymes
E3RE	E3 recruiting element
EDGs	Electron-donating groups
EDP	Event-driven pharmacology

EGFR	Epidermal growth factor receptor
eGFR	Estimated glomerular filtration rate
EMA	European Medicines Agency
EPR	Enhanced permeability and retention effect
ER	Estrogen receptor
ERK	Extracellular signal-regulated protein kinase
ERα	Estrogen receptor alpha
EWGs	Electron-withdrawing groups
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FRET	Fluorescence resonance energy transfer
GAN	Generative adversarial networks
GBM	Glioblastoma
GCN	Graph convolutional neural networks
GCP	Good Clinical Practice
GMP	Good manufacturing practice
GPLR	G protein-linked receptors
GPU	Graphical processing units
GSCs	GB stem-like cells
HBA	Hemoglobin A
HBAs	Hydrogen bond acceptors
HBDs	Hydrogen bond donors
HCC	Hepatocellular carcinoma
HD	Huntington's disease
HDAC6	Histone deacetylase 6
HDACs	Histone deacetylases
HECT	Homologous to E6-AP carboxyl 74 terminus
HER2/ErbB2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1-alpha
HIPK2	Homeodomain-interacting protein kinase 2
HM	Hematological malignancies
HNSCC	Head and neck squamous cell carcinoma
HPK1	Hematopoietic progenitor kinase 1
hs-CRP	High-sensitivity C-reactive protein
HTS	High-throughput screening
IAPs	Inhibitor of apoptosis proteins (IAPs)
ICFs	Informed consent forms
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICIs	Immune checkpoint inhibitors
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IGFALS	Insulin-like growth factor binding protein, acid labile subunit
IGFBP4	Insulin-like growth factor binding protein 4
IKBα	Inhibitor of nuclear factor kappa B

IKZF	IKAROS family zinc finger
IMDs	Immune-modulatory drugs
IND	Investigational new drug
IRBs	Institutional review boards
ITC	Isothermal titration calorimetry
IVIVE	*In vitro in vivo* extrapolation
JAK-STAT	Janus kinase-signal transducer and activator of transcription
KRAS	Kirsten rat sarcoma viral oncogene homologue
LSTM	Long short-term memory
LYTAC	Lysosome-targeting chimera
MAAs	Marketing Authorization Applications
mABs	Monoclonal antibodies
MAP4K1	Mitogen-activated protein kinase 1
MAPD	Model-free analysis of protein degradability
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
mCRPC	Metastatic castration-resistant prostate cancer
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal-epithelial transition factor receptor
MetAp-2	Methionine aminopeptidase-2
MI	Myocardial infarction
ML	Machine learning
MLP	Multi-layer perceptron
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MTD	Maximum tolerated dose
mTOR	Mammalian target of rapamycin
NAE	NEDD8-activating enzyme
NAPT	Nicotinamide phosphoribosyltransferase
NDAs	New drug applications
NDs	Neurodegenerative diseases
NEMO	NF-κB essential modulator
NF1	Neurofibromatosis type 1
NGAL	Neutrophil gelatinase-associated lipocalin
NPM1	Nucleophosmin
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung cancer
NVOC	Nitroveratryloxycarbonyl
ODP	Occupancy-driven pharmacology
ORM1	Alpha-1-acid glycoprotein 1
PAKs	p21-activated kinases
PARP	Poly (ADP-ribose) polymerase
PARP1	Poly (ADP-ribose) polymerase
PC	Prostate cancer
PD	Parkinson's disease

PD	Pharmacodynamics
PD-1	Protein cell death protein-1
PDGFRα	Platelet-derived growth factor receptor alpha
PD-L1	Protein cell death ligand-1
PDX	Patient-derived xenograft
PEG	Polyethylene glycol
PIM	Proviral integration sites for Moloney murine leukemia virus
PK	Pharmacokinetics
PK	Protein kinase
POI	Protein of interest
PPARγ	Peroxisome proliferator-activated receptor gamma
PPi	Protein–protein interaction
PPIA	Peptidyl-prolyl isomerase A
PPPI3K	Phosphoinositide 3-kinase
PR	Progesterone receptors
PROTAC-DB	Proteolysis-targeting chimera database
PROTACs	Proteolysis-targeting chimeras
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PTP1B	Protein-tyrosine phosphatase 1B
RA	Rheumatoid arthritis
RAF	Rapidly accelerated fibrosarcoma
RAS	Ras/Raf/MAPK pathway
RAS	Rat sarcoma virus
RBPs	Ribonucleic acid
RBR	Ring between ring
RIBOTACs	Ribonuclease-targeting chimeras
RING	Really interesting new gene
RING	Really interesting new gene finger
RIPK 1	Receptor-interacting protein kinase 1
RNF182	Ring finger protein 182
RNN	Recurrent neural networks
ROS	Reactive oxygen species
RP2D	Recommended phase 2 dose
RTKs	Receptor tyrosine kinases
RT-PROTAC	Radiotherapy-triggered PROTAC
SAEs	Serious adverse events
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
SASP	Senescence-associated secretory phenotype
SBD	Substrate binding domain
SCF	Skp1-Cullin-F-box
SELEX	Systematic evolution of ligands by exponential enrichment
SERD	Selective estrogen receptor degrader
SERDs	Selective estrogen receptor downregulators

SHP2	Src homology 2 domain-containing phosphatase 2
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering RNA
Sirt 2	Sirtuin 2
SMIs	Small molecule inhibitors
SOD1	Superoxide dismutase 1
SOPs	Standard operating procedures
STAT	Signal transducer and activator of transcription
STAT-3	Signal transducer and activator of transcription-3 system
TALEN	Transcription activator-like effector nuclease
TAMs	Tumor-associated macrophages
TBD	Target-based degradation
TC	Ternary complex
TCRs	T-cell receptors
TFs	Transcription factors
TGF-β	Transforming growth factor-β
TIS	Therapy-induced senescence
TKIs	Tyrosine kinase inhibitors
TLRs	Toll-like receptors
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP	Target protein
Tp53	Tumor protein p53
TPD	Targeted protein degradation
TPSA	Topological polar surface area
TRIM	Tripartite motif
TRK	Tropomyosin receptor kinase
Ub	Ubiquitin
UGT	Uridine diphosphate (UDP)-glucuronosyltransferases
UPS	Ubiquitin proteasome system
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel–Lindau
VHL1	Von Hippel–Lindau ubiquitin ligase-1
WHO	World Health Organization
ZFN	Zinc finger nuclease
β-TRCP	Beta-transducin repeats-containing proteins

1 Understanding the Ubiquitin Proteasome System: History and Revolution

Abdul Qadir, Amit Kumar, Riya Nagpal, Aqsa Khan, Abhishek Wahi, and Priti Jain

Abstract

The Ubiquitin Proteasome System (UPS) stands as a cornerstone in the realm of cellular biology, orchestrating the regulated degradation of proteins essential for cellular homeostasis. This chapter embarks on a journey through the historical milestones that have shaped our understanding of UPS, tracing its roots from early observations to contemporary breakthroughs. Moreover, the chapter delves into the transformative impact of UPS research on various fields, ranging from cancer biology to neurodegenerative diseases, highlighting how deregulation of UPS contributes to pathogenesis. Also, this chapter illuminates the historical journey and revolutionary insights that have propelled our comprehension of UPS, underscoring its pivotal role in cellular physiology and disease mechanisms.

Keywords

Ubiquitin Proteasome System (UPS) · Cancer biology · Neurodegenerative diseases · Cellular physiology · Chimeric antigen receptor T-cell therapy

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AP	Affinity purification
APC	Anaphase-promoting complex
CAR-T	Chimeric antigen receptor T-cell therapy
CDKS	Cyclin-dependent kinases
CRBN	Cereblon
DUBs	Deubiquitylating enzymes
E6-AP	E6-associated protein
HD	Huntington's disease
HECT	Homologous to E6-AP Carboxyl 74 Terminus
MDM2	Murine double minute 2
MHC	Major histocompatibility complex
NDs	Neurodegenerative diseases
NEMO	NF-κB essential modulator
PD	Parkinson's disease
POI	Protein of interest
PPI	Protein–protein interaction
PROTAC	Proteolysis-targeting chimera
PRRs	Pattern recognition receptors
RING	Really interesting new gene
RIPK 1	Receptor-interacting protein kinase 1
SMAD	Suppressor of mothers against decapentaplegic
SOD1	Superoxide dismutase 1
TCRs	T-cell receptors
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
Tp53	Tumour protein p53
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
VHL	von Hippel-Lindau

1.1 Introduction

The ubiquitin Proteasome System (UPS) regulates the turnover of numerous cellular proteins. It is a primary process of intracellular protein breakdown (Wang and Maldonado 2006). It is a precise and universal method for the cell to get rid of biologically useless proteins, such as mutant, misfolded, damaged, terminally altered, or excessively accumulated proteins. With its ability to quickly start substrate-specific proteolysis, UPS can function as a molecular switch in a range of signalling routes, swiftly stopping a target protein's activity. Preventing the build-up of potentially hazardous, non-functional proteins is one of the main roles of the

UPS. Furthermore, it is now evident that the UPS is engaged in the majority of eukaryotic biological mechanisms, encompassing transcriptional regulation, intracellular signalling, and control of cell death. In all eukaryotic cells, the UPS is the principal mechanism governing controlled peptide chain breakdown, and it is increasingly evident that abnormalities in this route are the root cause of many human diseases. A large range of proteins with half-life period ranging from hours to days can be progressively degraded in concert via the highly conserved and strictly regulated UPS pathway (Kleiger and Mayor 2014; Zolk et al. 2006). Herein, we intend to provide the historical turning points that have influenced our knowledge of UPS, from the earliest observations to the most recent innovations. Additionally, the chapter explores how UPS research has revolutionised a number of domains, from neurodegenerative diseases to cancer biology, emphasising how UPS dysregulation plays a role in pathogenesis. Furthermore, this chapter highlights UPS's critical function in cellular physiology and disease mechanisms while also illuminating the approaches to enhance the understanding of UPS and target UPS for treatment of diseases.

1.2 History of UPS

Cell biologists were first taken aback by the requirement of ATP for the process of cytosolic protein degradation because it is widely known that metabolic energy is not needed for peptide bond hydrolysis. Hershko and colleagues employed a method that included biochemically fractionating reticulocytes, which are terminally developed red blood cells devoid of lysosomes, purifying the reticulocytes, identifying the constituents, and reconstructing the ATP-dependent cytosolic protein breakdown in vitro. These discoveries were supported by information from other organizations and genetic research revealing the intracellular functions of cytosolic protein breakdown in numerous biological systems. Avram Hershko, Aaron Ciechanover, and Irwin Rose were granted the 2004 Nobel Prize in chemistry for their groundbreaking research on the function of protein ubiquitination during cytosolic protein breakdown. A rabbit reticulocyte system was created by Etlinger and Goldberg (1977) to investigate ATP-dependent and non-lysosomal protein breakdown (Etlinger and Goldberg 1977). After reticulocyte was fractionated in 1978 by Ciechanover et al., two fractions—active principle of fraction (APF)-I and (APF)-II—were identified (Ciehanover et al. 1978). By combining APF-I with APF-II in 1979, Hershko et al. reconstructed protein degradation. Two fractions, APF-IIa and APF-IIb, were created by subdividing APF-II. The E1-E3 ubiquitin-conjugating enzymes were present in APF-IIb. Later research revealed that APF-IIa contained proteasomes (Hough et al. 1986). Wilkinson et al. discovered that APF-I was ubiquitin (Wilkinson et al. 1980). Conjugating enzymes and ATP were required for the formation of high molecular conjugates between ubiquitin and substrate proteins (Hershko et al. 1980). Hershko et al. discovered deubiquitinating enzyme activity that could recycle ubiquitin bound to substrate proteins (Hershko et al. 1980). The E1 enzyme was identified to activate the ubiquitin carboxyl terminus

glycine (Hershko et al. 1981). According to Hershko et al., there is a connection between ubiquitination and protein degradation since reticulocytes produced more ubiquitin-protein conjugates when aberrant proteins were formed (Hershko et al. 1982). Further, A method for purification and identification of all three ubiquitin conjugating enzymes (E1, E2, and E3) was developed by Hershko et al. (1983) . The anatomic functions of the UPS were disclosed by Finley et al.'s 1984 genetic research. The absence of growth in the non-permissive temperature of the mutant mammary cell line ts85 was attributed to the E1 enzyme, indicating the potential role of ubiquitination in the advancement of the cell cycle. 1984 Ciechanover et al. At the non-permissive temperature, the ts85 cell line's ability to degrade short-lived proteins was hindered. Hershko and colleagues in 1986 discovered that an E3 ligase exhibited substrate specificity. A large molecular weight neutral protease that originated from rat liver, the pituitary gland of cows, and the archaebacterium *Thermoplasma acidophilum* was identified by numerous organizations. Since it was first believed that this protease was connected to RNA, it has been called "prosome". Notably, an allo-MHC serum was used to identify a large molecular weight complex made up of tiny subunits.

Several years later, it was found that the two subunits of the IFN-γ-inducible proteasome, β1i and β5i, were encoded by genes situated in the mammalian MHC region. In 1986, Hough et al. described a high molecular protease that broke down protein ubiquitin adducts but not untagged protein. Its proteolytic and particle characteristics led to the coining of the term proteasome. In the presence of ATP, isolated 20S proteasomes and 19S regulators were mixed to create active 26S proteasomes (Monaco and McDevitt 1984; Sijts and Kloetzel 2011).

1.3 Components of UPS

1.3.1 Ubiquitin

Ubiquitin is a tiny protein with a molecular weight of about 8.5 kDa that is made up of 76 amino acids folded into a compact globular shape. It exists in the cytoplasm either unbound or covalently attached to other proteins. All eukaryotic cells contain ubiquitin, a small, highly conserved peptide. Seven lysines are present in this molecule (K6, K11, K27, K29, K33, K48, and K63). K48 and K11 polyubiquitin chains mostly facilitate proteasomal breakdown. On the other hand, K63-related polyubiquitination, which is generally less prevalent in tumours, is generally linked to cellular signal building, transduction, and the repair of damaged cells rather than proteasomal breakdown (Huang et al. 2024; Park et al. 2020). A ubiquitin molecule is used to mark the substrate protein, which is then broken down by the 26 s proteasome. Ubiquitin attaches itself to proteins that need to be targeted by the proteasome. Targeted protein breakdown begins with ubiquitin's selective binding to specific proteins. The target protein and ubiquitin are connected via a branching isopeptide bond that forms between the lysine ε-amino group and the carboxyl-terminal glycine of ubiquitin. Polyubiquitin is created when a ε-amino group of

lysine from one ubiquitin molecule forms a similar isopeptide bond with the carboxy terminus of ubiquitin.

Monoubiquitination and polyubiquitination are two ways that ubiquitin is altered. Monoubiquitination is the result of adding a single ubiquitin molecule to the lysine residue of a substrate (Swatek and Komander 2016). In the process of polyubiquitination, one ubiquitin molecule is joined with additional ubiquitin molecules to create polyubiquitin chains. The bare minimum length of a chain required to effectively target a protein for proteasomal destruction is four ubiquitin adducts. There are three distinct steps of protein ubiquitination, commonly known as ubiquitinylation: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). In each phase, an enzyme is involved (Ebner et al. 2017). The ubiquitin chain is generated by the E1 and E2 enzymes and subsequently bound to proteins by the E3 enzyme.

1.3.2 E1, A Ubiquitin-Activating Enzyme

The ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme E1 starts the process of ubiquitination. E1-S ~ ubiquitin is created when ubiquitin is linked to an internal E1 Cys residue through an intermediary thiol ester between the target protein and the ubiquitin monomer from the E2 enzyme. Uba1 is the predominant version of this enzyme in yeast and humans (Schulman and Wade Harper 2009).

1.3.3 E2, A Ubiquitin-Conjugating Enzyme

Activated ubiquitin molecules are gradually added to the original ubiquitin proteins to form a polyubiquitin chain. Ubiquitin is trans-esterified to a conserved cysteine in an E2 enzyme subsequent to its activation by the E1. Yeast has 13 E2s, and vertebrates probably have a similar amount. The three-letter code 'Ubc' represents the genetic name of these enzymes. With the exception of Ubc9, an enzyme that conjugates SUMO, and Ubc12, an enzyme that conjugates NEDD8 and Rub1, the Ubcs have different genetic roles in ubiquitylation, albeit some of them overlap. Ubc3, or CDC34, is a crucial E2 enzyme in the Skp1, cullin, F-box (SCF) ubiquitin ligase complex (McKinnon and Tabrizi 2014).

1.3.4 E3, A Ubiquitin Ligase

The mechanism gains its specificity from the E3 ligase. The E3 ligase promotes the creation of a covalent link between the target protein and the ubiquitin monomer from the E2 enzyme by binding both the target protein and the complex E2-ubiquitin. To create a polyubiquitin chain, activated ubiquitin molecules are progressively added to the initial ubiquitin proteins. The 26S proteasome recognizes and targets proteins that are tagged with chains of four or more ubiquitins for destruction. By

attaching itself to a protein target preferentially, the E3 ligase gives the process selectivity (Grice and Nathan 2016). Hundreds of different E3 ubiquitin ligases are enciphered by the human gene pool and are grouped into three primary classes, according to structural similarities: the U-box proteins, the RING-finger proteins, which are the most prevalent class of E3 enzymes, and the HECT domain proteins. Prior to delivering the ubiquitin to the substrate, HECT domain proteins create a covalent (thiolester) link with it (Garcia-Barcena et al. 2020; Nakamura 2011). When RING finger (including amino acids cysteines and histidines responsible for co-ordination of two metal atoms) E3s connect to the E2-ubiquitin complex, they facilitate direct ubiquitin transfer to the targeted protein, eliminating the need for further thiol ester formation—a process that is seen in the HECT family of E3 ligases (Jackson et al. 2000).

1.3.5 HECT Domain Proteins

The 350-amino acid, which is similar to the E6-AP C terminus and was initially identified in E6-AP, defines HECT domain proteins. Eukaryotes, including yeast and humans, have HECT domain proteins. The oncoprotein of the papillomavirus in humans recruits E6-AP, a cellular ubiquitin ligase, which degrades the p53 tumour suppressor 4. A conserved cysteine combines with ubiquitin to create a thioester in the E6 HECT domain. This intermediate is necessary for the process of ubiquitination. HECT domain proteins are typically big (90–200 kDa) with extended N-terminal domains. The N-terminus of these ubiquitin ligases binds to the substrate, whereas the C-terminal HECT domain transfers ubiquitin directly by a thioester bond to the substrate (Eldridge and O'Brien 2010; Jackson et al. 2000). It was recently demonstrated that Xsmurf1, a novel HECT domain E3, may bind to Smad1, ubiquitylate it, and control its stability. Smad1 is a signalling regulator that is regulated by the TGF-β family member BMP. It is unknown if other types of protein–protein interaction domains have the ability to bind HECT domain proteins to substrates for ubiquitylation.

1.3.6 Skp1, Cullin, F-Box (SCF) Complexes

The SCF class of ubiquitin ligases comprises at least four proteins: Skp1, Cul1, Roc1/Rbx1/Hrt1, and an F-box protein. F-box proteins are adaptors that directly bind SCF substrates. These proteins link to substrates by protein–protein interaction domains and feature an F-box motif, which is around 45 residues long. The protein Skp1, which is significant in SCF complexes but may have additional functions, requires the F-box to bind to it. Skp1 then forms an association with either the human homolog Cul1's N-terminus or its counterpart Cdc53p in budding yeast. Cul1 belongs to the cullin family, which in humans consists of at least Apc2 and cullins Cul1–Cul5. In known E3 complexes, Cul2 and Apc2 have roles. Cullins seem to recruit the E2 ubiquitin-conjugating enzyme and help organise and activate

the E3 complex. It is possible that the remaining cullins will arrange ubiquitin ligase complexes in a similar way. For instance, Cul3 is involved in regulating cyclin E stability (McKinnon and Tabrizi 2014). Human Roc1/Rbx1 (Hrt1 in yeast), a protein with a RING-H2 finger domain, seems to increase ubiquitin ligase activity and facilitate the attachment of the Cul1 protein to the E2 enzyme (usually Ubc3/Cdc34 or Ubc5). One member of the class of finger proteins known as RING finger proteins, RING-H2 is composed of eight cysteine and histidine residues that aid in E2 binding and catalysis. These four components are sufficient to ubiquitylate certain substrates, based on biochemical reconstitution of SCF complexes in yeast and humans.

1.3.7 APC Ubiquitin Ligase

The APC, first multi-component ubiquitin ligase to be discovered, is required for the removal of cyclin B to allow for the escape from mitosis and for the degradation of substrates controlling the metaphase to anaphase transition. The APC includes a RING-H2 finger protein called Apc11 that is comparable to Roc1/Rbx1 and a cullin homologue named Apc2, which is similar to the SCF complex. Like the SCF, the APC binds to proteins to initiate its activation against particular substrates (Lehman 2009). The destruction box (also known as the D-box) and the KEN box are two destruction signals that have been found in substrates that the APC is aiming to destroy. All known APC substrates that use the Cdc20 adaptor, referred to as APC Cdc20 substrates, as well as certain APC Cdh1 substrates, referred to as APCCdh1, have the nine-residue D-box. The KEN box is a transposable motif consisting of seven residues that is known to target substrates to the APC Cdh1. Both D-box- and KEN-box-containing substrates, including Cdc20 itself, are susceptible to ubiquitylation by Cdh1 (Li and Zhang 2009).

1.3.8 Proteasomes

A hollow cylinder-shaped protein complex involved in UPS is called a proteasome. Adenosine triphosphate-dependent proteasomes are the primary non-lysosomal complexes accountable for the breakdown of most of the intracellular proteins. Proteases are essential for the survival of organisms and cells in eukaryotes. Proteasomes do not exist in eubacteria (Zolk et al. 2006). The ATP-dependent assembly of the 26S proteasome is facilitated by two primary subunits. There are several proteolytic sites in the 20S catalytic component, and several ATPases as well as a binding site for ubiquitin concatemers are present in the 19S regulatory component (Muratani and Tansey 2003).

The 26S proteasome catalytic activity is attributed to its four stacked rings, i.e. 20S subunit each of which has seven different subunits stacked one on top of the other. Two identical outside α rings and two inside β rings are present. Catalytic sites facing the hollow centre of the ring structure are located within the two inner

beta rings. Beneath the beta rings are the two alpha rings. The 20S β subunit possesses three unique proteolytic activities: activities similar to peptidylglutamyl, achymotrypsin, and trypsin. The alpha subunits' amino terminus prevents entry to the proteolytic chamber. Therefore, the small holes on either end of the cylinder are the sole ways to access the proteasome's inner cavity. While the β rings feature several catalytic sites, the outer two α-rings are unknown in purpose. To create the 26S proteasome, the 19S regulatory components come together at each pore of the 20S subunit. The two 19S regulatory caps that cap the ends of the 20S complex are also referred to as PA700.19S assemblies provide substrate recognition and insertion tasks. A 19S regulatory subunit, located at either end of the 20S proteasome, is made up of two distinct subcomplexes: a base made up of ten distinct proteins that attaches to the 20S proteasome and a lid made up of nine different proteins that binds and recognizes polyubiquitinated proteins. The regulatory caps not only identify the substrates of the 20S proteasome but also make it easier for the target proteins to enter the 20S proteasome by unfolding the substrate and opening the catalytic channel. Because the 20S subunit by itself is inactive, the 19S subunit is also necessary for proteolytic action. The 19S regulatory particle participates in protein translocation into the catalytic 20S chamber for degradation, as well as substrate preparation and selection. Many subunits, including six ATPases, make up each 19S particle. These subunits presumably supply the energy needed for substrate unfolding, which is necessary prior to entry into the 20S chamber. In order to process ubiquitin chains prior to substrate translocation and destruction, the 19S component's outer-lid subcomplex is engaged (Mani and Gelmann 2005). After proteasome degradation, ubiquitin monomers are released or actively eliminated by

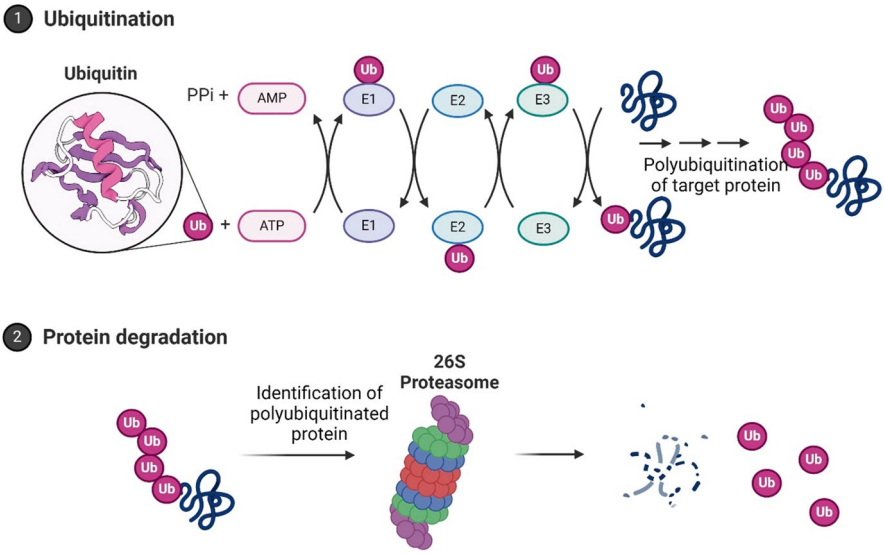

Fig. 1.1 Degradation of targeted protein through UPS

ubiquitin carboxyl-terminal hydrolases at the proteasome, where they are recycled for further usage (Fig. 1.1).

1.4 Role of UPS in Revolutionizing Cellular Biology

1.4.1 Functional Understanding

1.4.1.1 Protein Degradation

For cellular homeostasis and the control of numerous physiological functions, protein breakdown is necessary. UPS is an extremely controlled route responsible for selective protein degradation. It breaks down most of the proteins in a cell. The attachment of a polyubiquitin chain—a chain of ubiquitin molecules marks the target protein for destruction. A protein complex known as the proteasome then identifies this chain and carries the enzymatic activity necessary to break down the target protein. The enzymatic sequence of events that results in the initial attachment of the polyubiquitin chain to the target protein confers substrate specificity. Both the final protein breakdown and the transportation of polyubiquitinated proteins to the proteasome are tightly controlled processes (Bingol and Schuman 2005).

It requires three enzymatic components to attach chains of Ub onto proteins that are targeted for degradation. The preparation of Ub for conjugation is carried out by E1 which is the Ub-activating enzyme and E2, which is a Ub-carrier or conjugating protein. However, the most important enzyme in the process is E3, Ub-protein ligase, which identifies a particular protein substrate and catalyses the transfer of activated Ub to it (Lecker et al. 2006). Mechanistically, E3 enzymes can be divided into two primary types based on their RING (or RING-like) and HECT domains. The capacity of both varieties of E3 enzymes to establish specific substrate binding is similar (Ravid and Hochstrasser 2008).

There are two stages involved in the breakdown of a substrate protein: (1) covalent binding of ubiquitin to the substrate (also known as ubiquitination or ubiquitin conjugation); and (2) degradation of the polyubiquitinated substrate, which involves breaking down the polyubiquitin chain and recycling free ubiquitin (Li et al. 2022). Under stimulus, ubiquitin mediates the breakdown of protein clumps. Proteins in the endoplasmic reticulum accumulate in response to cell stimulation; these aggregates are tagged with ubiquitin when E1, E2, and E3 are present, and proteases subsequently are degenerated. Along with the hydrolysis of ATP, ubiquitin's C-terminal glycine binds to the active site of E1. Following its transfer to the E2 enzyme, the active ubiquitin forms a complex with the E2 conjugating activation. After that, this complex interacts with the specific enzyme E3, which causes the ubiquitin to go to the designated substrate (Li et al. 2022). Once the substrate protein is altered by a polyubiquitin chain including at least four ubiquitins (Ub), it can immediately bind to intrinsic Ub receptors in the 19S regulatory complex of the 26S proteasome. As an alternative, it can attach itself to adaptor proteins that include domains for both proteasome and polyubiquitin binding (Ravid and Hochstrasser 2008).