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Review

Control of p97 function by cofactor binding

Alexander Buchberger [a,*], Hermann Schindelin [b], Petra Hänzelmann [b,*]

[a] Department of Biochemistry, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany
[b] Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany

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ABSTRACT

p97 (also known as Cdc48, Ter94, and VCP) is an essential, abundant and highly conserved ATPase driving the turnover of ubiquitylated proteins in eukaryotes. Even though p97 is involved in highly diverse cellular pathways and processes, it exhibits hardly any substrate specificity on its own. Instead, it relies on a large number of regulatory cofactors controlling substrate specificity and turnover. The complexity as well as temporal and spatial regulation of the interactions between p97 and its cofactors is only beginning to be understood at the molecular level. Here, we give an overview on the structural framework of p97 interactions with its cofactors, the emerging principles underlying the assembly of complexes with different cofactors, and the pathogenic effects of disease-associated p97 mutations on cofactor binding.

1. Introduction

The covalent modification of proteins with the small protein ubiquitin (termed "ubiquitylation", "ubiquitination" or "ubi quitinylation") regulates numerous essential processes in all eukaryotic cells [1]. In particular, ubiquitylation controls protein degradation through three major proteolytic systems, the 26S proteasome, macroautophagy, and the endolysosomal pathway [2,3]. Moreover, ubiquitylation has diverse non-proteolytic roles in modulating protein function as well as the spatial and temporal organization of protein complexes [3].

Target protein ubiquitylation involves the formation of an isopeptide bond between the C-terminus of ubiquitin and lysine residues of target proteins. This process requires ATP and a cascade of three enzymatic activities, E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin protein ligase) [1,3]. The target protein specificity of this system is primarily determined by a large number of different E3 enzymes found in all eukaryotes. While target proteins can be modified with one or several individual ubiquitin molecules, in the majority of cases previously conjugated ubiquitin moieties are in turn ubiquitylated, resulting in target protein modification with ubiquitin chains of different lengths and linkage types [3]. Ubiquitin chains can also be subject to editing by de-ubiquitylating enzymes (DUBs), which further increases the plasticity of ubiquitin modifications [4].

In addition to the enzymes mediating the assembly of defined ubiquitylation marks on target proteins, the chaperone-related, ubiquitin-selective ATPase p97 (also known as Cdc48, Ter94 and VCP) has emerged as an important motor and regulator of many ubiquitin-controlled cellular processes. p97 is involved in the proteasomal degradation of protein quality control targets, cell cycle regulators, transcription factors and DNA repair proteins, but also in non-proteasomal proteolysis through macroautophagy and the endolysosomal pathway [5,6]. Moreover, p97 mediates the non-proteolytic mobilization of ubiquitylated proteins, *e.g.* transcriptional regulators and SNARE proteins. Its involvement in three major cellular proteolysis pathways makes p97 a central element of eukaryotic proteostasis (protein homeostasis). Importantly, mutations in the human *VCP* gene encoding p97 are causative of two fatal protein aggregation diseases ("proteinopathies"), Inclusion Body Myopathy with Paget's disease of the bone and Fronto-temporal Dementia (IBMPFD) and familial Amyotrophic Lateral Sclerosis (fALS) [7,8]. On the cellular level, both diseases are characterized by cytoplasmic protein inclusions (aggregates) positive

* Corresponding authors.
E-mail addresses: alexander.buchberger@biozentrum.uni-wuerzburg.de (A. Buchberger), petra.haenzelmann@virchow.uni-wuerzburg.de (P. Hänzelmann).

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for ubiquitin and the Tar DNA-binding Protein 43 (TDP-43). Analysis of IBMPFD patient-derived cell-lines and IBMPFD mouse models revealed that disease-causing point mutations in *VCP* affect autophagy and endolysosomal protein degradation [9,10].

p97 is a member of the AAA (ATPases Associated with diverse cellular Activities) protein family and forms homohexameric, ring-structured complexes [11,12]. It uses energy derived from ATP hydrolysis to extract or "segregate" ubiquitylated target proteins from stable protein assemblies, membranes and chromatin. The molecular mechanism underlying this "segregase" activity is still under debate, but is likely to rely on pronounced conformational changes induced by ATP binding and hydrolysis [6,11,12]. Since p97 is involved in highly diverse cellular processes, its segregase activity must be tightly controlled in time and space. This is achieved by a host of regulatory cofactors, which control substrate specificity and fate, subcellular localization and the oligomeric state of p97 [5,13,14]. In fact, the number and diversity of p97 cofactors is unique among the AAA ATPases, making p97 a prime example of a modular system comprising a relatively unspecific "engine" and a sophisticated "tool-kit" for specific cellular tasks.

Importantly, p97 has recently been recognized as a promising target of cancer chemotherapy [15]. Potent ATP-competitive and allosteric p97 inhibitors have been developed and are entering phase I clinical trials. Intriguingly, some inhibitors are specific for p97–cofactor complexes and may prove useful in blocking specific p97-dependent pathways *in vivo* [16].

In this review, we will first introduce the three-dimensional structure and nucleotide-dependent conformational states of p97. We will then explain in detail the molecular basis of cofactor binding as well as emerging principles governing the formation of distinct functional p97–cofactor assemblies. Finally, we will discuss the impact of disease-causing *VCP* mutations on the structure of p97 and its interactions with cofactors. More detailed information about the cellular functions of p97 can be found in several recent reviews [5,17–19].

2. p97 structure and ATPase activity

The p97 monomer starts with the N domain (residues 1–187 in mammalian p97), followed by the D1 (residues 209–460) and D2 (481–763) tandem ATPase domains and a disordered C-terminal region (residues 764–806) (Fig. 1A). Two linker regions (residues 188–208 and 461–480) connect the N and D1 domains as well as the D1 and D2 domains, respectively. From a protein taxonomy perspective, the three structural domains can be further divided into subdomains: The N domain forms a double Ψ β-barrel (Nn, residues 21–106) followed by a four-stranded β-barrel (Nc, residues 107–187), whereas each of the ATPase domains consists of a P-loop NTPase α/β subdomain adopting a Rossman fold and a smaller C-terminal α-helical bundle domain. The Rossman core of the ATPase domains features, besides the classical Walker A (P-loop; $G(x)_4GKT$) and Walker B (hhhhDE, h hydrophobic amino acid) motifs, a sensor 1 asparagine residue (Asn348 and Asn624, respectively), which assists the Walker B motif in coordinating the attacking water molecules, and an arginine finger (Arg359 and Arg635, respectively), which stabilizes the developing negative charge during ATP hydrolysis.

The three-dimensional structure of hexameric p97 in the absence of cofactors has been analyzed by X-ray crystallography, small angle X-ray scattering (SAXS) and electron microscopy (EM). Crystal structures of full-length murine p97 in the presence of the transition state analog ADP-AlF_x at 4.7 Å resolution [20] and in complex with ADP at 3.7 Å resolution [21] established an overall architecture featuring two hexameric rings consisting of the D1 and D2 ATPase domains, respectively, stacked in a head to tail arrangement, with the N domains protruding away from the 6-fold axis, in plane with the D1 domains (Fig. 1B). The interdomain linkers (N–D1 and D1–D2) are each located in close spatial proximity to the nucleotide binding site of the ensuing ATPase domain. Both structures as well as subsequently solved structures with various nucleotides at 3.5–4.4 Å resolution exhibited poor electron density in the D2 domain, thus prompting a separate analysis of the isolated D2 domain [22,23]. In all full-length structures, pre-bound ADP was invariably present in the D1 domain, whereas the D2 domain contained the nucleotides added during crystallization, indicative of a lower catalytic activity in the D1 domain and/or slow exchange kinetics. Structures of full-length p97 in the apo-state were also determined by cryo EM [24–27]. Unfortunately, however, the resolution remained rather low (around 20 Å) even in the latest study, perhaps suggesting that p97 poses problems to cryo EM that cannot be overcome by recent methodological advances in this field. One of these studies investigated structural differences between different nucleotide states, namely ADP, ADP-AlF_x AMP-PNP and a nucleotide-free state [26]. Finally, SAXS structures were derived for the same nucleotide states [28]. In contrast to full-length p97, the N–D1 fragment appears to be more amenable to high resolution structural analysis and has therefore been extensively investigated [25,29,30], including structures of N–D1 variants carrying IBMPFD-associated point mutations [29,30] (see below).

The p97 hexamer, at least to a first approximation, obeys C_6 symmetry, not only in the conformation of its subunits but also with respect to nucleotide occupancy where the same nucleotide is always present in all six subunits of the D1 (typically ADP) or D2 rings (different nucleotides). Along the sixfold axis, conically-shaped invaginations are present in both the D1 and the D2 rings with the one in the D2 ring being significantly more prominent, i.e. with a larger opening and depth. Between the two rings a central cavity exists (Fig. 1C) leaving open the possibility that at least under certain conditions a central pore traverses the hexamer along its sixfold axis. In the available structures of full-length p97, however, the pore is blocked by the inward facing side chains of the histidine residues at position 317 in the D1 ring [20]. The contacts between adjacent subunits are quite extensive with ~9% of the monomer surface per contact with an adjacent subunit in the AMP-PNP state and ~8% in the ADP-AlF_x and ADP states, in agreement with the predominantly hexameric state of p97 reported in all biochemical and structural studies so far.

The cryo EM [26] and SAXS [28] structures of p97 in different nucleotide states indicate large scale, nucleotide-dependent conformational changes. For example, the radii of gyration of the nucleotide-free, AMP-PNP, ADP-AlF_x and ADP states determined by SAXS are 61, 57, 55 and 58 Å, respectively. Furthermore, high speed atomic force microscopy revealed that ATP binding to the D2 domain induces forward clockwise and backward counterclockwise rotational movements (23 ± 8°) between the N–D1 and D2 hexameric rings [31]. The repeated back and forward rotational movements of p97 suggest a possible way of converting the chemical energy stored in ATP into mechanical energy required for the remodeling of substrates (see below). Similar rotational movements, albeit in the opposite direction, have been found in recent cryo EM studies [27]. Of note, these conformational changes were not observed in the corresponding crystal structures [23], where, in general, only small variations were found, and specifically the nucleotide-free state appeared to be significantly more compact than in the SAXS study. This discrepancy is probably the consequence of extensive crystal lattice contacts involving in particular the D2 and N domains, which may well prevent the conformational changes observed in solution. Nevertheless, despite these lattice contacts, rearrangements in the relative orientation of the domains could be observed in the crystal structures. For example, the

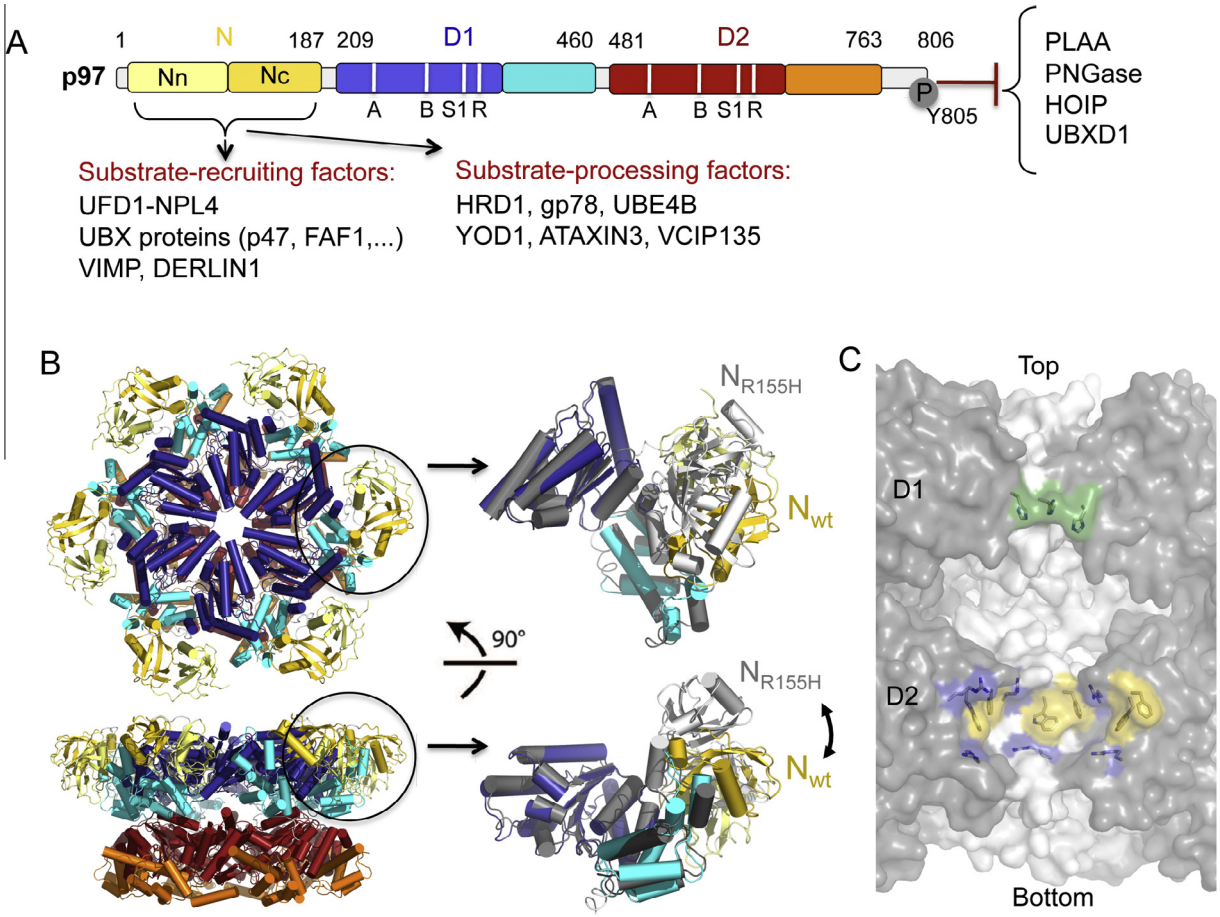

Fig. 1. p97 structure. (A) Domain architecture of p97 with the N domain in yellow and the two ATPase domains in blue/cyan (D1) and red/orange (D2), respectively. The Walker A (A) and Walker B (B) motifs as well as the Sensor 1 (S1) residues and arginine fingers are indicated. Representative substrate-recruiting and substrate-processing cofactors binding to the N domain and C-terminus of p97 are indicated. Note that phosphorylation of tyrosine residue 805 blocks cofactor binding to the C-terminus. (B) *Left:* Top and side view of the full-length p97 structure, color-coded as in (A) (pdb entry 3CF3). *Right:* Top and side view of N domain conformational changes (pdb entries 3CF3 and 3HU3) identified in the R155H mutant (black and gray). (C) Molecular surface representation of the p97 axial channel viewed from the side. For clarity, three monomers have been omitted. His317 in the D1 domain is shown in green. Residues in the D2 domain are colored as follows: Positively charged (Arg586, Arg599) in blue and hydrophobic residues in yellow (Trp551, Phe552).

maximal difference in the N–D1–D2 angle between different states is 11°, but also for these intra-protomer domain rearrangements it is unclear whether crystal contacts prevented more significant changes. Crystal structures of N–D1 fragments of the wild-type and IBMPFD-associated mutant variants, however, did reveal dramatic conformational changes in the position of the N domain [29]. It undergoes a displacement by ~12 Å and a rotation of more than 90° from its position in plane with the D1 ring ("down" conformation) as observed in all full-length crystal structures as well as in the ADP state of wild-type and IBMPFD mutant N–D1 fragments, to an out of plane ("up") conformation present in N–D1 structures of IBMPFD mutants in the presence of ATPγS (Fig. 1C). Coupled to this large rearrangement is a loop-to-helix transition in the linker connecting the N and D1 domains. While currently no crystal structure of the wild-type N–D1 fragment in the presence of ATPγS is available, SAXS studies suggest a similar nucleotide-dependent motion [29].

Even though the full-length crystal structures presumably do not reflect the full scale of conformational changes occurring in solution, they provide important insights into local structural changes in the vicinity of the nucleotide bound to the D2 domain. These changes affect the sensor 1 loop, the P-loop, the D1–D2 linker, the Arg finger and residues lining the central pore of p97. The latter include His317, which obstructs the pore, the aromatic tandem Trp551 and Phe552, located well within the D2 pore, and the basic residues Arg586 and Arg599, which are positioned at the entrance to the D2 pore (Fig. 1D) [23]. Mutational studies suggest that these pore-lining residues play critical roles for the segregase activity of p97 [32] and were interpreted to indicate that substrates do not pass through the D1 pore but instead interact with the much wider D2 pore. The cryo EM structures, on the other hand, indicate that the pore width changes during the ATPase cycle [26,27]. In particular, the pore appears much wider in the AMP-PNP state, consistent with the possibility that substrates are translocated along the central pore across the double D1–D2 ring, as in the case of Clp-type AAA ATPases possessing unfolding activity [12]. In this context it is interesting to note that molecular dynamics simulations of a substrate threading through the p97 pore suggest an energetically more favorable transport in the direction from the D1 to the D2 ring compared to the opposite direction [33]. However, in contrast to Clp-type unfoldases, no direct experimental evidence could be obtained that p97 substrates are threaded through the entire length of the central pore. An unfoldase activity of p97 was so far only observed upon simultaneous deletion of the N domain and mutation of D1 ring pore residues to tyrosines [34,35], perhaps suggesting that p97 evolved to use a mechanism of substrate turnover that is distinct from a Clp-like threading mechanism.

The ATPase activity of p97 is regulated in both an intramolecular (i.e. within the same subunit) and an intermolecular (i.e. between different subunits) manner. With respect to the intramolecular communication this involves the D1 and D2 subunits of the same polypeptide chain for which it could be shown that nucleotide binding to one ATPase domain is essential for the activity of the other domain [36,37]. The intermolecular effect is directly obvious from the structures: ATP is bound at the interface of two adjacent protomers, and the Arg finger is of critical importance for sensing the nucleotide state *in trans* [38]. As to the effects of these intramolecular and intermolecular signaling mechanisms, the communication between the D1 and D2 rings is inhibitory in nature so that ATP hydrolysis does not occur simultaneously in both domains (negative cooperativity) [39]. In contrast, the communication within each ring exhibits positive cooperativity since the Arg finger is essential for the catalytic mechanism [32,40]. Besides the Arg finger, the D1–D2 linker from the neighboring subunit has also been suggested to be involved in intermolecular communication within the p97 hexamer [41,42].

A recent systematic study of IBMPFD-causing p97 variants carrying point mutations at the N–D1 interface revealed increased ATPase activities [43]. The authors also showed for wild-type p97 that ATP hydrolysis by the D2 ring is suppressed in the coplanar/locked conformation (down conformation) of the N domain. When the N domain is released from the D1 plane and adopts a flexible state above the D1 ring (up conformation), D2 is competent for ATP hydrolysis. Experimental evidence for such a flexible state was provided by a cryo EM structure of the IBMPFD-causing A232E variant in which the N domains are indeed located above the D1 ring and interact with each other [43].

3. p97–cofactor interactions

p97 can interact with a large number of cofactors that regulate its function by recruiting it to different cellular pathways [5,17,44]. So far, about 40 cofactors have been identified in mammals, and the number is still growing. Most of the cofactors interact with the N domain of p97, while a small number binds to the C-terminal tail. Despite the large number of cofactors, the majority interacts *via* a small number of conserved binding modules (Fig. 2).

3.1. Interactions with the N domain

Cofactors interact with the N domain either *via* ubiquitin-related UBX (ubiquitin regulatory X) or UBXL (UBX-like) domains or *via* three short, linear binding motifs called VIM (VCP-interacting motif), VBM (VCP-binding motif) and SHP box (also called binding site 1 (BS1)) [44] (Fig. 2A–C). Functionally, these binding partners can be divided into substrate-recruiting and substrate-processing cofactors (Fig. 1A).

3.1.1. UBX and UBXL domains

The UBX domain is a module comprising approximately 80 amino acids and displays a β-grasp fold (β–β–α–β–β–α–β) closely resembling the structure of ubiquitin [45]. UBX domain containing proteins constitute the largest family of p97 cofactors [46,47]. A subset of UBX domain proteins is characterized by the presence of an ubiquitin-associated (UBA) domain, which mediates binding to ubiquitylated substrates [48,49]. These UBA-UBX proteins serve as prototypical substrate-recruiting cofactors or substrate "adaptors" of p97. So far, 13 UBX domain-containing proteins have been identified in mammals, all of which, with the exception of UBXD1 (see below), have been demonstrated to bind to p97 [46,47,49]. In addition, the related UBXL domain has been identified in a few p97 cofactors including the NPL4 subunit of the heterodimeric UFD1-NPL4 cofactor [50] and the two DUBs OTU1/YOD1 [51] and VCIP135 [52]. Due to their higher resolution, we will focus the following discussion on the UBX and UBXL domains of FAF1 and OTU1, respectively.

Molecular insights into p97-UBX domain interactions were obtained by crystal structures of the N domain in complex with the UBX domains of p47 [53] and FAF1 [54–56] (Fig. 2B). The FAF1 UBX domain interacts with the N domain *via* a conserved arginine from strand β1 and a Phe-Pro-Arg tripeptide located in the S3/S4 loop connecting strands β3 and β4. These four residues form the highly conserved R...FPR signature motif of UBX proteins [45]. The UBX domain inserts its S3/S4 loop into the hydrophobic cleft separating the two subdomains of the N domain, which represents a binding pocket common to the UBX and UBXL (see below) domains. Importantly, the FPR turn exhibits a *cis*-proline configuration and adopts a rarely observed *cis*-Pro touch-turn structure (also known as FcisP touch-turn motif) [57]. The *cis*-proline configuration is essential for N domain binding as it allows the aromatic ring of the phenylalanine to insert into the hydrophobic interdomain cleft [54–56]. In contrast, the *trans*-isomer would either produce severe steric clashes or prevent the phenylalanine residue from reaching the binding cleft. Interestingly, crystal structures of the free FAF1 UBX domain revealed that the S3/S4 loop displays conformational variability with the critical proline either in *cis*- or *trans*-configuration [54,57], suggesting an induced fit mechanism upon N domain binding. In addition to the phenylalanine, both arginine side chains of the R...FPR motif reach deep into the p97-binding pocket to anchor the UBX domain and to stabilize the complex by essential hydrophobic and electrostatic interactions.

The UBXL domains adopt a structure similar to the UBX domain despite a low sequence identity and the lack of the UBX signature motif [58]. The structure of the UBXL domain of NPL4 has been solved by NMR, and based on chemical shift perturbation analysis a structural model for the N domain–UBXL complex has been calculated [59] (Fig. 2B). Recently, the crystal structure of the yeast OTU1 UBXL domain in complex with the N domain of p97 has been solved, thus allowing for a detailed structural comparison of UBXL domains (NPL4, OTU1) and UBX domains (p47, FAF1) [58] (Fig. 2B). Although the UBX and UBXL domains share structural homology at the Cα level, the electrostatic surfaces are quite different between both families. While members of both families bind to the same site of the N domain, the interaction modes are specific for each domain. The most intriguing differences are found in the conformation of the S3/S4 loop. The UBXL domain of NPL4 resembles ubiquitin rather than UBX with respect to the length of its S3/S4 loop, which is extended by six residues, however, in contrast to ubiquitin it harbors a 3_{10}-helix. This loop provides specific contacts with p97, which distinguishes it from ubiquitin and the UBX domain. In the OTU1 UBXL domain the S3/S4 loop contains the motif YPP, with the central proline found in *cis*-configuration, similar to the corresponding proline in the FPR loop of the UBX domain. In contrast to the UBX domain, however, the central proline in OTU1 is involved in a stacking interaction with the preceding tyrosine, resulting in a different conformation of the S3/S4 loop. In addition, the first arginine of the R...FPR UBX signature motif is replaced by threonine in the OTU1 UBXL domain. Taken together, the UBX and UBXL domains bind in similar orientations to the N domain of p97 while exhibiting unique recognition features.

3.1.2. The VCP-interacting motif

The VCP-interaction motif (VIM) (Fig. 2A), a linear, α-helical polypeptide stretch composed of positively charged flanking residues separated by moderately hydrophobic alanine residues, was originally identified in the E3 ubiquitin ligase gp78 (also known as AMFR, Autocrine Motility Factor Receptor) [60] and in SVIP

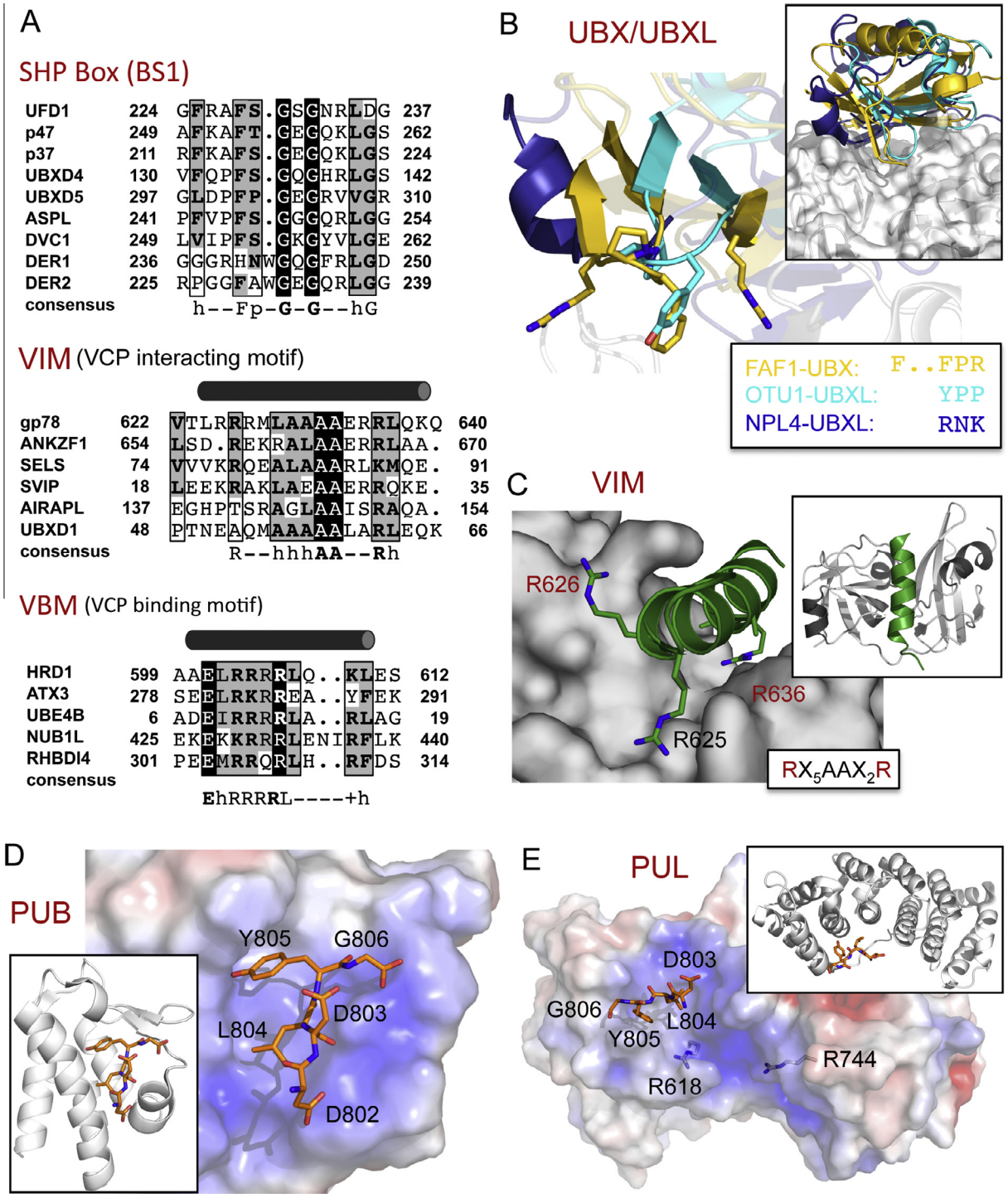

Fig. 2. p97 binding modules. (A) Sequence alignment of the SHP box, the VIM and the VBM (all protein sequences are from mammals). (B) Ribbon representations of the FAF1-UBX (pdb entry 3QQ8, colored in gold), NPL4-UBXL (pdb entry 2PJH, colored in blue) and OTU1-UBXL domains (pdb entry 4KDI, colored in cyan) in complex with the p97 N domain (molecular surface colored in gray). Details of the key interaction in the region of the S3–S4 loop are shown. (C) Ribbon and surface representation of the p97 N domain (colored in gray) in complex with the gp78 VIM peptide (pdb entry 1TIW, colored in green). Key interactions with the conserved arginines are shown. (D) Ribbon representation and molecular surface colored according to its electrostatic potential (electropositive in blue, electronegative in red, contoured at ±5 kT) of the PNGase PUB domain in complex with the C-terminal residues of p97 in stick representation (pdb entry 2HPL, colored in orange). (E) Ribbon representation and molecular surface colored according to its electrostatic potential (electropositive in blue, electronegative in red, contoured at ±5 kT) of the PLAA PUL domain in complex with the C-terminal residues of p97 in stick representation (pdb entry 3EEB, colored in orange).

(Small VCP/p97-Interacting Protein), a negative regulator of gp78-mediated ER-associated protein degradation [61,62]. A subsequent bioinformatic search defined a minimal VIM consensus sequence and identified several additional VIM-containing proteins [63]. These include ANKZF1 (ANKyrin repeat and Zinc Finger domain-containing-1, also known as ZNF744 or Vms1), a protein that has been implicated in mitochondrial protein degradation [64]; the UBX protein UBXD1, involved in sorting of ubiquitylated cargo in the endocytic pathway [10]; AIRAPL (Arsenite Inducible RNA Associated Protein Like Protein, also known as ZFAND2B), an

ER membrane-anchored protein which associates with and regulates the function of the 26S proteasome under arsenite stress [65]; SelS (also called VIMP, VCP-Interacting Membrane Protein), a protein involved in the recruitment of p97 to the ER membrane [66]; and the yeast protein Wss1, a metalloprotease involved in DNA–protein crosslink repair [67]. With the exception of SelS it was shown that these proteins interact *via* their VIM with p97 [63,64,67–69]. VIM containing cofactors can be grouped into two subfamilies [63] (Fig. 2A): (i) "RAAR" proteins, which contain the consensus sequence RX_5AAX_2R with two conserved arginine residues separated by a hydrophobic amino acid stretch including two conserved alanine residues as found in gp78, ANKZF1, SVIP and SelS, and (ii) "AAR" proteins as found in UBXD1 and AIRAPL, which contain the consensus sequence AAX_2R but lack the first conserved arginine.

The crystal structure of the p97 N domain in complex with a gp78-derived peptide spanning the VIM revealed that the VIM indeed adopts an α-helix and that it inserts into the hydrophobic pocket formed between the two subdomains on the N domain [68] (Fig. 2C). Besides the two conserved arginine residues of the signature motif, the VIM of gp78 contains a third, non-conserved arginine amino-terminal of the first consensus arginine (RRX_5-AAX_2R). All three arginine residues are important for binding, with the carboxy-terminal arginine being most critical. These arginines are not only involved in electrostatic interactions but also provide important hydrophobic contacts *via* their long aliphatic side chain regions. A detailed comparative study of VIM proteins from both subfamilies revealed that all bind to the hydrophobic interdomain cleft of the N domain [68]. A conserved binding mode of RAAR proteins is also supported by an NMR chemical shift analysis of the interaction of the ANKZF1 VIM with the N domain [63]. The binding mode of RAAR proteins involves the positively charged flanking residues separated by hydrophobic residues and results in a high affinity interaction [68]. However, differences due to variations in the amino acid composition are observed that translate into changes in affinity and might trigger a slightly altered orientation of the α-helix, thus resulting in a modified binding specificity. For example, despite a high degree of sequence homology with the VIMs of gp78 and ANKZF1, the RAAR protein SVIP requires for high affinity binding not only the N domain of p97, but also the N–D1 linker region and the adjacent D1 domain [68]. This is probably the consequence of the longer, VIM-containing α-helix in SVIP [70] and indicates that additional residues can determine affinity and specificity. In contrast, proteins of the AAR subfamily, which lack the first positively charged residue, bind with much lower affinity, and the interaction is mainly driven by hydrophobic contacts [68]. While it is conceivable that this binding mode with only one arginine residue as anchor allows for a high degree of rotational flexibility, this hypothesis has to await further support by a corresponding high resolution structure.

3.1.3. The VCP-binding motif

The VCP-binding motif (VBM) was first identified in the DUB ataxin-3 (ATX3) and the ubiquitin ligase UBE4B [71] and has subsequently also been found in the ubiquitin ligase HRD1 [72], in the ubiquitin-dependent ER-resident rhomboid protease RHBDL4 [73], and in NUB1L, which is involved in the proteasomal degradation of NEDD8 [70,74]. The VBM is characterized by a core of four positively charged residues (Arg/Lys) with a highly conserved arginine at position four (consensus sequence EhRRRRL; h, hydrophobic amino acid) (Fig. 2A). Mutational studies of the HRD1, ATX3 and UBE4B VBM motifs revealed that only the first and the last Arg/Lys residues are strictly required for p97 binding [72]. Solution structures of the HRD1 and NUB1L VBMs revealed an α-helix with the two critical positively charged residues located on the same face [70]. While the C-terminal leucine residue was found to be critical for p97 binding of the HRD1 and UBE4B VBMs, it is replaced in ATX3 by a glutamate residue that is not involved in p97 binding [72]. The function of the highly conserved N-terminal glutamate residue has not been studied so far. The VBM exhibits a roughly 10-fold lower affinity to the p97 N domain than the α-helical VIMs of gp78 and SVIP [70]. This may be due to the VBM forming a shorter helix and lacking additional positively charged residues besides the clustered arginines, leading to a more restricted binding interface compared to the VIM, where the positively charged residues are separated into two regions. Consistent with overlapping binding sites of VBM and VIM at the hydrophobic interdomain cleft of the p97 N domain, a mutually exclusive binding of the VBM from HRD1 and the VIM from SVIP was demonstrated [70].

3.1.4. SHP box/BS1

The SHP box binding motif (also called binding site 1, BS1) was initially identified in the yeast proteins Shp1 (the yeast ortholog of p47), Ufd1, Wss1 and the Derlin homolog Dfm1 [75,76], as well as in mammalian p47 and UFD1 [50]. It is a short hydrophobic sequence stretch containing two invariant glycine residues and is characterized by the consensus sequence $FxGxGx_2h$ (x, any amino acid, h, hydrophobic amino acid) (Fig. 2A). The binding sequence is quite variable and typically found in unstructured regions of p97 cofactors. In mammals, the motif is found in the SEP (Shp, eyes-closed, p47) domain-containing UBX proteins p47, p37, UBXD4 and UBXD5, where it is located N-terminally of the UBX domain [46], to which it is connected *via* a mainly unstructured linker. Further mammalian members are the UBX protein TUG (also known as ASPL) [77], UFD1 [50], DVC1 (also called Spartan, a homolog of yeast Wss1), a proteolytic enzyme that functions in DNA repair coupled to DNA replication [67,78], and the Derlin proteins DER1 and DER2 involved in endoplasmic reticulum associated protein degradation (ERAD) [66,79–81]. Currently, only limited structural information on the SHP box-p97 interaction is available, and the exact binding site of the SHP box on the p97 N domain remains unclear. While NMR data suggest that the binding region of the SHP box of UFD1 on the N domain partially overlaps with the binding site for the UBXL domain of NPL4 [59], pull-down experiments demonstrated that SHP box binding to p97 does not interfere with the binding of UBX/UBXL domains, suggesting different binding sites [50].

3.2. Interactions with the C-terminal tail

So far, the PUB (PNGase/UBA or UBX containing proteins; also known as PUG) and PUL (PLAP, Ufd3p, and Lub1p) domains are the only defined structural domains known to interact with the C-terminal tail of p97. Both modules had originally been linked to the ubiquitin system based on bioinformatic analyses [82–84].

3.2.1. The PUB domain

The PUB domain consists of a short, three-stranded β-sheet packed onto an α-helical core [85]. Three PUB-domain containing proteins have been described so far in humans and all interact with the p97 C-terminal tail: PNGase (peptide N-glycanase) [85,86], which is involved in the deglycosylation of misfolded glycoproteins [87], the UBX protein UBXD1 [68,85,88], and HOIP (HOIL-1-interacting protein) [89–91], the catalytic subunit of the E3 ubiquitin ligase LUBAC, which catalyzes the assembly of linear ubiquitin chains [92].

Molecular insights into the interaction of the PUB domain with p97 were revealed by crystal structures of the PNGase and HOIP PUB domains in complex with peptides comprising the five and four, respectively, C-terminal residues of p97 called PIM (PUB

Interacting Motif) [86,91] (Fig. 2D). The PUB-PIM complex is stabilized by a combination of hydrophobic and electrostatic interactions, with a key interaction mediated by insertion of the hydrophobic side chain of Leu804 and the aromatic side chain of Tyr805 of p97 into a hydrophobic pocket of the PUB domain called the Φ-Y pocket [91]. Interestingly, phosphorylation of the strictly conserved tyrosine residue within the PIM (Tyr805 in p97) completely blocks the interaction of p97 with the PUB domain and with the PUL domain of Ufd3 (see below) [86,90,91,93]. Thus, tyrosine phosphorylation appears to be a conserved mechanism to control protein–protein interactions of the C-terminal tail of p97.

3.2.2. The PUL domain

The PUL (PLAP, Ufd3p, and Lub1p) domain of human PLAA (phospholipase A2-activating protein; also know as PLAP) and its yeast homolog Ufd3/Doa1 has been shown to mediate binding to p97/Cdc48 [86,94,95]. The PUL domain adopts an extended fold composed of six armadillo repeats connected by short loops where each repeat is composed of three helices. The binding site of the p97 C-terminal tail on the PUL domain is still controversial. A detailed mutational and biochemical study of the yeast Ufd3 PUL domain revealed a conserved binding site on a concave surface, which represents the primary protein binding site of Armadillo domains [96]. This area forms a continuous, positively charged electrostatic surface, which could complement the highly negatively charged C-terminus of Cdc48/p97. Consistent with these biochemical data, Ufd3 variants with point mutations in this region failed to interact with Cdc48 *in vivo*. However, a crystal structure of the human PLAA PUL domain in complex with a p97 C-terminal peptide (TEDNDDDLYG) revealed a different binding site in an adjacent hydrophobic groove [97] (Fig. 2E). In this structure, only Leu804 and Tyr805 point into a hydrophobic pocket in a conformation that is strikingly similar to that when in complex with the PNGase PUB domain. Unfortunately, only the last four residues of the p97 peptide resembling the PIM (see above) are ordered in the crystal structure. It is therefore possible that the remaining negatively charged and polar residues reach into the neighboring positively charged ridge that has been identified by biochemical studies [96]. Thus, the binding mode between the PUL domain and the PIM of Cdc48/p97 may well be conserved from yeasts to mammals.

3.3. Non-canonical binding domains/motifs

The number of cofactors that associate with p97 is still growing. Although so far all cofactors bind either to the N domain or to the extreme C-terminus of p97, cofactor associations with the D2 domain are imaginable which could allosterically influence the translocation properties of the D2 domain. Furthermore, it would not be surprising if in the future additional binding domains or interaction motifs were identified. For example, neurofibromin 1 (NF1), a protein that is involved in synaptogenesis, has been shown to interact with the two ATPase domains (D1D2) in the absence of the N domain and the C-terminal tail *via* its LRD domain (leucine rich repeat domain) [98]. This domain neither displays structural similarity to any of the known p97 interacting domains nor does it contain any of the linear binding motifs. Other examples are the CSN5 subunit of the COP9 signalosome, which is proposed to interact *via* its MPN domain with the N domain [99] or SelS/VIMP, which instead of using its VIM binds *via* two proline residues [100].

4. Cofactors define functional p97 assemblies

One of the key questions regarding the biological function of p97 is how this highly abundant ATPase participates in so many dissimilar processes. The underlying principle for this functional diversity of p97 is its ability to associate with a large variety of cofactors mediating specificity for subcellular localization, pathways and/or substrates. To ensure that cofactor binding results in defined, productive p97 assemblies with specific cellular functions, it must be tightly controlled by multiple regulatory mechanisms. While a complete understanding will require further structural, biochemical and cellular studies of p97–cofactor interactions, some of the regulatory mechanisms underlying ordered cofactor binding, including competition for overlapping binding sites on the N domain, bipartite binding, hierarchical binding of major and auxiliary cofactors, and nucleotide-dependent conformational changes altering cofactor assembly have been described and are discussed below.

4.1. Competition for binding sites

As discussed above, the linear VIM and VBM motifs as well as the UBX and UBXL domains all bind to partially overlapping sites at the hydrophobic intersubdomain cleft of the p97 N domain, despite the absence of significant sequence similarity between them (Fig. 3A). This demonstrates that one N domain and hence one p97 protomer can only interact with one of these proteins at any given time, reducing the complexity of cofactor interactions to a combinatorial problem of six N domains per p97 hexamer. Competition for N domain binding has been experimentally verified for various combinations of cofactors possessing different binding modules, *e.g.* SHP/UBX – SHP/UBXL (p47 – UFD1-NPL4) [50,101], SHP/UBX – VIM (p47-UBXD1) [88], VIM – VBM (SVIP – HRD1) [70], VIM – SHP/UBXL (gp78 – UFD1-NPL4) [60]. The VIM protein SVIP is remarkable, as it is currently the only known p97 cofactor that binds with high affinity to all six N domains [68] and prevents the association of additional cofactors (see below). Consistent with its function as an inhibitor of the ERAD pathway [62] and its regulatory role in p97 subcellular localization and autophagy [102], SVIP forms a distinct complex with p97 and is an efficient competitor of the major N domain targeting cofactors UFD1-NPL4 and p47 [61,68,70]. It should nevertheless be noted that the basic ability of cofactors or their isolated binding modules to compete for p97 binding typically has been tested in biochemical titration experiments using purified proteins and peptides. The outcome of competitive binding in living cells is likely to be much more complex, as it depends, among other factors, on the relative intracellular concentrations and binding kinetics of many simultaneously present cofactors.

Competitive binding has also been described for the yeast substrate-processing cofactors Ufd2 and Ufd3, which bind to overlapping regions at the C-terminus of Cdc48 [95,103], but has not yet been analyzed for mammalian PUB and PUL domain containing cofactors.

4.2. Bipartite binding modes

Several p97 cofactors, including the three major cofactors p47, UFD1-NPL4 and UBXD1, contain more than one p97 binding module, enabling them to interact with p97 in a bipartite manner [50,59,63,88]. For p47, this involves binding *via* the UBX domain and the SHP box, whereas the UFD1-NPL4 heterodimer interacts *via* the UBXL domain of NPL4 and the SHP box of UFD1. Since p47 is a stable trimer, the bipartite binding mode results in the symmetric binding of three p47 subunits, which occupy all six N domains of the p97 hexamer [104]. By analogy, a similar symmetric, bipartite binding mode of the p47-related cofactors p37, UBXD4 and UBXD5 can be expected. In contrast, only one UFD1-NPL4 heterodimer binds to two adjacent protomers of a p97 hexamer [105]. Importantly, these binding modes provide the structural basis for the

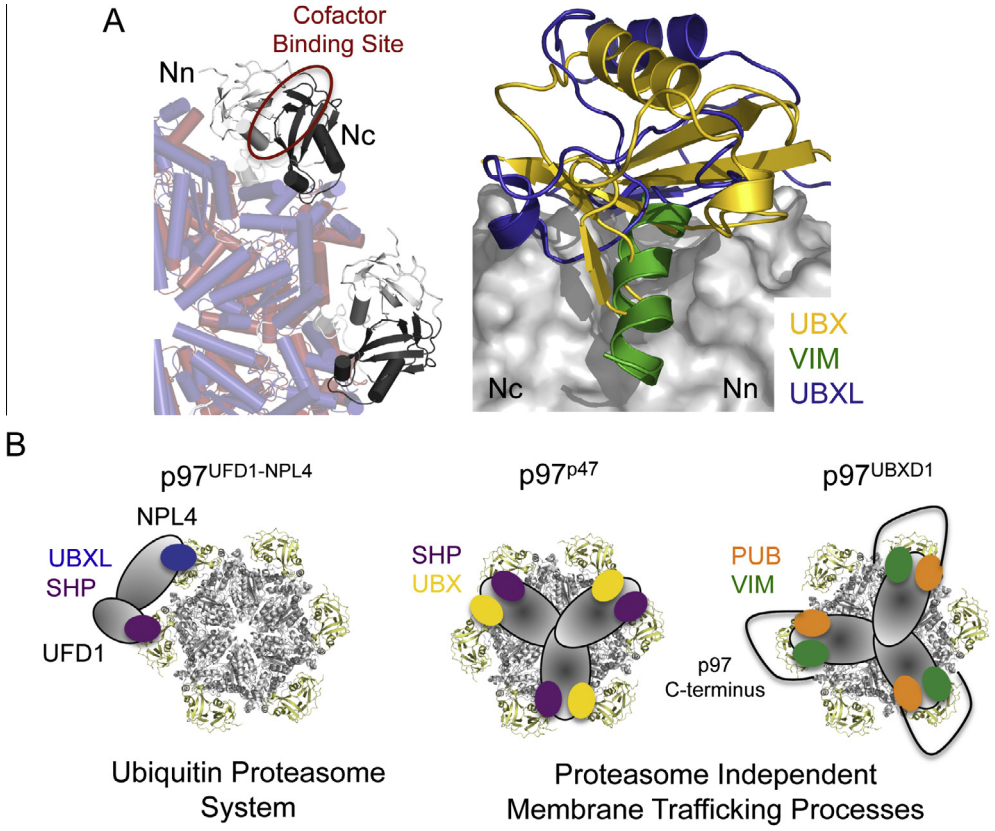

Fig. 3. Regulation of p97 cofactor assembly. (A) Competition for binding sites. Left, Ribbon representation of p97 and its cofactor binding site located at the intersubdomain cleft of the N domain (Nn in white, Nc in black, D1 in blue, D2 in red). Right, Ribbon representations together with the molecular surface of the p97 N domain in complex with the VIM of gp78 (pdb entry 3TIW, colored in green), FAF1-UBX (pdb entry 3QQ8, colored in gold) and NPL4-UBXL (pdb entry 2PJH, colored in blue). (B) Model for the bipartite binding of major p97 cofactor assemblies.

mutually exclusive binding of p47 (and its homologues) and UFD1-NPL4 to p97 [50,101], which is evolutionarily conserved and defines the two best-characterized p97 assemblies with distinct cellular functions. The $p97^{UFD1\text{-}NPL4}$ complex is required in a number of proteasomal degradation pathways such as ERAD, where it drives the dislocation of polyubiquitylated substrates from the ER membrane into the cytosol, degradation of proteins associated with chromatin and the outer mitochondrial membrane, and of important regulators of cell cycle progression and signal transduction (reviewed in [5,17–19]). In contrast, the $p97^{p47}$ complex functions mainly in proteasome-independent processes such as autophagy and the post-mitotic reassembly of Golgi stacks and the nuclear envelope [5,17,106].

In higher eukaryotes, the $p97^{UBXD1}$ complex represents a third, distinct p97 assembly with so far poorly defined function(s) in endolysosomal trafficking [10]. Even though UBXD1 belongs to the UBX family of p97 cofactors, it does not bind p97 *via* its UBX domain because the R. . .FPR signature motif (see above) is replaced by SGG. Instead, UBXD1 uses a bipartite binding mode where its PUB domain and VIM interact with the C terminus and N domain of p97, respectively [63,88]. This binding mode is unique among p97 cofactors in that the two major binding sites of p97, the N domain and the C terminus, are simultaneously contacted by a single cofactor, which presumably will restrict the conformational flexibility of p97. Similar to p47, isothermal titration calorimetry experiments indicate that UBXD1 binds as a trimer [68], which provides a rationale for the observed mutually exclusive binding with p47 and UFD1-NPL4 (Fig. 3B).

Even though the importance of bipartite binding modes for the assembly of the three major p97 complexes is well-established, it is not known whether the cofactors remain stably associated *via* both binding modules all the time. It is conceivable that ATP hydrolysis and/or substrate binding induce conformational changes that trigger dissociation at one site and lead to an increased conformational flexibility of the cofactor for subsequent catalysis. Such a model would be in accordance with the conformational changes observed in EM studies of $p97^{UFD1\text{-}NPL4}$ in different nucleotide states, where the heterodimer was found to be bound either *via* two binding sites in the apo state or one binding site in the ADP bound state [107]. EM studies of the $p97^{p47}$ complex in different nucleotide states also suggest that during ATP hydrolysis binding of trimeric p47 *via* its UBX domains involves only every second N domain in the ADP bound state or all six N domains with the UBX domain and its upstream linker bound between two adjacent N domains in the presence of AMP-PNP [104].

4.3. Higher order assemblies

Since only one heterodimeric UFD1-NPL4 cofactor binds to two adjacent protomers within the p97 hexamer, further cofactors could potentially bind to vacant N domains. Indeed, it was shown that immunoprecipitates of the UBA-UBX proteins FAF1, UBXD7, UBXD8 and SAKS1 contained not only p97, but also UFD1-NPL4 [49], an observation that is consistent with the existence of higher-order p97–cofactor complexes. A similar observation was made in yeast where the UBA-UBX protein Ubx2 was found to exist in a $Cdc48^{Ufd1\text{-}Npl4\text{-}Ubx2}$ complex [108]. These observations led to the concept of hierarchical cofactor binding where the general activity of the $Cdc48/p97^{Ufd1\text{-}Npl4}$ complex in proteasomal degradation pathways is further specified by the binding of additional,

pathway-specific cofactors [46]. Subsequently, a hierarchy in cofactor binding could be demonstrated directly with purified recombinant proteins [54,56]. Binding of FAF1 to p97 required the presence of UFD1-NPL4, resulting in the formation of a complex with a stoichiometry of 6:1:1 (p97:UFD1-NPL4:FAF1). Since no direct interaction of FAF1 with UFD1-NPL4 was observed in the absence of p97, it is conceivable that the binding of UFD1-NPL4 induces an asymmetry in the p97 hexamer such that only one of the vacant p97 N domains interacts tightly with FAF1. According to this model, FAF1 binding would strictly depend on the presence of the UFD1-NPL4 cofactor, but would not have to occur adjacent to the UFD1-NPL4 heterodimer. In one of these studies, UBXD7 was shown to also require UFD1-NPL4 for p97 binding [54].

In a recent study, however, the concept of a hierarchical binding of UFD1-NPL4 and FAF1 to p97 was challenged. According to an EM structure of FAF1 in complex with p97, FAF1 binds as a trimer on top of the p97 ring [109], reminiscent of the p97–p47 complex exhibiting a 6:3 stoichiometry described above. The reason for the conflicting data on the ability of FAF1 to bind p97 in the absence of UFD1-NPL4 *in vitro* is unclear. However, siRNA-mediated depletion of UFD1-NPL4 in HeLa cells almost completely abolished the co-immunoprecipitation of p97 with FAF1, suggesting that UFD1-NPL4 is indeed required for the stable binding of FAF1 to p97, in accordance with the hierarchical cofactor binding model [56]. While it presently cannot be ruled out that an UFD1-NPL4-independent $p97^{FAF1}$ complex can exist *in vivo*, further studies are required to determine physiological conditions supporting its assembly and to identify potential specific cellular functions.

In addition to the UBA-UBX proteins discussed above, the UFD1-NPL4 heterodimer was also found in quaternary complexes with p97 and Otu1/YOD1 [51,58], NUB1L [74] and SelS/VIMP [66]. Of these, only the $p97^{UFD1\text{-}NPL4\text{-}OTU1}$ complex has been studied in more detail. *In vitro* binding and EM studies showed that only one OTU1 molecule is recruited into the $p97^{UFD1\text{-}NPL4}$ complex [58], thus forming an oligomer with a stoichiometry of 6:1:1 similar to the $p97^{UFD1\text{-}NPL4\text{-}FAF1}$ complex discussed above. However, no hierarchical binding was observed, indicating an independent binding mode and suggesting that hierarchical binding may be restricted to substrate-recruiting cofactors of the UBA-UBX type.

The potential of the other two major p97 complexes, $p97^{p47}$ and $p97^{UBXD1}$, to form higher order complexes has not been explored yet. However, it was proposed that the DUB VCIP135 forms an unstable ternary complex with $p97^{p47}$ during post-mitotic Golgi reassembly, where p47 would be associated with p97 *via* only one of its two binding sites [52]. Given that the UBX domain of p47 and the UBXL domain of VCIP135 compete for the same binding site on the p97 N domain, this might indicate that in the ternary $p97^{p47\text{-}VCIP135}$ complex one or more p47 subunits stay associated with p97 *via* their SHP box.

4.4. Nucleotide-dependent control of cofactor interactions

The ability to interact with a large number of cofactor proteins in a highly regulated manner is critical for p97 to perform its diverse cellular functions. As described above, the N domains in wild-type p97 exist in a tightly regulated, mixed arrangement of up and down conformations (also called "locked" and "flexible" conformation, respectively) [27,43]. Since N domains are largely responsible for p97 interactions with various adaptor proteins, their conformational flexibility is believed to be critical for p97 to properly handle substrates bound to adaptor proteins. Although nucleotide-dependent N domain movements of p97 suggest that a mutual influence of cofactor binding and ATP binding or hydrolysis could exist [104], experimental data are still limited. Cryo-EM studies of the $p97^{p47}$ [104], $p97^{UFD1\text{-}NPL4}$ [107] and $p97^{FAF1}$ [109] complexes revealed that cofactor binding involves multiple N domains, and in all structures those N domains that are involved in cofactor binding are in the up conformation. A common model of cofactor binding to p97 has been proposed [109], according to which a relocation of the N domain during the ATP hydrolysis cycle from the up to the down conformation could provide the force necessary for target protein disassembly.

Furthermore, it was shown that ATP binding to the D1 domain regulates the recruitment of adaptor proteins to the N domain, which is triggered through binding rather than hydrolysis of ATP [110]. ATP was found to exert differential effects by strengthening the binding of UFD1-NPL4 while not affecting the binding of p47, and therefore significantly enhancing the capacity of UFD1-NPL4 to compete with p47 for p97 binding. Conversely, it has been demonstrated that cofactors affect the p97 ATPase activity in different ways: p47 and the related protein p37 are inhibitory and activating cofactors, respectively [111,112], whereas UFD1-NPL4 [111] and UBXD1 did not show an effect [113].

Recently, the link between the regulation of p97 ATPase activity by cofactors has been studied in more detail for p37 and p47 [112]. Zhang et al. identified a region of about 25 unstructured amino acids in p47 that is not involved in p97 binding and is missing in p37, which is responsible for the inhibitory effect of p47. In the D1 domain of each p97 monomer two ADP-bound states exist in a dynamic equilibrium: the ADP-locked state containing pre-bound, difficult-to-remove ADP [28,114], and the ADP-open state where ADP has a reduced affinity to the D1 site and is ready to be exchanged [29]. In the wild-type protein, the equilibrium is in favor of the ADP-locked state, whereas in IBMPFD mutants (see below), the ADP-open state is prevalent. p47 binding to p97 probably promotes an ADP-locked state, which prevents ATP from binding and consequently inhibits the activity of both the D1 and D2 domains. In contrast, p37 binding to the N domain most likely induces conformational changes which convert the D1 domain into the ADP-open state that allows ATP binding to D1, which in turn activates the D2 domain. These findings suggest that distinct cofactors can induce different conformational changes in N–D1 that regulate ADP/ATP binding to the D1 domain, which in turn controls both the D1 and D2 ATPase cycles [112].

Importantly, IBMPFD-associated p97 mutant proteins exhibit a dysregulation of the N domain conformational equilibrium as a consequence of abnormal nucleotide binding to the D1 domain [30]. Due to an altered inter-subunit communication in IBMPFD mutants the D1 domains fail to regulate their respective nucleotide-binding states as evidenced by a lower amount of pre-bound ADP and weaker affinity for ADP, resulting in a better accessibility for ATP in comparison to the wild-type protein [29,30]. The altered conformational equilibrium of the N domain in p97 disease mutants is accompanied by elevated ATPase activities *in vitro* [43,115,116] and altered interactions with some but not all cofactors in cells, which has been proposed to be key to the pathogenesis of IBMPFD [10,116–118] (for a recent review see [119]). Specifically, increased amounts of p47, UFD1-NPL4, and ataxin-3, but decreased amounts of UBXD1 and UBE4B have been found. Interestingly, the decreased binding to UBXD1 is associated with a blockage of the endolysosomal trafficking of caveolin 1 [10]. Although disease mutants and wild-type p97 interact with both cofactors in a similar manner *in vitro*, disease mutants no longer can be activated by p37, but are still inhibited by p47 [112]. In disease mutants the described conformational changes for the wild-type protein (see above) are already induced, as they are in the active ADP-open state, which might explain why no additional activation can be observed in these mutants with p37. In contrast, the inhibitory effect of p47 might be the consequence of promoting the ADP-locked state that prevents ATP binding as described for the wild-type protein (see above). Taken together,

the results of Zhang et al. for both wild-type and mutant proteins show that cofactors play a critical role in controlling p97 ATPase activity, and suggest that a lack of cofactor-regulated communication may contribute to p97-associated disease pathogenesis [112].

5. Conclusion

The number and complexity of p97–cofactor interactions is conceptually and experimentally challenging. While significant progress has been made in understanding the structural and mechanistic basis of cofactor binding, important open questions remain. For instance, a complete inventory of all physiologically relevant, distinct p97–cofactor assemblies is still lacking. How many major p97 complexes exist – just the known three ($p97^{UFD1\text{-}NPL4}$, $p97^{p47}$, $p97^{UBXD1}$), or are one or several more awaiting discovery? Is $p97^{SVIP}$ another major p97 complex and if so, what are its unique structural and functional features? How do all the underexplored "minor" cofactors modulate these major assemblies? Can the diversity of p97–cofactor complexes be fully understood on the basis of bipartite and hierarchical binding modes, or are additional, still unknown regulatory principles contributing to p97 function? Which are the limitations that prevent us from formulating a complete model describing the mutual influence of the D1 and D2 ATPase cycles, (sub)domain movements and cofactor interactions? Finally, will we be able to devise successful therapeutic strategies for patients suffering from IBMPFD once we fully understand the pathogenic consequences of disease-associated p97 variants on the structural, mechanistic and cellular levels?

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