

Review

Janitors at Work: The Multifaceted Role of the P97-UFD1-NPL4 Complex

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Abstract

The regulatory pathways governing protein homeostasis are a delicate balance of protein synthesis and degradation. At the core of proteolysis is a molecular “cleaning crew” composed of the P97 ATPase, ubiquitin fusion degradation 1 (UFD1), and nuclear protein localization 4 (NPL4). The ATPase activity of P97 drives the formation and activity of this complex, which is also regulated by post-translational modifications and ubiquitin regulatory X-domain proteins. Notably, the P97-UFD1-NPL4 complex plays a critical role in endoplasmic reticulum-associated degradation (ERAD), by retrotranslocating misfolded proteins from the endoplasmic reticulum (ER) to the cytosol. Beyond ERAD, this complex also targets proteins from mitochondria, ribosomes, and chromatin to regulate cellular processes such as organelle homeostasis, cell cycle progression, DNA replication, and DNA repair. The aberrant activity of the P97-UFD1-NPL4 complex can either accelerate or impair the removal of misfolded, unfolded, and regulatory proteins, disrupting cellular homeostasis and inducing various disorders. Significant efforts have been made to develop therapeutics that inhibit complex functions. However, to identify a suitable strategy, it is crucial to understand the molecular mechanisms linking the function of the complex to disease progression. In this review, we summarize the multifaceted roles of the P97-UFD1-NPL4 complex in protein quality control, discuss how its dysfunction leads to disease, and explore ongoing strategies to target the complex.

Keywords: P97-UFD1-NPL4; ERAD; CAD; proteostasis; RAD; UFD1; NPL4; VCP; cancer; P97

1. Introduction

In the intricate world of the cell, each molecule works together like a well-coordinated factory. Among them, proteins form and drive molecular machines, and their synthesis, folding, modification, and degradation must be tightly regulated [1]. Proteolysis, the selective degradation of proteins, is essential for proper cellular operations and relies on tightly regulated pathways. The endoplasmic reticulum (ER) is the site of major protein folding and assembly, participating in cellular functions such as synthesis and sorting of secretory and membrane proteins [2]. ER-associated degradation (ERAD) plays a critical role in removing improperly folded proteins to ensure protein quality [3]. P97, also referred to as valosin-containing protein (VCP) or cell division cycle 48 (Cdc48) in yeast, partners with ubiquitin fusion degradation 1 (UFD1) and nuclear protein localization 4 (NPL4), acting as a molecular “segregator” to extract and deliver aberrant proteins from the ER for proteasomal degradation [4,5,6].

In recent years, the P97-UFD1-NPL4 complex has become a focal point in research due to its wide-reaching implications in physiology and pathology. In physiological conditions, the P97-UFD1-NPL4 complex, in addition

to its role in ERAD, facilitates the removal of proteins in many pathways, such as mitochondrial-associated degradation (MAD), ribosome-associated degradation (RAD), and chromatin-associated degradation (CAD) [7,8,9]. The activity of this complex in these pathways is regulated by post-translational modifications (PTMs) and its cofactors, which can alter its conformation and impact substrate binding [10,11,12].

Pathologically, dysfunction of the P97-UFD1-NPL4 complex, whether through overexpression or mutations of one or multiple components, can alter cellular homeostasis and contribute to the development of a broad spectrum of diseases. While the P97-UFD1-NPL4 complex is integral to maintaining cellular order, it is often co-opted by cancer cells to mitigate proteotoxic stress, thus promoting tumor cell survival [13,14]. On the other hand, failure to remove misfolded and unfolded proteins by the complex leads to their accumulation and formation of toxic aggregates, a hallmark of neuronal disorders such as Alzheimer’s disease [15,16,17]. To effectively target this complex for therapeutic interventions, it is crucial to understand its roles in both physiological and pathological conditions. Here, we examine the structure, function, and regulation of the P97-UFD1-NPL4 complex, as well as mechanisms underlying



its dysfunction in disease and emerging therapeutic strategies targeting this complex in cancer.

2. Architecture of the P97-UFD1-NPL4 Complex

Within the P97-UFD1-NPL4 complex, UFD1 first forms a heterodimer with NPL4, enabling their binding to P97, which threads and processes substrates, preparing their delivery to the proteasome for degradation.

2.1 P97: the Power Engine

P97 is a hexameric AAA+ ATPase, consisting of six subunits arranged in a circular formation. Each subunit contains an N-terminal domain, two ATPase domains (D1 and D2), and a short C-terminus tail (Fig. 1A) [18]. The N-terminal domain of P97 is the primary recognition site for its cofactor proteins, while its D1 and D2 domains are involved in adenosine triphosphate (ATP) hydrolysis [19]. ATP hydrolysis of P97 induces conformational changes within its N-terminal domain, essential for substrate unfolding and releasing (Fig. 1B,C). The N-terminal domain of P97 starts in its “down” position when the D2 domain is bound to ADP and switches to an “up” position upon ATP binding and hydrolysis (Fig. 1B,C) [20]. This conformational change allows for the UFD1-NPL4 heterodimer to bind P97, priming the complex for substrate recognition (Fig. 2) [20]. As the N-terminal domain is locked in the ATP-bound state, ATPase activity in the D2 domain increases, unfolding substrates and translocating them into the cytoplasm (Fig. 2) [21,22]. After ATP hydrolysis within the D1 domain, the N-terminal domain returns to its “down” position, releasing unfolded substrates and the UFD1-NPL4 heterodimer (Fig. 1B, Fig. 2) [23,24]. The D1 and D2 domains are connected via a short polypeptide linker (D1-D2 linker), which induces asymmetry among the P97 subunits and increases the ATPase activity of the D1 domain and facilitates the capture and release of substrates (Fig. 2) [25]. The C-terminus is an unstructured, flexible tail at the end of P97 and serves as a secondary binding site for its cofactors (e.g., VCIP135), helping maintain the conformation of the D2 ring during substrate processing [26,27].

2.2 UFD1: Recognizing Substrates

While P97 provides energy to the complex, UFD1 facilitates the recognition and selection of substrates for retrotranslocation. UFD1 is composed of an N-terminal (UT3) and C-terminal (UT6) domain (Fig. 1A). UT3 has a primary binding site (residues 1-211) for ubiquitin and structurally resembles the N-terminal domain of P97. The UT3 domain of UFD1 thus interacts with ubiquitinated chains on target proteins, identifying and linking these proteins to P97 (Fig. 2) [8,11]. UT6 of UFD1 is home to two crucial sites, the SHP1 and SHP2 motifs (Fig. 1A) [28,29]. Previous studies have defined the amino acid sequence for SHP1 (residues 215-241) and SHP2 (residues 281-307) within UFD1 (Fig.

1A) [28,30]. The SHP1 motif allows UFD1 to anchor itself to the N-terminal domain of P97 (residues 112-185) and is critical for the formation and function of the P97-UFD1-NPL4 complex (Fig. 1A–C) [28,31]. However, little is known about how the SHP2 motif of UFD1 contributes to the formation and stability of the complex. UFD1 (residues 258-275) interacts with the Mpr1/Pad1 N-terminal (MPN) domain of NPL4 (residues 220-457) to form the UFD1-NPL4 heterodimer that subsequently binds to P97 (Fig. 1A).

2.3 NPL4: Guiding Substrates

NPL4 serves as the adaptor and protein cargo loader of the complex by bridging substrates from UFD1 to P97 and aligning them for extraction and translocation. NPL4 consists of an N-terminus in which a UBX-like (UBXL) domain resides, zinc finger motifs, an MPN domain, and a C-terminal domain (Fig. 1A) [32]. The UBXL domain (residues 1-96) is the primary binding site of NPL4 to the N-terminal domain of P97 (Fig. 1A), their strong interaction stabilizes complex formation. Meanwhile, the UBXL domain of NPL4 directly binds to polyubiquitin chains, facilitating substrate recognition (Fig. 2) [32]. The MPN domain (residues 220-457) of NPL4 interacts with UFD1 (residues 258-275) to form a heterodimer, which binds to P97 (Fig. 1A) [31,33]. Similarly, zinc finger motifs of NPL4 attach to the N-terminal domain of P97, which undergoes conformational changes upon ATP hydrolysis. This causes NPL4 to exhibit a seesaw motion driven by ATP hydrolysis, exposing the D1 domain of P97 to capture and unfold substrates through its central pore [29,32,34]. The interactions between UFD1-NPL4 and P97 occur through a bipartite binding mechanism, where UFD1 and NPL4 each bind to distinct regions of P97 using SHP-box motifs and UBX-like domains, respectively [35]. Hence, the three partners form a stable complex through the dual binding of UFD1 and NPL4 to each other and to P97.

2.4: Substrate Recognition and Processing

The P97-UFD1-NPL4 complex acts as a molecular “litter picker” and substrate-processing machine by selectively capturing polyubiquitinated proteins. The complex recognizes the substrates through the UFD1/NPL4 dimer, which binds to polyubiquitin chains on K48 (lysine 48) and unfolds the “initiator” ubiquitin on substrates [32]. They then position the substrate and feed it into the central pore of P97 for processing, extraction, and delivery to the proteasome for degradation (Fig. 2) [36]. The processing and extraction of substrates by P97 are powered by ATP/ADP binding and ATP hydrolysis, aided by additional cofactor proteins such as Rad23, Ubx5, and Shp1 [21,23,24,37].

2.4.1 Substrate Recognition

Post-translational modifications (PTMs), such as ubiquitination and sumoylation, serve as molecular signals

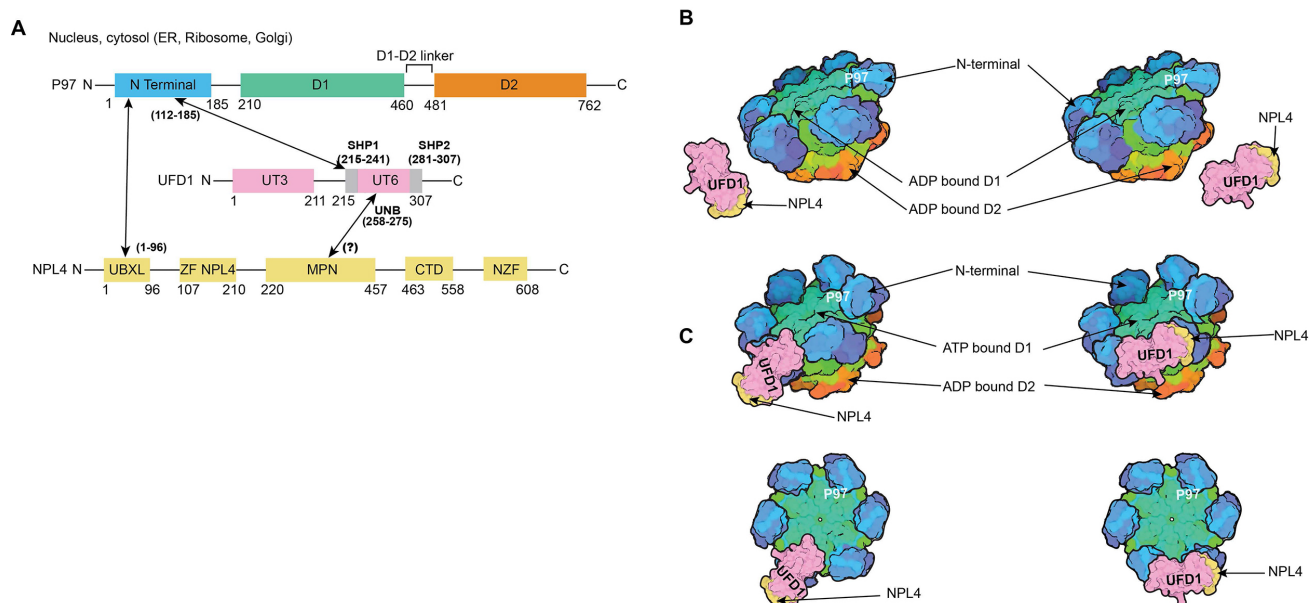


Fig. 1. Schematic of the P97-UFD1-NPL4 complex. (A) The P97-UFD1-NPL4 complex assembles through multiple conserved binding interfaces. UFD1 and NPL4 form a stable heterodimer via residues 258-275 of UFD1 and residues 220-457 of NPL4 and then bind to the N-terminal of P97 (blue, residues 112-185) through the SHP1 and SHP2 box motifs of UFD1 (grey, residues 215-241 and residues 281-307, respectively) and the UBXL-like (UBXL) domain of NPL4 (yellow, residues 1-96). (B,C) Crystal structural model illustrating the nucleotide-dependent conformational states of P97 and the potential modes of UFD1-NPL4 engagement. In the ADP-bound D1 (green) and D2 (orange) state, P97 adopts a “down” configuration without cofactor binding (B). In the ATP-bound D1 (green) and ADP-bound D2 (orange) state, P97 adopts an “up” configuration that facilitates cofactor recruitment (C). Two modes of UFD1-NPL4 engagement are proposed: binding to the same N-terminal domain (left) or spanning adjacent protomers (right) of P97 (B,C). Top-view of the P97-UFD1-NPL4 complex with the two possible modes of UFD1-NPL4 engagement: binding to the same N-terminal domain (left) or spanning adjacent protomers (right) (C). Created in BioRender. Kemet, C. (2026) <https://BioRender.com/mxovsiz>. UFD1, ubiquitin fusion degradation 1; NPL4, nuclear protein localization 4; UBXL, ubiquitin regulatory X; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

that enable selective recognition of substrates by the P97-UFD1-NPL4 complex. Ubiquitination involves an enzymatic cascade (E1, E2, and E3 enzymes) that results in the formation of ubiquitin chains on the lysine residues of substrates, tagging them for degradation [38]. This cascade is cyclic until the required chain length is reached, forming a K48-linked polyubiquitin chain on substrates. Upon its formation, K48 molds into a unique, compact shape and ends the enzymatic cascade. The unique shape of K48 forms the perfect pocket for UFD1 and NPL4 to release and guide misfolded and unfolded proteins into P97’s pore. The N-terminal domains of each P97 protomer assume an “up” or “down” position upon ATP or ADP binding, leading to the extraction of misfolded and unfolded proteins from the ER to the cytosol for proteasomal degradation [11,39,40]. In addition, ubiquitination regulates the complex function at sites of DNA damage. Specifically, K48-linked polyubiquitination of chromatin-binding proteins serves as a signal for their removal and recycling via the P97-UFD1-NPL4 complex [41,42]. Like ubiquitination, sumoylation is a cascade of enzymatic reactions leading to the attachment of a small ubiquitin-like modifier (sumo) tag on the ly-

sine residue of targeted proteins [43]. Wang et al. [44] showed that the sumoylation of P97 promotes the assembly of its hexameric structure, stability, and functionality. Additionally, it regulates the distribution of P97/VCP to cellular compartments in response to stress and promotes its binding to cofactors [12]. Lee et al. [43] found that Ufd1 in budding yeast contains a sumo-interaction motif within its C-terminus, allowing Ufd1 to recognize and create a non-covalent bond with sumo-tagged substrates. Additionally, interactions among sumo, ubiquitin, and the Cdc48/P97-UFD1-NPL4 complex were detected, indicating that sumoylation and ubiquitination can work in tandem to promote efficient extraction and degradation of substrates [43].

2.4.2 Substrate Processing

Substrate processing by the P97-UFD1-NPL4 complex involves the recognition, unfolding, and extraction of ubiquitinated proteins, which is ATP-driven and regulated by cofactors such as Otu1, Rad23, Ubx5, and Shp1 [37]. Otu1 or Yod1 in mammals was found to trim the ubiquitinated chain of substrates before their translocation

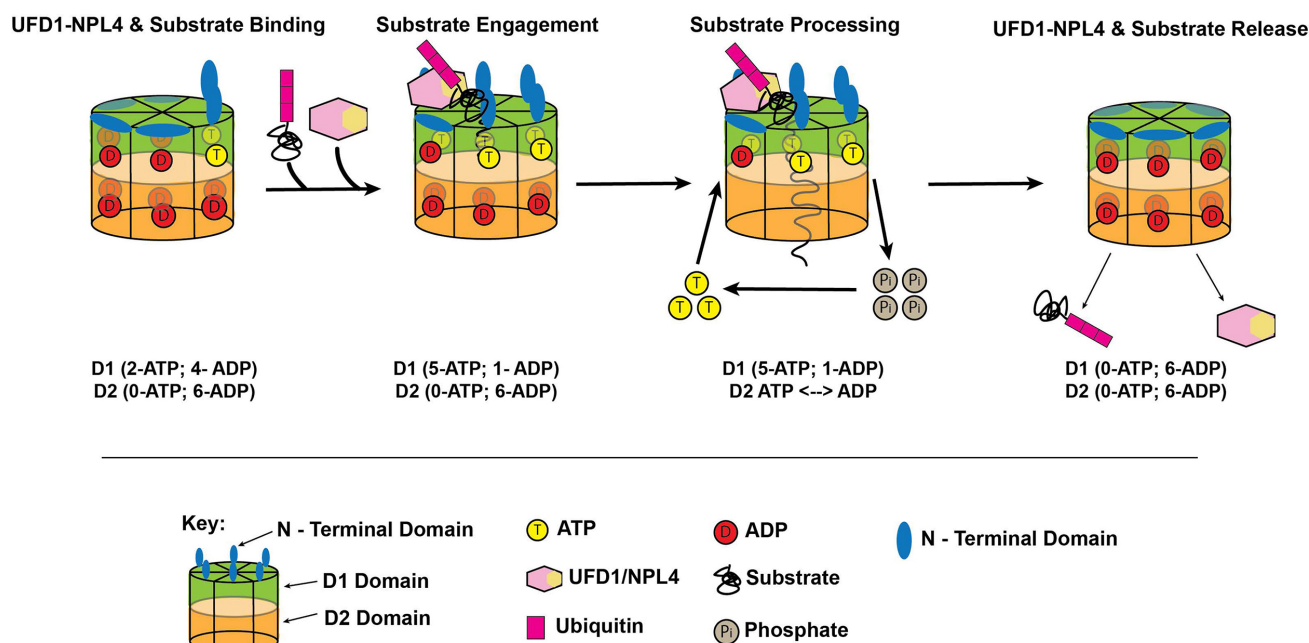


Fig. 2. Substrate extraction and processing by the P97-UFD1-NPL4 complex. The UFD1-NPL4 heterodimer is recruited to P97 when the N-terminal domains from two P97 protomers adopt the “up” conformation, triggered by ATP binding to the D1 domains. At this stage, the D2 ring remains ADP-bound, allowing initial substrate engagement. Upon the initial binding of UFD1-NPL4 to P97, N-terminal domains from five P97 protomers change to the “up” state, while the remaining “down” protomer interacts with the UBXL domain of NPL4 to guide substrate positioning. When a polyubiquitinated substrate binds to the complex, this triggers ATP hydrolysis within the D2 ring and substrate unfolding and translocation. Subsequently, ATP hydrolysis in the D1 ring mediates the release of the processed substrate and the UFD1-NPL4 heterodimer, returning P97 to its ground state. Figure modified from Arie et al. [20].

through the Cdc48/p97 pore [45]. To further facilitate substrate processing, studies have demonstrated that in yeast, the Cdc48/p97-Ufd1-Npl4 complex partners with cofactors Rad23 and Ubx5 to shuttle substrates from the complex to the proteasome [37]. Rad23 and Ubx5 recruit both the Cdc48/p97-Ufd1-Npl4 complex and the proteasome, leading to competition for polyubiquitinated substrates both before and after their unfolding [37]. Shp1 stimulates protein unfolding by the complex [37,46]. The ATP/ADP binding and ATP hydrolysis occurs in a cyclic matter, and powers the P97-UFD1-NPL4 complex during their formation and function, driving substrate unfolding and extraction [21,23,24]. As described in-depth above, ATP binding primes P97 by putting five N-terminal domains in an “up” position, allowing for proper docking of the UFD1-NPL4 heterodimer and interaction with ubiquitinated substrates (Fig. 1C, Fig. 2). Following ATP binding, ATP hydrolysis in the D2 domain puts P97 in an “active” state that drives substrate unfolding and translocation through its central pore. When ADP replaces ATP on the same binding regions, this moves P97 into its “down” position, facilitating substrate release and reset of the complex [21].

3. Cellular Janitor Management: Regulators of the P97-UFD1-NPL4 Complex

The assembly and activity of the P97-UFD1-NPL4 complex are primarily regulated by PTMs, ubiquitin regulatory X (UBX)-domain proteins, and ATP/ADP binding and hydrolysis [11,26]. PTMs of the complex components, including phosphorylation, and acetylation, can alter their cofactor binding, substrate engagement, and conformation [26]. Additionally, UBX proteins can stabilize the complex while assisting with substrate unfolding [11]. Finally, ATP/ADP binding followed by ATP hydrolysis regulates and drives complex formation and function [47].

3.1 PTMs

3.1.1 Phosphorylation

Phosphorylation is a reversible PTM wherein phosphate is added to specific amino acids on proteins. In the P97-UFD1-NPL4 complex, phosphorylation of P97 and UFD1 can regulate cofactor binding, as well as complex assembly and function [48,49,50]. Phosphorylation of a conserved penultimate Tyrosine (Tyrosine⁸⁰⁵) on P97 acts as a molecular switch by inhibiting its binding to cofactors that contain PUB (peptide:N-glycanase and UBA or UBX-containing proteins) or PUL (PLAP, UFD3 and Lub1) domains [10,51]. This provides P97 the flexibility to function in other cellular pathways besides ERAD. In addition, DNA

damage can induce phosphorylation of P97 at Serine⁷⁸⁴, which promotes the extraction of proteins from chromatin through accelerated substrate release during the late stages of DNA damage repair [49,52]. Similarly, Nguyen et al. [50] demonstrated that phosphorylation of UFD1 at Serine²²⁹, an amino acid within its SHP1 motif, directly disrupts the P97-UFD1 interaction and thus negatively regulates ERAD.

3.1.2 Acetylation

Apart from phosphorylation and sumoylation, lysine acetylation can also modify and regulate P97. Yuan et al. [53] revealed that high salt stimulates P97 acetylation at Lysine⁶⁶³, leading to the recruitment of the deubiquitinase, ubiquitin specific peptidase 33 (USP33), and its degradation by the proteasome. Additionally, intracellular accumulation of abnormal proteins, including polyglutamines in neuronal cells, can induce acetylation of P97 [54]. This allows P97 to enter the nucleus and suppress transcription, reducing the amount of misfolded proteins [54]. Proteomic studies have revealed at least 14 Serines, 6 Tyrosines, 14 Threonines, and 22 Lysines as potential acetylation sites on P97 [26]. These residues overlap with other PTM sites, suggesting a competition among different PTMs to regulate P97's function and cofactor binding.

3.2 UBX-Domain Proteins

UBX-domain proteins are the largest class of P97 cofactors, with 13 of them playing a central role in regulating the P97-UFD1-NPL4 complex [35,55,56]. They bind directly to the N-terminal domain of P97 to facilitate substrate recruitment and processing. Fujisawa et al. [11] demonstrated that UBX-domain proteins, specifically UBXL7, Fas-associated factor 1 (FAF1), and FAF2, increase the unfolding capacity of the P97-UFD1-NPL4 complex by reducing the required length of ubiquitin chains on substrates [57] (Table 1, Ref. [7,9,18,33,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91]). Additionally, these UBX-domain proteins stabilize the interaction between UFD1 and NPL4, which allows the complex to engage with the K-48 polyubiquitin chains on substrates [11].

4. Keeping Things in Order: The Complexity of Molecular Housekeeping

The P97-UFD1-NPL4 complex participates in several cellular processes, such as ERAD, RAD, CAD, and cell cycle regulation. The complex extracts ubiquitinated substrates from different cellular compartments and delivers them to the proteasome for degradation, maintaining protein homeostasis and regulating key events like DNA repair and cell cycle progression [32,35].

4.1 Endoplasmic Reticulum Associated Degradation (ERAD)

The P97-UFD1-NPL4 complex is a major player in ERAD, which can be divided into three pathways (ERAD-L, ERAD-M, and ERAD-C); each distinguished by substrate location. ERAD-L targets proteins in the ER lumen, ERAD-M targets transmembrane proteins within the ER, and ERAD-C targets proteins on the cytosolic side of the ER membrane [92]. After these substrates are ubiquitinated and retro-translocated to the cytosol, they are broken down into smaller peptides by the proteasome.

The function of the P97-UFD1-NPL4 complex in the ERAD-UPS axis requires its interaction with various adaptor proteins, such as Cdc48-associated UBL/zinc finger protein 1 (Cuz1), HMG-CoA reductase degradation protein 1 (Hrd1), VCP nuclear cofactor family member 1 (VCF1), small VCP interacting protein (SVIP), ataxin-3 (ATX3), tyrosinase (C89R), and glycoprotein 78 (Gp78) (Table 1). In yeast, Cdc48 (the P97 homolog) interacts with Cuz1 via its ubiquitin-like (UBL) domain, preventing the accumulation of ubiquitin-tagged proteins on the proteasome and Cdc48, ensuring proper UPS function (Table 1) [61]. The P97-UFD1-NPL4 complex can also partner with Hrd1, an E3 ubiquitin ligase, to recognize and degrade misfolded and unfolded proteins (e.g., tyrosinase C89R) (Table 1) [7]. Derlin Der1-like family member 1 (Dfm1) regulates Cdc48-driven retrotranslocation during ERAD [93].

4.2 Ribosomal Associated Degradation (RAD)

Similar to ERAD, the P97-UFD1-NPL4 complex also facilitates the clearance of ubiquitinated, tRNA-linked nascent peptides from ribosomes, ensuring proper ribosome recycling. When ribosomes stall during translation, incomplete nascent proteins accumulate. The ubiquitin ligases, listerin E3 ubiquitin protein ligase 1 (Ltn1) and ubiquitin protein ligase E3 component n-recognin 1 (Ubr1), partner with the P97-UFD1-NPL4 complex to ubiquitinate nascent polypeptides for degradation, a process referred to as RAD (Table 1) [71,94].

4.3 Mitochondrial Associated Degradation (MAD)

Within the outer mitochondrial membrane, the P97-UFD1-NPL4 complex facilitates MAD to maintain mitochondrial homeostasis [95] (Table 1). Parallel to ERAD, the complex removes misfolded and damaged proteins from the mitochondrial outer membrane and delivers them to the proteasome for degradation. PTEN-induced kinase 1 (PINK1) is a protein responsible for monitoring mitochondrial health. When mitochondria are damaged, PINK1 accumulates on the outer mitochondrial membrane. The accumulation of PINK1 then triggers ubiquitination of damaged proteins and the phosphorylation and activation of Parkin, an E3 ubiquitin ligase, in a process known as the PINK1/Parkin pathway [96,97,98].

Table 1. Cellular functions of the P97-UFD1-NPL4 complex.

Cellular processes	Co-factors	Substrates	Functions	Experimental models	Reference
ERAD	SVIP	Ubiquitinated substrates	SVIP binds to P97 and downregulates gp78-mediated ERAD.	HEK 293T cells	[58,59]
	ATX-3	Ubiquitinated substrates	ATX-3 interacts with P97-bound substrates to shorten them, preparing them for proteasomal degradation.	HEK 293T cells	[60]
	Cuz1	Ubiquitin conjugates	Cuz1's UBL domain and Cdc48/P97's N-terminal domain interact to promote ERAD. Cuz1 prevents buildup of ubiquitin-tagged proteins on the proteasome and Cdc48/P97.	<i>S. cerevisiae</i>	[61]
	HRD1	Tyrosinase (C89R)	HRD1 regulates the degradation of its substrate, Tyrosinase (C89R).	HEK 293T cells	[7]
	gp78	Cluster of differentiation (CD3), α 1-antitrypsin Z (ATZ), and Cystic fibrosis transmembrane conductance regulator (CFTR) delta-F508	Hrd1-mediated ERAD facilitates the gp78-mediated ERAD pathway, and degrades CD3, ATZ, and CFTR delta-F508.	HEK 293T cells	[7]
	N/A	Major histocompatibility complex (MHC) class I heavy chains	US2 recruits P97 to facilitate the translocation of MHC class I heavy chains from the ER to cytosol for its degradation.	HEK 293T cells	[62]
	Lethal (3) malignant brain tumor-like protein 1 (L3MBTL1)	N/A	RNF8 and RNF168 recruits P97 to remove L3MBTL1 and reveal the binding sites of 53BP1, allowing for DNA damage repair.	Mouse NIH2/4 cells, U2OS cells, and <i>C. elegans</i>	[63]
	N/A	Ubiquitin(K48)-conjugates	DNA lesions recruit P97 to remove K48-Ub conjugates from DNA damage sites, activating DNA repair mechanisms.	U2OS and HEK293T cells	[64]
	UBXD9	Cockayne syndrome group A and B (CSB)	P97 extracts ubiquitin-conjugated CSB from chromatin at DNA lesion sites and presents it to the 26S proteasome.	HCT116, HCT116-USP7 ^{-/-} , CS1AN, HeLa, and GM22764 fibroblasts	[65]
	UBXN7, Cullin 4 (CUL4)	Damage specific DNA binding protein 2 (DDB2), Xeroderma pigmentosum complementation group C (XPC)	Regulates the recognition and extraction of DNA damage subunits (DDB2 and XPC) from chromatin.	U2OS, HEK293T, and HeLa cells	[66]
	UBX4, UBX5, CUL3	Retinol binding protein 1 (Rbp1)	Cul3 ubiquitinates Rbp1 that is extracted from DNA damage sites and subsequently degraded.	<i>S. cerevisiae</i>	[67]
	N/A	Ku80	DNA-locked Ku70/80 complex is extracted during DNA double-strand break repair.	<i>X. Laevis</i> egg extract	[68]
	N/A	centromere-specific histone H3 variant (CenH3)	CenH3 is removed from centromeres, which regulates the release of rRNA genes into the nucleolus for ribosome production in plants.	Arabidopsis pollen vegetative cells	[69]
RAD	VCF1		VCF1 binds to P97 and facilitates UFD1-NPL4 interactions with ubiquitin conjugates.	U2OS and HEK293-EBNA1-6E cells	[70]
	N/A	N/A	The Cdc48/p97-Ufd1-Npl4 complex facilitate Ltn1 and Ubr1-tagged nascent polypeptides extraction and translocation from stalled ribosomes.	<i>S. cerevisiae</i>	[71]

Table 1. Continued.

Cellular processes	Co-factors	Substrates	Functions	Experimental models	Reference
MAD	Doa1	Cdc48/p97	Doa1 recognizes substrates and binds to the C-terminal region of Cdc48/p97 to recruit its cofactors, UFD1 and NPL4.	HEK293T, HGC-27, mice, and <i>in vitro</i> system	[72]
	WIPI2	VCP-UFD1-NPLOC4	WIPI2 recruits the VCP/P97-UFD1-NPLOC4 complex to damaged mitochondria, mediating the MAD pathway.	<i>in vitro</i> system	[73]
CAD	N/A	CDT1, CDC-45/GINS	CDC48/P97 breaks down CDT1 and releases the CDC-45/GINS complex from the replication fork of DNA.	<i>X. Laevis</i> egg extracts	[74]
	N/A	LexA-VP16, methionine requiring protein 4 (Met4), receptor- regulated Smads (R-Smads)	Cdc48/p97-Ufd1-Npl4 inhibits transcription by extracting LexA-VP16 from DNA.	<i>S. cerevisiae</i>	[75]
	UBXN-3/FAF1	CDT1	UBXN-3/FAF1 coordinates DNA replication fork progression by promoting CDC-48/p97-dependent turnover of replication factor complexes.	<i>C. elegans</i>	[76]
	UFD1	Sumo-Rad52	Cdc48/p97-Ufd1 binds to sumoylated Rad52, leading to the removal of RAD52-RAD51 from chromatin.	<i>S. cerevisiae</i> , U2OS cells	[77]
Cell cycle regulation	N/A	CDC25A	Proteasomal degradation of CDC25A, activating the G2/M checkpoint.	HeLa, HCT116 cells	[78]
	N/A	Decapping and exoribonuclease (DXO)	NPL4 promotes the degradation of DXO and upregulates cell proliferation.	T24 (Bladder cancer) cells	[79]
	N/A	N/A	Binding to spindle assembly factors (XMAP215, TPX2, and PIX1) regulates spindle disassembly.	<i>X. Laevis</i> egg extracts and <i>S. cerevisiae</i>	[80]
	N/A	Condensin	Cdc48/p97-Ufd1-Npl4 releases condensin, which regulates chromosome decondensation.	<i>S. cerevisiae</i>	[81]
Nuclear envelope assembly	N/A	N/A	P97-P47 facilitates nuclear envelope strengthening and growth.	<i>X. Laevis</i> egg extracts	[82]
	N/A	Aurora B	P97 mediates Aurora B deactivation, its removal from chromatin, and stimulates nucleus reformation.	<i>C. elegans</i>	[83]
	UBXD1 w/out UFD1-NPL4	K48-Ub proteins	P97-UBXD1 activity modulates the accumulation of K48-Ub and FG-nucleoporins (FG-Nups).	Torsin-deficient and WT HeLa cells	[84]
	P47	N/A	P47 forms a complex with P97 to promote and facilitate nuclear envelope growth.	<i>X. Laevis</i> egg extracts	[18]
Post-mitotic golgi membrane fusion	P47	Syntaxin 5 (SYN5)	SYN5 recognition enables VCIP135-mediated deubiquitination and SNARE complex formation.	HeLa cells	[85,86]
	VCPIP1, P47	Soluble NSF attachment protein receptors (SNARE)	VCPIP1 associates with P97 to promote its binding to SNARE substrates for golgi fusion	A2058, HEK293T, and HeLa cells	[87,88]
DNA replication	UBXN-3	CMG helicase	Binding to UBXN-3 facilitates CMG removal from DNA at the end of replication.	<i>C. elegans</i>	[89]

Table 1. Continued.

Cellular processes	Co-factors	Substrates	Functions	Experimental models	Reference
DNA replication termination	UBXN7 (UBA-UBX)	MCM7	UBXN7 binding regulates MCM7 recognition, complex assembly, and function.	HeLa Flp-in TRex (HFT) cells	[33]
	N/A	Centromeric histone 3 (CenH3)	Binding to CenH3s removes them from centromeres, regulating the termination of DNA replication.	<i>A. thaliana</i>	[69]
DNA damage repair	Rfp, Pli1	Sumo/Ubiquitin conjugates	UFD1 interacts with STUbLS, facilitates the removal of sumoylated proteins, regulating genome stability and repair.	<i>S. pombe</i>	[9]
		Sumo/Ubiquitin conjugates	Complex binding to sumo, which allows STUbLS-tagged substrate recognition and regulates genome stability.	<i>S. cerevisiae</i>	[90]
Autophagy	UFM1	VCP/P97	UFM1 attaches to VCP/P97 via UFMylation to stabilize Beclin-1, regulating autophagy.	HEK293 cells and HeLa cells	[91]

UFD1, ubiquitin fusion degradation 1; NPL4, nuclear protein localization 4; ERAD, endoplasmic reticulum-associated degradation; SVIP, small VCP interacting protein; ATX-3, Ataxin-3; Cuz1, Cdc48-associated UBL/zinc finger protein 1; HRD1, HMG-CoA reductase degradation protein 1; gp78, glycoprotein 78; N/A, not applicable; UBXD9, UBX domain-containing protein 9; VCF1, VCP nuclear cofactor family member 1; RAD, ribosome-associated degradation; MAD, mitochondrial-associated degradation; CAD, chromatin-associated degradation; UBA, ubiquitin-like modifier activating enzyme; UFM1, ubiquitin-fold modifier 1; VCP, valosin-containing protein; MCM7, minichromosome maintenance complex component 7; NSF, N-ethylmaleimide-sensitive factor; CDT1, chromatin and DNA licensing factor 1; Cdc48, cell division cycle 48; HEK, human embryonic kidney.

In yeast, Doa1 (also known as ufd3) plays an important role in regulating substrate recognition and recruitment of the Cdc48/p97-Ufd1-Npl4 complex to the mitochondrial outer membrane [99]. Mechanistically, Doa1 recognizes ubiquitinated substrates through its WD-domain, followed by its binding to the C-terminal region of Cdc48/p97 (Table 1). This signals the recruitment of cofactors, Ufd1 and Npl4, to form the Cdc48/p97-Ufd1-Npl4 complex [72,99]. In addition, Lu et al. [73] demonstrated that WD repeat domain phosphoinositide-interacting protein 2 (WIPI2), a key protein for initiating autophagy, can enhance the recruitment of the P97-UFD1-NPLOC4 complex, which ultimately mediates MAD and mitophagy (Table 1).

4.4 Chromosomal Associated Degradation (CAD)

CAD maintains genome integrity by targeting chromatin-associated proteins, including ubiquitinated regulators of DNA replication or transcription factors, for proteasome-mediated degradation [100,101]. Prior to DNA replication, chromatin and DNA licensing factor 1 (CDT1) facilitates the formation of the pre-replication complex (PRC) by recruiting the minichromosome maintenance complex component 2-7 (MCM2-7) helicase to the origin recognition complex, Cdc6, Cdt1, and Mcm2-7 (OCCM) complex, which includes the origin replication complex and the initiator protein CDC6 [102,103]. The PRC is activated through phosphorylation by cyclin-dependent kinase 2 CDK2/cyclin E, CDK2/cyclin A, and Dbf4-dependent kinase, leading to the formation of the active CDC45-MCM-GINS (CMG) helicase and the initiation of DNA unwinding [104,105] (Table 1). When DNA damage occurs, CDT1 is ubiquitinated by UBXN-3/FAF1. The P97-UFD1-NPL4 complex subsequently targets CDT1 for degradation, safeguarding cells against replication errors [76,106]. In *C. elegans*, Ubiquitination of Mcm-7 in the CMG helicase triggers the recruitment of Cdc48/P97, Ufd1, Npl4, and Ubxn-3, enabling the unfolding and degradation of Mcm-7 and the disassembly of CMG [89] (Table 1). In addition, Ubxn7 enables substrate recognition and unfolding via its attachment of polyubiquitin chains to the P97-UFD1-NPL4 complex, facilitating replication termination (Table 1) [56,107]. Interestingly, Ndoja et al. [108] suggested a non-proteolytic pathway in CAD that regulates gene transcription. They demonstrated in budding yeast that mono-ubiquitinated LexA-VP16, a transcription activator, can recruit the Cdc48/p97-ufd1-npl4 complex to inhibit transcription without undergoing degradation [108].

4.5 Cell Cycle Regulation

Outside the nucleus, the P97-UFD1-NPL4 complex controls cell division through the timely removal of key cell cycle regulators. Among them, CDC25 dephosphorylates and activates CDK2/cyclin E and CDK2/cyclin B complexes, promoting the G1/S and G2/M transition

[109,110,111,112]. In response to DNA damage, the Skp1-Cullin 1-F box protein complex works in tandem with the P97-UFD1-NPL4 complex to target and degrade CDC25A, activating the G2/M checkpoint to prevent the mitotic entry of cells with DNA damage (Table 1) [78,113,114,115,116].

During mitotic entry, coordinated structural changes of chromatin must occur to ensure chromosome morphogenesis. Specifically, the ring-shaped condensin complex compresses chromatin using a loop extrusion mechanism [117,118]. In yeast, Cdc48/p97-ufd1-npl4 has been found to release condensins from chromatin entrapment (Table 1) [81]. After chromosome compacting, spindle fibers attach centromeres to mediate chromosome segregation during mitosis and meiosis [119,120]. Cao et al. [80] found that the Cdc48/P97-Ufd1-Npl4 complex binds directly to spindle assembly factors, xenopus microtubule associated protein (Xmap215), targeting protein for Xklp2 (Tpx2), and proteome of centriole protein (Pix1), and regulates their interactions with microtubules, leading to spindle disassembly and mitotic exit (Table 1).

During mitotic exit, P97 binds and inactivates ubiquitinated Aurora B, a serine/threonine kinase that enables the attachment of the mitotic spindle to the centromere, leading to subsequent chromatin condensation and nuclear envelope formation (Table 1) [83]. P97 works with its cofactors to sequentially regulate nuclear envelope assembly: sealing the nuclear envelope to enclose chromosomes by the P97-UFD1-NPL4 complex and driving nuclear envelope growth by the P97-P47 complex (Table 1) [82].

4.6 Autophagy Regulation

Besides its prominent role in protein quality control, P97 can also coordinate DNA damage repair and autophagy regulation through multiple mechanisms. During autophagy initiation, P97 stabilizes Beclin-1, a master regulator of autophagy, through ATXN3-dependent deubiquitination, thus promoting Beclin-1-PI3K complex I assembly and activity. Successful assembly of this complex enables the production of phosphatidylinositol 3-phosphate, a signaling lipid that recruits autophagy machinery and supports autophagosome formation [91]. To efficiently stabilize Beclin-1, P97 is modified at K109 through the attachment of ubiquitin-fold modifier 1 (UFM1) by UFM1-specific ligase 1, a process known as UFMylation. Interestingly, impaired UFMylation is associated with P97 mutations in multiple types of neurological disorders and cancers [121]. Beyond autophagy initiation, P97 is also required for lysosomal positioning and autophagosome maturation. Pharmacological inhibition of P97 by kushenol E disrupts these steps, leading to the accumulation of immature autophagosomes and subsequent apoptosis [122]. During DNA damage repair, P97 serves as a key executor by binding to the autophagy receptor testis expressed 264, which senses and processes TOP1cc DNA-protein lesions via selective autophagy [123]. In addition, both P47

and P37 were identified as P97 cofactors, with P47 stabilizing P37 to regulate P97's nucleocytoplasmic shuttling and autophagy [124]. Together, these studies highlight the critical role of P97 in integrating protein quality control, DNA damage repair, and autophagy regulation.

5. Human Diseases: A Messy House With a Dysfunctional P97-UFD1-NPL4 Complex

The essential function of the P97-UFD1-NPL4 complex in regulating protein turnover, stress responses, and genome stability makes it a guardian of cellular homeostasis. When dysregulated, diverse pathologies, such as neurodegenerative diseases, viral infections, and cancer, develop [14]. Gene mutations of the complex components can disrupt proteostasis and cause the accumulation of misfolded/unfolded proteins, leading to neuronal cell death and genetic disorders [125]. Conversely, overexpression of *P97*, *UFD1*, and/or *NPL4* enhances the function of this complex, promoting the degradation of tumor suppressors or misfolded/unfolded proteins to sustain the survival of stressed cancer cells.

5.1 Neurodegenerative Diseases

The discovery of mutations encoding *P97* not only shed light on the structural and mechanistic underpinnings of the P97-UFD1-NPL4 complex but also revealed its association with disease. Particularly, mutations in the N-terminal, D1 and D2 domains, and the D1-D2 linker of P97 have been identified in patients with multisystem proteinopathy (MSP or inclusion body myopathy with Paget's disease of bone and frontotemporal dementia, IBMPFD), familial amyotrophic lateral sclerosis (FALS), charcot-marie-tooth disease type 2Y (CMT2Y), and parkinson's disease [126,127,128]. These mutations reveal a multitude of structural differences between wild-type and pathogenic P97 [13,129,130,131,132,133].

MSP/IBMPFD is a multisystem disease affecting the muscles, bones, and brain. Patients commonly present with muscle weakness, large and weak bones, and dementia. More than 20 amino acid substitutions on P97, resulting from missense mutations, have been identified among these patients, all located in the N-terminal and D1 domains of the protein [13,134]. The most prevalent mutation is an Arginine to Histidine substitution at position 155 (R155H) in the N-terminal domain [135]. The R155H mutation induces deficiencies in the autophagy of lysosomes, and motor neurons with this substitution are unable to clear lysosomes [121,136]. Apart from the R155H mutation, the other MSP P97 mutants are able to consistently accelerate the unfolding rate of this segregase [137,138]. While these mutants increase P97 function, they do not change the abundance of the P97-UFD1-NPL4 complex. Instead, they enhance the binding affinity of P97 to the UFD1-NPL4 heterodimer [137]. In addition, R95G, R155H, R191Q, L198W, A232E, and T262A mutations weaken the ability of P97 to bind to

ADP [139,140]. Structural and biochemical studies suggest that this can destabilize the "down" conformation at the N-terminal, which can increase its ATPase activity [141,142]. Pathogenic mutations associated with IBMPFD, including R155H, L198W, and A232E, can increase the interaction between P97 and UFD1-NPL4, thereby enhancing substrate recognition and processing [143].

FALS is a neurodegenerative disease affecting motor neurons both in the brain and spinal cord. While the majority of ALS cases are sporadic, 10% of FALS cases harbor mutations in *P97* and other 17 genes [126,144]. Early sequencing studies revealed that mutational variants clustered in the N-terminal domain and the D1 ATPase region of P97, such as R155H and R191Q, may interfere with the movement of these regions after ATP hydrolysis [126,144]. Klickstein et al. [136] demonstrated that the R155H mutation disrupts lysophagy in motor neurons.

CMT2Y is an autosomal dominant genetic disorder characterized by muscle weakness, atrophy, and sensory loss. This disease is caused by a glutamic acid to lysine substitution at the amino acid 185 site (E185K) on P97, residing within the linker between the NTD and the D1 ATPase domain [132]. This mutation does not impact the ATPase activity of P97 [132]; however, it increases levels of LC3II, similar to other P97 mutations that disrupt autophagosome maturation [132,145]. These disease-associated *P97* mutations may cause the protein's failure in maintaining axonal integrity, leading to progressive neuronal dysfunction and neurodegenerative diseases. The fact that a variety of neurodegenerative syndromes trace back to *P97* mutations shows how delicately P97's activity is balanced for neurons to survive, making the P97-UFD1-NPL4 complex both a vulnerability and a focus for therapies aimed at restoring their function.

5.2 Genetic Disorders

22q11.2 deletion syndrome, also referred to as CATCH 22 syndrome, encompasses several distinct conditions, including DiGeorge syndrome, velocardiofacial syndrome, conotruncal anomaly face syndrome, autosomal dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome [146]. These syndromes present a variety of developmental abnormalities, including cardiac defects, abnormal facies, T-cell deficit, cleft palate, and hypocalcemia. Additionally, it was thought that these defects were a result of impaired neural crest development. The strong expression of UFD1 in developing facial and ocular structures supports its potential role in regulating normal craniofacial and cardiac morphogenesis [147]. *UFD1* haploinsufficiency has been associated with heart and craniofacial defects similar to those observed in 22q11 deletion syndrome [147,148]. Mutations or deletions of *UFD1* within the 22q11.2 locus have been linked to impaired proteostasis, abnormal cell cycle regulation, and altered neuronal differentiation [148,149]. However, these heart and craniofacial

phenotypes are also observed in patients without detectable rearrangements or mutations in *UFD1*, indicating that it is not the sole genetic contributor [148,149].

5.3 Viral Diseases

Several viruses directly exploit the P97-UFD1-NPL4 complex to enhance their replication and evade host defenses [12,150]. In HIV, the accessory protein Vpu utilizes UFD1 and NPL4 to recognize ubiquitinated CD4, hand it to P97, and promote its degradation by the proteasome [151,152]. Polyomaviruses, such as simian vacuolating virus 40 (SV40), mimic misfolded ERAD substrates during entry to host cells, tricking the ERAD machinery to extract viral capsid proteins and enable its genome release [153,154,155]. Recently, it was found that classical swine fever virus (CSFV), a flavivirus, uses VCP for viral uncoating and CSFV core protein degradation [156]. Flaviviruses, such as Japanese encephalitis virus and dengue virus, recruit NPL4 to bridge a viral nonstructural protein, NS4B, to P97 [157]. The P97/NPL4 complex promotes stress granule disassembly in infected cells, thereby ensuring continued viral protein synthesis [157].

Beyond the examples above, many viral families utilize P97 to support their life cycle. It has been demonstrated that endosomal trafficking in certain viruses is dependent on P97 mainly through its binding to syntaxin 5 and other fusion proteins [158,159]. Flaviviruses, including Zika, West Nile, and Yellow Fever viruses, are known to use P97 in the early stages of viral infection [160,161,162]. In mosquito cells, P97 interacts with the capsid proteins of Zika virus and facilitates early infection [163,164]. An RNAi screen using green fluorescent protein (GFP)-tagged Sindbis virus, an alphavirus, demonstrated that VCP/P97 and protein transport protein sec16 (SEC16A) were required for efficient infection of *Drosophila* cells [164,165,166].

P97 plays an important role in viral RNA and DNA replication by assembling replication factories, regulating viral nonstructural proteins, and processing host/viral protein complexes [167]. In alpha viruses, inhibition of P97 blocks RNA replication after their entry into host cells without affecting initial RNA templates [168]. Knock-down or inhibition of P97 in flaviviruses reduces viral RNA levels and disrupts replication factory assembly [160,169,170,171]. In picornaviruses (Enterovirus 71 (EV71) and poliovirus), P97 co-localizes with replication proteins and supports organelle assembly [172,173]. EV71 can inhibit ERAD, cleaving factors such as ubiquitin conjugating enzyme E2 6 (Ubc6e) and Ubx8, while still requiring P97 ATPase activity. In DNA viruses, P97 is essential for RNA splicing, replication compartment formation, and capsid trafficking. Baculoviruses similarly rely on P97 for DNA replication and protein production [174,175]. Taken together, the P97-UFD1-NPL4 complex is hijacked by viruses to remodel membranes, degrade host restriction factors, and bypass stress responses, promoting viral repli-

cation. Hence, selectively targeting the complex or disrupting its virus-specific interactions offers a promising therapeutic avenue to treat infection.

5.4 Cancer

Cancer cells are reliant on elevated protein quality control to survive chronic proteotoxic stress due to heightened protein synthesis. As such, cancer cells frequently upregulate *P97*, *UFD1*, and/or *NPL4* and co-opt the P97-UFD1-NPL4 complex to maintain protein homeostasis, and disruption of its function tips the balance toward unresolved proteotoxic stress, apoptosis, and tumor suppression [176,177].

P97 is frequently overexpressed across tumor types, including breast, non-small cell lung cancer, clear cell renal cell carcinoma, and colorectal cancer, where it functions as a driver of aggressiveness and therapeutic resistance [178,179,180,181,182,183,184,185,186,187,188,189,190]. In breast cancer, high *P97* mRNA levels predict shorter overall survival, with the strongest association in triple-negative breast cancer patients, a subgroup lacking targeted therapies and with intrinsically high proteotoxic stress [191]. Zhang et al. [192] observed that both P97/VCP and E74-like factor 2/new Ets-related factor (ELF2/NERF) are upregulated in clinical breast cancer specimens, which is associated with poor patient survival. In hematological malignancies, such as B-cell lymphomas, high *P97* expression is associated with shorter overall and disease-free survival [193]. Importantly, pharmacologic inhibition of P97 not only induces apoptosis but also suppresses lymphoma invasion and metastatic potential.

UFD1 is broadly overexpressed across cancers with variable levels between tissue types; however, relatively few studies have investigated the upstream mechanisms driving its expression in these cells [194,195]. In T-cell acute lymphoblastic leukemia (T-ALL), *UFD1* is transcriptionally upregulated by MYC and drives leukemia aggressiveness [196]. Genetic inactivation of *UFD1* disrupts ERAD, amplifies ER stress, and triggers apoptosis in T-ALL cells [196]. In breast cancer, post-transcriptional regulation of UFD1 further promotes tumor progression: the RNA-binding protein, poly (rC) binding protein (PCBP2), directly interacts with the 3' untranslated region of *UFD1* mRNA to enhance its expression, revealing how cancer cells exploit RNA stability mechanisms to upregulate this essential adaptor [197]. After implantation of the MCF-7 breast cancer cell line and subsequent radiation, Kul Köprülü et al. [198] observed significant upregulation of ERAD pathway genes, including UFD1, indicating that radiotherapy may alter the ERAD pathway.

NPL4 is overexpressed in various cancers, such as bladder cancer and clear cell renal cell carcinoma, where high expression correlates with poor prognosis and more aggressive tumors [79,199]. Mechanistically, NPL4 not only coordinates substrate delivery to P97 but also regu-

lates protein turnover that is essential for rapid cancer cell proliferation. Pharmacologic blockade of NPL4 causes an accumulation of insoluble protein aggregates, collapse of proteostasis, and apoptosis, highlighting its indispensable role in sustaining malignant growth [200]. The central role of the P97-UFD1-NPL4 complex in enabling cancer cells to endure proteotoxic stress and sustaining malignant growth highlights it as a compelling therapeutic target.

6. Therapeutic Targeting of the P97-UFD1-NPL4 Complex

P97, UFD1, and NPL4 are often overexpressed in various tumor contexts [196]. Strategies aimed at disrupting its function, through inhibiting the ATPase activity of P97 or by interfering with cofactor binding, have been tested in preclinical and clinical studies (Table 2, Ref. [201,202,203,204,205,206,207,208,209,210,211,212,213,214,215,216,217,218,219,220,221,222,223,224,225,226]).

6.1 Targeting P97

Enhanced P97 expression has been shown to predict disease aggressiveness and poor prognosis in multiple cancers [227]. Early studies revealed that siRNA knockdown of P97 causes unmanageable ER stress, triggering the unfolded protein response and subsequent apoptosis in HeLa cells [228]. This finding led to the hypothesis that P97 inhibition could selectively kill cancer cells with heightened protein synthesis. Subsequently, many generations of P97 inhibitors have been developed, although few have made it to clinical trials [229].

6.1.1 ATP-Competitive Inhibitors

P97 hydrolyzes ATP in its D1 and D2 domain; compounds that bind to either domain can inhibit ATP hydrolysis. DBEq (N2,N4-dibenzylquinazoline-2,4-diamine) was discovered during a high-throughput screening based on ATPase activity (Table 2), but it acts at a μM dose [202]. The development of more potent ATP-competitive inhibitors soon followed. ML-240 and ML-241 both inhibit ERAD; however, ML-240 also inhibits autophagy [203] (Table 2). ML-240 exhibited broad antiproliferative activity in a panel of 60 cancer cell lines and synergized with the proteasome inhibitor MG132 in colon cancer. It also shows a promising safety profile in porcine, macaque, and human retinal explants [230]. FQ393 has similar structural units found in DBEq, both containing quinazoline and phenyl-methanamine [201] (Table 2). FQ393 inhibited ATP hydrolysis in a dose-dependent manner and prohibited proliferation in a variety of cancer cell lines. Despite being valuable research tools, these compounds lack sufficient potency and pharmaceutical properties required for clinical applications [201].

CB-5083 was identified as a P97 inhibitor through the optimization of DBEq and ML240 (Table 2). *In vivo* and *in vitro* studies confirmed that CB-5083 binds to the D2 do-

main of P97 to inhibit its ATPase activity with outstanding cellular potency and bioavailability [222,231,232]. Importantly, CB-5083 treatment not only decreases cell viability, but it also synergizes with other treatments, including histone deacetylase (HDAC) inhibitors and ionizing radiation [219,233,234,235,236]. Due to its promising preclinical data, Cleave Biosciences took CB-5083 into Phase I clinical trials in 2014 to evaluate the safety, toxicity, and maximum tolerated dose in patients with lymphoid hematological malignancies (NCT02223598). In 2018, CB-5083 was tested in Phase I clinical trials to study the efficacy in patients with refractory, advanced, metastatic solid tumors (NCT02243917). Interestingly, when CB-5083 was delivered together with immunomodulators imiquimod and miR-142 using nanoparticles, it resulted in immunogenic cell death and reprogramming of the tumor microenvironment [237] (Table 2). In colorectal cancer, treatment with CB-5083 enhanced immunotherapy response [238]. These findings suggest a role of P97 in tumor immunity regulation. Unfortunately, clinical testing for CB-5083 was terminated due to adverse effects on vision, such as photophobia and dyschromatopsia. In 2021, a study was conducted using *in vivo* and *ex vivo* electroretinography and demonstrated that treatment of CB-5083 inhibits phosphodiesterase (PDE)-6, which mediates signal transduction in photoreceptors [220]. Although this effect on vision is potentially reversible, tumors treated with CB-5083 can quickly develop resistance due to gatekeeper mutations [239].

Owing to CB-5083's unsatisfactory results in clinical trials, Cleave Therapeutics subsequently developed its second-generation analog, CB-5339 (Table 2). This analog has 15-times less activity in PDE6 biochemical assays with 30-times decreased activity on rat retinas compared to CB-5083 [223]. In pet dogs bearing naturally occurring tumors, CB-5339 had minimal adverse effects, with a few mainly in the gastrointestinal system. Pharmacokinetic and pharmacodynamic data indicate a correlation between exposure and the modulation of targets associated with the induction of the unfolded protein response, but not with the agent's tolerability [240]. CB-5339 entered Phase I clinical trials in 2020 to test its safety in patients with solid tumors or lymphomas (NCT04372641), but it was soon withdrawn. CB-5339 was also evaluated for its treatment of acute myeloid leukemia and myelodysplastic syndrome (NCT04402541). Although this clinical trial was completed, its clinical application remains uncertain. Using the 5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine core of CB-5339, Wang et al. [204] designed and synthesized compound V12 and metabolite V13, with V12 demonstrating *in vivo* and *in vitro* antitumor efficacy.

6.1.2 Covalent Inhibitors

Covalent inhibitors form a stable, irreversible bond with P97, disrupting its function and offering potential therapeutic benefits. Eeyarestatin (EerI), one of the co-

Table 2. Inhibitors targeting the P97-UFD1-NPL4 complex.

Type	Inhibitors	Stages of development	Mechanisms of action	References
P97				
ATP competitive	FQ393	Preclinical	Binds active site of P97 D2 catalytic domain	[201]
	DBeQ		Binds active site of P97's D2 catalytic domain; known to be reversible	[202]
	ML-240		Binds active site of P97's D2 domain	[203]
	ML-241		Binds active site of P97 D2 domain	[203]
	V2		Binds active site of P97 D2 domain	[204]
Covalent	Eeyarestatin I	Preclinical	Binds the D1 catalytic domain	[205,206]
	NMS-859		Modifies P97/VCP at Cysteine522 blocking ATP binding; known to be irreversible	[207]
	Dehydrocurvularin		Binds Cysteine522 in D2 ATP-binding pocket	[208]
	Withaferin A		Binds Cysteine522 in D2 ATP-binding pocket	[209]
	PPA		Binds Cysteine522 in D2 ATP-binding pocket	[210]
	UTE-156		Binds Cysteine522 in D2 ATP-binding pocket	[211]
Allosteric	NMS-873	Preclinical	Binds in a lateral tunnel between D1 and D2 domains of adjacent protomers, connecting nucleotide pockets	[212]
	NMS-862		Binds in a lateral tunnel between D1 and D2 domains of adjacent protomers, connecting nucleotide pockets	[207]
	MSC1094308		Binds the D2 domain; reversible	[213]
	UPCDC30245		Binds at the junction between the D1 and D2 domain	[214,215]
	NSC819701		Binds interface between D1 and D2 domains of adjacent P97 monomers	[216]
	NSC799462		Binds interface between D1 and D2 domains of adjacent P97 monomers	[216]
	NSC804515		Binds interface between D1 and D2 domains of adjacent P97 monomers	[216]
	UPCDC30766		Binds interface between D1 and D2 domains of adjacent P97 monomers	[217]
	Compounds 25 and 38		D2 domains of adjacent P97 monomers	[218]
ATP competitive	CB-5083	Clinical	Binds the nucleotide-binding pocket of D2 domain	[219,220,221,222]
	CB-5339		Binds the nucleotide-binding pocket of D2 domain	[223]
NPL4				
NPL4 competitive	Thonzonium Bromide	Preclinical	Binds Tyrosine143 of P97	[224]
NPL4 aggregator	Disulfiram	Clinical	Converts to CuET that binds NPL4 to induce its aggregation	[225]
UFD1				
UFD1 competitive	NBM Peptide (IPNYEFKLGKITFIRNSR)	Preclinical	Competes with endogenous UFD1 for its binding to NPL4	[226]

valent inhibitors for P97, targets its D1 domain and effectively inhibits the translocation of ER substrates (Ta-

ble 2). Lactones, including curvularins and steroidal lactones, have been shown to inhibit cell division [241]. De-

hydrocurvularin (DHC) and its monochloro variant interfere with both the proteasome and P97, while its dichloro analogue demonstrates P97 specificity (Table 2). DHC interacts with the D2 ATP-binding pocket of P97 and increases the amount of the ERAD substrate, T-cell receptor α [242]. Besides lactones, pyrimidines (e.g., PPA) can inhibit the proliferation of HCT116, HeLa, and RPMI8226 cell lines, as well as those resistant to CB-5083 (Table 2) [210]. Using a structure and fragment-based screening, Tamayo-Jaramillo et al. [211] developed scaffolds positioned within the nucleotide binding pocket of Cysteine 522 of the D2 ATPase domain. Through further optimization, UTE-156 was found to bind Cysteine 522 and inhibit the ATPase activity of VCP [211] (Table 2).

6.1.3 Allosteric Inhibitors

The first allosteric inhibitor of P97, NMS-873, was discovered during a high-throughput screen of covalent and allosteric modifiers of P97 (Table 2) [243]. The chemical expansion of an allosteric class of inhibitors yielded NMS-873, which maps into the regions spanning the D1 and D2 domains of adjacent P97 protomers. NMS-873 activated the unfolded protein response and induced cancer cell death with varied potencies (Table 2) [207,244]. The allosteric compound, MSC1094308, is a reversible inhibitor with less specificity than NMS-873 (Table 2) [207,213]. MSC1094308 targets predominantly the D2 domain of P97, leading to inhibition of proteolysis and the accumulation of polyubiquitinated proteins. More recently, Green et al. [218] developed *in vivo* active allosteric compounds demonstrating oral bioavailability and moderate efficacy in inhibiting tumor growth in xenografts (Table 2).

6.2 Targeting NPL4

Despite the advances in understanding the role of the P97-UFD1-NPL4 complex in malignant diseases, much of the effort in developing therapeutics has been directed against P97. Disulfiram (tetraethylthiuram disulfide, DSF) is an FDA-approved medication for alcohol-use disorder; its applications have recently been expanded to include cancer (Table 2). DSF has shown anti-cancer activity in pre-clinical models and has been used in a clinical trial for the treatment of breast cancer (NCT03323346). DSF penetrates cancer cells and chelates intracellular copper (Cu). Many cancers have elevated copper levels, alluding to its anti-cancer effects [245,246,247]. The generation of DSF-Cu complexes induces phenotypic features similar to proteasome inhibitors, such as Bortezomib, resulting in the inhibition of NF- κ B [245,248]. Treatment of DSF prevents the degradation of CDC25A and the cleavage of nuclear respiratory factor 1 (NRF1), both known as P97 targets, suggesting that the P97 pathway is compromised. DSF treatment has been shown to immobilize NPL4 in nuclear clusters and cytoplasmic areas. Immobilized NPL4 attracts ubiquitinated proteins, leading to the co-localization with

heat shock protein 70 (HSP70), induction of the cellular heat shock response, and eventually cell death [225]. In colorectal cancer cells, DSF was shown to promote autophagy in through proteasomal inhibition and transcriptional upregulation of beclin-1 [249].

6.3 Targeting the P97-NPL4 Interaction

In addition to identifying compounds that selectively inhibit each complex component, recent efforts seek to target their interactions. Through an amplified luminescent proximity homogenous assay of FDA-approved drugs, thonzonium bromide (TB) was identified as a selective inhibitor of the P97-NPL4 interaction [224] (Table 2). Nie et al. [224] indicate that the complex can promote the development of immune cells such as tumor-infiltrating T regulatory (TI-T_{reg}) and T helper 17 (TH17) cells, both of which suppress antitumor responses. Disruption of the P97-NPL4 interaction, by TB, can block TI-T_{reg} development while promoting TH17 development (Table 2). Interestingly, peripheral T_{reg} cells and T_{conv} cells, CD4⁺ T cells in particular, are not affected [224]. The exact mechanism by which the P97-NPL4 interaction regulates the development of TI-T_{reg} and TH17 cells remains unknown. Recent efforts in targeting TI-T_{reg} cells have focused on distinguishing TI-T_{reg} from T_{conv} cells [250]. The interaction between P97 and NPL4 has been implicated in TI-T_{reg} differentiation and recruitment. The amino acids F52, D55, and Y110 of P97 are essential for binding to NPL4; mutations in these residues abolish their interaction. TB interacts with Tyrosine¹⁴³ of P97 and inhibits tumor growth more than its ATPase inhibitor, CB-5083. Treatment with TB was able to enhance the tumor microenvironment, resulting in increased CD8⁺ T cells, B cells, monocytes, and macrophages.

6.4 Targeting the UFD1-NPL4 Interaction

Besides developing small molecule inhibitors, researchers also developed peptide inhibitors for targeting interactions within the P97-UFD1-NPL4 complex. As previously mentioned, UFD1 and NPL4 form a heterodimer before binding to P97 (Fig. 1A–C). Targeting the interactions within the complex is an understudied avenue. Using the interaction motif on UFD1 that binds to NPL4, encompassing residues 258–275, researchers designed peptide mimetics [226] (Table 2). These peptides prevent UFD1-NPL4 from binding to P97, inhibiting the unfolding activity of the complex [226]. Using peptides to probe and disrupt specific interfaces within the P97-UFD1-NPL4 complex offers valuable insight into how targeting distinct sites can differentially modulate its activity, guiding the development of more precise therapeutic strategies.

7. Summary and Future Perspectives

As the janitor of the cell, the P97-UFD1-NPL4 complex plays a central role in protein quality control. The complex maintains protein homeostasis by facilitating the

extraction of ubiquitinated substrates from various cellular compartments for proteasomal degradation, with ERAD as the representative pathway. P97 utilizes ATP hydrolysis to power the complex for substrate unfolding and retrotranslocation. UFD1 interacts with NPL4 to bind to P97 through its SHP-box motifs and UBX-like domains, respectively, aiding in substrate recognition. The P97-UFD1-NPL4 complex works synergistically in major proteolytic pathways. Apart from ERAD, the complex also regulates RAD, MAD, CAD, and other cellular processes, including DNA replication, cell cycle regulation, and DNA damage repair. PTMs, UBX-domain proteins, and ATP/ADP binding and hydrolysis regulate the interactions within the complex and their cofactors and their function.

Recent studies have shown that mutations of the complex components, *P97* in particular, lead to the accumulation of misfolded and unfolded proteins, which have been associated with neurodegenerative diseases, such as Paget's disease, FALS, and CMT2Y. In contrast, the enhanced function of the P97-UFD1-NPL4 complex mitigates cellular stress, allowing for cancer cell survival. Progress has been made to inhibit the P97-UFD1-NPL4 complex, with inhibitors primarily targeting the ATPase activity of P97 for the treatment of cancer. Despite their advancement to clinical trials, such inhibitors failed to progress further in clinical testing. Notably, a recent study identified a novel peptide inhibitor that disrupts the interaction between UFD1 and NPL4, and in turn disassembles the P97-UFD1-NPL4 complex. However, to develop therapeutics with minimal toxicity, we must distinguish the function of the P97-UFD1-NPL4 complex in normal versus pathological contexts. Selecting the most suitable strategy to target the complex will pave the way for the development of inhibitors that can specifically treat diseases, like cancer, without evoking systemic toxicities. Furthermore, understanding the molecular mechanisms that link P97-UFD1-NPL4 complex function with disease progression can serve as a guide to uncover biomarkers and inform targeted therapies.

Availability of Data and Materials

Not applicable.

Author Contributions

CK, MU, and HF conceptualize the work; CK, MU, and XQ wrote the manuscript; SM conceptualized and generated the illustrations; XQ brainstormed the manuscript and helped with Figures; and SM and HF edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

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