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# The complexities of p97 function in health and disease

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## Abstract

p97 is a homohexameric, toroidal machine that harnesses the energy of ATP binding and hydrolysis to effect structural reorganization of a diverse and primarily uncharacterized set of substrate proteins. This action has been linked to endoplasmic reticulum associated degradation (ERAD), homotypic membrane fusion, transcription factor control, cell cycle progression, DNA repair, and post-mitotic spindle disassembly. Exactly how these diverse processes use p97 is not fully understood, but it is clear that binding sites, primarily on the N- and C-domains of p97, facilitate this diversity by coordinating a growing collection of cofactors. These cofactors act at the levels of mechanism, sub-cellular localization, and substrate modification. Another unifying theme is the use of ubiquitylation. Both p97 and many of the associated cofactors have demonstrable ubiquitin-binding competence. The present review will discuss some of the current mechanistic studies and controversies and how these relate to cofactors as well as discussing potential therapeutic targeting of p97.

## 1. Introduction

The AAA+ (ATPase associated with various cellular activities) ATPase p97 (also called valosin-containing protein (VCP)) is a homohexameric ring shaped molecular machine comprising four domains: the flexible N-terminal domains responsible for cofactor binding and possibly for substrate binding, two AAA ATP-binding domains, and a short C-terminal domain that binds some of the substrate modifying factors.[1–3] p97 is an essential protein with homologues in archaea and all eukaryotes, that has been linked to a diverse set of biological processes including post-mitotic homotypic membrane fusion[4], endoplasmic reticulum associated degradation (ERAD)[5], mitotic spindle disassociation[6], transcription factor regulation[7], and, perhaps, aggregation prevention and disassembly[8]. To coordinate this diverse collective of functions, p97 co-opts a collection of cofactors.[9] These factors seem to serve one of three purposes: mechanistic control, sub-cellular localization, and/or substrate modification. First, mechanistic control is exerted either by controlling the ATPase cycle or by blocking other cofactors from binding p97. It is possible that further mechanistic control might be exerted at the level of domain motions and substrate processing as well. Second, many of the p97-associated cofactors are membrane localized or have affinity for localized proteins this acts to sequester p97 to the required cellular location to exert the needed function. Finally, there are many seemingly antagonistic cofactors that modify p97 substrates at the level of their post-translational modification state. These substrate-modifying cofactors seem to decide the fate of a given substrate, deciding perhaps between deubiquitylating and releasing or ubiquitylating and sending to the proteasome for processing.[10, 11]

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The many cellular pathways in which p97 plays a central role implicate p97 in a number of disease states.12 Compromised p97 function has been linked to cell death through a series of events initiated by poor protein quality control, which ultimately leads to mitochondrial dysfunction, oxidative stress, and apoptosis.13 This has possible implications in a number of neurodegenerative diseases, as p97 has been shown to be sequestered by ubiquitylated pathological protein fibrils.14 Further, the role played by p97 in transcription factor processing and DNA maintenance offers possible links to cancer survival and metastasis.[15] There is also a rare, lethal, autosomal-dominant condition called inclusion body myopathy associated with Paget's disease of bone and frontal temporal dementia (IBMPFD) that has been linked directly to subtle, single amino acid mutations in p97.[16] Designing or discovering small molecule inhibitors or regulators of p97 presents many challenges. The most likely target is the ATP-binding pocket, but this is a highly conserved pocket with many off-pathway possibilities. p97 is also one of the most highly abundant cytosolic proteins, potentially further confounding drug discovery. This being so, there are already researchers looking for inhibitors. The present review will discuss some of the issues raised here, including structure, mechanism, cofactor regulation, and p97 as a drug target. There have been many excellent reviews on some of these topics, so this review will try to focus on the ongoing and current unresolved issues.

## 2. Structure

The AAA+ proteins are divided into two classes based on the number of conserved AAA cassettes they possess. The class I AAA+ ATPases have one AAA cassette and the class II AAA+ ATPase have two ATP-binding cassettes.17¯19 p97 is a class II AAA+ comprising four domains: the N-terminal domains which have been definitively shown to interact with many of the p97 cofactors required for cellular positioning and activity9 and which may also interact directly with substrate proteins, but this remains uncertain in a physiological context; the more N-terminal AAA binding cassette (D1) is believed to be primarily structural, facilitating hexameric assembly20, however, a substantial increase in ATPase activity obtains at elevated temperatures and allostery between D1 and D2 has clouded interpretation of data21, 22; a D2 domain which is likely responsible for the majority of ATPase activity at physiological temperatures as well as housing conserved, essential Ar-Φ-Gly pore loops (in this case Trp-Phe-Gly)23; and the disordered C-terminal domains which bind substrate modifying cofactors to alter the post-translational modification state of substrates10. The C-terminal domain also contains a phosphorylation site that is blocked from cofactor binding when phosphorylated, potentially playing a critical regulatory role.24 The overall structure of p97 is shown in Figure 1A.[25] The crystallographically resolved domains of one protomer are color-coded as indicated in the figure legend.

The AAA-ATP binding cassettes are made up of six defining elements: the Walker A motif, the Walker B motif, sensors 1 and 2, the second region of homology (SRH), and the pore loops (not highlighted in Figure 1B).[17] The Walker A and Walker B motifs are responsible for ATP binding and hydrolysis, respectively. Walker A motifs have the consensus sequence GxxxxGK(T/S), where x is any amino acid and mutations of the conserved lysine generate ATP-binding deficient proteins. Walker B motifs have the consensus sequence (R/K)xxxGxxx(L/V)hhh(D/E), where x is any amino acid, h is a hydrophobic amino acid, and mutations of the acidic residue block ATP hydrolysis. Sensor 1 interacts with the Walker B motif and the γ-phosphate of ATP. Mutations of sensor 1 are also hydrolysis deficient. Sensor 2 (interchangeably called arginine fingers (R-fingers)) interacts directly with the γ-phosphate of ATP on a neighboring subunit (Figure 1B) via a conserved arginine and is essential for ATP hydrolysis and binding. These R-fingers are also believed to be the primary line of communication between the protomers. The SRH contains these critical arginine fingers. Finally, the pore loops are often involved in substrate binding and

processing, which has been shown for the D2 Ar-ϕ-Gly loops, however the pore loops in D1 are a bit mysterious in the case of p97.[23] The mechanism of p97 function and whether or not each of the pore loops comes into direct contact with the substrate remains controversial. Two of the proposed mechanisms do not explain the function of the D1 pore loops (see below). Details of a protomer are shown in Figure 1B, with the conserved AAA+ structural elements being color-coded as described in the figure legend.

Structural characterization of p97 has been the driving force for many of the important biochemical studies, but has also led to some confusion about the mechanism of polypeptide processing. Particularly as pertains to the central pore as a function of nucleotide state. Cryo-electron microscopy (Cryo-EM) was used to generate 3-dimensional reconstructions of the apo-, AMP-PNP-bound (a non-hydrolysable ATP analogue – ATP state), ADP-$AlF_x$-bound (a mimic of the transition state), and the ADP-bound states.[26] In these studies the D2 pore switched from an open to an occluded state in going from the ATP state to the transition state and then closing again post-hydrolysis. Whereas the D1 pore is open until after ATP is hydrolyzed. Small angle x-ray scattering (SAXS) studies indicate an open pore conformation for both D1 and D2 followed by narrowing of the D2 pore after ATP binding. 27 The D2 pore widens in the transition state and the post-hydrolysis state is similar to the ATP-bound state. Crystallographic analysis shows only the D2 side of the pore to be open in the ATP-state and narrowly open in the post-hydrolysis state with the D1 occluded throughout.28 Further, the x-ray crystallography studies demonstrated the presence of divalent zinc coordinated by six histidines at the top of the D1 pore. This residue was shown to be critical; leading to the idea that occlusion is necessary and providing credence to the “denaturation collar”23 and “molecular ratchet”29 models of substrate interaction (see below).

Studies of the general mechanism of action of AAA+ proteins have been carried out on a number of different proteins, but structural data with bound substrate has been difficult to collect. There are EM studies from ClpA[30] and ClpX31 a type II and type I AAA+, respectively, with bound substrate that helped demonstrate a translocation mechanism, but these were low resolution. No high resolution structures with bound substrate have been collected, however, high resolution asymmetric structures of a viral RNA packaging motor were used to generate a translocation model.32 This structure shows large scale, asymmetric movement of the RNA-binding domains which were modeled in contact with the nucleobases, threading the substrate through the pore in a corkscrew fashion. It remains to be seen if this type of asymmetric mechanism of action is utilized in polypeptide-modifying AAA+ proteins. Structures of other type II AAA+ proteins both by CryoEM and x-ray have been able to capture asymmetric complexes with the pore loops in various positions around the pore.[33–35] This remains to be verified for p97, as does the mechanism of action (see below). Although isothermal titration calorimetry (ITC) studies show substoichiometric binding of nucleotide, indicating asymmetric binding and/or hydrolysis.[36, 37]

There is also some confusion about the location and use of the N-domains. It is clear these domains are quite mobile, as many of the solved structures failed to resolve them all together or only partially resolved them. All crystallography studies of full-length p97 showed the N-domains to be coplanar with D1, as did EM studies.[26, 28, 29] SAXS data showed the N-domains to be coplanar with D1 except in the ATP-bound state when they were below the plane of D1.[27] These domains have been shown both biochemically and structurally to be the primary site of substrate-recruiting cofactor interaction and possibly substrate interaction.[38–41] Further, the N-domains are known from IBMPFD N-domain mutant characterization and from cofactor binding studies to influence ATP hydrolysis rates.[38, 42] IBMPFD mutations are found invariably at the interface between the N-domains and D1 and cause acceleration of the rate of ATP turnover.[37, 42] This change in ATP cycle is

clearly deleterious as IBMPFD is lethal, and the mutations have been shown to be deleterious in cell lines43, a fly model44, and a mouse model45, but the biochemical underpinnings remain obfuscated. Recent structural studies using the N- and D1-domains of wild-type (wt) p97 and three p97 variants harboring IBMPFD mutations (R155H, R95G, and R86A) bound to ATPγS as well as wt p97 and R155H bound to ADP provided much needed insight into how these mutants operate as well as potential cofactor regulation and general p97 operation.[37] It was noted that there are two primary, nucleotide bound states, the open (up) state and the closed (down) state, however, no below D1 plane state was observed as in SAXS studies. In the open state, the N-domains rotate 93°, swinging up and away from D1 by 12.5 Å relative to the closed state. This allows for nucleotide to exchange more freely. These structural studies, coupled with biochemical studies displayed dysregulation of the open and closed states for the IBMPFD mutants, explaining differences in the ATPase rates. R155H bound ATPγS seven times more tightly and ADP five times more weakly than wt p97. Moreover, the mutant had higher average nucleotide occupancy. X-ray scattering studies were also used in an attempt to correlate these domain movements, which have yet to be observed crystallographically, to wt p97. All of the studies conducted used p97 variants without D2 domains and recent biochemical studies showed structural alterations in D2, arguing D2 is responsible for the altered ATPase activity.37 Strangely, the authors of the later study also argue that heat influences the mutants more strongly than wt p97, again arguing for D1, however, their data show identical relative changes in ATPase activity between wt p97 and A232E and only a modest change between wt and R155P. Interestingly, A232E shows the most severe phenotype of the IBMPFD lesions.42 These data indicate the complexities of characterizing the domains in isolation.

## 3. Mechanism

### 3.1. ATPase activity

As discussed above, p97 is a type II AAA+ ATPase with two ATP-binding domains. Both of these domains are highly similar to each other and to the D1 domain of *N*-ethylmaleimide-sensitive factor (NSF). NSF is an analogous type II AAA+ protein which has been shown to have one AAA domain required for assembly, D2, and one with ATPase activity, D1.[46] Thus the similarity between the p97 domains and NSF D1 made it seem likely both domains may be utilized for ATP turnover. It was also shown that the ATPase activity produced a sigmoidal curve, arguing for allosteric interactions, although the Hill coefficients were modest.21 Genetic dissection of the two domains using mutations in the Walker A motif to block ATP binding or in the Walker B motif to block ATP hydrolysis demonstrated a greater loss of ATP hydrolysis when D2 was compromised. This was interpreted by one group to indicate D2 is the major ATP hydrolyzing domain21 and by another group to indicate that D1 and D2 alternate in ATP hydrolysis and depend on each other.22 It was later shown that blocking ATP-binding in D1 slowed the rate of hexamerization slightly, but to a far less extent than blocking binding in both D1 and D2.[20] This has lead to many people stating that the D1 domains are required for assembly, but the data do not support this. It seems more likely the presence of ATP accelerates folding and assembly of the protomers as has been demonstrated for other macromolecular machines.[47] This process is augmented by having both domains binding competent, showing the whole is greater than the sum of the parts. A number of type II AAA+ proteins have been analyzed in a similar fashion with similar results.[48–50] It must be pointed out, however, that each of these ATPases displays multiple levels of allostery both between ATP binding sites of a single domain and between the two ATP-binding domains. This may confound interpretation of the observed ATPase activities. It should be further mentioned that the in vitro data are borne out in vivo using both yeast22 and PC12 cells[51]. Mutations in the Walker A or Walker B motifs of D2 are dominantly lethal, whereas mutations in D1 are not always lethal, but do show severely compromised ability to handle expanded polyglutamine (ex-polyQ). Curiously, a recently characterized

mutant unable to release ATP from D1 proved to be exceptionally lethal, again confounding a straightforward assignment of ATPase function by domain.[52] Interestingly, screening for suppressors primarily produced mutations with allosteric implications: the linker between D1 and D2, the D1 SRH, and the D1 pore.

It was further shown that mutations blocking ATPase activity in D2 were temperature sensitive, increasing the rate of ATP hydrolysis up to a maximum at ~60°C.[21] This effect was not observed in the case of the D1 ATPase mutants, implicating D1 as a temperature regulated ATPase. It remains unclear what the precise biological significance of this heightened D1 activity may be. These studies were carried out using metazoan p97 and similar studies were not conducted using p97 homologues from yeast or archaea. It is possible this extra activity helps metazoans under cell stress conditions. Yeast contain a heat shock regulated Hsp104p molecule that is lacking in metazoans, perhaps the heightened D1 activity helps facilitate disaggregation in a manner similar to Hsp104p, although there is no heightened expression of p97 under stress.[53] Interestingly, recent studies have shown the type II AAA+, ClpA, requires only D1 for unfolding single domain proteins of low stability, D2 for unfolding proteins of moderate stability, but both domains for processing highly stable proteins.[50] Perhaps, similar to ClpA, the heightened function of both domains assists p97 under severe stress.

### 3.2. Substrate interaction

Central to the function of p97 is the nature of p97-substrate interaction. The interaction between p97 and its substrates is the source of some controversy, however, on a number of levels. First, it is unclear if p97 initiates substrate interaction directly or if this is always mediated by cofactors. Second, the mode of interaction and the relationship to the ATPase cycle is not clear. Several models exist, as outlined in Figure 2. One model, the translocation model (Figure 2C), posits substrates are engaged at the level of the N-domains and then threaded through the central pore where they can be further modified, recycled, or degraded. Another model put forth is the “denaturation collar” model (Figure 2B).[23] In this case, the substrate is not threaded through the entire pore, but only interacts at the level of the D2 pore, entering and exiting through the bottom aspect of the ring and being denatured by the guanidinium groups of two arginines in the D2 pore. Finally, there is the “molecular ratchet” model and its variants, which propose substrates are remodeled through secondary structural rearrangements while interacting with the outer aspect of the barrel.[29] The “molecular ratchet” models require asymmetric D1 and D2 nucleotide states and movements to generate the required mechanical force.

As discussed above in the section on structure, the collective of high and low resolution structures has produced different results regarding the nature of the central pore. X-ray structures indicate the pore opening at the level of D1 is occluded28, but SAXS and EM data both indicate this is dependent on the nucleotide state.26, 27 The biochemical studies conducted to date provide no definitive demonstration of the true mechanism. High-resolution structural studies, coupled with the ATPase data indicating asymmetric nucleotide states, led to the molecular ratchet model and its variants.[1] These models were based on the observation that the largest domain movements were at the level of the outer aspect and not in the pore loop. Ultimately, these models all argue that substrate remodeling takes place outside of the barrel. Interestingly, a similar model was initially proposed for the type II AAA+ ClpB (Hsp104p).54 This was based on similar evidence showing asymmetric nucleotide states and large protuberances between the D1 and D2 domains that seemed to be mobile. This was termed the “molecular crowbar” model, but biochemical studies55 coupled with recent CryoEM data56 have eliminated this model, demonstrating substrates are translocated through the pore. Studies looking at the effects of pore loop mutants, driven by high resolution structures, indicate only mutations of the conserved hydrophobic and aryl

amino acids in the D2 pore have an effect on p97 substrate interaction.23 Further, the two guanidinium groups, R586 and R599, proposed to denature proteins, were also shown to be important for substrate processing. This coupled with x-ray studies led to the proposal of the "denaturation collar" mechanism. In this model, the substrate can be engaged at the level of the N-domains, but the ultimate remodeling would take place via the D2 pore. Substrates would enter through the opening of the D2 pore and exit through the same pore or perhaps through transient openings in the p97 walls. In the ERAD pathway, it has been shown p97 interacts with ER membrane localization factors (e.g. Ubx2) at the level of the N-domains and it is likely ubiquitylated proteins interact with substrate recruiting cofactors (Ufd1/Npl4) at the level of the N-domains or the substrate interacts directly with p97 via the N-domains. In either case, the interaction would be through the N-domains. The substrate would then be required to interact with D2 where it would be extracted and further processed by substrate processing factors (e.g. Ufd2, Ufd3, PNGase) before being handed off to the proteasome or released into the cytosol. Early structural studies of ClpB indicated an occluded D1 pore loop[54] and biochemical studies of the pore loops of both ClpB and ClpA demonstrated much greater effects of pore loop mutants at the level of the conserved Ar-ϕ-Gly pore loop in D2, but it has been shown that both of these use a translocation mechanism driven by the D2 pore loops and assisted by the D1 loops.55, 57 The translocation model, however, is the least supported by direct p97 related evidence. Low-resolution structural studies do argue against occlusion of D1, but no biochemical studies support this model, although they also do not disprove it.27 Clearly, the strongest evidence can only be drawn from studies carried out on the other type II AAA+ machines with quite divergent biological functions. This being said, the best structural evidence for the translocation mechanism comes from the viral packaging motor, P4 from bacteriophage ϕ12, but this structure agrees with the collective of biochemical and structural studies of polypeptide processing AAA+ proteins.[32]

## 4. Cofactors

Certainly one of the most remarkable aspects of p97 is the diversity of cellular activities carried out in which it plays a critical role. Initially discovered in yeast as a cell division control protein (thus the name Cdc48p) and in metazoans as 'pro-valosin' (thus the misnomer valosin containing protein), p97 was rapidly demonstrated to occupy a place of prominence in a number of areas.[58] It has been shown over the last ~15 years that this diversity of activity is carried out by co-opting a large collective of cofactors (see Table 1). The first of these to be characterized was the founding member of the ubiquitin regulatory X (UBX) domain containing proteins, p47.[59] This cofactor binds the p97 N-domains as a trimer, blocking other cofactors from binding.38, 59 p47 is required for homotypic membrane fusion of the nuclear membrane and Golgi cisternae after mitosis.60 The interaction between the N and D- domains of p97 and p47 is the only p97-cofactor interaction to be characterized at high resolution, demonstrating p47 binds to the N-domains in a hydrophobic groove formed at the interface of the two N-domain sub-domains.61 This binding site has proven to be a general site of interaction for many p97 cofactors binding both UBX62 domains and ubiquitin D domains (UBD)[9]. This also demonstrated a coplanar (closed) relationship between p47 and the N- and D1 domains of p97. Although initial structural studies produced some confusion26, the binding stoichiometry has been demonstrated to be three p47 per p97 hexamer with p47 occupying alternating N-domains. 59 It has also been shown that p47 causes nearly complete loss of p97 ATPase activity.38 The biological significance of this remains to be demonstrated, as it has been shown via in vitro reconstitution that homotypic membrane fusion is an ATP dependent process,63 so perhaps the ATPase activity is regulated by another cofactor or the as yet unidentified substrate of this process. It is nearly certain that the p97-p47 substrate is ubiquitylated, as p47 also contains an ubiquitin-associated (UBA) domain.

Seemingly the most functionally diverse of cofactors is the heterodimeric complex Ufd1/Npl4. These bind to a single subunit of p97 in a p47 excluding manner.9 Exactly how this exclusion takes place is not known, but it is clearly a means of controlling p97 function. Ufd1 was characterized originally in a genetic screen of effectors of the ubiquitin fusion degradation (UFD) pathway[64] and Npl4 in a screen of nuclear protein localization (NPL) effectors65. It was later shown that these two components are required for transcription factor regulation and ERAD.5, 7 Both proteins bind p97; Ufd1 via its UBX domain and Npl4 via its UBD domain in a synergistic fashion, as both proteins bind more tightly as a dimer with Npl4 binding requiring prior Ufd1 interaction.38 The mechanism by which the various p97-Ufd1-Npl4 programs are regulated in vivo remains to be determined, but it is likely through further cofactors that act as p97 localizing factors and substrate modification factors. Interestingly, a recent study showed extraction of the major histocompatibility complex (MHC) class 1 heavy chain (HC) is facilitated by the human cytomegalovirus (HCMV) protein US2 in a p97/ubiquitin/ATP dependent manner without the use of Ufd1/Npl4.[66] A similar extraction of MHC HC facilitated by US11 requires the Ufd1/Npl4 heterodimer. These studies have interesting implications for cofactor mediated pathway differentiation for both ERAD and potentially other critical pathways.

Along with the substrate recruiting and localization cofactors are substrate-modifying cofactors. These function by altering the post-translational modification state of the substrates. This can be in the form of extending the ubiquitin chain (e.g. Ufd2)67, blocking the ubiquitin chain from further modification (e.g. Ufd3)68, removing the ubiquitin chain (e.g. Otu1)69, or removing carbohydrates (PNGase)70. This is a truly fascinating aspect of p97 biology, as many of these cofactors have antagonistic functions and thus seemingly decide the fate of the protein. Indeed this processing has led to p97 being likened to a “gear box” and “molecular purgatory”; further ubiquitylation leading to further proteasomal processing, blocking of further ubiquitylation, and deubiquitylation.10, 11 How these decisions are made remains unknown. It is known that Ufd2 and Ufd3 bind in a mutually exclusive manner, Ufd3 and PNGase seem to be mutually exclusive, and Ufd2 and Otu1 are mutually exclusive.10 The identification of the binding site(s) has not been without controversy, but it has been shown that Ufd3 and PNGase bind to the last ten residues of the p97 C-domain via the PNGase/ubiquitin-associated (PUB) domain.71 It is speculated that Ufd2 binds to the same domain, but this has not been unambiguously shown and Ufd2 lacks the PUB domain and instead binds via the VCP interacting motif (VIM). What is known is that Ufd2 does not bind to the N-domains but somewhere in the D2 or C-domain.10 Interestingly, Ufd3 binding is often associated with the binding of the deubiquitinase (DUB), Otu1, which binds the N-domains, but Ufd2 is not, thus separating counter-indicated functions.10 This again speaks to the degree of communication between the domains. So far, the most likely mode of control is post-translational modification (PTM) of p97.24 p97 is subject to a variety of PTMs, but many of these do not have demonstrated function with the exception of phosphorylation of Tyr805 near the C-terminus that has been shown, in vitro, to block binding of PUB domain containing proteins. This particular phosphorylation, as a means of cofactor regulation, remains to be demonstrated in vivo. Another possibility could be a combination of stochastic and genetic control. It has been shown that Ufd3 binds more tightly than Ufd2 and that Ufd2 is upregulated under stress conditions, but Ufd3 is not.72, 73 Perhaps the Ufd3 pathway is a regularly explored path under normal conditions, when the expense of protein destruction is to be avoided, but the Ufd2 pathway is operative when a large collection of troubled and/or heat shock and regulatory proteins must be processed.

## 5. p97 in disease

### 5.1. IBMPFD

The diversity of p97 functions carried out in the cell implicates p97 in a large number of diseases, especially as they relate to protein quality control, offering a potentially interesting therapeutic target. As discussed above a genetic link between p97 and the rare autosomal dominant, multi-system disorder called inclusion body myopathy associated with Paget's disease of bone (IBMPFD) exists.16 There have been thirteen mutations found in the D1 and N-domains of p97, exclusively at the interface between these two domains.16, 37 The mutations that have been studied have an ~3-fold increase in ATPase activity and altered nucleotide affinity, increasing ATPγS affinity and decreasing ADP affinity.37 This leads to a shift in the ratio of nucleotide dependent orientation of the N-domains relative to D1 (see above), but exactly how these biochemical and structural observations relate to pathology remains an area of intense study. Disease models have been generated in cell lines43, fruit flies44, and mice45. These studies have illuminated a number of potential paths to disease that are linked by problems with protein quality control. Models have demonstrated that loss of p97 function through mutation or depletion leads to ER stress, ER expansion, mitochondrial malfunction, release of cytochrome c, production of reactive oxygen species, caspase activity, and apoptosis.13 The exact aspect of protein quality control compromised in IBMPFD is not certain. Some studies claim both the ubiquitin proteasome system (UPS) and autophagy are compromised[74], while other studies claim only autophagy is lost75. It was shown that expression of the IBMPFD mutants R155H and A232E both lead to defective autophagosome maturation and accumulation of aggregated proteins. At what point in the developmental cycle the autophagosomes fail to mature is not clear. One study indicates it is during fusion with lysosomes and another study indicates fusion is intact, but some other process is defective.75, 76 In either case, the required cofactor and substrate of this process are unclear, but a correlation between p97-HDAC6 and aggresome formation has been established. Further, HDAC6 interacts strongly with ubiquitylated proteins, strengthening the link between p97 and aggresome formation.[78] The structural and biochemical correlation between HDAC6 and p97 has not been determined, but it is possible IBMPFD mutations compromise this interaction and interfere with the balance between HDAC6 and p97.

### 5.2. Neurodegenerative proteinopathies

It has been known for some time that polyQ regions lead to aggregate formation and diseases such as Huntington's disease and Machado-Joseph disease (MJD) (also called spinocerebellar ataxia Type 3 (SCA3). PolyQ aggregates from cultured cells have been shown to co-localize with p97 and aggregates isolated from patients with Huntington's or MJD have been shown to have p97 associated with them.79 This is possibly unrelated to pathology, as other pathological fibrils isolated from patients have been shown to be ubiquitylated and to have p97 associated. However, there is evidence that p97 is either directly or indirectly linked to pathology. Conflicting studies have been put forth regarding ataxin-3 with an ex-polyQ region, the protein linked to MJD. Some studies claim p97 interacts directly with the ex-polyQ region in a size dependent manner80, but biochemical studies indicate there is a VCP/p97 binding motif (VBM) present on ataxin-3, this region is recognized by p97, and fibrillogenesis is accelerated by the proximity of the ex-polyQ regions in a length dependent manner due to the fibril forming properties of ex-polyQ.81 This study further shows that p97 can prevent or catalyze fibril formation in a concentration dependent manner, similar to Hsp104p and the yeast prion protein Sup35p.53 Another ex-polyQ protein that has been shown to interfere with ERAD is huntingtin, the causative agent of Huntington's disease. This protein has not been shown to interact with p97 directly, but it does interact with the Cue domain of gp78 and an unidentified Hrd1 domain. These are both

ER-membrane localized E3 ubiquitin ligases that also interact with p97 in the ERAD pathway.[82, 83] Gp78 binds to huntingtin and ubiquitylates it for proteasomal degradation, however, the ex-polyQ region sterically interferes with p97 binding to gp78 in a size dependent manner, thus large amounts of huntingtin ex-polyQ lead to ER stress and apoptosis via the path illuminated above. There is also indication that TAR DNA binding protein (TDP-43) proteinopathies are related to p97, as compromised p97 function leads to movement of TDP-43 from the nucleus to the cytosol with concomitant fibril formation and disease phenotype.[84] However, how exactly these are connected remains an area of active research.

### 5.3. Cancer

The most likely exploitation of p97 as a therapeutic target is in cancer treatment. Elevated levels of p97 have been linked to a poor prognosis in many different forms of cancer including prostate cancer, non-small-cell lung carcinoma, pancreatic cancer, breast cancer, and leukemia.[12] The strongest correlation between p97 and cancer is in NFκB signaling.[85] NFκB is inhibited by IκBα until needed by the cell. Removal of IκBα has been shown to be carried out by p97, which can be upregulated by pre-B-cell leukemia transcription factor 1 or E74-like factor 2 as well as other possibly unidentified transcription factors.[12] In any case, these transcriptional programs lead to increased survival and proliferation. Another possible factor implicating p97 has to do with the cellular senescence pathway. Studies have linked p97 depletion to activation of the p53 pathway and activated senescence.[15]

## 6. Inhibition

Given the success of bortezomib (Velcade – marketed by Millenium Pharmaceuticals) a proteasome inhibitor used to treat multiple myeloma and mantle cell lymphoma, it seems targeting other aspects of the UPS could offer further avenues of cancer treatment. Not surprisingly, p97 has emerged as one possible target. To date, there have been two published studies conducted on p97 inhibitors with two more meeting abstracts available.[86–89] One of these studies was on the ERAD inhibitor eeyarestatin I (EerI), a small molecule inhibitor discovered in a small screen for molecules that stabilized a fluorescent ERAD substrate (EGFP-tagged MHC class I).[86, 90] One study of the complex function of this molecule indicates EerI is a pro-drug that is processed to an unidentified metabolite inhibitor of p97-DUB interaction.[86] Other studies indicate other modes of action and this is a source of controversy[91], but it has been shown EerI is effective in killing the hematological cancer cell lines JEKO-1 and HBL-2[92]. Two studies from the Deshaies lab have looked at developing specific p97 inhibitors by targeting the D2 active site cysteine.[88, 89] The first of these studies used the pyrozololpyrimidine, PP1, from the kinase field as a starting point. This was appended with electrophiles in an attempt to discover a molecule that makes a covalent linkage to Cys522 of murine p97. This molecule was shown to have an $IC_{50}$ ~600 nM against the target molecule with $IC_{50}$s 500 and 150 times higher for Cdc48p and NSF, respectively. Mass spectrometry was used to demonstrate specific cross-linking to Cys522 in the D2 domain. The second study from this group screened a small library for inhibitors of p97 ATPase activity. This study produced a molecule with an $IC_{50}$ ~300 nM. Importantly, a p97 variant with a C522A mutation showed an $IC_{50}$ ~100 fold higher than wt p97. Cell-based assays showed an accumulation of poly-ubiquitylated proteins, providing evidence of p97 inhibition, but not ruling out other targets. Finally, a recent screen for ATPase inhibitors revealed 2-anilino-4-aryl-1,3-thiazoles as nanomolar p97 inhibitors, after medicinal chemistry optimization of the lead compound.[87] The most potent of these molecules were then demonstrated to effectively stabilize a reporter construct expressed in HeLa cells. This study and the previously discussed study both explore the inhibition of ATPase activity to good effect, but in both cases there are many off-pathway targets that could lead to the cell-based observations. The p97 ATP-binding pocket is, after all, a member of one of the most

highly conserved pockets. Further, the abundance of p97 may necessitate reasonably large quantities of compound, increasing the likelihood of off pathway targeting. If, to put forth one model, the compound were to interfere with the protein kinase implicated in C-terminally binding cofactor modulation, this would also interfere with UPS, leading to identical results. There are also other ATP-binding proteins in the UPS that could be inadvertently targeted.

## 7. Conclusion

The importance of p97 is undeniable. This central chaperone acts to connect a large collective of critical biological processes through the use of a growing list of cofactors. These cofactors seem to serve one of two roles: 1) regulation of p97 at the level of ATP turnover or substrate interaction or 2) modification of the substrate engaged by p97. This later function seems to have the power to dictate the fate of substrates deciding between proteasomal destruction or perhaps release for reuse. Developing a better mechanistic understanding of p97 should help us understand how this is achieved as well as providing insight into p97 function in health and disease. Many questions remain to be answered including definition of ATPase function, substrate processing, and cofactor interaction to name a few. Not to mention the need to discover missing cofactors and substrates involved in the more poorly defined pathways. Progress in the area of therapeutic design must also continue, as p97 offers a promising target in cancer treatment. Better understanding of other mechanistic and cofactor interaction aspects of p97 might also lead to the development of therapeutics for other diseases including some of the lethal proteinopathies, but this is unlikely to require inhibition, instead this will need regulation. For instance, in the case of IBMPFD, perhaps a small molecule that restores the interaction between the N-domains and D1, perhaps only to a small extent, may be a useful treatment. In any case, p97 offers many exciting areas of exploration and possible therapeutic intervention.

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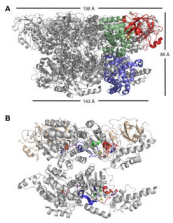

**Figure 1. p97 structure (PDB 3CF1)**

A) The overall structure of full length p97 from *Mus musculus* is shown. One of the protomers is color coded to display the domains; the N-domains are red, the D1 domains are green, the D2 domains are blue, and the N-D1 and D1-D2 linkers are both colored brown. The C-terminal domains were not resolved. This structure was solved in the presence of ATPγS but this is only seen in the D2 domains, the D1 domains are occupied by nucleotide most likely not released during purification. B) The bound nucleotides are shown explicitly in two contiguous protomers in order to illustrate the AAA+-defining structural elements. The elements for both D1 and D2 are shown and colored according to element as follows: the Walker A motifs are red, the Walker B motifs are green, the second regions of homology (SRH) are blue, sensors 1 are yellow and shown as sticks, and sensors 2 (R-fingers) are magenta and also shown as sticks. The N-domains have been colored tan for orientation.

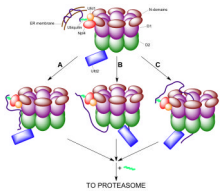


**Figure 2. Possible substrate interaction models**

The mode of substrate interaction remains unclear. There are three primary models as illustrated here. In each case an ubiquitylated substrate in the ER membrane is engaged by Ufd1, bound to Npl4 and the N-domain of p97, which is then extracted from the membrane using one of the proposed models. The first model, designated A, is what we are calling the molecular ratchet model. There are several subdivisions of this model, but the basic premise is that structural rearrangements of the outer aspect of p97 generate the force to dislodge a polypeptide interacting with the outside of p97. The second model, B, is the denaturation collar model. In this case the polypeptide interacts with the D2-pore loops, entering and exiting through the D2 pore, and is 'melted' by the guanidinium groups of Arg586 and Arg599, while the D2 pore loop movement extracts the polypeptide. The third model, C, is the translocation model. In this case the polypeptide is thread through the pore loop from D1 to D2, using the motion of the D1 and D2 pore loops to generate the required force. In any case, once the polypeptide is extracted from the membrane it can be further processed for salvaging or degradation. In the figure, the 'E4' ligase, Ufd2, is shown, which will transfer a four to five ubiquitin chain, targeting the substrate to the proteasome for degradation.

**Table 1**

p97 Cofactors

| Cofactor | p97 Binding Domain | p97 Interaction Motif | Ubiquitin Binding Domain | Structural Notes | Function |
|---|---|---|---|---|---|
| **Substrate Recruiting** | | | | | |
| Ufd1-Npl4 | N[22] | BS1, UBD, UBX[93] | Ufd1: UT3[40] Npl4: NZF[94] | NZF not in yeast[94], heterodimer: one heterodimer per p97 hexamer[95] | Retrotranslocation[5], transcription factor regulation[96] |
| p47 | N[61] | UBX[61] | UBA[94] | One trimer per p97 hexamer[59] | Golgi regrowth, membrane fusion[59] |
| Ubx2 | N[62] | UBX[97] | UBA[97], UIM[98] | | ERAD[97] |
| FAF1 | N[62] | UBX[99] | UBA[99] | | Protein degradation regulator[99] |
| VIMP | | Cytosolic Domain[100] | | | May be ER receptor during retrotranslo cation[100] |
| **Substrate Processing** | | | | | |
| Ufd2 | C[101] | VIM[71][a] | | U-box, RING finger-related ubiquitin-ligase domain[102] | E4 enzyme, involved in extending ubiquitin chains, maybe retrotranslocation[103] |
| Ufd3 | C[101] | PUL[104], PUB[105] | PFU[104] | WD-40 domain, protein-protein in teracting domain[104] | Control of ubiquitin concentration in the cell[64] |
| Otu1 | N[72] | UBX[72] | Zinc Finger[3] | Catalytic OTU domain[71] | Deubiquitylating enzyme[72] |
| VCIP135 | N[62] | UBX[106] | | OTU, deubiquitylating enzyme domain[106] | Deubiquitylation, Golgi reassembly[69] |
| Ataxin-3 | N[81] | VBM[81] | UIM[108] | Josephin domain[109] | Retrotranslocation[110] |
| PNGase | C[110] | PUB[112] | | Transglutaminase domain[113] | Deglycosylation, ERAD[114] |
| HDAC6 | | | ZnF-UBP[115] | Duplicate HDAC domains[116] | Required for aggresome formation[117] |
| gp78 | C[118] ND1[119][b] | VIM[119] | Cue[118] | RING finger, E2-binding site[120] | Membrane associated E3, retrotranslocation[118] |
| Hrd1 | | VIM[83] | | RING finger[121] | Membrane associated E3, retrotranslocation[83] |
| SVIP | ND1[122] | VIM[119] | | | Inhibits ERAD[123] |

[a] The only proposed p97-binding domain of Ufd2, however, the VIM domain is not known to bind the C domain of p97

[b] Both the C and the ND1 domains of p97 have been presented as being the binding site of gp78

## Table 2

p97 Related Diseases

| Disease | Involved Protein | Mutation or Cause | Symptoms | Pathology |
|---|---|---|---|---|
| IBMPFD[12,37,124] | p97 | R93C, R95G, R95C, R155H, R155P, R155C, R159H, R159C, R151Q, A232E, L198W, N387A, T262A[37,124,125,126,127] | Progressive muscle weakness and atrophy, increased osteoclastic bone resorption, early onset FTLD[126,127] | UPS and ERAD disruption, impaired aggresome formation, impaired autophagy leading to apoptosis[16,77,126,127] |
| FTLD (FTLD-U)[12,128,133] | TDP-43[44,129,130] | TDP-43 mislocalization | Movement disorder, (parkinsonism or motor neuron disease) behavioral or language dysfunction | Mislocalization of TDP-43, decreased proteasome activity, induced ER stress, increased apoptosis, impaired cell viability[43,131,133] |
| Huntington's disease[82] | Huntingtin protein (*htt*) | Ex-polyQ | Uncontrolled movement, cognitive decline, dementia | *htt* binds gp78 Cue domains, ex-polyQ sterically occludes p97 leading to ER stress and ultimately apoptosis[79,82] |
| Machado-Joseph disease [81,132] | Ataxin-3[81] | Ex-polyQ | Cerebellar ataxia, peripheral nerve palsy, facial and lingual fasciculation | p97 modulates fibrillogenesis of pathogenic forms of Atx-3 in a concentration dependent manner[81,132] |
| Alzheimer's disease[132] (AD) | Amyloid-β[139] | Aggregation and accumulation of misfolded A-β | Global cognitive decline, memory impairment | Erasin[a] (UBXD2) colocalizes with p97 in AD affected brain[145] |
| Creutzfeldt-Jakob disease (CJD)[141] | Prion protein isoform ($PrP^{Sc}$)[141] | $PrP^{Sc}$ aggregates and inclusions | Progressive dementia, personality change, hallucinations | Ubiquitin positive inclusions colocalize with p97 in CJD neurons[132] |
| Parkinson's disease (PD)[14,132] | α-Synuclein[142] | α-Synuclein aggregates | Bradykinesia, tremors | Dorfin and p97 colocalize in Lewy neurites of PD affected neurons[143,146,] [b] |
| Colorectal carcinomas[134] Pancreatic cancer[135] Follicular thyroid cancer[136] Breast cancer[144] Gingival squamous cell carcinoma[138] Gastric carcinoma[137] | | | | Upregulation of p97 is associated with a poor prognosis[134,135,136,] [c] p97 is associated with anti-apoptotic and metastatic activity via NFκB signaling pathway[12,137,138] |

[a] It has been shown that an extensive amount of Erasin was present in the AD affected brain compared to the non affected brain, however, the precise relationship between Erasin and AD has not been identified, yet.[145]

[b] Dorfin (RING-IBR type ubiquitin ligase, E3) interacts directly with p97 through its C-terminal region.

[c] It has been shown that many different cancers are related by p97 regulated NFkB signaling pathways.