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STRUCTURE AND FUNCTION OF THE AAA+ ATPASE p97/Cdc48p

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Abstract

p97 (also known as valosin-containing protein (VCP) in mammals or Cdc48p in *S. cerevisiae*) is an evolutionarily conserved ATPase present in all eukaryotes and archaebacteria. In conjunction with a collection of cofactors and adaptors, p97/Cdc48p performs an array of biological functions mostly through modulating the stability of 'client' proteins. Using energy from ATP hydrolysis, p97/Cdc48p segregates these molecules from immobile cellular structures such as protein assemblies, membrane organelles, and chromatin. Consequently, the released polypeptides can be efficiently degraded by the ubiquitin proteasome system or recycled. This review summarizes our current understanding of the structure and function of this essential cellular chaperoning system.

A genetic screen conducted three decades ago in *S. cerevisiae* identified several alleles of *Cdc48* that affects cell growth at non-permissive temperatures due to a cell cycle arrest at the G2-M transition stage (Moir et al., 1982). The mammalian homolog of Cdc48p was later reported as a 97 kDa protein precursor for the small peptide valosin, and therefore named as valosin-containing protein (VCP) or p97 (Koller and Brownstein, 1987). Although it turned out that valosin is a purification artifact unrelated to p97 (Gill et al., 1989), the VCP nomenclature is still being used in the literature. In some species, the name transitional endoplasmic reticulum ATPase (TER ATPase) is used given the localization and function of a fraction of this enzyme at the endoplasmic reticulum (ER) (see below). In this review, we use p97 and Cdc48p for the mammalian and yeast homologs, respectively.

p97/Cdc48p belongs to a large ATPase family termed AAA+ (extended family of ATPases associated with various cellular activities) ATPase. Enzymes of this family function in all species from bacteria to humans, often as essential chaperones that promote protein folding or unfolding. p97/Cdc48p is a type II AAA+ ATPase because it has two AAA ATPase domains in tandem (named D1 and D2, respectively) (Figure 1A). A short polypeptide linker

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(D1–D2 linker) connects the two ATPase domains and another linker (N-D1 linker) joins the D1 domain to a large amino-terminal domain (N-domain). The carboxyl-terminus of the D2 domain is appended with a short tail containing ~40 residues. Interaction of p97/Cdc48p with its partners is mostly mediated by the N-domain, although a few proteins bind p97/Cdc48p via the C-terminal tail (Buchberger et al., 2015; Ogura and Wilkinson, 2001). The D1 and D2 domains are homologous both in sequence and in structure. However, they have distinct functions. For example, the hexameric assembly of p97 only requires the D1 but not the D2 domain (Wang et al., 2003).

In mammalian cells, p97 is localized mainly to the cytoplasm with a fraction associated with the membranes of subcellular organelles such as the ER, Golgi, mitochondria, and endosomes (Acharya et al., 1995; Latterich et al., 1995; Rabouille et al., 1995; Ramanathan and Ye, 2012; Xu et al., 2011). The membrane localizations are probably mediated by membrane-associated receptors, whose identity is largely unknown in most cases. A fraction of p97/Cdc48p is also present in the nucleus and serves essential roles in chromatin-associated events and nuclear protein quality control (PQC) (see below) (Madeo et al., 1998).

As one of the most abundant proteins in eukaryotic cells, p97 is ubiquitously expressed in multicellular organisms. In humans, the mRNA expression of p97 was moderately increased in certain types of cancer, and the expression level to some extent correlates with the sensitivity of cancer cells to a potent p97 inhibitor that is currently evaluated as a potential anti-cancer drug (Anderson et al., 2015).

Most known substrates of p97/Cdc48p are conjugated with ubiquitin chains and degraded by the 26S proteasome. Accordingly, many p97/Cdc48p cofactors/adaptors are capable of recognizing ubiquitin conjugates (Ye, 2006). It has been thought that the interplay between ubiquitin and the p97 system is critical for p97 functions, although the precise role awaits further elucidation. Some p97 cofactors are ubiquitin ligase or deubiquitinase that can process ubiquitin chains, but the vast majority serves as adaptors that link p97/Cdc48p to specific subcellular compartment or substrate.

More recently, genetic studies have linked mutations in p97 to several human diseases including IBMPFD (Inclusion Body Myopathy associated with Paget's disease of the bone and Frontotemporal Dementia) and amyotrophic lateral sclerosis (ALS). These findings have stimulated a flurry of investigations on the structure and function of p97/Cdc48p.

Structural organization of p97

Structural Features of p97

Structural information on p97 was initially obtained with negatively stained samples examined by electron microscopy (EM). The results provided the basic shape of a ring-like hexameric molecule (Peters et al., 1990; Zhang et al., 1994). Higher resolution structures were later obtained by cryo-EM (Rouiller et al., 2000) and by crystallization of a N-D1 fragment (Zhang et al., 2000). These studies confirmed the hexameric assembly of p97, but

also showed that unlike many bacterial AAA+ proteins, the assembly of p97 does not depend on the presence of nucleotide.

Subsequent high resolution structural studies were primarily carried out with X-ray crystallography (Table 1) focusing on full-length wild-type p97 (Davies et al., 2008; DeLaBarre and Brunger, 2003; DeLaBarre and Brunger, 2005; Huyton et al., 2003) as well as several disease-associated mutants (Tang et al., 2010; Tang and Xia, 2012; Tang and Xia, 2013). More recently, three additional reports on the structure of full-length p97 appeared. One featured crystal structures of the full-length p97 bearing mutations in the D2 domain with nominal improvements in resolution (Hanzelmann and Schindelin, 2016b). The other two studies used the latest development in electron microscopic technology to obtain higher resolution structures of p97 (Banerjee et al., 2016; Schuller et al., 2016). The interactions of p97 with adaptors were also extensively studies with X-ray crystallography (Dreveny et al., 2004; Hanzelmann et al., 2011; Hanzelmann and Schindelin, 2011; Hanzelmann and Schindelin, 2016a; Kim et al., 2011; Kim and Kim, 2014; Lee et al., 2013; Qiu et al., 2010; Schaeffer et al., 2014; Zhao et al., 2007). These studies show that p97 forms two concentric rings (Figure 1B, C); the N-D1 ring has a larger radius than the D2 ring owing to the laterally attached N-domain. Like other AAA+ ATPases, the AAA module of p97 features a highly conserved RecA-like domain and a characteristic helical domain (Figure 1D). Each RecA-like domain in a protomer bears an active site, which is situated at the interface between two adjacent promoters in the hexameric assembly. The active site is formed by the classical Walker A (P-loop; G(x)4GKT) and Walker B motifs (hhhhDE, h for hydrophobic amino acid), responsible for nucleotide binding and hydrolysis, respectively. The configuration of the active site allows an arginine-finger residue (R359 for the D1 ring and R635 for the D2 ring) from an adjacent subunit to stimulate ATP hydrolysis.

Nucleotide-Driven Conformational Changes in p97

It is generally believed that p97 undergoes dramatic conformational changes during the nucleotide hydrolysis cycle (Beuron et al., 2006; Beuron et al., 2003; DeLaBarre and Brunger, 2005; Rouiller et al., 2002; Tang et al., 2010). Mechanical force generated by these conformational changes would be applied to substrate molecules to influence their stability and function. As a type II AAA+ ATPase, each p97 hexamer contains 12 ATPase domains and 6 N-domains. If each nucleotide-binding site were capable of producing 3 distinct conformations for apo-, ADP- and ATP-state, there would be a total of 3^{12} different conformations assuming each subunit operates independently. Reported structural conformations and interactions among different subunits are far less than the theoretical possibilities due to inter-subunit communications and coordination. Nevertheless, the conformational dynamics of p97 is difficult to study because the six ATPase domains within each ring are not synchronized in ATP hydrolysis (see below).

The conformational changes driven by ATP hydrolysis have been sought by various biochemical and biophysical methods (Davies et al., 2005). Initially, low-resolution cryo-EM structures revealed that upon ATP hydrolysis moderate rotational movement occurs between the two ATPase rings, associated with either closure or opening of the D1 and D2 central pores (Rouiller et al., 2002), but subsequent studies suggested other modes of domain

movement (Beuron et al., 2003). Due to resolution limitation, domain assignment in EM-reconstruction has been unreliable, and therefore, it is not feasible to consolidate these structural data into a consistent model that explains the action of p97. Moreover, because insufficient resolution prevents accurate determination of the p97 nucleotide-binding state, the interpretation of the EM results relied on the assumption that all 12 ATP-binding sites are occupied by the added nucleotide in a homogenous manner. Consequently, the EM studies failed to consider the heterogeneity in nucleotide association, not to mention the nucleotide pre-bound to the D1 ring.

Crystallographic studies of wild-type p97, using either full-length or N-D1 fragment, showed that D1 domains of all six subunits always have ADP bound and all N-domains are coplanar with the D1-ring, forming the so called Down conformation (Davies et al., 2008; DeLaBarre and Brunger, 2003; DeLaBarre and Brunger, 2005; Zhang et al., 2000). On the other hand, nucleotide bound to the D2 domains could be either ADP, AMP-PNP or ADP*AlFx. The D2 domain can also exist with no nucleotide bound (Apo state) (Huyton et al., 2003). Thus, structural studies of wild-type p97 by crystallography could only reveal conformational changes associated with the D2 nucleotide cycle. To date, one of the most significant structural changes associated with the ATP cycle of the D2 domain is the opening of the D2 pore, but whether pore opening is triggered by ATP binding or hydrolysis in the D2 ring has been controversial (Banerjee et al., 2016; Davies et al., 2008; Davies et al., 2005; Hanzelmann and Schindelin, 2016b; Pye et al., 2006; Rouiller et al., 2002; Schuller et al., 2016). Part of the D2 domain also seems to undergo an order-to-disorder transition, propelling small movements of the other domains in the molecule. In this regard, it is worth noting that even under the same nucleotide-binding conditions, conformational asymmetry exists among different subunits in D2-domain, and the magnitude of such difference can be as large as that experienced by the same subunit undergoing nucleotide exchange (Davies et al., 2008). Although structural studies fail to generate a conclusive model on how ATP hydrolysis in D2 triggers motion, it has become clear that at any given time, the six D2 domains in a p97 hexamer can exist in different nucleotide-binding states, suggesting that these ATPase domains do not act in a concerted manner. Indeed, biochemical studies of wild-type p97 ATPase activity demonstrated positive cooperation between subunits of the D2 domain (Nishikori et al., 2011).

Despite the repeated observations that p97 D2 mutants with an intact D1 domain have a low but discernable ATPase activity, there had been a tendency to ignore the D1 ATPase activity. Accordingly, the D1 domain has been assumed to function only in structural assembly of p97/Cdc48p without the need to hydrolyze ATP. However, recent studies using D2 specific p97 ATPase inhibitors showed convincingly that in the presence of an energy regenerating system, the D1 domain contributes significantly to the overall ATPase activity of p97 (~30%) (Anderson et al., 2015; Chou et al., 2014). The discrepancy may be caused by the accumulation of ADP when the assay was carried out without simultaneously converting it back to ATP because ADP seems to inhibit the D1 ATPase activity (Anderson et al., 2015). Thus, the new findings have a far reaching impact because it implicates a D1-dependent ATP hydrolysis cycle in p97 function, which is consistent with genetic evidence that Cdc48p D1 mutants with a functional D2 domain is unable to complement the growth defect of *Cdc48* temperature sensitive mutant cells (Ye et al., 2003). Nevertheless, conformational changes

associated with D1 ATP hydrolysis has not been observed by X-ray crystallographic studies for wild-type p97, mainly because purified recombinant p97 has ADP exclusively bound to the D1 domain (Davies et al., 2005; Tang and Xia, 2013), which cannot be removed completely to obtain a homogeneous sample with the D1 subunit being all occupied by supplemented nucleotides.

Recently, by genetic manipulation of certain regions in the D2 or N domain, the nucleotide-binding sites of the D1 ring can be altered to either empty or ATPγS-occupied state (Hanzelmann and Schindelin, 2016b; Tang et al., 2010). Strikingly, by simultaneous mutating 4 residues (N750D/R753D/M757D/Q760D) or deletion of a disordered loop in the D2 domain, Hanzelmann and colleagues were able to determine the structures of p97 with ATP binding sites all in either Apo or ATPγS-bound state. A comparison of the conformations of these two states suggests that ATP binding can open the D2 pore and also generate a rotational movement between the two ATPase rings (Hanzelmann and Schindelin, 2016b). However, the physiological relevance of these conformational changes remain to be determined because to what extent the mutations might affect p97 function is unclear (The deletion mutant has a lower ATPase activity compared to wild-type p97). Moreover, because no biochemical evidence suggests that the two ATPase rings in p97 hydrolyze ATP in a synchronized manner, intermediate conformations with the two ATPase rings in different nucleotide binding states are missing. In this regard, structural studies using another approach to remove D1 nucleotide seems to provide more biologically relevant insights into how the ATP cycle in the D1 domain might influence p97 function. The approach involved determining the structures of mutant p97 proteins carrying a single missense mutation that is associated with the IBMPFD syndrome. Because patients bearing a copy of the mutation do not suffer any developmental defects, the mutations seem to only cause suboptimal performance in the ATPase cycle that leads to gradual impairment of certain p97-dependent biological function and late on-set of the diseases in adulthood (Kimonis et al., 2000). Intriguingly, many IBMPFD mutations occurring at the interface between N- and D1-domain can weaken the affinity of ADP to the D1-domain (Tang et al., 2010). Consequently, these mutants can be purified with D1 bound by exogenously added nucleotides, permitting crystallographic studies of conformational changes associated with the D1 ATP hydrolysis cycle. Strikingly, compared to the structures in which D1 is in the ADP-bound state, when the ATP analog ATPγS occupies the D1 domain, the center of gravity of the N-domain undergoes a hinged translational movement of 13 Å with the angle (α angle in Figure 2) of 11°, followed by a further 92° rotation. As a result, the far edge of the N-domain represented by the residue Pro178 moves up by a distance of 38 Å, forming the Up-conformation (Figure 2) (Tang et al., 2010). This conformational change was also detected in wild-type p97 in solution by small-angle X-ray scattering (SAXS) (Tang et al., 2010). The difference between wild-type and mutant p97 lies in that in mutant p97, all six N-domains undergo a uniform conformational change, allowing study of p97 by X-ray crystallography, whereas in the wild-type p97 only a few subunits have their N-domains in the Up-conformation in the presence of ATP due to occluded ADP in some D1-domains. Thus, unsynchronized ATP hydrolysis appears to be a common feature for both the D1 and D2 domains. This feature may be functionally linked to the observed asymmetric binding of certain adaptor proteins to the p97 N-domain (Buchberger et al., 2015).

The abovementioned nucleotide-dependent N-domain conformational change is further confirmed recently by structural studies of full-length wild-type p97 by cryo-EM. One of these studies showed that in the presence of ATPγS, p97 can exist in three different nucleotide-binding states in solution: one has all 12 sites occupied with ADP and the N-domains in the Down conformation, one has the six sites of the D1-ring bound with ADP and the six sites of the D2-ring taken by ATPγS with the N-domains still in the Down conformation, while in the third conformation, all 12 sites are occupied by ATPγS and the N-domains are in the Up-conformation (Banerjee et al., 2016). In another report, p97 in a solution containing AMP-PNP displays both the Up- and Down-conformations from different protomers within a hexamer as well as significant asymmetric domain movement (Schuller et al., 2016). However, it is noteworthy that the structures obtained with full-length p97 bearing D2 mutations do not reveal a similar conformational change in the N-domain between the apo and ATPγS-bound state (Hanzelmann and Schindelin, 2016b). One possible explanation is that the introduced D2 mutations may have inhibited this conformational change.

p97-interacting proteins

A large collection of p97/Cdc48p-interacting proteins has been identified through proteomic studies. These proteins either function as adaptors that link p97/Cdc48p to a specific subcellular compartment or substrate, or serve as cofactors that help to process substrates. Cofactors usually have enzymatic activities that can process protein modifiers such as N-glycan or ubiquitin conjugates that are appended to p97 substrate (e.g. N-glycanase, ubiquitin ligase, and deubiquitinase).

Although a few proteins such as PLAA/Ufd3, PNGase, HOIP, and Ufd2 bind p97/Cdc48p through its short C-terminal tail (Bohm et al., 2011; Murayama et al., 2015; Qiu et al., 2010; Rumpf and Jentsch, 2006; Schaeffer et al., 2014; Zhao et al., 2007), the vast majority of p97-interacting proteins bind it through its N-domain (Buchberger et al., 2015). Representative N-domain-interacting proteins include Ufd1, Npl4, p47, ataxin3, and FAF1. Sequence analyses identified several frequently occurring p97-interaction patterns: such as the UBX motif (ubiquitin regulatory X) (Schuberth and Buchberger, 2008), the VIM (VCP-interacting motif) (Stapf et al., 2011), VBM (VCP-binding motif) (Boeddrich et al., 2006) and SHP box (also known as binding site 1, bs1) (Bruderer et al., 2004).

The UBX domain is an 80-residue module structurally homologous to ubiquitin, whereas the VCP-interacting motif (VIM) is a linear sequence motif (RX_5AAX_2R) found in a number of p97 cofactors or adaptors including gp78 (Ballar et al., 2006), SVIP (small VCP-inhibiting protein) (Ballar et al., 2007) and VIMP (VCP-interacting membrane protein) (Ye et al., 2004). The VBM domain features a different, highly polarizing linear sequence motif (RRRRXXYY) found in ataxin-3, Ufd2 and hrd1 (Boeddrich et al., 2006). The SHP box is a short amino acid stretch enriched in hydrophobic residues, which can be found in p47 (Kondo et al., 1997), Ufd1-Npl4 (Meyer et al., 2000) and Derlin-1 (Greenblatt et al., 2011; Lilley and Ploegh, 2004; Ye et al., 2004).

Biochemical studies showed that some adaptors and cofactors bind p97/Cdc48p in a mutually exclusive manner (Meyer et al., 2000; Rumpf and Jentsch, 2006), raising the possibility that cells may possess distinct populations of p97/Cdc48p complexes, each bearing a unique partner for a specific function. However, some adaptors and cofactors can bind p97 simultaneously, and for those who bind p97 in a competitive manner, they do not necessarily have to act against each other because conceptually, a hierarchical binding system that allows ordered interaction with p97 to fulfill a specific function may be orchestrated (Hanzelmann et al., 2011; Meyer et al., 2012).

Molecular insights on adaptor or cofactor binding have been mostly obtained through crystallographic studies using isolated domains or segments in complex with either the p97 N-domain or with its C-terminal tail. One of the better characterized interactions reported to date is the p47-N-D1 complex (Dreveny et al., 2004). The structure showed that the p97 N-domain is comprised of two sub-domains of roughly equal size: an N-terminal double Ψ-barrel and a C-terminal β-barrel (Figure 3A). p47 uses a C-terminal UBX domain to bind to the N-domain of p97 at a cleft between these two sub-domains. The interaction of p47 with p97 occurs asymmetrically as only two out of the six p97 subunits are occupied by p47. Interestingly, although VIM is structurally unrelated to the UBX domain, it binds p97 at a similar location (Figure 3B) (Hanzelmann and Schindelin, 2011). More recently, Hanzelmann and colleagues described an additional surface on the N-domain of p97 for the SHP box binding, suggesting a bipartite interaction with cofactors carrying both the UBX domain and the SHP box (e.g. p47) (Hanzelmann and Schindelin, 2016a).

Adaptors or cofactors binding to the C-terminal tail of p97 are illustrated by several crystallographic studies, exemplified by a structure of the PUB domain of the peptide-N-glycanase (PNGase) in complex with a 10-residue peptide derived from the C-terminus of p97 (Figure 3C) (Zhao et al., 2007). PNGase is responsible for the removal of N-glycosylated sugars from misfolded glycoproteins prior to their degradation by the proteasome (Blom et al., 2004). In higher eukaryotes, PNGase has acquired an additional N-terminal PUB (PNGase/UBA) domain that binds the C-terminus of p97. The latter was termed PUB-interacting motif (PIM). Crystallographic studies showed that the PUB domain forms a complex with PIM in a 1:1 stoichiometry, and the last six residues of the PIM peptide sit in a conserved surface depression of the PUB domain (Allen et al., 2006; Zhao et al., 2007). Intriguingly, the conserved residue Y805 in p97 that is essential for interaction with PNGase is subject to phosphorylation in cells, which provides a means to regulate p97-PNGase interaction (Zhao et al., 2007). Another example of adaptor recruitment by the p97 C-terminal peptide is illustrated by the structure of the complex between PLAA and the C-terminal peptide of p97 (Qiu et al., 2010). PLAA (also named Ufd3) is a phospholipase A2-activating protein implicated in maintaining cellular ubiquitin level (Johnson et al., 1995). In this structure, the critical residue Y805 is also located at the binding interface, which may allow a similar regulation by phosphorylation and de-phosphorylation (Figure 3D).

Structural studies on p97 complexes have also been attempted by cryo-EM (Bebeacua et al., 2012; Beuron et al., 2006; Pye et al., 2007; Rouiller et al., 2000), but inconsistent sample quality and insufficient resolution have led to some controversies. For example, in one study, the stoichiometry of p97 in complex with p47 was determined as six p47 to one hexameric

p97 (Rouiller et al., 2000), whereas in another study (Beuron et al., 2006), p47 was shown to be a trimer in solution and the binding stoichiometry is one trimeric p47 to one hexameric p97, a ratio that is more consistent with previously published biochemical data (Kondo et al., 1997). Two EM studies on the complex of p97 and Ufd1-Npl4 (Bebeacua et al., 2012; Pye et al., 2007), a heterodimeric complex known for their involvement in the endoplasmic reticulum-associated degradation (ERAD) (see below) (Ye et al., 2001), were also reported. Collectively, these EM studies cast a general impression that the N-domain-binding adaptors contact both the N- and the D1-domain simultaneously as they sit on top of the N-D1 ring. This conclusion is further supported by an EM study on the interaction of Fas-associated factor-1 (FAF1) with p97 (Ewens et al., 2014).

A comparison between the crystal structure of wild-type N-D1 (PDB:1E32) and that with p47 bound (PDB:1S3S) shows no major domain movement on the part of p97 upon p47 binding (Dreveny et al., 2004). However, adaptor- or cofactor-induced conformational changes may have escaped detection thus far because almost all structural studies were conducted with isolated N-domains or C-terminal PIM (Hanzelmann et al., 2011; Hanzelmann and Schindelin, 2011; Isaacson et al., 2007; Kim et al., 2011; Qiu et al., 2010; Schaeffer et al., 2014; Zhao et al., 2007). On a related note, ATP-dependent conformational changes, particularly those associated with the D1 ATP hydrolysis cycle will certainly influence the position of adaptors and cofactors that bind to the N domain, which may be important for the physiological function of p97.

Biological functions of p97/Cdc48p

The diverse biological functions of p97 have been extensively reviewed (Dantuma and Hoppe, 2012; Franz et al., 2014; Meyer et al., 2012; Meyer and Weihl, 2014; Yamanaka et al., 2012). Therefore, we only highlight a few key established functions in this review. In general, p97 uses ATP hydrolysis to segregate polypeptides from large protein assemblies or immobile cellular structures such as membranes or chromatin, and therefore, facilitates the degradation of the released polypeptides by the 26S proteasome. To date, the major functions of p97 can be summarized into the following three categories (Figure 4).

Roles in protein homeostasis regulation

A key function of p97/Cdc48p is to maintain protein homeostasis through a network of protein quality control processes (Meyer et al., 2012). In this context, the best studied process is endoplasmic reticulum-associated degradation (ERAD), a pathway that degrades misfolded proteins of the endoplasmic reticulum (ER) (Christianson and Ye, 2014; Ruggiano et al., 2014; Smith et al., 2011). In ERAD, p97/Cdc48p is recruited to the ER membrane via association with membrane adaptors including Derlins and VIMP in mammalian cells or Ubxd2 in *S. cerevesiae* (Lilley and Ploegh, 2004; Neuber et al., 2005; Schuberth and Buchberger, 2005; Ye et al., 2004). Membrane-associated p97 captures misfolded proteins once they have emerged through a putative retrotranslocation channel (Carvalho et al., 2010). Misfolded proteins are then ubiquitinated and extracted from the membranes upon p97 ATP hydrolysis (Bays et al., 2001; Braun et al., 2002; Flierman et al., 2003; Garza et al., 2009; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001; Ye et al., 2003; Zhong et al., 2004), and subsequently targeted to the proteasome for degradation (Figure 4A) (Zhang

and Ye, 2014). Besides misfolded ER proteins, p97 and Cdc48p are both capable of releasing certain membrane-bound transcription factors (Hitchcock et al., 2001; Radhakrishnan et al., 2014; Rape et al., 2001; Shcherbik and Haines, 2007). These factors apparently are not degraded by the proteasome. Instead, once released from the membrane, they translocate into the nucleus to influence gene expression in response to specific environmental insults.

Besides ERAD, ATP hydrolysis by p97 is also involved in extracting polypeptides from the mitochondrial outer membrane to facilitate mitochondria-associated degradation (Figure 4B) (Hemion et al., 2014; Heo et al., 2010; Xu et al., 2011). This process can regulate mitochondrial protein homeostasis if the client proteins to be degraded are aberrant molecules. Additionally, factors controlling the turnover of damaged mitochondria by the mitophagy process (e.g. mitofusin) can be degraded in a p97-dependent manner (Tanaka et al., 2010). Intriguingly, p97 and its co-factors Ufd1 and Npl4 are highly enriched on the surface of mitochondria that are damaged by a protonophore, and loss of either p97 or these co-factors causes a defect in mitophagy-mediated clearance of damaged mitochondria (Kimura et al., 2013). These findings suggest a critical role for p97 in mitochondrial homeostasis regulation.

p97 can also release defective translation products stalled on ribosome in a process termed ribosome-associated degradation (Figure 4C) (Brandman et al., 2012; Defenouillere et al., 2013; Verma et al., 2013). It appears that only after extraction from ribosome, can these aberrant polypeptides be degraded efficiently by the proteasome. In addition to the proposed “segregase” function, p97 might play a role in shuttling aberrant polypeptides to the proteasome for degradation. This chaperoning function seems to be particularly important for degradation of certain aggregation-prone misfolded proteins in the nucleus in budding yeast (Gallagher et al., 2014).

Several lines of evidence also implicate p97 and Cdc48p in autophagy, a stress adaptation process that turns over cellular proteins (including misfolded ones) by engulfment of cytosol into double-membrane-surrounded vesicles named autophagosome. However, the precise role of p97 in this process is unclear (Bug and Meyer, 2012; Ju et al., 2009; Ju and Weihl, 2010). In *S. cerevisiae*, the Cdc48p adaptor Shp1p interacts with Atg8p, an essential autophagy regulator, and the complex of Cdc48p and Shp1p is required for macroautophagy (Krick et al., 2010). Whether the homologous p97-p47 complex interacts with the Atg8 homolog LC3 to regulate autophagy in mammalian cells is unclear. Lastly, p97 was recently demonstrated to play a pivotal role in clearance of non-translating messenger ribonucleoprotein complexes accumulated in stress granules (Buchan et al., 2013).

Chromatin-associated functions

By releasing protein molecules from chromatins in a manner analogous to that in ERAD, p97 also participates in a series of degradation processes in the nucleus collectively known as chromatin-associated degradation (Figure 4D) (Dantuma et al., 2014). Established nuclear substrates of p97/Cdc48p include transcriptional repressor α2 (Wilcox and Laney, 2009), RNA polymerase (Pol) II complex (Verma et al., 2011) and CMG DNA helicase (Maric et al., 2014) in budding yeast, and the DNA replicating licensing factor CDT1 (Franz et al.,

2011; Raman et al., 2011), a replisome component Mcm7 (Moreno et al., 2014), DNA repairing proteins DDB2, XPC, and Rad52 (Bergink et al., 2013; Puumalainen et al., 2014), mitosis regulator Aurora B (Ramadan et al., 2007), certain DNA polymerases (Davis et al., 2012; Mosbech et al., 2012) and the polycomb protein L3MBTL1 (Acs et al., 2011) in mammals. These substrates have linked p97 function to diverse nuclear events ranging from gene transcription to DNA replication and repair.

Membrane fusion and vesicular trafficking

Biochemical and genetic evidence also implicated p97 in fusion of vesicles that lead to the re-formation of the Golgi apparatus at the end of mitosis (Uchiyama and Kondo, 2005). This process depends on the ubiquitin binding adaptor p47 (Kondo et al., 1997; Meyer et al., 2002), a related adaptor p37 that lacks ubiquitin binding activity (Uchiyama et al., 2006), and a p97-associated deubiquitinase named VCIP135 (Uchiyama et al., 2002). However, the precise role of p97 in vesicle fusion is unclear due to lack of information on its substrate(s).

A role in endocytosis for p97 has also been revealed two recent studies that used a proteomic approach to explore p97-interacting proteins in mammalian cells (Bug and Meyer, 2012; Kirchner et al., 2013; Ramanathan and Ye, 2012). One study identified the early endosome-associated antigen 1 (EEA1) as a p97 interacting protein and showed that inhibition of p97 results in a delay in lysosomal targeting of an endocytosis cargo. p97 inhibition also generated an endosome clustering and swelling phenotype, which appeared to be caused by increased EEA1 oligomerization and thus uncontrolled endosome tethering and fusion (Ramanathan and Ye, 2012). In a second study, the plasma membrane protein caveolin was found to interact with p97 and its cofactor UbxD1. In p97-inhibited cells, enlargement of endosome was similarly observed and the trafficking of caveolin to late endosome is affected (Ritz et al., 2011). These results not only suggest a general function for p97 in regulating the clustering and fusion of membrane vesicles, but also imply that it can control the trafficking itinerary of a specific endocytic cargo, perhaps by regulating its partition between different lipid domains.

Molecular basis of force generation

Although intensely studied, the molecular mechanism underlying the “segregase” activity of p97/Cdc48p remains poorly defined. Major unresolved issues are whether or not p97/Cdc48p unfolds its client proteins, and therefore disrupting their interactions with protein assemblies, membranes, or chromatin; if so, whether or not it can act as a ‘translocase’ that moves substrates through the central pore.

The ATP hydrolysis cycle of p97/Cdc48P

Isolated full-length p97 has a moderate ATPase activity that turns over 1–5 ATP molecules per hexmer per second (Meyer et al., 1998; Song et al., 2003; Tang and Xia, 2013; Ye et al., 2003). Mutations in the D1 Walker A (K251A/T) motif led to a huge reduction in ATPase activity, indicating that nucleotide binding in the D1 domain is important for the activity of the D2 domain (Nishikori et al., 2011; Tang and Xia, 2013; Ye et al., 2003). Conversely, Walker A or Walker B mutations in the D2 domain almost completely abolish the p97

ATPase activity (Nishikori et al., 2011; Tang and Xia, 2013; Ye et al., 2003), suggesting that nucleotide binding and hydrolysis by the D2 domain are required for activating the D1 domain. Apparently, there is a communication mechanism between the D1 and D2 domains. Based on these observations, it was proposed that the two ATPase rings may alternate in ATP hydrolysis (Ye et al., 2003). The feedback from the D2 domain to the D1 domain is likely mediated by the D1–D2 linker, because a p97 mutant carrying both the N- and D1-domains shows no ATPase activity when this linker is absent (Chou et al., 2014; Tang and Xia, 2013; Tang and Xia, 2016; Ye et al., 2003).

The N-domain appears to suppress p97 ATPase activity via communications with the D1 domain. Deletion of the N-domain in VAT, a VCP-like ATPase from *Thermoplasma acidophilum*, increases its ATPase activity significantly (Rothballer et al., 2007), and fixing the N-domain to the Down-conformation by an engineered disulfide linker reduces p97 ATPase activity (Niwa et al., 2012). p97 mutants carrying single IBMPFD mutations (see below) have 2–3 fold increase in ATPase activity. These mutations are all mapped to the N-D1 interface, and therefore likely affect the N-D1 interactions (Halawani et al., 2009; Niwa et al., 2012; Tang and Xia, 2013; Weihl et al., 2006; Zhang et al., 2015). The slow deteriorating pathology associated with these mutations suggests that the p97 ATPase activity needs to be tightly regulated to achieve optimal function in cells.

The ATPase activity of p97 can also be influenced by many other factors *in vitro*. For example, it can be stimulated by elevated temperature (Song et al., 2003) or by a putative substrate protein (DeLaBarre et al., 2006). Association with different cofactors can have either positive or negative impact on p97 ATPase activity (Meyer et al., 1998; Zhang et al., 2015), but the physiological relevance of these observations is unclear. How cells regulate the p97 ATPase activity is also largely unknown.

Is p97 an unfoldase?

By definition, a protein unfoldase unfolds substrates into linear peptides. A “segregase” may act with or without unfolding its client proteins (Yamanaka et al., 2012). Despite that many hexameric ring-like AAA+ proteins are classical examples of protein unfoldases (e.g. ClpA and ClpX) that unfold polypeptides by threading them through the central pores (Singh et al., 2000), full-length wild-type p97 cannot unfold GFP-ssrA, a model aberrant protein (Rothballer et al., 2007). Nevertheless, it is interesting to note that VAT, a *thermoplasma acidophilum* p97 homolog, is able to unfold GFP-ssrA (Gerega et al., 2005), but it does so with high efficiency only when the N-domain is removed (Barthelme and Sauer, 2012; Gerega et al., 2005). In addition, a N-domain-deleted VAT variant can cooperate with the 20S proteasome to efficiently degrade GFP-ssrA *in vitro*. A comparison of the sequences between p97 and VAT identified a motif (KYYG) at the D1 pore of VAT, which is replaced with KLAG in p97. When combined with the removal of the N-domain, tyrosine substitution to either Leu or Ala allows p97 to unfold GFP-ssrA, which causes its degradation by the 20S proteasome (Barthelme and Sauer, 2013; Rothballer et al., 2007). These findings suggest that the widely observed cooperation between AAA rings and the 20S proteasome is an ancient molecular design for unfolding and degradation of aberrant polypeptides, but the

divergent evolution of the N-domain and the D1 pore of p97 seems to allow p97 to operate by a new mode.

Although eukaryotic p97/Cdc48p does not appear to thread substrates through the central pore, it may still act as an unfoldase to promote protein turnover. In cells, the requirement of p97/Cdc48 for degradation of a ubiquitin fusion protein can be bypassed if a flexible peptide was appended to the C-terminus of this substrate (Beskow et al., 2009). This observation was taken to indicate that p97/Cdc48p acts to initiate protein unfolding, which exposes a loosely-folded initiation peptide for engaging the proteasome. Intriguingly, the involvement of Cdc48p in the ubiquitin fusion degradation pathway coincides tightly with the requirement for Lys48- and Lys29-linked ubiquitination in the ubiquitin fusion part (Godderz et al., 2015), suggesting that the function of p97/Cdc48p is intimately linked to substrate ubiquitination. This is consistent with the observation that many p97/Cdc48p interacting-proteins contain independent ubiquitin binding site.

Mechanism of force generation

How do conformational changes in p97 account for the proposed “segregase” activity? By far, the most consistent conformational changes observed are the close-and-open of the D2 pore, which seems to be associated with a rotational movement between the D1 and D2 rings during the D2 ATPase cycle, and more dramatically, the up-and-down swing motion of the N-domain, driven by nucleotide hydrolysis in the D1 domain (Figure 5A). If the two ATPase domains indeed alternate in ATP hydrolysis, it is conceivable that the opening of the D2 ring, if coupled to the ATP-bound state of the D1 ring and therefore to the Up-conformation (Banerjee et al., 2016; Tang et al., 2010), may allow substrates to bind to the D2 pore, as suggested by a mutagenesis study (DeLaBarre et al., 2006). The alternate ATPase cycle could then lead to a piston-like, up-and-down motion for the D1–D2 rings, which could be coupled to substrate binding and release. Taking ERAD as an example, since the N-domain is anchored to the immobile ER membranes via adaptors, the downside swing of the N-domain would naturally be translated into a pulling force that moves the D1–D2 rings toward the membrane, leading to the extraction of substrates bound to the D2 pore (Figure 5B, top panel). The model is equally applicable if p97 engages substrates with only the D1 ring (bottom panel). Although these models provide a plausible means for p97 to separate a client protein from its interaction partners, at the moment, we cannot rule out other models, as proposed previously (Pye et al., 2006).

p97 inhibitors and cancer therapy

By screening and characterizing compounds that inhibit the degradation of a fluorescence-labeled ERAD substrate, the first p97 inhibitor Eeyarestatin (EerI) was reported a few years ago (Fiebiger et al., 2004; Wang et al., 2008; Wang et al., 2010). Structure-activity relationship studies suggested that EerI contains two functional modules: a nitrofuran ring that binds the D1 domain of p97 and an aromatic ring-containing module that recruits EerI to cellular membranes including the ER. Once localized to the ER membrane, the target selectivity of EerI is significantly enhanced. Consequently, it mainly targets p97 that is membrane-bound (Wang et al., 2010). Although the mechanism of p97 inhibition by EerI is unclear, it induces several key phenotypes associated with p97 inhibition such as

polyubiquitinated protein accumulation, ERAD inhibition, ER stress induction, and apoptosis (Wang et al., 2009). Importantly, EerI displays significant cancer-killing activity *in vitro* preferentially against cancer cells isolated from patients, and it can synergize with the proteasome inhibitor Bortezomib to kill cancer cells (Wang et al., 2009). These observations raise the possibility of targeting p97 as a potential cancer therapy. Subsequent studies on several ATP competitive and allosteric inhibitors of p97 confirmed this hypothesis (Chou et al., 2011; Chou et al., 2013; Magnaghi et al., 2013). Importantly, a potent and specific p97 inhibitor CB-5083 has been developed recently, which elicits promising anti-cancer effect in mouse xenograft tumor models (Anderson et al., 2015; Zhou et al., 2015). The mode of interaction by p97 inhibitors has been studied by both structural modeling as well as by cryo-EM technology (Anderson et al., 2015; Banerjee et al., 2016; Magnaghi et al., 2013). The information may be helpful for design of more potent and specific p97 inhibitors.

Relevance to human diseases

p97 has received much attention recently also because genetic studies have linked mutations in p97 to pathogenesis of several human diseases including Inclusion Body Myopathy associated with Paget's disease of the bone and Frontotemporal Dementia (IBMPFD) and amyotrophic lateral sclerosis (ALS) (Johnson et al., 2010; Watts et al., 2004).

IBMPFD is an autosomal dominant, progressive, and ultimately fatal disorder with initial symptoms typically appearing in adulthood (Kimonis et al., 2000). The disease mainly affects one or more of the following tissue types: the muscles (myopathy), the bones (Paget's disease of the bone), and the brain (frontotemporal dementia). Among them, myopathy is the most common clinical menifestation found in approximately 85% of the patients, while the bone pathology and frontotemporal dementia are observed in approximately 50% and 30% of the patients, respectively. However, there is no clear genotype-phenotype corrletion as patients from the same family with the same mutation can show different symptoms.

Phenotypic characterizations of muscle samples from IBMPFD patient revealed rimmed vacuoles that were stained positively for both ubiquitin and p97 (Watts et al., 2004). In brain tissues from patients, nuclear inclusions were detected in neurons and they also contain ubiquitin and p97 (Kimonis and Watts, 2005). More recent studies found inclusions positive for TAR DNA-binding Protein-43 (TDP-43) in patient samples (Weihl et al., 2008). These findings suggest that defects in p97-mediated PQC network may contribute to the etiology of this disease.

To date, more than 30 missense mutations in *p97* have been reported in IBMPFD patients, involving amino acids at 17 different positions (Mehta et al., 2013; Nalbandian et al., 2011). Intriguingly, these mutations are all mapped to the interface between the N- and D1-domain of p97, suggesting that communications between the N-domain and the D1 domain may be crucial for p97 function.

Autosomal dominantly inherited amyotrophic lateral sclerosis (ALS) is another disorder genetically linked to mutations in *p97*. ALS is clinically characterized by progressive dysfunction in motor neurons, resulting in death from respiratory failure. Unlike IBMPFD,

ALS-associated p97 mutations account for ~1%–2% of all familial ALS cases (Johnson et al., 2010). The pathological hallmark of the disease, loss of motor neurons, is often associated with the presence of ubiquitin-positive inclusions or deposition of TDP-43 aggregates in motor neurons (Johnson et al., 2010), once again, linking the ALS pathology to defects in quality control of misfolded proteins. However, how mutations in *p97* cause ALS has remained unclear.

Conclusions and Perspective

Through years of studies, we have significantly deepened our understanding on the structure and function of p97/Cdc48p. Most noticeable is the rapid expansion in our knowledge on cofactors and adaptors and the corresponding new functions of this essential chaperone system. Nonetheless, several fundamental questions regarding the mechanistic action of p97/Cdc48p remain unanswered. The most important one is whether the conformational changes observed in the reported studies are truly physiologically relevant, and if so, how these conformational changes generate force to carry out the “segregase” function. To better address this question, we would need a robust *in vitro* assay that recapitulates an *in vivo* function of p97. This appears to be an extremely challenging task as it has apparently been tried by many laboratories without much success. The recently identified p97 substrates may offer new hope along this direction. Another key issue is to understand the hierarchical organization of adaptors/cofactors binding in the context of a given p97/Cdc48p dependent pathway. Animal studies are also in urgent need to better understand the mechanistic links between p97 and human diseases. Finally, given the demonstrated favorable anti-cancer activity in mouse model for a p97 inhibitor, it is anticipated that more p97 inhibitors may be sought, and studies along this line may one day lead to a new class of anti-cancer agents.

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Abbreviations

VCP	valosin-containing protein
TER ATPase	transitional endoplasmic reticulum ATPase
AAA+	extended family of ATPases associated with various cellular activities
ER	endoplasmic reticulum
PQC	protein quality control
IBMPEF	Inclusion Body Myopathy associated with Paget’s disease of the bone and Frontotemporal Dementia
ALS	amyotrophic lateral sclerosis
EM	electron microscopy

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP-PNP	Adenylyl-imidodiphosphate
ERAD	ER-associated protein degradation
VAT	VCP-like ATPase

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Highlights

- p97/Cdc48p participates in various cellular pathways by functioning as a “segregase” to separate polypeptide substrates from large immobile cellular structures.
- Mutations in p97 have been associated with various human diseases.
- Significant progress has been made in obtaining atomic resolution structures of p97 in different conformations.
- Controversy remains as to the mechanism underlying force generation by p97.

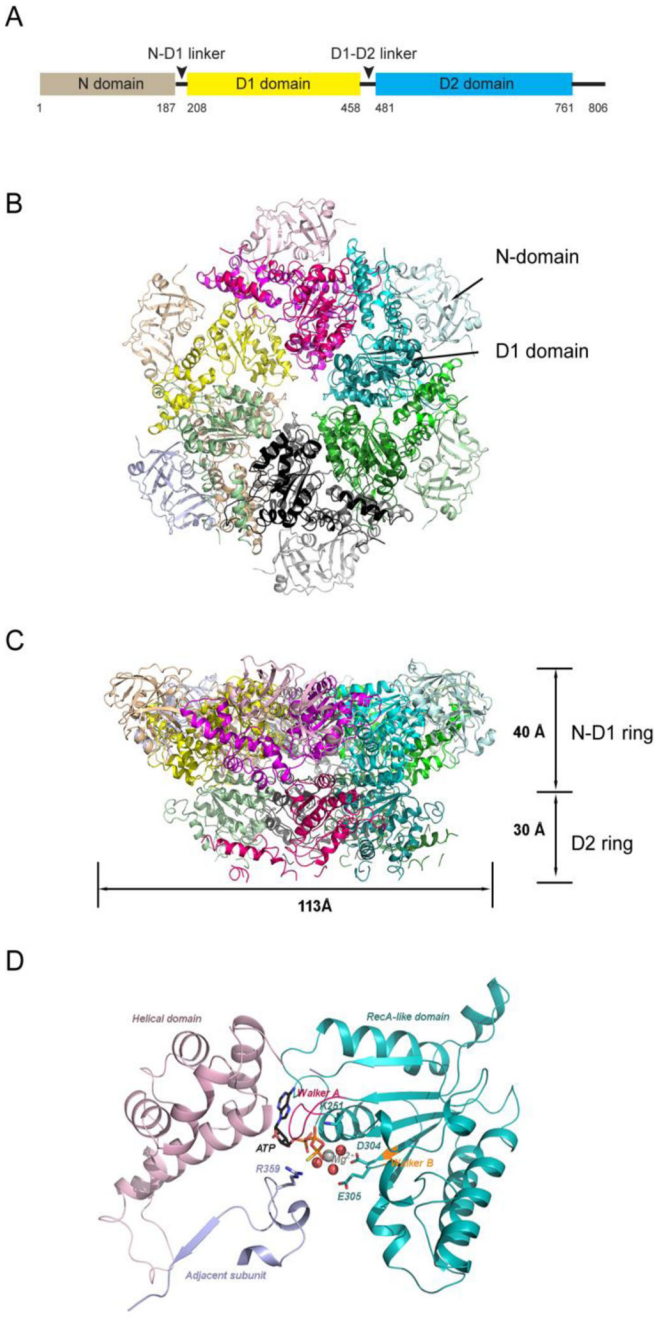

Figure 1. Structure of p97/Cdc48p

(**A**) The schematic domain organization of p97/Cdc48. (**B**) The structure of hexameric p97 (PDB: 3CF2 in the ADP-bound form) is viewed down the 6-fold symmetry axis showing the N-D1 ring. The six subunits are shown as cartoon diagrams in different colors. Domains of each subunit are also shaded differently. The D1 domain and the N-domain are indicated with arrows and labeled for one of the six subunits. (**C**) The side view of p97 is presented with indicated width and height. (**D**) The structure of the D1 AAA domain of a p97 subunit with bound ATPγS is presented in the ribbon format (PDB:4KO8). An AAA domain

consists of a RecA-like domain (cyan) and a characteristic helical domain (purple). An ATPγS, bound at the interface between the two domains, is shown as stick model. The Mg^{2+} ion and three conserved water molecules are shown as silver and red balls, respectively. The Walker A motif or P-loop is highlighted in red and the conserved lysine residue K251 is shown as stick model and labeled. The Walker B motif is shown in orange and the two conserved acidic residues D304 and E305 are represented by stick models. The nucleotide-binding site communicates with a neighboring subunit through the SRH (second region of homology, in light blue) motif, where a conserved Arg-finger residue R359 is in contact with the bound nucleotide.

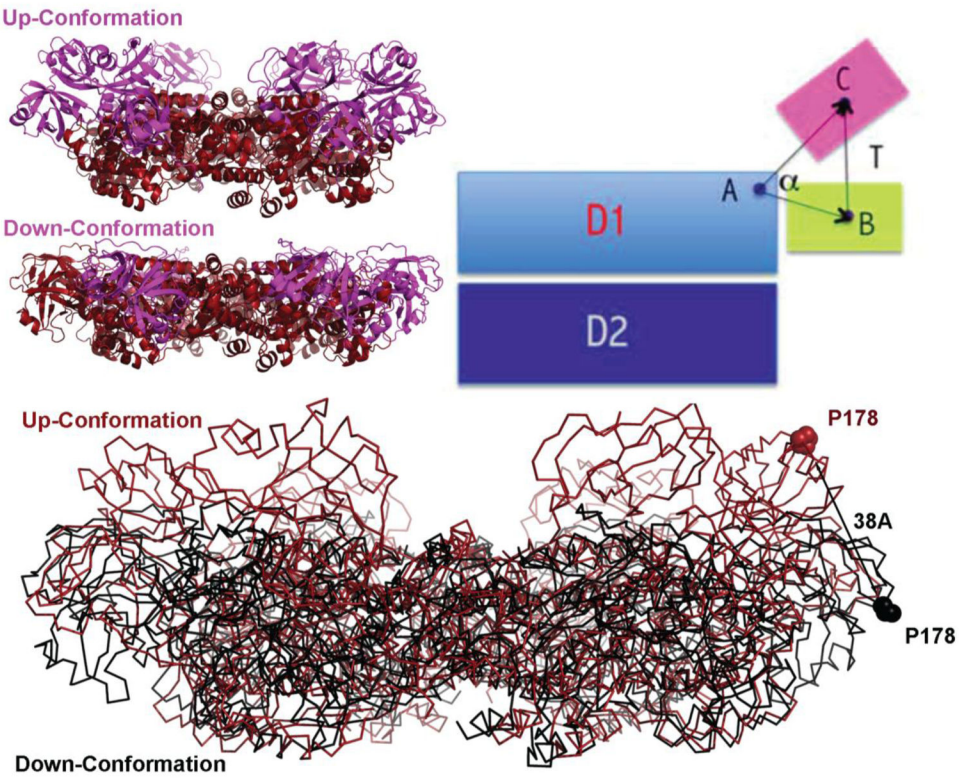

Figure 2. Nucleotide-dependent N-domain conformational change

A large N-domain conformational change has been observed, being driven by the nucleotide cycle of the D1 domain. When ATP is bound, the N-domain, illustrated in ribbon diagram in magenta, goes to the Up-conformation, whereas it moves to the Down-conformation when ADP is bound to the D1 domain. During the transition between the UP- and Down-conformation, the center of gravity of the N-domain (B for the Down-conformation and C for the Up-conformation) translated by 13 Å and the α angle is 11° as defined by the triangle ABC (A is the position of G208 of the D1 domain). Additionally, to adopt the Up-conformation configuration, the N-domain needs a further 92° rotation. As a result, the residue P178 moves up by 38 Å.

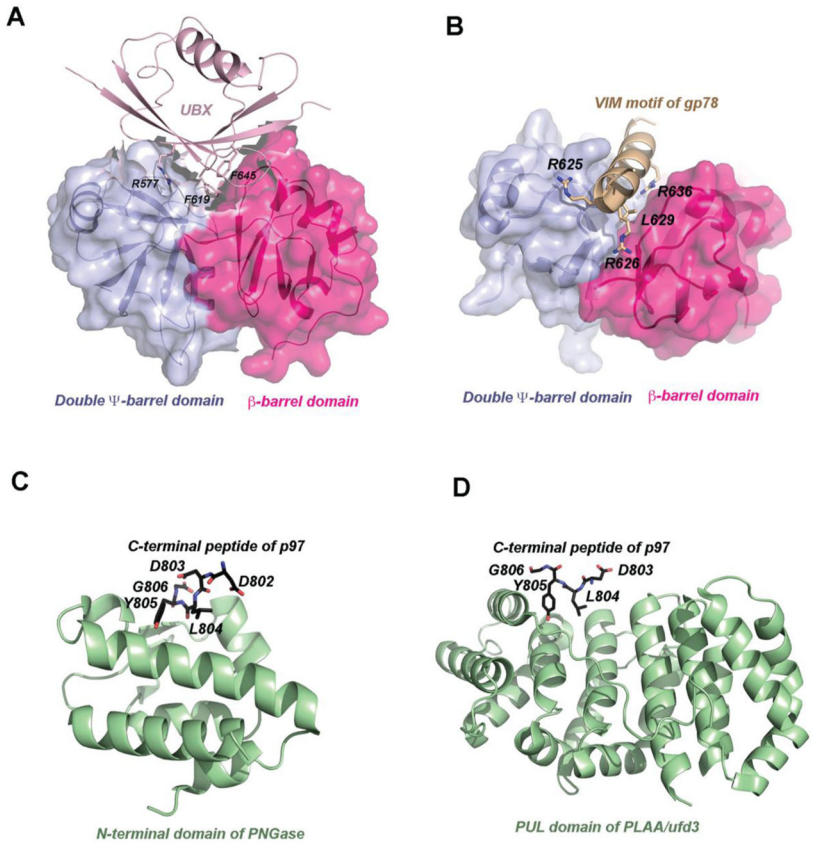

Figure 3. The interactions of p97 with adaptors and cofactors

(**A**) Structure of the p97 N-domain in complex with the UBX domain of FAF1 (PDB:3QC8). The N-domain, depicted as a molecular surface overlaid to a ribbon representation, consists of two subdomains: N-terminal double Ψ-barrel domain (purple) and C-terminal β-barrel domain (red). The UBX domain of FAF1 is depicted as ribbon diagram in magenta. Critical residues for interaction are shown as ball-and-stick models and labeled. (**B**) Structure of the p97 N-domain in complex with the VIM motif of gp78 (PDB:3TIW). Here the VIM motif is shown as helix in brown and its binding to the N-domain is mostly mediated by charged residues. (**C**) Structure of the N-terminal domain of PNGase in complex with a C-terminal peptide of p97 (PDB:2HPL). The PNGase N-terminal domain is shown in cartoon representation in green. The bound peptide is shown as a stick model with five residues (labeled) seen in the structure. The carbon atoms are colored in black, nitrogen in blue and oxygen in red. (**D**) Structure of the PUL domain of FLAA/Ufd3 in complex with a C-terminal peptide of p97 (PDB:3EBB). The PLAA PUL domain is shown in cartoon representation in green. The bound peptide is shown as a stick model with four residues visible in the structure. The carbon atoms are colored in black, nitrogen in blue and oxygen in red.

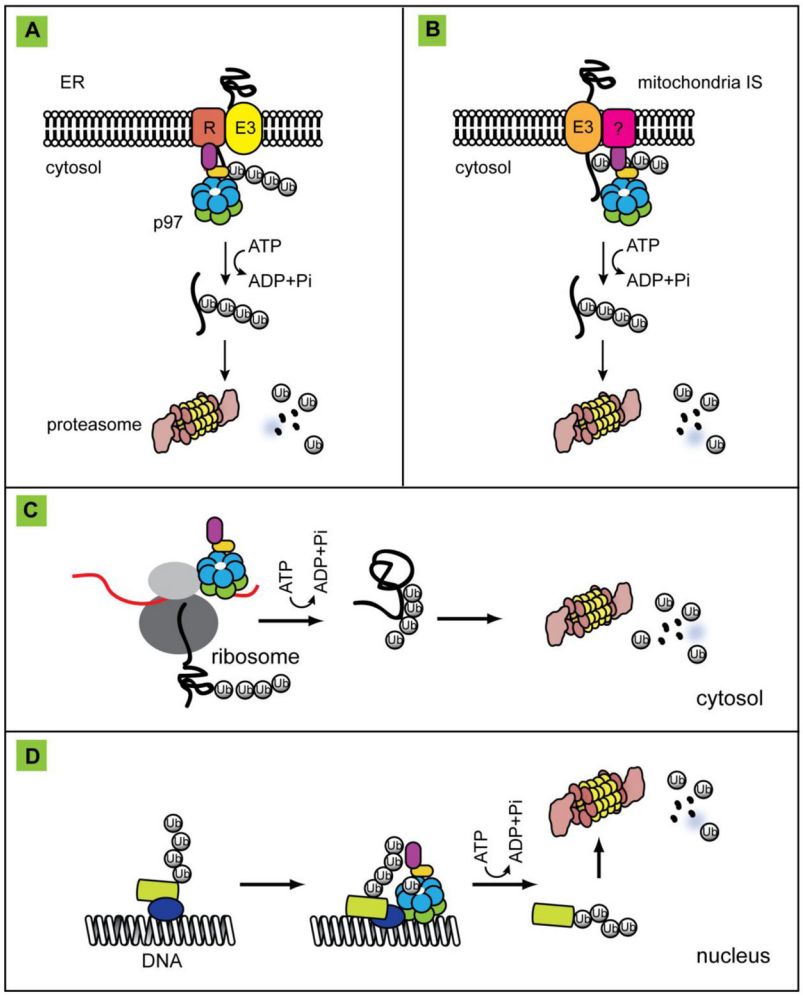

Figure 4. The established segregase function of p97/Cdc48p

p97 collaborates with the proteasome in degradation of misfolded ER proteins (the ERAD pathway) (**A**), misfolded proteins in the mitochondrial outer membrane (**B**), defective translocation products (**C**), and chromatin-associated proteins (**D**). In each scenario, p97 uses energy from ATP hydrolysis to release polypeptides from either the membranes, the ribosome, or DNA, and then target them to the proteasome for degradation. E3, ubiquitin ligase, R, retrotranslocation complex, mitochondrial IS, mitochondrial inter-membrane space.

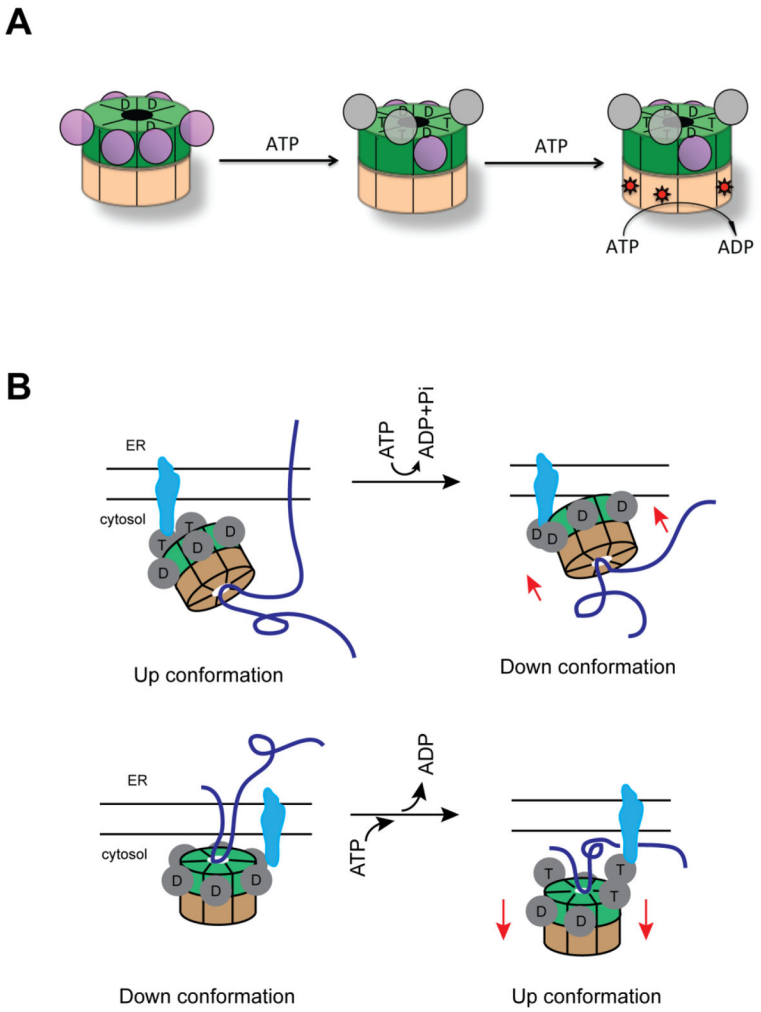

Figure 5. Force generation coupled to ATP hydrolysis

(**A**) An ATP hydrolysis model for p97. A p97 hexamer is represented as two concentric rings with D1 ring in green and D2 ring in brown. The N-domains in the Down-conformation are shown as magenta balls. D1 domains with pre-bound ADP are labeled with the letter D. ATP molecules introduced into the system will first go to the D1 domains with no pre-bound nucleotide, which leads the N-domains to the Up-conformation. Occupation of ATP to the D1 domain renders the cognate D2 domain capable of hydrolyzing ATP, which is labeled with a red *. The D1 domain probably hydrolyzes ATP once a few D2 domains have been converted to the ADP bound state. (**B**) A proposed model of force generation by a N-domain conformational change in ERAD. p97 is anchored to the ER membrane by association with a membrane adaptor (blue) using its N-domain. If substrate is bound to the central pore of the D2 domain when the D1 domain is in the Up-conformation (top panel), the swing of the N-domain to the Down-conformation after ATP hydrolysis will pull the D1 ring closer to the membrane, leading to the extraction of substrate out of the membranes. Alternatively, if the substrate is bound to the D1 domain (bottom panel), a switch from the Down-conformation

to the Up-conformation following nucleotide exchange in D1 will move the ATPase domains away from the membrane, pulling substrate out of the membrane.

Table 1

Available structures of p97 deposited in the PDB.

PDB	Construct	Mutant	N-Conform	Bound Adaptor/Inhibitor	Bound Ligand	Res (Å)	Date Deposit	Note	References
3CF3	FL	WT	Down	-	ADP(D1,D2)	4.25	2008	1YQI[a]	Davies et al., 2008; DeLaBarre & Brunger, 2005
1R7R	FL	WT	Down	-	ADP(D1)	3.6	2003	D2 PlyA[b]	Huyton et al., 2003
3CF1	FL	WT	Down	-	ADP(D1), ADP•AlF (D2)	4.4	2008	1OZ4[a] 1YQ0[a]	Davies et al., 2008; DeLaBarre & Brunger, 2003 & 2005
3CF2	FL	WT	Down	-	ADP (D1) AMP-PNP (D2)	3.5	2008	1YPW[a]	Davies et al., 2008; DeLaBarre & Brunger, 2003 & 2005
3C19	FL	α9-D4[c]	Down	-	None(D1, D2)	4.2	2016		Hanzelmann et al., 2016a
3C1A	FL	α9-D4[c]	Down	-	ATPγS (D1,D2)	3.8	2016		Hanzelmann et al., 2016a
3C18	FL	Δ(709–728)[d]	Down	-	ATPγS (D1,D2)	3.30	2016		Hanzelmann et al., 2016a
5C1B	FL	Δ(709–728)[d]	Down	SHP	ATPγS (D1,D2)	3.08	2016		Hanzelmann et al., 2016b
5FTJ	FL	WT	Down	-	ADP(D1,D2)	2.4	2016		Banerjee et al., 2016
5FTK	FL	WT	Down	UPCDC30245	ADP(D1,D2)	2.3	2016		Banerjee et al., 2016
5FTL[e]	FL	WT	Down	-	ADP(D1,D2)	3.3	2016		Banerjee et al., 2016
5FTM[e]	FL	WT	Down	-	ADP(D1), ATPγS(D2)	3.2	2016		Banerjee et al., 2016
5FTN[e]	FL	WT	Up	-	ATPγS (D1,D2)	3.3			Banerjee et al., 2016
3QQ7	N	WT	-	-		2.65	2011		Hanzelmann et al., 2011a
3QQ8	N	WT	-	FAF1-UXB	-	2.0	2011		Hanzelmann et al., 2011a
4KDI	N	WT	-	OTU1-UBX	-	1.86	2013		Kim et al., 2014
4KDL	N	WT	-	OTU1-UBX	-	1.81	2013		Kim et al., 2014
3QC8	N	WT	-	FAF1-UBX	-	2.2	2011		Kim et al., 2011
3QWZ	N	WT	-	FAF1-UBX	-	2.0	2011		Lee et al., 2013
3TIW	N	WT	-	Gp78-VIM	-	1.8	2011		Hanzelmann et al., 2011b
1E32	N-D1	WT	Down	-	ADP (D1)	2.9	2000		Zhang et al., 2000
4KO8	N-D1	R155H	Up	-	ATPγS (D1)	1.98	2014		Tang et al., 2013
4KLN	N-D1	A232E	Up	-	ATPγS (D1)	2.62	2014		Tang et al., 2013
4KOD	N-D1	R155H	Down	-	ADP (D1)	2.96	2014		Tang et al., 2013
3HU3	N-D1	R155H	Up	-	ATPγS (D1)	2.2	2009		Tang et al., 2010

PDB	Construct	Mutant	N-Conform	Bound Adaptor/Inhibitor	Bound Ligand	Res (Å)	Date Deposit	Note	References
3HU1	N-D1	R95G	Up	-	ATPγS (D1)	2.81	2009		Tang et al., 2010
3HU2	N-D1	R86A	Up	-	ATPγS (D1)	2.85	2009		Tang et al., 2010
5DYG	N-D1	L198W	Down	-	ADP (D1)	2.20	2015		Tang et al., 2016
5DYi	N-D1	WT	Down	-	ADP (D1)	3.71	2015		Tang et al., 2016
1S3S	N-D1	WT	Down	P47-UBX	ADP (D1)	2.9	2004		Dreveny et al., 2004
3CF0	D2	WT	-	-	ADP (D2)	3.0	2008	7mer[f]	Davies et al., 2008
3EBB	C-10mer	WT	-	PLAA PUL	-	1.9	2008		Qiu et al., 2010
2HPL	C-10mer	WT	-	PNGase	-	1.8	2006		Zhao et al., 2007
4POA	C-10mer	WT	-	HOIP PUB		2.3	2014		Schaeffer et al., 2014
2PJH	N	WT	-	Npl4-UBD			2011	NMR	Isaacson et al., 2007

[a] superseded. b – D2 domain is a polyA model. c – isolated D2 domain forms heptamer in solution.

[b] D2 domain is a polyA model.

[c] full-length p97 with four mutations N750D/R753D/M757D/Q760D.

[d] full-length p97 with the deletion in a loop in the D2 domain from residues 709–729.

[e] in the presence of 5 mM ATPγS during experiments.

[f] isolated D2 domain forms a heptamer in crystal.

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