SUPPLEMENTARY METHODS

Cloning procedures. The coding region of human p97 was cloned into the KasI and HindIII sites of the bacterial expression vector pPROEX-HTa (Invitrogen), resulting in the replacement of residue Met1 with Gly. For ectopic expression in HEK293T cells, the coding region of p97 was cloned into the BamHI and HindIII sites of pCMV-Tag2B (Stratagene). Point mutations in p97 were introduced using the QuickChange XL II kit (Stratagene) according to the manufacturer's instructions. The coding region of human Ufd1 was cloned into the NdeI and HindIII sites of the bacterial expression vector pET28a (Novagen). The coding region of human Npl4 was cloned into the BamHI and EcoRI sites of pGEX-4T1 (GE Healthcare) for expression as GST fusion protein, or into the NcoI and EcoRI sites of pET21d (Novagen) for expression without tag. The coding region of human E4B was cloned into the BamHI and EcoRI sites of pGEX-4T2 (GE Healthcare) for bacterial expression. The plasmid for hexahistidin-tagged p47 was previously described (Allen et al., 2006). Construction details are available from the authors upon request.

Protein purification. Recombinant proteins were expressed in *E. coli* BL21 pRIL. Hexahistidin-tagged p97 was purified by Ni-NTA affinity chromatography followed by size exclusion chromatography on a Superdex S200 column (GE Healthcare). Untagged p97 was generated by incubation with TEV protease and recovered as flow-through from a Ni-NTA agarose column. GST fusion proteins of p47 and E4B were purified by glutathione sepharose affinity chromatography, using standard protocols. Purification of heterodimeric Ufd1-Npl4 complex and of hexahistidine-tagged p47

was performed as described (Allen *et al.*, 2006; Pye *et al.*, 2007). Purified proteins were dialyzed against 50 mM HEPES-NaOH pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol (p97, E4B), or 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT (p47, Ufd1-Npl4), respectively.

Antibodies. Western blots were performed using antibodies against p97 (A300-588 and A300-589, Bethyl Laboratories), E4B (BD Biosciences), Npl4, Ufd1 and p47 (kind gifts from O. Stemmann), ataxin-3 (MJD; kind gift from H. Paulson; 1H9, Millipore), Hrd1 (kind gift from E. Wiertz), and PNGase (kind gift from T. Suzuki).

Size exclusion chromatography. Purified p97 (6 μM) was loaded onto a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with 50 mM HEPES/NaOH pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol. Eluted fractions (1 ml) were precipitated with trichloroacetic acid, resuspended in SDS-PAGE loading buffer, and silver-stained after SDS-PAGE.

Circular Dichroism. Far-UV spectra of p97 (0.3 mg/ml in 20 mM PBS, pH 8) were recorded at 20°C on a Jasco-715 spectropolarimeter equipped with a thermoelectric cell holder. The collection parameters were 0.5 mm path length, 1 nm band, 50 nm/min scan rate, and 1 s response time. For each spectrum, ten scans were accumulated.

ATPase assay. ATP hydrolysis activity was determined by a colorimetric assay (Lanzetta et al., 1979). Briefly, p97 (50 nM monomer) was incubated with 2 mM ATP for 10 minutes at 37°C. 800 µl of a malachite green solution was added, and colour development was stopped after 1 minute by addition of 100 µl of 34% citric acid. Absorbance at 640 nm was measured after 20 minutes of equilibration at room temperature. Inorganic phosphate release was calculated from a calibration curve set up with known concentrations of potassium dihydrogen phosphate.

ESI-MS. p97 (75 µg) was incubated with 3.125 µg trypsin for 15 minutes at 25°C. The reaction was stopped by flash-freezing in liquid nitrogen, and samples were kept at -80°C until further analysis. 10 µl sample was loaded onto a Symetry C4 column (300 Å, 3.5 mm; Waters) and washed with 0.05% TFA/H_2O at a flow rate of 250 µl/min at 20°C. Tryptic fragments were eluted with a linear gradient from 20% to 80% acetonitrile, and electrospray mass spectra were acquired using a MicroTOF-LC mass spectrometer (Bruker Daltonics). Spectra were deconvoluted using a maximum entropy algorithm.

Edman degradation. 20 µl of the samples prepared for ESI-MS as described above were separated by SDS-PAGE and electroblotted onto a PVDF membrane. Proteins were stained with Amido Black solution (Sigma), and the 52 and 54 kDa fragments were excised for Edman analysis by standard procedures.

References

Allen, M.D., Buchberger, A. and Bycroft, M. (2006) The PUB domain functions as a p97 binding module in human peptide N-glycanase. *J Biol Chem* **281:** 25502-25508

Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem* **100:** 95-97

Pye, V.E., Beuron, F., Keetch, C.A., McKeown, C., Robinson, C.V., Meyer, H.H., Zhang, X. and Freemont, P.S. (2007) Structural insights into the p97-Ufd1-Npl4 complex. *Proc Natl Acad Sci USA* **104:** 467-472

SUPPLEMENTARY DATA

Supplementary Table 1: Masses of tryptic p97 fragments in the presence of ATP.

	Masses expected for cleavage at R487 (Da)	Masses determined by ESI-MS (Da)
p97	54,103.9	-[a]
	52,416.1	52,415.6
$p97^{K524A}$	54,103.9	-[a]
	52,416.1	52,413.0
$p97^{R95G}$	54,004.8	54,004.8
	52,317.0	52,314.1
$p97^{R155H}$	54,084.9	54,078.8
	52,397.1	52,397.7
$p97^{A232E}$	54,162.0	54,164.7
	52,474.1	52,472.5

Reaction mixtures of partial tryptic digests in the presence of ATP were subjected to LC-ESI-MS. Shown are masses identified in the HPLC elution peak containing the 54 and 52 kDa fragments. The upper and lower values correspond to fragments starting with residues 1 and 19, respectively. Expected masses were calculated using the ProtParam tool on the ExPASy proteomics server (http://www.expasy.ch/tools/protparam.html) [a] no 54 kDa fragment identified.

Supplementary Figure Legends (S1-S5)

Supplementary Figure S1. IBMPFD-causing p97 mutant proteins retain a wildtype-like hexameric structure

Size exclusion chromatography of wildtype and mutant p97 proteins was performed on a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with 50 mM HEPES/NaOH pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol. Fractions (1 ml) were precipitated with trichloroacetic acid, resuspended in SDS-PAGE buffer and silver-stained after SDS-PAGE. The void volume and elution peaks of molecular weight standards are indicated.

Supplementary Figure S2. IBMPFD-causing p97 mutant proteins possess wildtype-like secondary structure content

Far-UV circular dichroism spectra of wildtype and mutant p97 proteins were recorded in 20 mM PBS pH 8.0. black, wildtype p97; blue, $p97^{R95G}$; red, $p97^{R155H}$; green, $p97^{R191Q}$; pink, $p97^{A232E}$.

Supplementary Figure S3. IBMPFD-causing p97 mutant proteins possess wildtype-like ATPase activity

ATPase activities of wildtype and mutant p97 proteins were determined from the release of inorganic phosphate. The ATPase-deficient $p97^{K524A}$ mutant protein was included as a negative control. Error bars represent the standard deviation of the mean of three independent measurements.

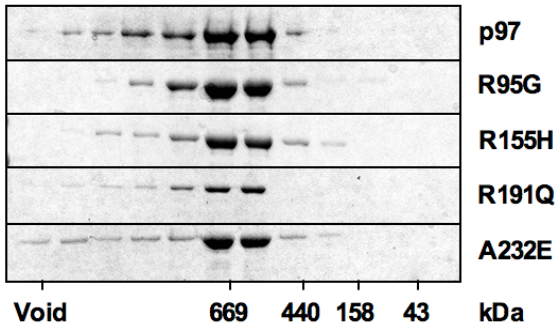
Supplementary Figure S4. Edman degradation of tryptic p97 fragments

Reaction mixtures of preparative tryptic digests of wildtype and mutant p97 proteins as described in Supplementary Methods were separated by SDS-PAGE, blotted onto a PVDF membrane and stained with amido black. The 52 and 54 kDa bands subjected to Edman degradation are marked by arrows.

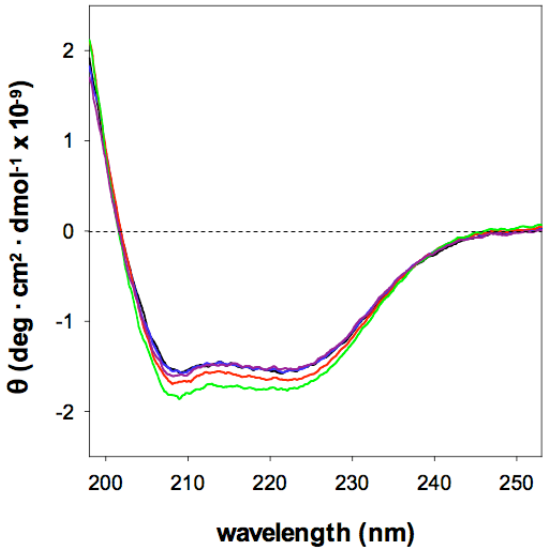
Supplementary Figure S5. Direct binding of E4B to the N domain of p97

Binding of recombinant, full-length p97 or the indicated p97 variants lacking the carboxy-terminal ten residues, the D2 domain, the D1 and D2 domains, or the N domain to glutathione sepharose beads decorated with recombinant GST-E4B (PD: E4B) or GST (PD: GST) was analyzed by Western blot (WB) using a mixture of p97 antibodies recognizing amino- and carboxy-terminal epitopes, respectively. The bottom panel shows a Coomassie Brilliant Blue (CBB)-stained gel demonstrating equal decoration with GST-E4B and GST, respectively. Shown are the results of the same Western blot exposure and stained gel, respectively, where irrelevant lanes have been removed. Note that the apparently reduced E4B binding of the p97 N domain reflects its monomeric state, whereas all other p97 variants form hexamers. The weak binding of the p97 fragment lacking the N domain to GST-E4B is unspecific, because darker processing of the blot reveals binding to the GST negative control as well.

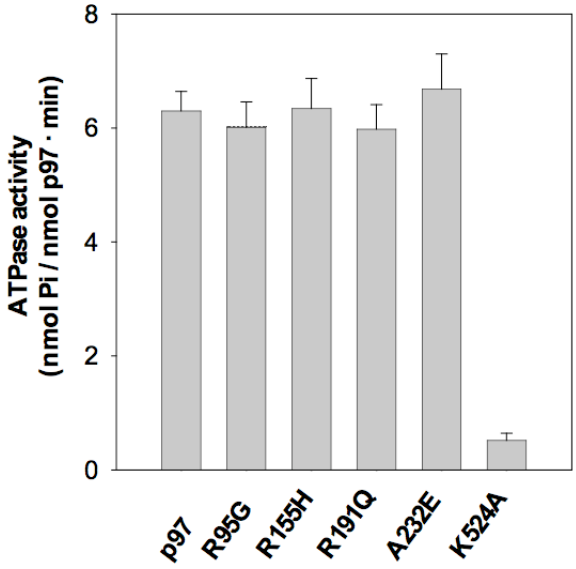
Supplementary Figure S1



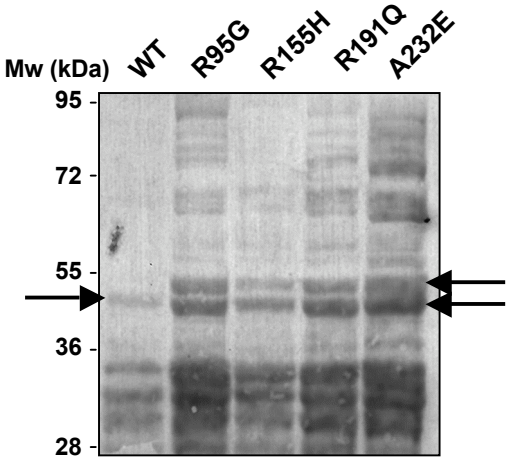
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5