

## Review Article

# The AAA+ ATPase p97, a cellular multitool

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The AAA+ (ATPases associated with diverse cellular activities) ATPase p97 is essential to a wide range of cellular functions, including endoplasmic reticulum-associated degradation, membrane fusion, NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation and chromatin-associated processes, which are regulated by ubiquitination. p97 acts downstream from ubiquitin signaling events and utilizes the energy from ATP hydrolysis to extract its substrate proteins from cellular structures or multiprotein complexes. A multitude of p97 cofactors have evolved which are essential to p97 function. Ubiquitin-interacting domains and p97-binding domains combine to form bi-functional cofactors, whose complexes with p97 enable the enzyme to interact with a wide range of ubiquitinated substrates. A set of mutations in p97 have been shown to cause the multisystem proteinopathy inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia. In addition, p97 inhibition has been identified as a promising approach to provoke proteotoxic stress in tumors. In this review, we will describe the cellular processes governed by p97, how the cofactors interact with both p97 and its ubiquitinated substrates, p97 enzymology and the current status in developing p97 inhibitors for cancer therapy.

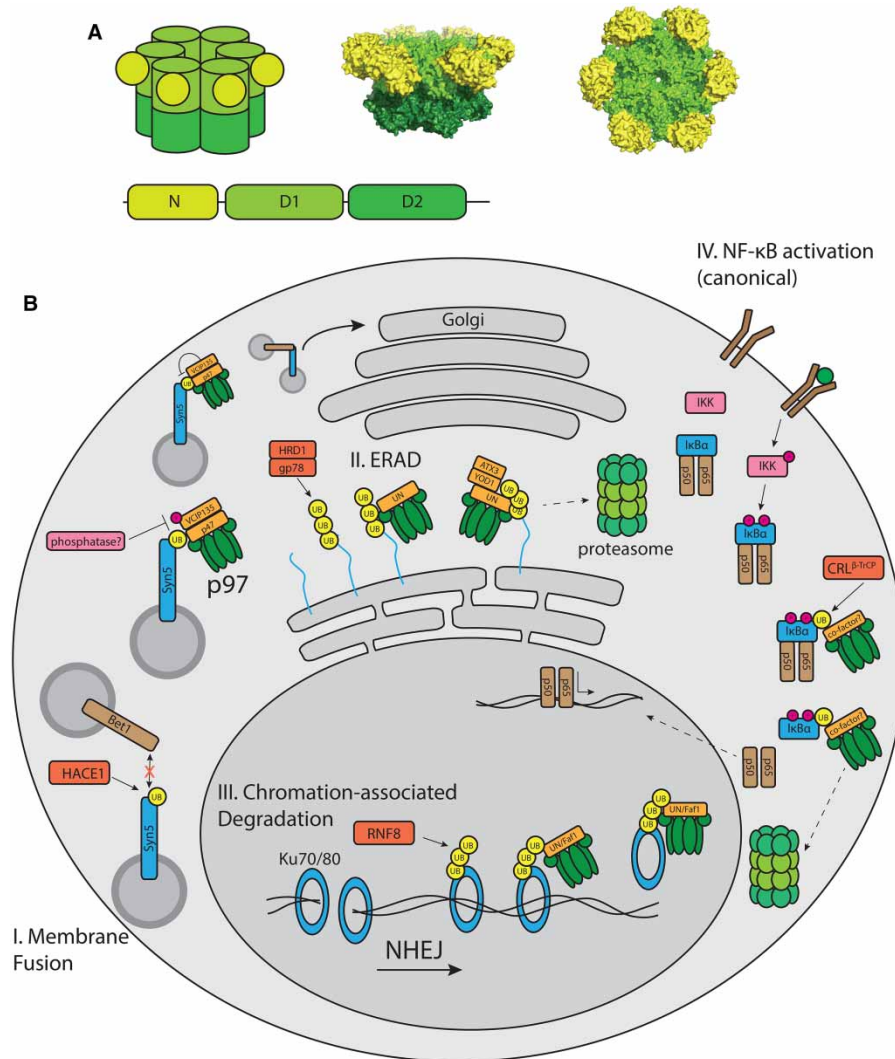
## Introduction

The human AAA+ (ATPases associated with diverse cellular activities) ATPase p97, also known as valosin-containing protein (VCP) and homologs Cdc48 (cell division cycle protein 48) in *Saccharomyces cerevisiae*, Ter94 (transitional endoplasmic reticulum ATPase) in *Drosophila melanogaster* and VAT (VCP-like ATPase) in *Thermoplasma acidophilum*, is highly conserved across all domains of life [1]. It contains an amino-terminal N-domain, two AAA ATPase domains and a C-terminal unstructured extension. Six p97 protomers form a homohexamer with two planar ATPase rings stacked on top of each other (Figure 1A) [2,3]. The enzyme performs many important cellular functions, highlighted by its conservation across diverse species and its abundance [4]. In *Xenopus leavis*, the protein makes up ~1% of cytosolic protein [5,6].

The cellular processes in which p97 is implicated include protein degradation via the ubiquitin proteasome system (UPS), DNA repair, DNA replication, cell cycle regulation, NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, endoplasmic reticulum-associated degradation (ERAD), mitochondria-associated degradation, Golgi formation and autophagy, reviewed in ref. [7]. In all of these processes, ubiquitination of p97 substrates is a common theme. The enzyme converts the energy of ATP hydrolysis into mechanical energy to extract ubiquitinated proteins from lipid membranes or macromolecular complexes. While p97 itself has a weak affinity for ubiquitin, it interacts with its targets primarily via cofactors. It is the variety of these cofactors that allow a single machine such as an ATPase to play a role in the diverse processes mentioned above, reviewed in ref. [8,9]. The large proportion of p97 cofactors identified so far are part of the ubiquitin system, either ubiquitin ligases or DUBs (deubiquitinases) or ubiquitin-binding proteins that function as adaptors [7]. Mutations in p97 have been associated with familial amyotrophic lateral sclerosis and inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD), a multisystem disease that is associated with abnormally active p97 on a

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**Figure 1. Cellular processes governed by p97.**

(A) Top: schematic of the p97 architecture, side and top views of the cryo-EM structure of ADP-bound p97 (PDB: 5FTK). Bottom: domain organization of p97, color-coded as above. (B) Well-characterized cellular process requiring p97 activity. (I) Post-mitotic membrane fusion via the p97/p47 pathway. (II) Endoplasmic reticulum-associated degradation. (III) Removal of KU70/80 from chromatin. (IV) NF- $\kappa$ B activation via the canonical pathway. Proteins are color-coded according to their function. p97 and the proteasome are in green, ubiquitinated p97 substrates are blue, cognate E3 ubiquitin ligases decorating p97 substrates are red, phosphorylation signaling proteins are in pink, ubiquitin is in yellow and other proteins are in brown.

molecular level leading to neurodegenerative defects and muscular weakness at the physiological level [10,11]. This review will provide an overview of the cellular processes p97 is involved in, what role its cofactors play in such processes, the molecular mechanism and regulation of the enzyme, as well as efforts to target p97 in cancer chemotherapy. Where possible, connections will be made between these different aspects to provide a more integrated picture.

## Cellular processes associated with p97

The p97 AAA ATPase has been found to be involved in a large variety of cellular processes. In this section, we will describe some of the main pathways and cellular functions that p97 has been directly implicated in.

## Endoplasmic reticulum-associated degradation

The ERAD pathway serves as a quality control system for nascent proteins entering the secretory pathway, a pathway in which the UPS and p97 play central roles (Figure 1B) [12]. Nascent peptide chains that fail to fold properly in the ER require retrotranslocation into the cytosol where they are degraded by the proteasome. It is in the energetically unfavorable retrotranslocation event in which p97 plays its role in ERAD [13,14].

Ubiquitin binding, ATP hydrolysis and p97 cofactor binding all play a role in retrotranslocation. The heterodimeric UFD1–NPL4 (ubiquitin fusion degradation protein 1 and nuclear protein localization protein 4 homolog) complex possess both ubiquitin-binding domains and p97-interacting motifs [15–18]. It recruits p97 to ubiquitinated substrates at the ER membrane [19]. Another ERAD-associated p97 cofactor is Ubx2 [20]. The protein also recruits p97 to the ER membrane, and specifically, it facilitates the interaction between p97 and Hrd1 (Hmg2-regulated degradation), a membrane-anchored ubiquitin ligase which ubiquitinates ERAD substrates and also contains a p97-interacting VBM (VCP-binding motif) [21–24].

The ubiquitin E3 ligase gp78 (also known as the RING-type E3 ubiquitin transferase autocrine motility factor receptor) and the DUBs Ataxin-3 (spinocerebellar ataxia type 3 protein) and YOD1 (also known as ubiquitin thioesterase OTU1 in yeast) are examples of p97 ubiquitin catalytic cofactors. The E3 ligase interacts with p97 through its VIM (VCP-interacting motif) domain and has been shown to act downstream from Hrd1 in the degradation of ERAD substrates [25–27]. The role of the DUB Ataxin-3 is less well defined. This protein contains a poly-glutamine (poly-Q) domain, which causes its aggregation in genetic variants where this sequence is extended [28]. Similar to gp78, Ataxin-3 also interacts with p97 through the arginine/lysine-rich VBM [29,30]. Functionally, the ubiquitin chain-trimming activity of this enzyme has been suggested either to slow down substrate processing [31] or to facilitate the transport of substrates from p97 to the proteasome [32]. The DUB YOD1, which binds to p97 via its UBX (ubiquitin regulatory X) domain, is also associated with ERAD [33]. Its role in ERAD was proposed to be the trimming of polyubiquitin chains to optimize p97 recruitment or the removal of ubiquitin to allow the substrate to be threaded through the p97 pore for unfolding [34].

The complexes at the ER membrane formed by p97 interact primarily with K11- and K48-linked ubiquitin chains. Upon disruption of p97 function or overexpression of a catalytically dead mutant of YOD1, the model ERAD substrate CD3 $\delta$  (T-cell surface glycoprotein CD3 delta chain), decorated with these types of polyubiquitin, accumulates in the cell [35].

## Chromatin-related functions of p97

The functions of p97 are not limited to extraction of substrates from lipid bilayers such as in ERAD, as p97 has also been shown to be essential in the extraction of proteins from chromatin (reviewed in ref. [36]). Chromatin-related processes where p97 is essential include the disassembly of protein complexes at sites of DNA damage and the unloading of stalled replication helicases. The regulation of DNA double-strand break repair is regulated by extensive post-translational modification events, particularly protein phosphorylation and ubiquitination, which facilitate the assembly of large multiprotein complexes essential to DNA repair [37,38]. Proteins that interact with both types of modification, such as the ubiquitin E3 ligase RNF8 (RING finger protein 8), which contains a pThr-binding FHA (forkhead-associated) domain, facilitate cross-talk between these modifications [39]. Ubiquitination plays a role in complex assembly and disassembly, a process where p97 is required [36]. The removal of proteins from chromatin by p97 is not only limited to the final disassembly of complexes, but also includes the removal of K48-ubiquitinated proteins from complexes to facilitate the binding of downstream factors. Recently discovered examples of protein extraction from chromatin by p97 will be introduced in the following section.

The Ku70/Ku80 (70/80 kDa subunit of Ku antigen) complex, which binds the open ends of DNA double-strand breaks in a first step towards break repair via non-homologous end joining, is a recently discovered p97 substrate (Figure 1B) [40,41]. The Ku70/Ku80 complex forms a ring-like structure with a central cavity for DNA [42]. Following joining of the DNA ends, the protein is trapped around the double helix like a hose clamp and forms a barrier to further processing of the break. Thus, the protein complex is decorated with K48-ubiquitin chains by RNF8, triggering the recruitment of p97 to the site and subsequent removal of Ku70/Ku80 from DNA [41]. There is some redundancy as to which cofactors play a role in this process. Both FAF1 (FAS-associated factor 1) and UFD1–NPL4 have been shown to be sufficient for this process, with UFD1–NPL4 being the more efficient [41].

In another study, it was shown that the presence of p97, recruited to DNA double-strand breaks by RNF8-generated K48 chains, is a prerequisite for efficient recruitment of downstream DNA damage response proteins BRCA1 (breast cancer type 1 susceptibility protein), 53BP1 and Rad51 [43]. This process is dependent on the NPL4 cofactor [43]. Disruption of p97 by the inhibitor NMS873, or RNA interference of p97, NPL4 was shown in both studies mentioned above to cause foci of DNA damage to persist, indicating that p97 in concert with UFD1–NPL4 is essential for efficient DNA damage repair [41,43].

Analogous to the extraction of Ku from DNA, p97 is also required for the removal of CMG replicative helicases (comprised of Cdc45, MCM2-7 and GINS), those stalled by interstrand cross-links as well as under physiological conditions [44,45]. The stalled replication fork is resolved via a ubiquitin-dependent process where p97 is essential [46]. Again, removal of a protein complex by p97 from chromatin is a prerequisite for access to the DNA lesion and appropriate repair [46]. As a response to a stalled fork, the MCM7 subunit of the helicase is decorated with K48-linked ubiquitin chains, which recruits p97 to extract the helicase. The E3 ubiquitin ligase CRL2<sup>Lrr1</sup> generates K48-ubiquitinated MCM7, which promotes p97 binding via its UFD1–NPL4 cofactor [47].

In *Caenorhabditis elegans*, an RNAi screen of UBX-containing proteins identified essential p97 cofactors [48]. Both UFD1 and NPL4 are essential for development. In addition, the silencing of FAF1 homolog UBXN3 (UBX-containing protein 3) compromises *C. elegans* survival rates, particularly in p97-depleted cells and those treated with the DNA-damaging agent hydroxyurea [48]. More specifically, UBXN3 binds CDT-1, a DNA replication licensing factor. While CDT-1 is required for replication initiation, it needs to be extracted from chromatin for replication completion. In the absence of p97, or the FAF1 or UFD1–NPL4 cofactors, CDT-1 remains bound to chromatin and severe replication defects are observed [48,49].

In addition to the examples mentioned above, p97 has also been shown to be central to numerous chromatin-related processes beyond the scope of this review, such as extraction of SUMOylated proteins from chromatin and Cockayne syndrome protein extraction to resolve stalled RNA polymerase [50,51], all comprehensively reviewed by ref. [36]. From the studies introduced above, it is apparent that p97 plays a role in the extraction of DNA-binding proteins from different types of DNA damage. The active removal of proteins from chromatin to facilitate access to sites of DNA damage for downstream repair factors, or to allow helicase and polymerase activity to proceed, is a central function of p97. The ATPase is therefore an essential factor in genome stability, reviewed by ref. [52].

## NF-κB activation

The transcription factor NF-κB controls the expression of cytokines, immunoreceptors and other components in the immune system (Figure 1B) [53]. Stimulation of Toll-like receptors or interleukin-1 receptors on the cell surface triggers a cell signaling event utilizing both protein phosphorylation and K63-linked ubiquitination, which leads to the release of NF-κB from the cytosol into the nucleus, where it can affect transcription [54].

In its basal state, the NF-κB heterodimer, consisting of proteins p50 and p65, is kept in an inactive state via association with the inhibitory protein IκBα (NF-κB inhibitor alpha) or related proteins [55]. For the transcription factor to be active, IκBα needs to be degraded, a process which is dependent on p97 [56]. As part of the signaling cascade, both p65 and IκBα become phosphorylated. Subsequent to phosphorylation, which is regulated by an unknown mechanism, the cullin-RING ubiquitin ligase (CRL) CRL1<sup>β-TrCP</sup> ubiquitinates IκBα and thus recruits p97 [57]. It has been shown that both a functional E3 ubiquitin ligase and active p97 are required for efficient degradation of IκBα and subsequently activation of NF-κB, indicating that p97 is essential for the degradation of ubiquitinated IκBα [57]. There is so far no evidence as to which p97 cofactors, if any, are essential in this pathway. However, the cofactors p47 and FAF1 have inhibitory effects on NF-κB activation [58,59].

## Membrane fusion

The ATPase p97 also plays a role in membrane fusion of most parts of the endomembrane system (Figure 1B). It has functions in the biogenesis of the ER, the Golgi, nuclear membrane assembly and in the fusion of lysosomes. The first cellular functions assigned to p97 were the membrane fusion events essential to Golgi and ER formation [60,61]. The cofactor required for formation of the Golgi, which undergoes disassembly and re-assembly during the cell cycle, was subsequently identified to be p47 [62]. This cofactor contains an N-terminal UBA (ubiquitin-associated) domain, which allows it to bind ubiquitin as well as a C-terminal UBX domain, which allows it to bind p97 [16]. Ubiquitination drives Golgi membrane dynamics [63]. The enzymes driving these ubiquitination events are the E3 ubiquitin ligase HACE1 (HECT domain and ankyrin repeat-



containing E3 ubiquitin protein ligase 1) and the DUB VCIP135 (VCP-interacting protein 135 kDa), which act on the t-SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor) Syn5 (syntaxin5) [64–66]. During early mitosis HACE1 ubiquitinates Syn5, which prevents the interaction of Syn5 with its corresponding v-SNARE Bet1 [66]. A multiprotein complex containing p97 and p47 binds the ubiquitinated SNARE via the UBA domain of p47. In late mitosis, the DUB VCIP135, which associates with p97 via its UBXL (UBX-like) domain, deubiquitinates Syn5, which permits interaction between the SNAREs, membrane fusion and finally the formation of Golgi cisternae [66].

VCIP135 activity is regulated by protein phosphorylation [67,68]. Phosphomimetic mutants at known VCIP135 phosphorylation sites prevent the association of VCIP135 with p97 [68]. More significantly, VCIP135 phosphorylated at Ser130 displays severely reduced DUB activity [65]. During early mitosis, phosphorylated VCIP135 is inactive and may not be able to bind p97. During late mitosis, VCIP135 becomes dephosphorylated and associates with the p97–p47–Syn5 complex. The active DUB then deubiquitinates Syn5, releasing the SNARE allowing membrane fusion.

This model for membrane fusion differs from the p97 functions described above, as p97 only functions as a scaffold, the role of its ATPase activity has not been fully investigated. There is some circumstantial evidence to support this theory. p47 inhibits p97 ATPase activity by up to 5-fold [69,70]. However, it has also been shown that ATP is required for the p97–p47–VCIP135 complex to dissociate from Syn5 [71].

The p97 cofactor p37, closely related to p47, has also been shown to play a role in Golgi formation. This cofactor contains the C-terminal UBX domain, but has no ubiquitin binding UBA domain [8]. The cofactor is also required for Golgi formation, but a siRNA knockdown of p37 does not affect ERAD [72]. The precise role of p37 in membrane fusion is not as well established as that of p47, but it has been shown that the process requires the tethering protein p115 and its binding partner GM130 [72].

p97 not only has a role in Golgi formation, but is also involved in the assembly of other parts of the endomembrane system. p97 plays a role in the assembly of the nuclear envelope and the ER membrane. The p97 cofactor UFD1 binds CHMP2A (charged multivesicular body protein 2a), a protein that is part of the membrane abscission ESCRT-3 (endosomal sorting complexes required for transport 3) complex. A knockdown of UFD1 prevents correct localization of CHMP2A to the nuclear envelope, suggesting that p97 plays a role in nuclear envelope formation [73]. Analogous to Golgi formation, p97 and its cofactors p47 and VCIP135 are essential to ER formation following mitosis [71].

The p97 cofactors p37, p47 and the DUB VCIP135 play essential roles in membrane fusion. Here, p97 appears to act as a scaffold primarily. While p97 ATPase activity may well have a role in membrane fusion, it has not been described. This is unlike the roles described earlier, where the mechanical energy generated from ATP hydrolysis was used for the extraction of proteins from chromatin, lipid bilayers and protein complexes. Conversely, the ATPase NSF, which is closely related to p97, has been shown to play a central role in membrane fusion. Its ATPase activity is used to disassemble SNAP (soluble NSF attachment protein)–SNARE complexes [74]. It remains to be determined which role p97 ATPase activity has in membrane fusion. Interestingly, it has been shown that p47 inhibits p97, while p37 activates p97 [70].

## Additional roles of p97

There are several additional functions of p97 which do not fall into any of the above categories. In addition to ERAD, p97 also plays a role in the degradation of proteins from the mitochondrial outer membrane. Silencing of the *cdc48* gene leads to a slowdown of protein turnover in the mitochondrial membrane and accumulation of ubiquitinated proteins [75]. Analogous to ERAD, the Cdc48–UFD1–NPL4 complex plays a role in mitochondrial degradation [75]. The cofactor Doa1 (degradation of alpha 1), a homolog of human PLAA (phospholipase A-2-activating protein), is essential in cells under mitochondrial oxidative stress, but not under ER stress [75]. This suggests that Doa1 is a cofactor specific to mitochondria-associated degradation.

In another degradation-associated function, p97 is essential for the degradation of aberrant, nascent proteins from ribosomes. mRNAs lacking stop codons lead to stalled ribosomes as the translated poly-A tail produces poly-Lys, which interacts with the ribosomes. The E3 ubiquitin ligase ltn1 (listerin 1) has been identified to ubiquitinate the nascent peptide on stalled ribosome [76,77]. A complex containing p97, UFD1 and NPL4 extracts the peptide from the ribosomes for proteasomal degradation [78].

Just like with many other cellular processes, viral proteins have evolved to take advantage of and hijack p97 function. The influenza A matrix protein M2 undergoes retrotranslocation from the ER lumen to the cytosol [79]. This transport mechanism of M2, which does not rely on M2 ubiquitination, is reliant on the ATPase

activity of p97 [79]. Hepatitis E virus protein ORF2 is another viral protein that relies on p97 to be transported from the ER into the cytosol [80]. In both cases, the protein is not readily degraded by the proteasome and remains in the cytosol for several hours.

The protein p97 also has a function in lysosomal trafficking, illustrated by the example of Cav-1 (caveolin-1), a protein which introduces invaginations in the plasma membrane called caveolae [81]. Overexpression of GFP-tagged Cav-1 causes its aggregation in aggresomes in the perinuclear region, which are cleared by a combination of lysosomal and proteasomal degradation. Inhibition of p97, which is found in large amounts in the aggresomes, prevents these structures from being cleared [81]. The lysosomal degradation pathway is also required for the clearance of endogenous Cav-1, which localizes correctly to caveolae [82]. This lysosomal pathway is dependent on p97 and a class of cofactors called Ankrd13 proteins, most of which contain ubiquitin-interacting motifs [82]. The Ankrd13 (ankyrin repeat domain-containing protein 13) UIMs (ubiquitin-interacting motifs) have been shown to bind preferentially to K63-linked ubiquitin chains [82].

## p97 and its cofactors

### Modular architecture of p97 cofactors

The ATPase p97 is central to a diverse array of cellular processes despite only exhibiting one enzymatic function, the generation of mechanical energy from ATP hydrolysis. The p97 cofactors and their different domain architectures and cellular localizations permit p97 to fulfill different roles depending on the context (Figure 2).

Furthermore, different p97 cofactors have specificity for different ubiquitin linkages. In polyubiquitin, the carboxyl-terminus of a distal ubiquitin is ligated to an amine on the proximal ubiquitin. Ubiquitin contains eight primary amines, seven lysine side chains plus its N-terminus, all of which can be used to form polyubiquitin. Depending on which amine is used, different ubiquitin chains are produced with discrete physical properties and cellular functions, reviewed in ref. [83].

The best characterized polyubiquitin types include K48-, K63- and M1-linked chains. Polyubiquitin linked via K48, the first linkage identified, is a powerful degradation signal [84]. Conversely, K63-linked chains do not constitute a degradation signal, but have a role in signal transduction [85]. Linear polyubiquitin, where a regular peptide bond is formed to link 2 ubiquitin molecules together, plays a role in NF- $\kappa$ B activation and apoptosis [86]. In addition, mixed chains can be formed which may be branched, and ubiquitin itself can be the target of post-translational modifications such as phosphorylation and acetylation [87–90], reviewed in ref. [91].

The cofactors can be divided into two groups depending on what part of p97 they interact with. The larger group of cofactors binds the p97 N-domain, via UBX (ubiquitin regulatory X), UBX-L, VIM, VBM or SHP (binding segment 1) motif, reviewed in ref. [8,9]. A smaller group binds the very C-terminus of p97, via PUB (PNGase/UBA- or UBX-containing proteins) and PUL (PLAA, Ufd3p and Lub1p) domains. As the list of proteins containing p97 recognizing motifs is rather extensive, this review will focus on cofactors also containing ubiquitin-interacting domains.

### UBA–UBX proteins

Of the ~30 UBX-containing proteins encoded in the human genome, five display a common architecture of an N-terminal UBA domain, which binds ubiquitin, and a C-terminal UBX domain [7]. The proteins UBXD7, UBXD8, FAF1, SAKS1 (SAPK substrate protein 1) and p47 show this domain arrangement. Using immunoprecipitation experiments with all five proteins, the UBX–UBA proteins were shown to be somewhat promiscuous, binding to K11-, K33-, K48- and K63-linked ubiquitin chains to varying degrees, with K11 and K48 chains the most prevalent [92].

The cofactor p47, essential for membrane fusion in Golgi biogenesis, binds p97 via a canonical UBX–N-domain interaction [66,93]. In addition to the UBX domain, p47 also contains an SHP motif. The SHP motif is a more recently discovered p97-interacting sequence, which provides an additional interacting surface for p97 cofactor interactions [17,18]. The UBA domain of p47 has been shown using pull-down experiments to preferentially bind K63 and M1 ubiquitin chains over K48 chains [59].

The adaptor SAKS1 plays a role in ERAD, where it has an inhibitory effect on the clearance of some ERAD substrates, for example  $\alpha$ -TCR (T-cell receptor alpha) and NHK-HA (Null Hong Kong variant 1-antitrypsin) [94,95]. The protein recognizes both K48 ubiquitin chains and atypical K6 linked ubiquitin, which can be found on auto-ubiquitinated BRCA1 [94,96].

	Co-factor	Associated Cellular Process	UB-linkage
UBX	FAF1  650aa	ERAD, chromatin associated	recognises K48
	UBXD7  468aa	nucleotide excision repair, HIF1 $\alpha$ turnover	unclear/multiple
	UBXD8  445aa	ERAD, lipid droplet turnover	unclear/multiple
	SAKS1  297aa	ERAD	recognises K6, K48
	p47  370aa	membrane fusion	recognises K63, linear
	p37  331aa	membrane fusion	N/A
UBX-L	NPL4  608aa	multiple	recognises K48
	UFD1  307aa		
	VCIP135  1220aa	membrane fusion	DUB K11, K48
	YOD1  348aa	ERAD, autophagy	DUB K11, K27, K29, K33
VIM/VBM	gp78  648aa	ERAD	E3 K48
	Hrd1  617aa	ERAD	E3 K48
	ATX3  364aa	ERAD	DUB K63
	Ube4b  1302aa	ERAD, chromatin associated	E4 K48
	Rhbd14  315aa	ERAD	recognises K48
PUB/PUL	PLAA  795aa	ERAD, autophagy	recognises K48
	Rnf31  1072aa	NF- $\kappa$ B activation, apoptosis	E3 linear
Other	Ankrd13a  590aa	caveolae formation	recognises K63
	Ubx9  553aa	disassembles p97 hexamers	N/A

Ub - interacting  
UB ligase  
DUB  
p97 - interacting  
other

Transmembrane helix  
 VIM/VBM-motif  
 SHP-motif

**Figure 2. Table of ubiquitin-associated p97 cofactors.**

Left: domain organization of cofactors, color-coded according to domain function, organized according to p97-interacting motif. Middle: cellular processes the cofactors have been implicated in. Right: types of polyubiquitin the cofactors have been associated with or possesses activity against.

The UBXD7 cofactor has been implicated in the degradation of cockayne syndrome group protein B, a protein essential in nucleotide excision repair [97]. It also has a role in the turnover of HIF1 $\alpha$  (hypoxia-inducible factor 1 $\alpha$ ) [98]. UBXD7 is required for the interaction of p97 with its substrate HIF1 $\alpha$ . In addition, it also interacts with cullin-2, the cognate ubiquitin E3 ligase of HIF1 $\alpha$ .

The function of UBXD8 is in the regulation of intracellular fatty acid storage [99]. The protein can be found both in the ER membrane and in cytosolic lipid droplets, where it has a role in the regulation of ATGL (adipose triglyceride lipase) turnover, an enzyme central to triglyceride hydrolysis [100]. The roles of ubiquitin, as well as the molecular role of p97 in this pathway, are still to be determined. UBXD7 and UBXD8 have both been immunoprecipitated with K11, K48 and K63 ubiquitin peptides, but the lack of biochemical data makes it difficult to make firm predictions about the ubiquitin specificity of their UBA domains [98].

The adaptor FAF1, and its interactions with p97 and ubiquitin, have been characterized more comprehensively. A crystal structure of the UBX–N-domain complex shows a canonical interaction [101]. The protein specifically binds K48-linked di-ubiquitin with low micromolar affinity [102]. Pull-down experiments confirm the interaction of FAF1 with K48 ubiquitin and suggest that FAF1 may also bind K63 chains [103]. The coiled-coil region of

FAF1 is required for its trimer formation, which may not readily form in solution, but is thought to be formed in complex with p97 [104]. FAF1 also plays a role in ERAD and chromatin-associated degradation [41,48,105]. A p97–FAF1 complex is also essential in the degradation of TGF $\beta$  (transforming growth factor beta). Disruption of the FAF1–p97 interaction due to FAF1 phosphorylation by AKT is thought to drive cancer metastasis [106].

### UBX-like domain-containing cofactors

The UFD1–NPL4 complex is an example of a UBX-L domain-containing cofactor. The protein Npl4 contains a UBX-L domain, which binds to the same groove on the p97 N-domain as the UBX domain, but possesses a slightly different fold and employs a distinct binding mode [107]. In addition to the UBX-L of NPL4, UFD1 contains an SHP motif that further stabilizes the interaction with p97 [17,18]. The UFD1–NPL4 heterodimer has been implicated in a multitude of p97-associated processes, primarily ERAD, but also chromatin-associated degradation and ribosome-associated degradation [19,41,78]. Both UFD1 and NPL4 possess ubiquitin-interacting motifs — the NPL4 zinc finger and the UFD1 UT3 domain both contribute to ubiquitin binding. The heterodimer binds to K48-linked ubiquitin chains, but discriminates strongly against K63-linked chains [19].

The DUB VCIPI35, central to the regulation of membrane fusion, also binds p97 via a UBX-L domain [71]. It is specifically active against K11- and K48-linked polyubiquitin [108]. In the regulation of membrane fusion however, its substrate is not thought to be polyubiquitin, but the monoubiquitinated t-SNARE Syn5 [66].

Another UBX-L-containing DUB, YOD1, has been associated with lysosomal autophagy and ERAD [33,109]. This DUB is specific against atypical K27, K29 and K33 chains [108].

### VIM/VBM-containing proteins

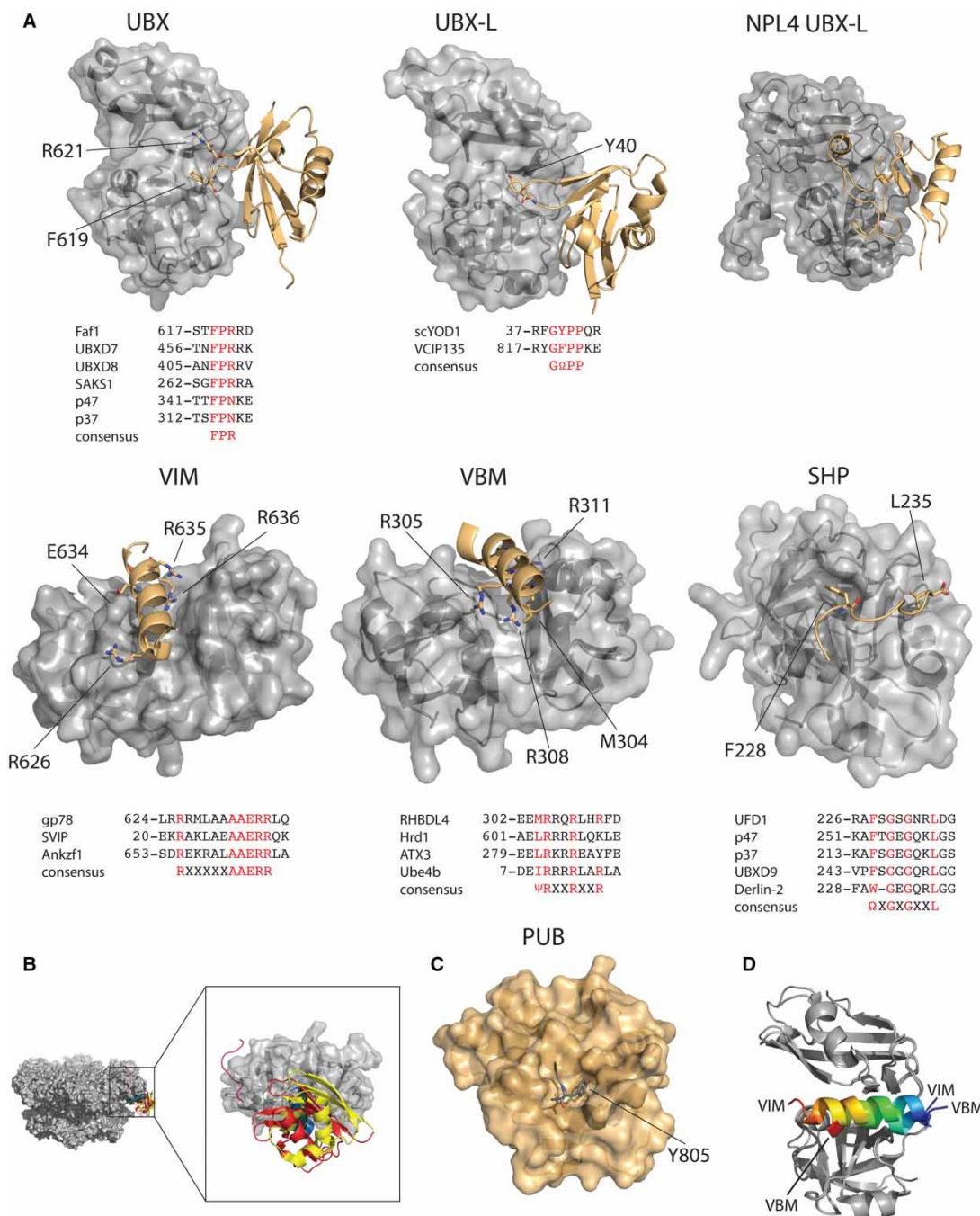
Unlike the globular UBX and UBX-L folds, the VIM and VBMs are small peptide-like interaction motifs consisting of a single  $\alpha$ -helix. The ubiquitin E3 ligases gp78 and Hrd1 both possess such a helical motif — a VIM in the case of gp78 and VBM for Hrd1, reviewed in ref. [9]. Tethered to the ER membrane via their multiple membrane domains, these enzymes play central roles in ERAD [21,25]. In agreement with its role in protein degradation, Hrd1 has E3 ubiquitin ligase activity specific for K48 chains [22]. The E3 ligase gp78 acts on ERAD substrates downstream from Hrd1, presumably after retrotranslocation [27]. This enzyme also catalyzes the formation of K48 chains [19]. The role of this ligase is thought to be the elongation of K48 chains on ERAD substrates. gp78 specifically elongates K48 chains, but does not introduce K48-linked polyubiquitin to other chain types producing mixed chains [27], with its CUE domain acting as a proof-reader in this mechanism [27].

The ubiquitin elongation factor Ube4b is another p97-associated ubiquitin ligase. Its conserved *S. cerevisiae* homolog Ufd2 co-localizes with p97 and proteasomes at sites of DNA damage and has been shown to be essential for the timely removal of Rad51 from such sites [110]. The enzyme also plays a role in ERAD [111]. Ube4b interacts with p97 via its N-terminal VBM [24]. While there is little information about the substrate specificity of Ube4b, there is extensive information about the close homolog Ufd2. The Ufd2 enzyme has the ability to produce various chain types, but most efficiently produces K48 polyubiquitin [112–116]. Interestingly, it strongly interacts with K29 polyubiquitin and adds K48 chains to proximal ubiquitins in such chains to produce branched chains, giving it a ubiquitin-editing function when taking into account the numerous p97-interacting DUBs [116].

The DUB Ataxin-3, which contains a C-terminal VBM, plays a role in ERAD, but its specific function is less clear [30,32]. Its catalytic activity has been reported to negatively regulate ERAD [31], but also be essential for the avoidance of ER stress [32]. The DUB efficiently cleaves K63-linked chains and discriminates against K48 chains; it is particularly effective against longer chains and mixed chains [117]. Ubiquitination of Ataxin-3 itself activates the enzyme, as does binding to p97 [118,119]. Aggregation of Ataxin-3, caused by a polyglutamine sequence C-terminal to the VBM, is known to cause a wide range of neurodegenerative diseases [120].

The protein Rhbdl4 (rhomboid-related protein 4) is a rhomboid protease essential for the ERAD of integral membrane proteins [121]. The protease is embedded in the ER membrane via its seven transmembrane helices and has been shown to cleave ERAD substrates which contain transmembrane helices, in a process that is essential for their efficient degradation [121]. Like other rhomboid proteases, Rhbdl4 is an intramembrane protease, its active site buried in the lipid bilayer where one of its function is the release of growth factors [122]. The protease has been shown to efficiently form a ternary complex with p97 and K48-linked polyubiquitin





**Figure 3. Structural details of p97-cofactor interactions.**

(A) Top: crystal structures of p97 N-domains (gray) in complex with cofactor (gold). FAF1 UBX (PDB: 3QQ8), OTU1 UBX-L (PDB: 4KDL), Npl4 UBX-L (PDB: 2PJH), gp78 VIM (PDB: 3TIW), Rhbd14 VBM (PDB: 5EPP) and UFD1 SHP (PDB: 5C1B). Conserved residues are indicated. Below: sequence alignment of the conserved interacting motifs, first line in the alignment shows the sequence of the protein shown in the structure above. (B) Composite model of UBX (red), UBX-L (yellow), VIM (green), VBM (blue) and SHP (pink) overlaid on the cryo-EM structure of ADP-bound ATP (5FTK). (C) Crystal structure of PNGase PUB domain (gold) in complex with p97 C-terminal peptide (gray) (PDB: 2HPJ). (D) Overlay of the VIM and VBMs complex structures, with the VIM and VBMs rainbow colored from N-terminus (blue) to C-terminus (red). N-domains in gray.

[123,124]. A VBM, whose interaction with p97 has been comprehensively characterized biochemically, is located at its C-terminus, next to a ubiquitin-interacting motif [123,124].

## PUB/PUL

A small subset of cofactors binds a conserved peptide at the C-terminal end of p97. There are two identified cofactors which bind this peptide and are also associated with the ubiquitin system. The protein PLAA (phospholipase A-2-activating protein) binds p97 via its C-terminal PUL domain and interacts with ubiquitin via its WD40 (WD-repeat) and PFU (PLAA family ubiquitin binding) domains [125–127]. The PLAA cofactor acts in concert with YOD1, Ubx1 and p97 in the autophagy of damaged lysosomes [109]. PLAA was shown to be required for the clearance of K48-labeled lysosomes, suggesting that it is this linkage type which PLAA recognizes. However, YOD1 depletion also caused K48 lysosome accumulation, an enzyme, which has negligible activity against K48 chains *in vitro* [108,109].

The protein Rnf31 (RING finger protein 31), called HOIP (HOIL-1-interacting protein) in humans, is part of the LUBAC (linear ubiquitin chain assembly complex), the only human UB E3 ligase which catalyzes the formation of linear ubiquitin chains [86,128]. These linear ubiquitin chains are associated with various cellular processes, primarily NF- $\kappa$ B activation and cell death [129]. Rnf31 contains a PUB domain, which recognizes the p97 C-terminal peptide [130]. In addition to p97, the DUB OTULIN also possesses a peptide which interacts with the PUB-binding pocket in the same manner [130]. The physiological significance of these interactions remains to be determined.

## Other significant cofactors

While not *bona fide* ubiquitin-binding p97 cofactors, there are two interesting p97-associated proteins worth introducing. The correct endolysosomal trafficking of the protein caveolin-1 relies on p97 [131]. A ternary complex formed by K63-ubiquitinated Cav-1, p97 and the putative p97 cofactor Ankrd13a is required for correct trafficking [82]. The Ankrd13a protein contains four ubiquitin-interacting motifs which bind K63-linked polyubiquitin [82]. The interaction of Ankrd13a with p97 on the other hand is thought to be indirect, and a cofactor connecting Ankrd13 and p97 has not yet been identified.

Structural biology studies of full-length p97 have identified the enzyme to form stable hexameric rings [3,5]. The p97 cofactor Ubx9, also called ASPL (alveolar soft part sarcoma locus) or TUG (tether-containing UB domain for GLUT4), which is located at the ER exit site, has been found to disassemble p97 hexamers, and its overexpression affects an accumulation of ubiquitinated species, consistent with p97 depletion, suggesting that Ubx9 is a p97 antagonist [132]. Mechanistically, an extended UB domain is essential for disassembly, the canonical UB–N-domain interaction forms the initial contact, while  $\alpha$ -helical lariats target D1 interprotomer interfaces and cause disassembly [92].

## Structural details of cofactor interactions

A wealth of structural information is available describing p97 and p97-cofactor interactions. While the interactions of all the p97-binding domains have been visualized by X-ray crystallography, the ubiquitin-interacting domains of p97 cofactors unfortunately have not. The mechanisms by which the cofactors discriminate against some ubiquitin chain types still remain to be identified. The following section will therefore focus on the interactions of the p97 N-domain and its binding partners.

## UBX

The UB domain, displaying a ubiquitin-like  $\beta$ -grasp fold with a  $\beta$ - $\beta$ - $\alpha$ - $\beta$ - $\beta$ - $\alpha$ - $\beta$  topology, binds the p97 N-domain via a conserved loop (Figure 3A). Crystal structures of the complex between the p97 N-domain and the FAF1 and Ubx7 UB domain, and between the p97 ND1 and the p47 UB domain, explain the conserved binding mechanism [93,101,105,133,134]. The binding of the extended UB domain of Ubx9 with p97 also includes a canonical UB–N-domain interaction [92]. The loop connecting  $\beta$ 3 and  $\beta$ 4 contains an FPR motif, which fits into the groove and is conserved in all UB domains. The side chain of the conserved phenylalanine residue fits into a hydrophobic pocket located in the groove. The arginine makes contact with an acidic patch on the C-terminal lobe of the N-domain. The central proline takes the *cis*-configuration to enable a rare touch turn to be formed at the end of the loop. The resulting *cis*-peptide has unusual  $\Psi$  and  $\Phi$  angles that enable the Phe and Arg side chains to interact with their respective binding pockets within the N-domain. Using the p47 and FAF1 UB domains as examples, the affinity of this interaction has been shown to be  $\sim 1 \mu\text{M}$  [104,135].

## UBX-L

The UBX-L domain also folds into a  $\beta$ -grasp, but with  $\beta$ - $\beta$ - $\alpha$ - $\beta$ - $\beta$ - $\alpha$ - $\alpha$ - $\beta$ , revealed by the solution structure of rat VCIP135 (PDB:2MX2) and the crystal structure of the *S. cerevisiae* homolog of YOD1 called OTU1 in complex with human p97 (Figure 3A) [136]. The most significant differences between UBX and UBX-L is the insertion of a third helix and the conformation of the  $\beta$ 3/ $\beta$ 4-turn. Instead of the FPR motif, the UBX-L of OTU1 contains a YPP motif. The tyrosine fits into the same hydrophobic pocket as the phenylalanine of the UBX domain. Again, the central proline is in the *cis*-configuration. The second proline is in the *trans*-configuration. Taken together, the *cis*-proline and its preceding tyrosine residue stabilize the conformation of the touch-turn by  $\pi$ -stacking interactions with each other [136]. The  $K_D$  of the OTU1–p97 interaction was shown by ITC to be 700 nM, indicating that UBX–p97 and UBXL–p97 interactions are similar in strength [136]. It also confirms that this interaction is highly conserved between humans and yeast. In the VCIP UBX-L domain, the tyrosine is replaced by phenylalanine, suggesting a conserved motif of G $\Omega$ PP, where  $\Omega$  stands for an aromatic residue [137].

The UBX-L domain of Npl4 shows a similar overall fold, but does not contain a touch-turn motif (Figure 3A) [107]. The  $\beta$ -grasp fold shows a  $\beta$ - $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  topology, and instead of the  $\beta$ 3/ $\beta$ 4-turn, the interaction with p97 is facilitated by the  $\beta$ -sheet and its adjacent loops. While the binding is considerably weaker than the other OTU1–p97 interaction with a  $K_D$  of 18  $\mu$ M [105], the overall orientations of the two binding partners are comparable to the other UBX–N-domain interactions.

## VIM

The VIM motif consists of a single  $\alpha$ -helix, rich in arginine residues. The crystal structure of the gp78 VIM domain in complex with p97 has revealed the mode of the interaction (Figure 3A) [138]. The helix binds in the groove between the two N-domain lobes, in an interaction that is similar in strength to the UBX interaction. Using the VIM domain of SVIP as an example, the  $K_D$  of this VIM with p97 was in the low micromolar range, similar to UBX and UBX-L binding [138]. The consensus sequence for VIM is RX<sub>5</sub>AAERR, where all three conserved arginines contribute to the binding, the most C-terminal arginine residue contributes to the interaction the most. While the two alanine residues and the glutamate are highly conserved, they are not required for binding, replacement of E634 in the gp78 VIM with a leucine in fact increases the affinity for p97 [138].

## VBM

The VBM, also a helical motif rich in arginine residues, is related to the VIM. A crystal structure of the VBM of RHBDL4 with the p97 N-domain has revealed the interaction of VBM to be highly similar to the VIM–p97 interaction (Figure 3A) [123,124]. The VBM also binds to the groove between the two N-domain lobes, in the same direction as the VIM (Figure 3D). In addition to three conserved arginine residues important for binding, there are also hydrophobic residues that make contact with the same hydrophobic pocket as the conserved phenylalanine of the UBX domain. The consensus motif of the VBM is  $\Psi$ RXXRXXR, where  $\Psi$  stands for an aliphatic residue.

## SHP

The SHP motif is a short linear motif that interacts with the p97 N-domain, but uniquely and not in the aforementioned groove. The consensus motif of SHP is  $\Omega$ XGXGXXL, where  $\Omega$  stands for an aromatic residue (Figure 3A) [9,17,18,139,140]. The crystal structures of both the UFD1 and Derlin-1 SHP motifs in complex with the p97 N-domain reveal the interaction to be driven primarily by the two aromatic residues and the conserved leucine, which fit into distinct hydrophobic pockets on the C-terminal lobe of the N-domain [17,18,123,124,140,141]. The second glycine residue and its following phenylalanine make main chain hydrogen bonds with a  $\beta$ -sheet of the N-domain, forming an additional antiparallel  $\beta$ -strand. The binding constant of the SHP–p97 interaction has not been determined, but mutation of essential SHP residues decreases the dissociation constant of the UFD1–NPL4 interaction by a factor of 6- to 17-fold [17,18]. Given that no cofactors have been identified that rely solely on the SHP motif for binding, it is likely that the role of SHP is that of an auxiliary interaction motif only.

From the interactions described, all cofactors bind in the same cleft between the two N-domain lobes. It is likely that *in vivo* there would be competition for the six N-domain-binding sites on each p97 hexamer which

could lead to multiple cofactors binding to a single p97 hexamer. While all cofactors described above have been shown to bind to p97 independently, there is evidence that FAF1 and UBXD7 bind preferentially to p97 in complex with UFD1/NPL4 [101]. This suggests that there may be core complexes formed by p97, for examples with UFD1/NPL4 or p47, which are prerequisite to the binding of some ‘secondary’ cofactors [142]. However, it is also possible that temporal and/or spatial differences in the expression and localization of the various cofactors could dictate which p97 complexes are formed [8]. In support of this, p97 can form different complexes *in vitro* with FAF1 and UFD1/NPL4 depending on the concentrations of both cofactors [104].

All of UBX, UBX-L, VIM and VBM bind in the same groove on the N-domain, suggesting that there is competition between cofactors for p97 N-domains, and also placing the cofactors spatially to the ‘side’ of the p97 hexamer, towards the N–D1 interface in the ADP-bound form of p97 (Figure 3B). In the ATP-bound form, the cofactors would be located ‘on top’ of the hexamer.

## PUB/PUL

The PUB domain binds a conserved peptide at the extreme C-terminus of p97. The leucine and tyrosine residues that form part of a DDLYG-COOH sequence conserved in eukaryotes bind a hydrophobic pocket on the PUB domain [143]. Protein phosphorylation at Y805 totally abolishes binding of p97 to the PUB domain of PNGase [peptide-N(4)-(N-acetyl- $\beta$ -glucosaminyl) asparagine amidase] and also disrupts ERAD (Figure 3C) [143,144]. The observation that Y805 fits tightly into the hydrophobic pocket, with no space for an additional phosphate, provides a clear mechanism as to how this interaction is regulated by phosphorylation. The PUL domain of PLAA, which is structurally unrelated to the PUB domain, binds the C-terminal p97 peptide in a highly similar interaction [127]. While the interaction of the Rnf31 PUB domain with the p97 C-terminal peptide is only  $\sim 50 \mu\text{M}$ , the DUB OTULIN binds this PUB domain with a dissociation constant  $\sim 300 \text{ nM}$ , indicating that this mode of binding can facilitate a wide range of specificities [130].

## Regulation of p97 enzymatic activity by cofactors and *vice versa*

A subset of cofactors do not just bind to p97, but also alter its enzymatic activity. This regulation is effective in both ways, for example the DUB Ataxin-3 has been shown to be activated by p97, while keeping its specificity for K63 chains [119]. This stimulatory effect is absent from disease-associated Ataxin-3 with an extended polyglutamine sequence. The lack of DUB activation and potentially inefficient processing of ERAD substrates may be one mechanism by which the expanded poly-Q of Ataxin-3 may contribute to disease phenotypes [119].

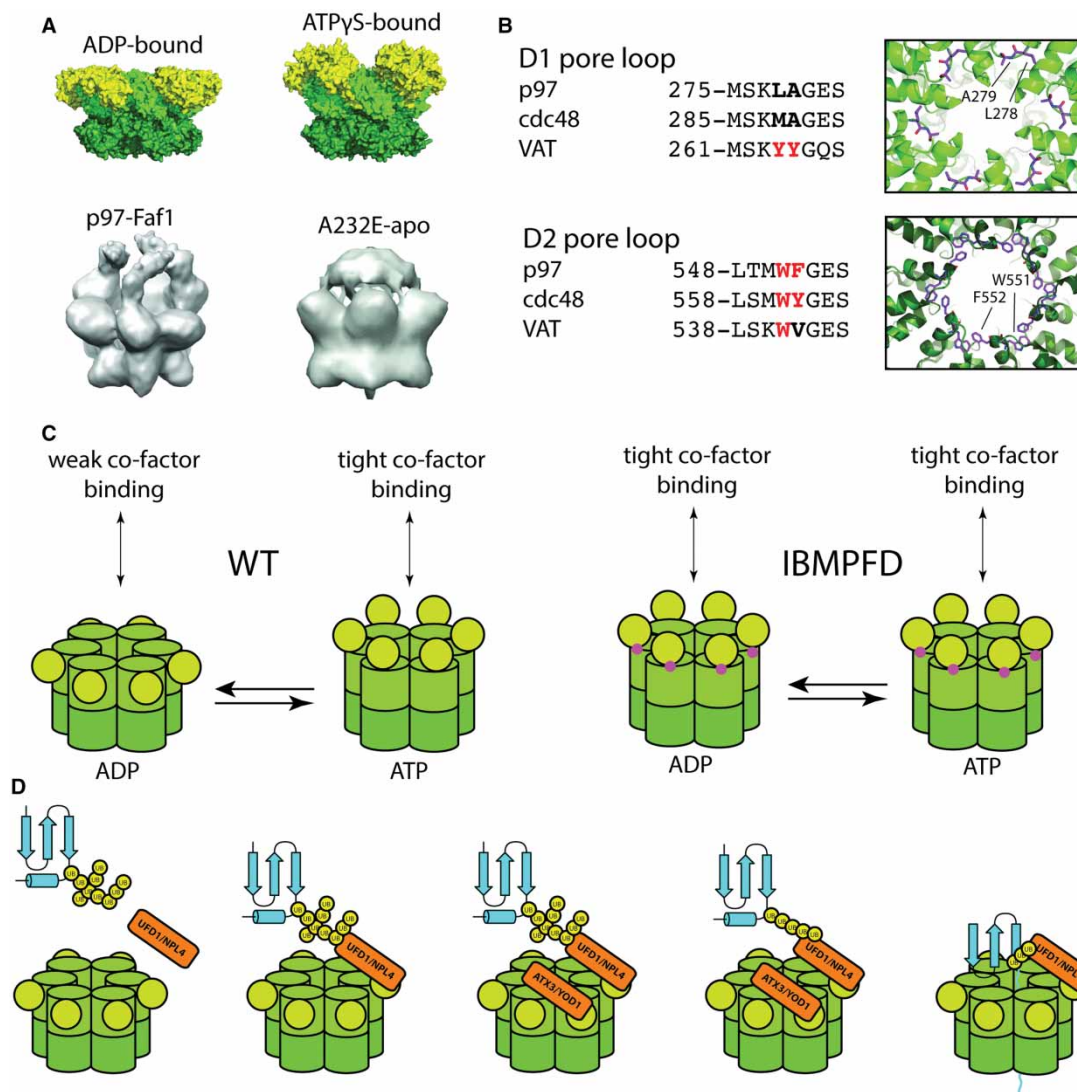
The p37 cofactor activates p97, while p47 decreases ATPase activity [69,70]. Both cofactors result in a tighter  $K_M$  of p97 for its substrate ATP [70]. Interestingly, deletion of an N-terminal region in p47 that lacks homology to p37 turns p47 from an inhibitory cofactor into an activator. These data indicate that UBX domains in general have a stimulatory effect on p97, but an N-terminal region of p47 can counter this effect [70]. Some mutations in the interface between N-domain and D1 domain of p97 are associated with the multisystem disease IBMPFD, defects in ERAD as well as other p97-associated functions, and on a molecular level with altered (increased) ATPase activities [7,145–147].

## Molecular mechanism and enzymology of p97

### Structure

Initially identified as a homohexameric particle by negative stain electron microscopy, p97 was shown to possess ATPase activity that is dependent on its oligomeric state and the presence of  $\text{Mg}^{2+}$  [5,6]. Subsequently, the crystal structure of the N-domain and first ATPase domain (ND1) was solved by crystallography, followed by full-length p97 [2,3,148]. The hexamer displays a mushroom-like shape where two rings of ATPase domains stack on top of each other and the N-domain is co-planar to the D1 ATPase domain in ADP-bound p97 and in an ‘up’ conformation in the ATP-bound state (Figure 4A) [149]. The D1 and D2 ATPase domains both fold into typical AAA domains with an  $\alpha/\beta$ -subdomain followed by a helical subdomain. Of the 12 ATPase domains in a p97 hexamer, the D1 domains are primarily required for oligomerization, while the D2 domains play a larger role in ATP hydrolysis [150,151]. There is some evidence that the D1–D2 linker, which can introduce asymmetry in the protein, is required for activity — p97 ND1 displays negligible ATPase activity, but a slightly longer protein containing the 20 aa D1–D2 linker possesses ATPase activity roughly half of full-length p97 [146,147]. The six D2 domains of p97 have been shown to bind less than six molecules of ATP, providing an additional link between asymmetry and ATPase activity





**Figure 4. Enzymology of p97.**

(A) EM structures of p97. ADP bound (PDB: 5FTK), ATP $\gamma$ S (PDB: 5FTN), in complex with FAF1 (EMDB-2319) and the IBMPFD mutant A232E (EMDB-2038). (B) Left: conservation of D1 and D2 pore loop residues in p97, Cdc48 and VAT. Right: structure of pore loops with residues highlighted on table on the left shown as sticks (PDB: 5FTN). (C) Models of p97 conformations in different nucleotide states. p97 domains color-coded as before. IBMPFD mutant positions highlighted in purple. (D) Model for p97 activity. p97 forms a complex with UFD1–NPL4 and a polyubiquitinated substrate. Additional cofactors containing DUB activity remove polyubiquitin leaving K48-chains only. The substrate is then threaded through the central pore of p97 and unfolded.

[152]. ATP hydrolysis is also regulated by inter-protomer interactions between the D2 domain and the C-terminal tail of the neighboring protomer [17,18].

## Molecular function of p97

Some indications as to the molecular function of p97 are provided by its physiological functions and the enzymatic activity of its archaeal homolog VAT. The *T. acidophilum* VAT ATPase, from a prokaryote without a ubiquitin system, has unfoldase activity [153]. Tagging of a protein with an ssrA peptide, a C-terminal degradation tag added to protein lacking an in-frame stop-codon, is essential and sufficient for unfolding by

VAT [154]. In VAT, removal of its N-domain increases unfolding activity, indicating that the N-domain may have an autoinhibitory role [153]. Electron microscopy structures of VAT in different nucleotide states suggest that in an ATP-bound VAT, the two ATPase rings form planar rings [155]. However, upon ATP hydrolysis, the 12 ATPase domains can form a continuous spiral. The large structural rearrangement between planar rings and screw-like conformation was proposed to provide the mechanical energy required for protein unfolding [155].

Native human p97 does not possess activity against *ssrA*-tagged proteins [156]. However, through mutagenesis of VAT, it has been shown that aromatic residues are required in both the D1 and D2 pore for unfolding activity against *ssrA*-tagged proteins. p97 lacks aromatic residues in the D1 pore, and only through the introduction of such residues and removal of the N-domain could VAT-like unfolding activity be reconstituted (Figure 4B) [156]. Recently, two studies have independently succeeded in reconstituting p97 and Cdc48 unfoldase activity *in vitro*. Both studies used K48-ubiquitinated GFP as a p97 substrate. These were engineered *in vitro*, by enzymatically K48-ubiquitinating either a degron sequence [157] or a ubiquitin [158] linearly fused to the GFP. UFD1–NPL4 was required for efficient unfolding, and YOD1 for substrate release, confirming that p97 relies on this cofactor to recognize K48-ubiquitinated substrates, but removal of ubiquitin is required for complete processing and release [157,158].

The ATPase activity of Cdc48 is reduced to 50% by the presence of Ufd1–NPL4, but increases to 300% by the additional presence of a K48-ubiquitinated substrate, a regulatory mechanism effective at preserving the ATP consumption of such an abundant ATPase [157]. Conversely, the presence of UFD1–NPL4 does not affect human p97 activity [158]. An assay using the engineered substrate also confirmed Cdc48 as a *bona fide* unfoldase whose substrates are threaded through the central pore, as in its prokaryotic homologs (Figure 4D) [157,159]. Given that important pore residues are conserved between Cdc48 and p97, this strongly suggests a highly similar mechanism for mammalian p97 (Figure 4B). These substrates may also aid structural studies and biochemical work to determine the mechanism that provides the mechanical energy for unfolding activity.

## ATPase cycle

Several studies have linked the control of the ATPase cycle to the movement of the N-domain, a regulatory mechanism that appears to fail in IBMPFD mutants, where the ‘up’ conformation is favored even in the apo-form. Structural data on p97 have shown the N-domains to be co-planar with the D1 domain in ADP-bound p97, but in an ‘up’ position in the ATP-bound form [149]. When in complex with the UBX-containing cofactor FAF1, the N-domain also occupies the ‘up’ position (Figure 4A) [104]. The apo-form of the IBMPFD mutant A232E also takes this conformation (Figure 4A) [160]. Biochemical data also support these structural observations. When the N-domain is locked in a co-planar orientation, the ATPase activity is compromised [160]. The cofactors p37 and p47 bind more tightly to the ATP-bound form of p97 than the ADP-bound form. In IBMPFD mutants, the affinity of these cofactors for p97 is independent of nucleotide state, and the interaction is always as tight as for the wild ATP-bound form [161]. Binding of UBX-containing cofactor p37 also increases ATP activity, while p47 inhibits the enzyme [70]. IBMPFD mutants also show higher activity, both in terms of ATP hydrolysis and unfolding activity [158,160]. Interestingly, neither p37 nor p47 is capable of increasing the ATPase activity of IBMPFD mutants of p97 further, providing additional evidence that the N-domain is somehow ‘uncoupled’ from ATP hydrolysis in these disease-linked mutants [70].

Cofactors bind more tightly to hexameric p97 than monomeric N-domain constructs [101]. Given that the N-domain UBX interaction is located on the top of the hexamer, the ‘up’ conformation may favor cofactor binding by virtue of enabling oligomerization of cofactors on ‘top’ of the p97 hexamer. This model has been suggested for the FAF1–p97 and p47–p97 complexes [104].

There also seems to be a correlation between bound ATP, bound cofactor, increased ATP hydrolysis and N-domains in the ‘up’ position, and conversely no ATP bound, weakly binding cofactors and co-planar N-domains (Figure 4C). While there is a strong correlation, it is less clear as to what is the cause and what is the effect. The ATPase activity is also further regulated by extensive post-translational modifications, reviewed by ref. [9]. However, it is clear that the p97 ATP hydrolysis cycle is tightly co-ordinated with cofactor binding and substrate engagement which result in significant conformational changes between the N-domain and D1 and the D1 and D2 AAA domains. It will be important in future studies to try and capture intermediate states of p97 throughout the ATP hydrolysis cycle.

## Therapeutic potential of p97 inhibitors

### Proteotoxic stress in tumour cells

One characteristic of many tumor cell lines is their proteotoxic stress caused by an abundance of misfolded proteins [162]. Owing to their rapid growth and excessive genetic abnormalities, cancer cells suffer from this stress naturally. Enabling an accumulation of misfolded proteins and escalating the proteotoxic stress is an established method to target cancer cells in chemotherapy, proven effective by the proteasome inhibitor bortezomib [162]. However, cancer cells can overcome this selective pressure and develop resistance, for example by up-regulating expression of proteasome subunits as well as chaperones to relieve some of the proteotoxic stress, reviewed in ref. [163]. One molecular mechanism through which this is achieved is via the transcription factor Nrf1 (nuclear respiratory factor 1). The absence of proteasome activity leads to an accumulation of this transcription factor, which triggers increased transcription of proteasome subunits [164]. Targeting other components of the ubiquitin system, such as E3 ubiquitin ligases, DUBs or the interaction surfaces of ubiquitin-binding proteins, has proved challenging, as these proteins rarely possess defined catalytic pockets [165].

Since the ATPase activity of p97 is essential for its function and p97 possesses clearly defined catalytic centers for ATP hydrolysis, inhibition of p97 offers a promising avenue through which proteotoxic stress can be induced. The dependence of tumor cells on p97 activity is further illustrated by the overexpression of this enzyme in NSCLC (non-small-cell lung cancer) cells [166]. Both ATP-competitive and allosteric inhibitors of p97 have been developed with the competitive inhibitors being at a much later stage of development.

### A drug-like ATP-competitive inhibitor of p97

From a high-throughput screen, DBEQ (N2,N4-dibenzylquinazoline-2,4-diamine) was identified as an ATP-competitive p97 inhibitor *in vitro* (Figure 5A). It also successfully inhibited known p97-dependent processes such as ERAD and autophagy [167]. The molecule was developed further using SAR (structure–activity relationship) to produce the more potent inhibitors ML240 and ML241 and finally CB-5083, a potent p97 inhibitor with drug-like properties (Figure 5A) [168–170]. In addition to the inhibition of known p97-dependent cellular processes, CB-5083 was shown to effectively stop tumor growth in both solid and hematological cancer xenografts and has entered Phase I trials in the clinic [170]. Interestingly, CB-5083 reduces the unfolding activity of IBMPFD mutant p97 to that of wild-type p97 [158]. Dendrimer-coated DBEQ has been shown to be more effective in triggering an accumulation of ubiquitinated species than DBEQ by itself, suggesting that coating of a p97 inhibitor, with polymers, may increase effectiveness [171].

### Discovery of noncompetitive inhibitors of p97

Several different classes of allosteric p97 inhibitors have been discovered by high-throughput screening. One of the classes discovered were alkylsulfanyl-1,2,4-triazoles [172]. Following SAR, the most promising lead compound (numbered 116) possessed an ATPase  $IC_{50}$  of 24 nM and displayed antiproliferative properties with an  $IC_{50}$  value of 380 nM (Figure 5A). The SAR experiments also showed that the  $IC_{50}$  values of the ATPase activity and of the antiproliferative effect were not always closely related. Some compounds screened showed strong ATPase inhibition but little antiproliferative effect. The optimized compound showed strong specificity for p97 when tested against other ATPases such as NSF or kinases, but suffered from high clearance when used in an animal model [172].

The inhibitor NMS873 is among the best characterized allosteric p97 inhibitors and was derived from the triazole inhibitors described above (Figure 5A). It has an ATPase  $IC_{50}$  comparable to CB5083 [173]. In addition to the p97 inhibitory effect, NMS873 also triggers known phenotypes of p97 inhibition, such as accumulation of polyubiquitin and CHOP induction [173]. While NMS873 is an effective p97 inhibitor *in vitro*, it is at an earlier stage of development than CB5083 in terms of drug-like properties.

In another screen, trifluoromethyl and pentafluorosulfanyl indoles were identified as allosteric p97 inhibitors [174]. Following SAR, the most powerful compound from this class (compound 23) inhibited p97 ATPase activity with a nanomolar  $IC_{50}$  (Figure 5A). The structure of p97 in complex with one compound of this class (UPCDC30245) was solved by cryo-EM, confirming that the indole part of the inhibitor interacts with p97 at the D1, D2 interface (Figure 5B) [149].

A third class of allosteric p97 inhibitors discovered by high-throughput screening were indole amides, which act as uncompetitive inhibitors (Figure 5A) [175]. Following SAR, the strongest of the optimized compounds (compound 3) inhibited p97 with an  $IC_{50}$  value of up to 500 nM at an ATP concentration of 0.1 mM. In terms

of solubility and stability, these indole amides are among the most promising allosteric inhibitors being developed. They do, however, require higher concentrations of compound to effectively inhibit p97. The indole amide failed to display robust antiproliferative effects, but a more potent inhibitor may induce the desired cellular responses [175]. An ATPase  $IC_{50}$  considerable lower than 500 nM has been shown to be required for eliciting an antiproliferative effect [172].

In addition to the molecules identified by high-throughput screening, the natural products withaferin A and dehydrocurvularin have been shown to inhibit the enzyme [176,177].

### ATP competitive versus allosteric inhibitors of p97

There are some caveats with ATP competitive inhibition of p97. It has been shown that binding of some p97 cofactors, for example p37 and p47, results in a decreased  $K_M$  for ATP and thus may decrease the potency of a competitive inhibitor [70]. Subsequently, it was reported that for all ATP-competitive inhibitors tested, the  $IC_{50}$  of p97–47 is at least 4-fold higher than that of p97 alone. Conversely, the allosteric inhibitor NMS-873 does not lose potency in the presence of p47 [178]. The loss of inhibitor efficacy has so far only been observed for the p97–p47 complex. Subsequent research on the highly similar p97–p37 complex, as well as the ternary p97–UFD1–NPL4 complex, showed that these complexes are equally sensitive to ATP competitive p97 inhibitors as p97 is itself. Furthermore, the discovery that different p97 inhibitors target different complexes with varying efficacies may be the first step towards developing complex-specific inhibitors [178].

### Perspectives on p97 drug discovery efforts

There is a wealth of information on a large number of inhibitors and their interaction with p97 from both *in vivo* and *in vitro* experiments, with the notable absence of any crystal structures of p97-inhibitor complexes which may aid medicinal chemistry efforts. The sole interaction between p97 and an inhibitor has been visualized by high-resolution cryo-electron microscopy [149]. The recent development of *in vitro* substrates for p97 may aid the future development of novel p97 inhibitors [158].

### Trends and conclusions

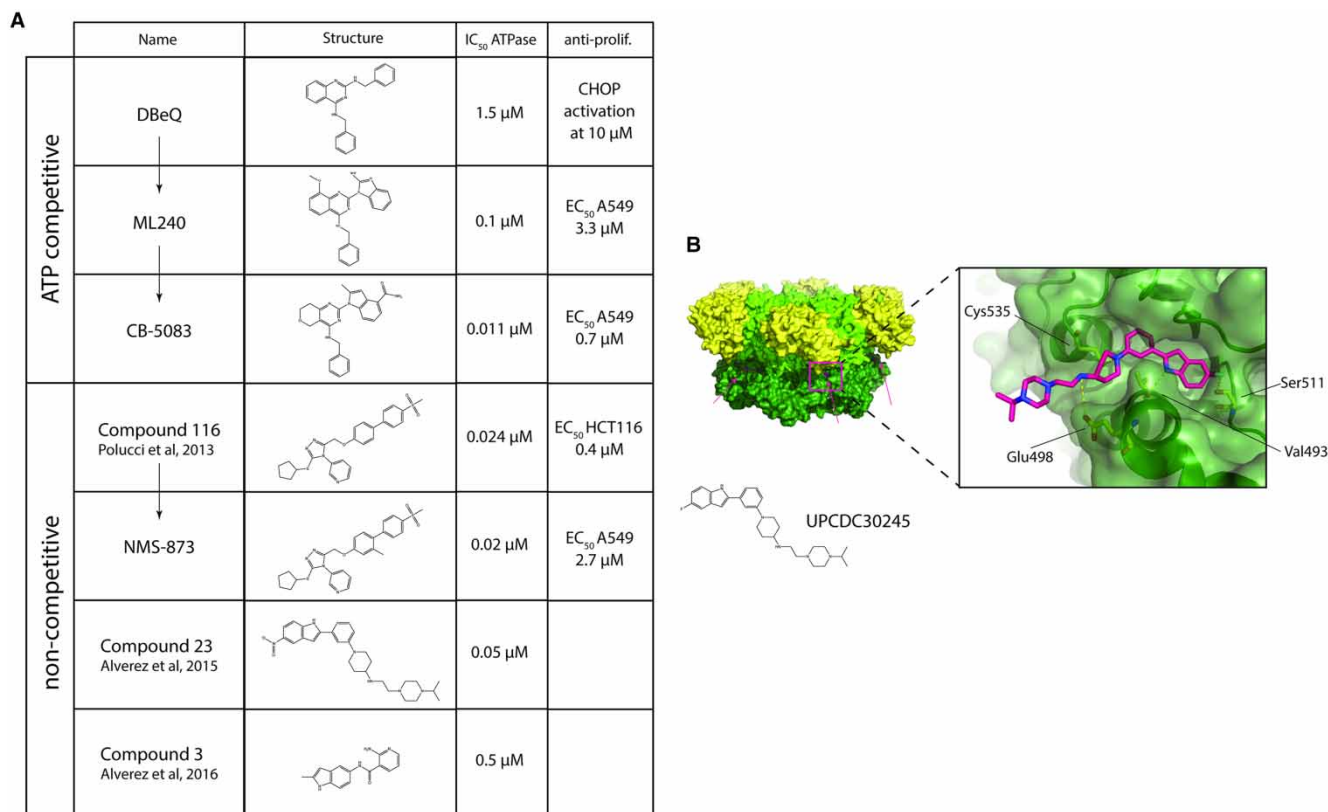
The diversity of cellular processes listed above illustrates the versatility of p97. The enzyme plays a role in protein degradation from the ER and mitochondrial membrane, primarily in concert with its cofactors UFD1 and NPL4. In addition, it is used to extract ubiquitinated protein complexes from chromatin in DNA repair pathways, DNA replication and transcription. A pathway where its ATPase activity is less well understood is in membrane fusion, where p97 regulates the formation of the endomembrane system. From these well-described processes, there is a common theme among them, which is the recruitment of p97 by ubiquitinated substrates. The vast majority of p97 substrates described are ubiquitinated and their interaction with p97 is mediated by bi-functional cofactors, which contain both p97-interacting and ubiquitin-interacting domains.

Several common themes also become apparent when summarizing the cellular and molecular functions of p97 cofactors. With the exception of the common UBA–UBX architecture, the ubiquitin-associated domains and the p97-interacting motifs occur in many different combinations, highlighting the modularity of these protein domains and binding motifs. While there are a multitude of p97-interacting motifs, most bind the same cleft in the p97 N-domain suggesting competition between cofactors for N-domains. As there are six potential binding sites, there is the possibility of mixed cofactor binding as well as co-operative binding and hierarchical complex assembly. It will be interesting to determine whether multiple complexes can function in similar biochemical pathways or if there is functional specificity assigned to specific sub-complexes.

The different p97-interacting motifs can be found at different positions within p97 cofactors. The UBX and VIM/VBMs are primarily found at the extreme N- and C-termini of the respective cofactor, while the SHP motif is located more centrally in the linear polypeptide chain. Looking at the 3D structures of p97 cofactor complexes reveals that the majority of N-domain-interacting motifs, UBX, UBX-L, VIM and VBM, bind the same groove between the N-domain lobes. Different cofactors thus compete for p97 binding. Hydrophobic and basic residues dominate the consensus sequences of the N-domain-interacting motifs.

In terms of ubiquitin specificity, K48 chains appear the most intimately associated with p97 functions. The ubiquitin-binding cofactors UFD1/NPL4, FAF1, SAKS1, UBXD7 and UBXD8 are all capable of binding K48 polyubiquitin. Only p47, which is not associated with protein degradation or segregation of any kind, but essential to the regulation of membrane fusion, bucks the trend and shows specificity for K63 and linear chains. The ubiquitin specificities of the ubiquitin E3 ligases and DUBs fit into the same pattern as the





**Figure 5. p97 inhibitors.**

(A) Table of a selection of known p97 inhibitors, including name, chemical structure, IC<sub>50</sub> against p97 ATPase activity and ability to cause an antiproliferative effect in a cell-based assay, e.g. CHOP activation, EC<sub>50</sub> of cell death against cancer cell lines A549 and HCT116. (B) Cryo-EM structure of p97 in complex with the UPCDC30245 inhibitor (pink) (PDB: 5FTJ).

adaptors. The three ubiquitin ligases Hrd1, gp78 and Ube4b all catalyze the formation of K48-linked chains. The DUBs are somewhat more promiscuous, but neither YOD1, nor ATX3, cleaves K48 isopeptide bonds efficiently. Only VCIP, associated with membrane fusion like p47, is active against K48 polyubiquitin. The cofactor complexes formed by p97 favor the catalysis of K48-linked chains and the hydrolysis of any other type of polyubiquitin. These complexes appear to have evolved to strip p97 substrates of polyubiquitin signals other than K48, from upstream signaling or other processes, and generate or amplify a K48 signal.

Following recruitment of p97 to its substrate, the substrate is unfolded and it is proposed that the unfolded substrate is threaded through the central pore. However, the energy requirements of unfolding larger proteins for threading through the p97 pore are still an open question. It is equally plausible that partial threading results in destabilization of target proteins or protein complexes and disassembly or extraction. For a continuous threading mechanism, multiple rounds of ATP hydrolysis would be required and p97 would need to be anchored in such a way as to apply the necessary force for unfolding or extraction. Although *in vitro* using model substrates there is some evidence for threading in Cdc48, it is still unclear how cofactors and ATP hydrolysis orchestrate the disassembly, unfolding process.

In terms of targeting p97 as a drug target for cancer, various screening efforts have produced promising leads with CB5083 being the most developed. Some allosteric inhibitors, for example NMS873, inhibit p97 with comparable potency and elicit antiproliferative responses in cells but are not drug-like, yet. The availability of an *in vitro* substrate may now allow screening for additional inhibitors, which do not interfere with ATPase hydrolysis, but nonetheless disrupt the enzymatic function of p97.

In summary, p97 continues to be a fascinating enzyme and while much has been learned about its structure function and mechanism, there are still a large number of unanswered questions which need to be addressed given the potential clinical importance of p97 as a drug target for cancer treatments.

## Abbreviations

AAA+, ATPases associated with diverse cellular activities; Ankrd13, ankyrin repeat domain-containing protein 13; Ataxin-3, ataxia type 3 protein; BRCA1, breast cancer type 1 susceptibility protein; Cav-1, caveolin-1; Cdc48, cell division cycle protein 48; CDT-1, CDC10-dependent transcript 1; CHMP2A, charged multivesicular body protein 2a; CHOP, C/EBP-homologous protein; CRL, cullin-RING ubiquitin ligase; CUE, coupling of ubiquitin conjugation to ER degradation; DBE, N<sub>2</sub>,N<sub>4</sub>-dibenzylquinazoline-2,4-diamine; Doa1, degradation of alpha 1; DUBs, deubiquitinases; ERAD, endoplasmic reticulum-associated degradation; FAF1, FAS-associated factor 1; HACE1, HECT domain and ankyrin repeat-containing E3 ubiquitin-protein ligase 1; HIF1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; Hrd1, Hmg2-regulated degradation; IBMPFD, inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia; I $\kappa$ B $\alpha$ , NF- $\kappa$ B inhibitor alpha; NF- $\kappa$ B activation, nuclear factor kappa-light-chain-enhancer of activated B cells; NSF, N-ethylmaleimide-sensitive fusion protein; OTU1, ovarian tumour domain containing protein 1; PLAA, phospholipase A-2-activating protein; PUB, PNGase/UBA- or UBX-containing proteins; PUL, PLAA, Ufd3p and Lub1p; Rhd1, rhomboid-related protein 4; Rnf31, RING finger protein 31; RNF8, RING finger protein 8; SAKS1, SAPK substrate protein 1; SAR, structure–activity relationship; SHP, suppressor of high-copy PP1 protein; SVIP, small VCP-interacting protein; Syn5, syntaxin5; t-SNARE, soluble NSF attachment protein receptor; UBA, ubiquitin-associated; UBXL, UBX-like; UBXN3, UBX-containing protein 3; UFD1–NPL4, ubiquitin fusion degradation protein 1 and nuclear protein localization protein 4 homolog; UPS, ubiquitin proteasome system; VAT, VCP-like ATPase; VBM, VCP-binding motif; VCIP135, VCP-interacting protein 135 kDa; VCP, valosin-containing protein; VIM, VCP-interacting motif; WD40, WD-repeat; YOD1, yeast OTU domain containing protein.

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## Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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