Structure of the Cdc48 ATPase with its ubiquitin-binding cofactor Ufd1–Npl4

Nicholas O. Bodnar1,5, Kelly H. Kim2,5, Zhejian Ji1, Thomas E. Wales3, Vladimir Svetlov4, Evgeny Nudler4, John R. Engen3, Thomas Walz5,2* and Tom A. Rapoport1,2*

Many polyubiquitinated proteins are extracted from membranes or complexes by the conserved ATPase Cdc48 (in yeast; p97 or VCP in mammals) before proteasomal degradation. Each Cdc48 hexamer contains two stacked ATPase rings (D1 and D2) and six N-terminal (N) domains. Cdc48 binds various cofactors, including the Ufd1–Npl4 heterodimer. Here, we report structures of the Cdc48–Ufd1–Npl4 complex from Chaetoctomium thermophilum. Npl4 interacts through its UBX-like domain with a Cdc48 N domain, and it uses two Zn2+–finger domains to anchor the enzymatically inactive Mpr1–Pad1 N-terminal (MPN) domain, homologous to domains found in several isopeptidases, to the top of the D1 ATPase ring. The MPN domain of Npl4 is located above Cdc48’s central pore, a position similar to the MPN domain from deubiquitinase Rpn11 in the proteasome. Our results indicate that Npl4 is unique among Cdc48 cofactors and suggest a mechanism for binding and translocation of polyubiquitinated substrates into the ATPase.

Certain polyubiquitinated substrates cannot be directly degraded by the proteasome because they are well folded or located in membranes, chromatin, or multimeric complexes. These proteins are generally extracted from such assemblies and unfolded by a conserved ATPase, called Cdc48 in yeast and p97 or VCP in mammals (denoted Cdc48/p97 hereafter), before being transferred to the proteasome. Cdc48/p97 belongs to the AAA family of ATPases. The Cdc48/p97 hexamer contains two ATPase rings (D1 and D2) and N-terminal (N) domains that can be captured in different conformations. Upon ATP binding by the D1 ATPases, the N domains transition from a ‘down conformation’ coplanar with the D1 ring to an ‘up conformation’ above the D1 plane. Structural studies also indicate that the D1 and D2 ATPase rings undergo relative rotations upon ATP binding by D2. Cdc48/p97 binds various cofactors, which determine substrate specificity, target the ATPase to different cellular locations, or modify the ubiquitin chain attached to the substrate. The precise functions of these cofactors are poorly understood. All known cofactors bind either the N domain of Cdc48/p97 or its unstructured C-terminal tail. The Ufd1–Npl4 heterodimer (UN) is one of the most important cofactors; it participates in many Cdc48-dependent processes, including endoplasmic reticulum–associated protein degradation, a process in which misfolded proteins are extracted from the endoplasmic reticulum membrane and degraded by the proteasome. Like Cdc48, both Ufd1 and Npl4 are evolutionarily conserved and essential for cell viability. Npl4 is a target of the potential cancer drug disulfiram.

Npl4 contains an N-terminal UBX-like domain that binds the N domain of Cdc48 and is predicted to have a Zn2+–finger (zf-Npl4) followed by an MPN domain (Fig. 1a). MPN domains are found in several Zn2+-dependent isopeptidases, including AMSH and AMSH-LP, the COP9 signalosome subunit CSN5, and the proteasomal deubiquitinase (DUB) Rpn11 (refs. 10–11). Apart from the zf-Npl4 domain, mammalian Npl4 contains a C-terminal Zn2+–finger domain that binds ubiquitin, but this domain is absent in yeast orthologs. Ufd1 has two short SHP motifs that bind Cdc48, and a ubiquitin-binding UT3 domain with homology to the N domain of Cdc48 (ref. 15). Ufd1 interacts with Npl4 through its UT6 domain, a segment predicted to be unstructured (Fig. 1a). Cdc48 and Npl4 can also interact with Vms1, instead of Ufd1, and Vms1 then recruits the ATPase complex to mitochondria. Recent in vitro experiments with purified Cdc48, UN cofactor, and a polyubiquitinated model substrate have yielded some mechanistic insight. After interaction of the polyubiquitin chain with UN, Cdc48 uses ATP hydrolysis in the D2 domain to move and unfold the polypeptide substrate through its central pore. ATP hydrolysis in the D1 domain is involved in substrate release from Cdc48, a process that requires the cooperation of the ATPase with a DUB. The DUB trims the polyubiquitin chain, and the remaining oligoubiquitin chain is then also translocated through the pore. These experiments have indicated that at least two strands of the translocating polypeptide chain can be present in the central pore, as has also been found for other hexameric AAA ATPases.

The mechanism by which translocation of a polypeptide chain through Cdc48 is initiated is unclear. One unresolved issue is how the polyubiquitin chain is recognized by the UN complex. The only well-characterized ubiquitin-binding site is in the UT3 domain of Ufd1 (ref. 23). How a polypeptide chain is moved into the central pore of Cdc48 is even less understood. A substrate segment must move through the D1 ring before the D2 ATPases can use their loop residues to grab the polypeptide and pull it through the pore. This requirement is particularly puzzling, because Cdc48 can act on a large variety of folded substrates. In contrast, initiation of translocation by the ATPase ring of the 19S subunit of the proteasome is much easier to understand. Here, the substrate needs a flexible polypeptide segment that inserts into the pore of the single ATPase ring and serves as the initiation site.
An understanding of the mechanism of Cdc48 requires structural information. To date, several structures of the ATPase itself are available3,4, but there is only limited information on the UN cofactor and its interaction with Cdc48. Previous EM structures show density for the cofactor near the N domains of the ATPase, but the resolution of the reconstructions is insufficient to derive molecular models23,24. Here, we report single-particle cryo-EM and crystal structures that clarify the interaction of the UN cofactor with the Cdc48 ATPase.

Results
Cryro-EM structures of the Cdc48 ATPase complex. We decided to use Cdc48 and the UN cofactor from the thermophilic fungus C. thermophilum, reasoning that the flexibility of protein segments might be limited compared with that of orthologs from mesophilic organisms. We first determined cryo-EM structures of Cdc48 alone. C. thermophilum Cdc48 was expressed in Escherichia coli and purified as a hexamer (Supplementary Fig. 1a,b). Structures of Cdc48 were determined in the presence of ADP or ATP-γS, and, after 3D classification and refinement, reached overall resolutions of 7.2 Å and 8.2 Å, respectively (Table 1 and Supplementary Figs. 2, 3, 4a and 5). As reported for mammalian p97 (ref. 3), both structures showed stacked D1 and D2 ATPase rings, and the best-refining classes had the N domains in the down conformation in the ADP-bound state and in the up conformation in the ATP-γS-bound state (Supplementary Figs. 2, 3 and 5a). Some classes in ATP-γS were in the down conformation, perhaps because of slow nucleotide hydrolysis. The conformational switch of the N domains is probably triggered by the change in the nucleotide state of the D1 ring3. The thermophilic Cdc48 protein thus recapitulates essential features of the mammalian p97 ATPase.

Next, we purified a complex of Cdc48 and UN. The UN complex from C. thermophilum was again expressed in E. coli and had the expected 1:1 stoichiometry after purification (Supplementary Fig. 1c). A complex of hexameric Cdc48 and UN (Supplementary Fig. 1d) was subjected to single-particle cryo-EM analysis in the presence of ADP or ATP-γS (Table 1 and Supplementary Figs. 4–7). The refined structures had overall resolutions of 6.7 Å and 4.3 Å with ADP and ATP-γS, respectively. The presence of the cofactor had only a small effect on the structure of the ATPase rings (Supplementary Figs. 5–7). However, even in the ADP-bound state, a sizable population of the Cdc48 molecules had their N domain in the up conformation, although the percentage was lower than that in the ATP-γS-bound state (~60% versus ~95%; Supplementary Figs. 6 and 7). Thus, ATP and cofactor binding together move the N domains into the up conformation, a state probably required for initiation of substrate processing17.

The most obvious density contributed by the cofactor complex is a central tower that lies above the D1 ATPase ring (Fig. 1b). In addition, some 3D classes show density close to one of the N domains a
result that can be attributed to the UBX-like domain of Npl4. This domain is known to bind a hydrophobic cleft on the N domain of Cdc48, and a previously determined structure of Npl4UBXL–p97N fits well into this region of our cryo-EM density map9 (Fig. 1c and 618). 

The Cdc48–UN complex was treated with a previously determined structure of Npl4UBXL–p97N. This result that can be attributed to the UBX-like domain of Npl4. This domain is known to bind a hydrophobic cleft on the N domain of Cdc48, and a previously determined structure of Npl4UBXL–p97N fits well into this region of our cryo-EM density map9 (Fig. 1c and 618). 

Identification of Npl4 domains in the density map. Although the density map of the Cdc48–cofactor complex permitted the visualization of helices, the resolution was insufficient to build a molecular model for the cofactor. We therefore first identified cofactor regions near the D1 ATPase ring. The Cdc48–UN complex was treated with bis[sulfosuccinimidyl] suberate (BS3), a bifunctional amine-reactive cross-linker. The sample was then digested with trypsin, and cross-linked peptides were identified by mass spectrometry. The data showed that the N terminus of Ufd1 interacts promiscuously with multiple locations in the ATPase and Npl4 (Supplementary Fig. 8a,b). Thus, these domains were the best candidates to form the base of the central tower. In agreement with the location of the cofactor density, no cross-links were discovered between the Cdc48 D2 domain and either Ufd1 or Npl4. Using limited proteolysis, we found that the zf-Npl4 and MPN domains of Npl4 cross-linked specifically to residues on the surface of the D1 ATPase ring (Supplementary Fig. 8a,b). Thus, these domains were the best candidates to form the base of the central tower. In agreement with the location of the cofactor density, no cross-links were discovered between the Cdc48 D2 domain and either Ufd1 or Npl4.

Fig. 2 | Interactions between Cdc48 and its cofactor. a, A crystal structure of Npl4 (light blue) and a homology model of Cdc48 (D1 in pink; D2 in yellow) were docked into the cryo-EM map. Unassigned density (orange) probably belongs to parts of the UT6 domain of Ufd1. b, Close-up view of the Npl4 crystal structure docked into the cryo-EM map. The MPN domain is yellow, and D1-interacting regions extending from it are highlighted: the two Zn²⁺ fingers (ZF; red), an N-terminal bundle (NTB; light green), and the β-strand finger (blue). c, Top view of D1-interacting regions, colored as in b, with the D1 ring shown as a white-gray surface. For clarity, D2 is omitted. ATPase subunits of the Cdc48 hexamer are numbered. d, Close-up views of the Zn²⁺ fingers, with Zn²⁺-coordinating residues in stick representation. The interacting FFF sequence in Cdc48 is highlighted.
Cdc48 binding. The bottom of the tower is formed by the zf-Npl4 domain, the central portion by the MPN domain, and the top portion by a C-terminal domain (CTD) of five α-helices (Fig. 2a,b). A small unassigned region of the central tower density probably corresponds to a segment of UT6 in Ufd1 (Fig. 2a). Indeed, hydrogen/deuterium-exchange mass spectrometry experiments showed that several Npl4 peptides in this region were protected when Ufd1 was present (Supplementary Fig. 8d,e). The Npl4-interacting region of UT6 is likely to be located between the two SHP motifs that anchor Ufd1 to the N domains of Cdc48 (ref. 25). The UT3 domain of Ufd1 might be fixed only when they bind to ubiquitinated substrate.

The MPN domain of Npl4 is anchored to the top of the D1 ATPase ring of Cdc48 via the preceding Zn$^{2+}$-finger domains, both of which are of the CHCC type, in which one histidine residue and three cysteine residues are used for coordination of the Zn$^{2+}$ ion (Fig. 2b–d). The Zn$^{2+}$ fingers form two ‘stalks’ that project into grooves between adjacent subunits of the Cdc48 D1 ring (Fig. 2b,c). When numbered from the position of the N-terminal Zn$^{2+}$ finger, the interacting grooves are between ATPase subunits 1 and 2 and between subunits 3 and 4 (Fig. 2c). A third stalk is formed by segments preceding the first Zn$^{2+}$ finger as well as residues located between the two Zn$^{2+}$ fingers. This N-terminal bundle makes only a few contacts with the surface of ATPase subunit 3 and is less conserved than the Zn$^{2+}$ fingers. Finally, a fourth stalk is formed by two β-strands with a loop at their tip. This loop projects over the axial pore and faces ATPase subunit 6 (Fig. 2c), but it makes no clear contact with the D1 ring. The use of several contact sites precludes the binding of a second cofactor molecule (Supplementary Fig. 9), thus explaining why one Cdc48 hexamer binds only one UN heterodimer.

**Functional tests of Npl4 segments.** To test the functional role of the Zn$^{2+}$ fingers, we used *Saccharomyces cerevisiae* Cdc48, Npl4, and Ufd1 in an in vitro unfolding assay. A fusion between a short degron and the fluorescent protein mEos3.2 was polyubiquitinated and incubated with the ATPase complex; the loss of fluorescence is an indication of Eos unfolding17. The results showed that mutation of the central histidine and cysteine residues in either of the individual Zn$^{2+}$-finger domains had little effect on unfolding, but a defect was observed when both domains were mutated together (Fig. 3a). Similarly, mutants in individual Zn$^{2+}$ fingers were able to rescue the temperature-sensitive growth phenotype of an npl4-1 yeast strain, but a mutant in both Zn$^{2+}$ fingers was not able to do so (Fig. 3b). We also tested mutations in the Cdc48 ATPase in the unfolding assay. Both Zn$^{2+}$-finger domains are close to a conserved triphenylalanine (FFF) sequence in the D1 domain.
Indeed, mutation of the first or third phenyalanine decreased the
unfolding activity of Cdc48 without affecting hexamer formation
(Fig. 3c and Supplementary Fig. 10a). Mutation of the central
phenylalanine in the FFF motif abolished unfolding completely
but also decreased hexamerization, a result consistent with this
residue facing a hydrophobic pocket in the ATPase domain. The
importance of the Zn²⁺ fingers of Npl4 is supported by a recent
report in which these domains have been identified as a target
of the drug disulfiram⁵. The role of the ‘β-strand finger’ of Npl4
remains unclear. Although there is a highly conserved tyrosine
residue at the tip of the β-strand finger, its mutation or deletion
did not alter unfoldase activity in vitro or affect the ability to com-
plement the cleft of the MPN domain with ubiquitin, as has been observed for AMSH and several
other MPN family members. Given its similarity with Rpn11 and
AMSH, the cleft in Npl4’s MPN domain is also likely to accom-
modate the C-terminal tail of a ubiquitin molecule, but in our
structure, the groove is covered by a segment of UT6 of Ufd1, thus
suggesting that the UT6–MPN interaction may be broken to allow
ubiquitin binding.

Ubiquitin binding by Npl4. To test whether the cleft of the MPN
domain has a role in ubiquitin binding, we incubated a polyubiqui-
tinated substrate with a streptavidin-binding peptide (SBP)-tagged
version of the Npl4 construct used for crystallization. In agreement
with previous results, this construct indeed bound polyubiqui-
tin, as shown after pulldown with streptavidin beads (Fig. 3d,e). However, mutagenesis showed that the cleft of the MPN domain
does not substantially contribute to the interaction. Although this
result is at variance with findings from a previous report, perhaps
because the bait in the previous experiments was destabilized by
its fusion with glutathione S-transferase, it is consistent with only
a few residues in the MPN cleft of Rpn11 contacting ubiquitin. Most of the affinity for ubiquitin may be provided by the inter-
face between the MPN and CTD domains rather than by the cleft,
because neither domain alone was active (Fig. 3d). Alternatively,
both domains might have weak affinity, and the combination may
be required for avid substrate recognition. Although the elucidation
of the exact binding mode of polyubiquitin to the UN complex
requires a structure of the Cdc48 complex with a ubiquitin chain of
defined length, it is clear that the UN cofactor has ubiquitin-binding
sites in both Ufd1 and Npl4, similarly to the multiple receptors
present in the proteasome⁶⁻⁸.

The MPN domains of Rpn11 and AMSH-LP accommodate the
C-terminal tail of ubiquitin in a cleft between insert 1 and helix 2,
thus positioning the C terminus of ubiquitin next to the active
site (Fig. 4c). Insert 1 of Npl4 closely resembles the structure
assumed by the corresponding region of Rpn11 in the presence
of ubiquitin, but it adopts this conformation even in the
absence of ubiquitin, as has been observed for AMSH and several
other MPN family members. Given its similarity with Rpn11 and
AMSH, the cleft in Npl4’s MPN domain is also likely to accom-
modate the C-terminal tail of a ubiquitin molecule, but in our
structure, the groove is covered by a segment of UT6 of Ufd1, thus
suggesting that the UT6–MPN interaction may be broken to allow
ubiquitin binding.

Comparison with other MPN domains. Like other members of
the MPN family, the Npl4 MPN domain consists of a core MPN fold
with two inserts (inserts 1 and 2) (Fig. 4a). Npl4 is enzymatically inactive, because it lacks the Zn²⁺-binding motif in the core, which
is essential for the hydrolytic activity of other MPN domains. The
position of the Npl4 MPN domain above the D1 ATPase ring
(Fig. 2a–c) is similar to that of the MPN domain in the 19S regulatory
particle of the proteasome. In the case of Rpn11, an enzymati-
cally active MPN domain is located over a ring of six homologous
ATPase subunits. The Rpn11 MPN domain dimerizes with the
enzymatically inactive MPN domain of Rpn8. The Npl4 MPN
domain, however, is a monomer, with its helical CTD occupying the
site that mediates dimerization in Rpn11 (Fig. 4b).

Table 2 | Data collection and refinement statistics
zf-Npl4-MPN-CTD (PDB 6CDD)

| Data collection |        |
|-----------------|--------|
| Space group     | P121   |
| Cell dimensions |        |
| α, β, γ (Å)     | 58.858, 72.221, 193.543 |
| α, β, γ (°)     | 90, 96.714, 90 |
| Resolution (Å)  | 96.11–2.582 (2.675–2.582) |
| Rmerge          | 0.06434 (2.031) |
| I/αI            | 9.82 (0.49) |
| Completeness (%)| 95.15 (77.79) |
| Redundancy (%)  | 2.9 (2.7) |
| Refinement      |        |
| Resolution (Å)  | 2.582 |
| No. reflections | 94,503 (7,751) |
| Rmerge/Rmerge   | 0.1918 / 0.2287 |
| No. atoms       | 7,515 |
| Protein         | 7,283 |
| Ligand/ion      | 4 |
| Water           | 228 |
| B factors       | 96.95 |
| Protein         | 97.44 |
| Ligand/ion      | 120.87 |
| Water           | 80.87 |
| R.m.s. deviations |        |
| Bond lengths (Å)| 0.002 |
| Bond angles (°) | 0.53 |

Data were collected from a single crystal. Highest-resolution shell is in parentheses.

Fig. 4 | MPN domain of Npl4. a, Crystal structure of the MPN and CTD
domains of Npl4. The core MPN region is tan, the insert 1 (Ins-1) region
is magenta, the insert 2 (Ins-2) region is purple, and the CTD is orange.
The dashed line indicates a nine-residue acidic loop unresolved in the
crystal structure. b, As in a, but with the Rpn11–Rpn8 structure overlaid
(PDB 5U4P). Rpn11 is green, and Rpn8 is gray. c, As in a, with the Rpn11
MPN (green) and its associated ubiquitin (cyan) overlaid (PDB 5U4P).
The conserved tyrosine at the tip of insert 2 is shown in stick
representation in red. H2, MPN helix 2; Ub, ubiquitin.
but it would allow for the accommodation of the C terminus of this ubiquitin molecule in a similar manner as seen in Rpn11 of the proteasome. However, in the Cdc48 complex, the putative ubiquitin-binding groove and central ATPase pore are at an approximately right angle, whereas they are more closely aligned in the proteasome (Fig. 5c). Furthermore, the catalytic site of Rpn11 is located immediately above the pore in the substrate-engaged proteasome, whereas in the Cdc48–UN complex, there is an ~40 Å gap between the putative C terminus of ubiquitin and the pore (Fig. 5c). The presence of this gap raises the question of how substrates might initially be inserted into the ATPase. One possibility is that the gap is bridged by an additional ubiquitin moiety proximal to the one bound to the MPN cleft. In this case, Cdc48 might begin translocation not on a segment of the substrate but instead on a segment of this proximal ubiquitin molecule. This model is attractive in that ubiquitin could serve as a universal initiating signal for translocation into the pore, regardless of the substrate to which it is attached. This mechanism would eliminate the need for a flexible region to mediate pore entry and explain why Cdc48, unlike the proteasome, has no requirement for a preunfolded segment. Although the details of pore insertion remain to be clarified, ideally with a structure of a substrate-associated Cdc48 complex, our data show that Npl4 is unique among the known Cdc48 cofactors in that it binds directly to the ATPase ring and probably serves as a universal gatekeeper for all Cdc48-dependent reactions that require translocation through the central pore.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-018-0085-x.

Received: 2 March 2018; Accepted: 31 May 2018; Published online: 2 July 2018

References
1. Stach, L. & Freemont, P. S. The AAA+ATPase p97, a cellular multitool. *Biochem. J.* **474**, 2953–2976 (2017).
2. Zhang, X. et al. Structure of the AAA ATPase p97. *Mol. Cell* **6**, 1473–1484 (2000).
3. Tang, W. K. et al. A novel ATP-dependent conformation in p97 N-D1 fragment revealed by crystal structures of disease-related mutants. *EMBO J.* **29**, 2217–2229 (2010).
4. Banerjee, S. et al. 2.3Å resolution cryo-EM structure of human p97 and mechanism of allosteric inhibition. *Science* **351**, 871–875 (2016).
5. Nuzhmann, P. & Schindelin, H. The interplay of cofactor interactions and post-translational modifications in the regulation of the AAA+ATPase p97. *Front. Mol. Biosci.* **4**, 21 (2017).
6. Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D. & Warren, G. A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J.* **19**, 2181–2192 (2000).
7. Ye, Y., Meyer, H. H. & Rapoport, T. A. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414**, 652–656 (2001).
8. Skrotz, Z. et al. Alcohol-abuse drug disulfiram targets cancer via p97 segregase adaptor NPL4. *Nature* **552**, 194–199 (2017).
9. Isaacson, R. L. et al. Detailed structural insights into the p97-Npl4-Ufd1 interface. *J. Biol. Chem.* **282**, 21361–21369 (2007).
10. McCullough, J., Cлагаue, M. J. & Urbé, S. AMSH is an endosome-associated ubiquitin isopeptidase. *J. Cell. Biol.* **166**, 487–492 (2004).
11. Cope, G. A. et al. Role of predicted metalloprotease motif of Jab1/Can5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608–611 (2002).
12. Maytal-Kiviy, Y., Reis, N., Hofmann, K. & Glickman, M. H. MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem.* **3**, 28 (2002).
13. Alam, S. L. et al. Ubiquitin interactions of N2P zinc fingers. *EMBO J.* **23**, 1411–1421 (2004).
14. Hänselmann, P. & Schindelin, H. Characterization of an additional binding surface on the p97 N-terminal domain involved in bipartite cofactor interactions. *Structure* **24**, 140–147 (2016).
15. Hetzer, M. et al. Distinct AAA-ATPase p97 complexes function in discrete steps of nuclear assembly. *Nat. Cell Biol.* **3**, 1086–1091 (2001).
16. Heo, J.-M. et al. A stress-responsive system for mitochondrial protein degradation. *Mol. Cell* 40, 465–480 (2010).

17. Bodnar, N. O. & Rapoport, T. A. Molecular mechanism of substrate processing by the Cdc48 ATPase complex. *Cell* 169, 722–735.e9 (2017).

18. Lee, C., Prakash, S. & Matouschek, A. Concurrent translocation of multiple polyubiquitin chains through the proteasomal degradation channel. *J. Biol. Chem.* 277, 34760–34765 (2002).

19. Burton, R. E., Siddiqui, S. M., Kim, Y. I., Baker, T. A. & Sauer, R. T. Effects of protein stability and structure on substrate processing by the ClpXP unfolding and degradation machine. *EMBO J.* 20, 3092–3100 (2001).

20. Park, S., Isaacson, R., Kim, H. T., Silver, P. A. & Wagner, G. Ufd1 exhibits the AAA-ATPase fold with two distinct ubiquitin interaction sites. *Structure* 13, 995–1005 (2005).

21. DelaBarre, B., Christianson, J. C., Kopito, R. R. & Brunger, A. T. Central pore residues mediate the p97/VCP activity required for ERAD. *Mol. Cell* 22, 451–462 (2006).

22. Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E. & Matouschek, A. An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat. Struct. Mol. Biol.* 11, 830–837 (2004).

23. Pye, V. E. et al. Structural insights into the p97–Ufd1–Npl4 complex. *Proc. Natl Acad. Sci. U. S. A.* 104, 467–472 (2007).

24. Bebeacua, C. et al. Distinct conformations of the protein complex p97–Ufd1–Npl4 revealed by electron cryomicroscopy. *Proc. Natl Acad. Sci. U. S. A.* 109, 1098–1103 (2012).

25. Bruderer, R. M., Busse, C. & Meyer, H. H. The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *J. Biol. Chem.* 279, 49609–49616 (2004).

26. Blok, N. B. et al. Unique double-ring structure of the peroxisomal Pex1/Pex6 ATPase complex revealed by cryo-electron microscopy. *Proc. Natl Acad. Sci. U. S. A.* 112, E4017–E4025 (2015).

27. Ambroggio, X. L., Rees, D. C. & Deshaies, R. J. JAMM: a metalloprotease-like zinc site in the proteasome and signalosome. *PLoS Biol.* 2, E2 (2004).

28. Lander, G. C. et al. Complete subunit architecture of the proteasome regulatory particle. *Nature* 482, 186–191 (2012).

29. Woerdon, E. J., Dong, K. C. & Martin, A. An AAA motor-driven mechanical switch in Rpn11 controls deubiquitination at the 26S proteasome. *Mol. Cell* 67, 799–811.e8 (2017).

30. Sato, Y. et al. Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* 455, 358–362 (2008).

31. Davies, C. W., Paul, L. N., Kim, M.-I. & Das, C. Structural and thermodynamic comparison of the catalytic domain of AMSH and AMSH-LP: nearly identical fold but different stability. *Mol. Cell* 413, 416–429 (2011).

32. Tsuchiya, H. et al. In vivo ubiquitin linkage-type analysis reveals that the Cdc48-Rad23/Dsk2 Axis contributes to K48-linked chain specificity of the proteasome. *Mol. Cell* 66, 488–502.e7 (2017).

33. Woerdon, E. J., Padovani, C. & Martin, A. Structure of the Rpn11–Rpn8 dimer reveals mechanisms of substrate deubiquitination during proteasomal degradation. *Nat. Struct. Mol. Biol.* 21, 220–227 (2014).

34. Shi, Y. et al. Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. *Science* 351, aad9421 (2016).

35. Matsukiela, M. E., Lander, G. C. & Martin, A. Conformational switching of the 26S proteasome enables substrate degradation. *Nat. Struct. Mol. Biol.* 20, 781–788 (2013).

36. Chen, S. et al. Structural basis for dynamic regulation of the human 26S proteasome. *Proc. Natl Acad. Sci. U. S. A.* 113, 12991–12996 (2016).

Acknowledgements
We thank X. Wu and L. Li for assistance with crystallography, D. Finley for critical reading of the manuscript, the SBGrid consortium at Harvard Medical School, and the IC CB Longwood for use of equipment. We thank M. Ebrahim and J. Sotiris at the Rockefeller University Evelyn Gross Lipper Cryo-Electron Microscopy Resource Center for assistance with microscope operation. This work is based on research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the NIH/NIGMS (P41 GM103403). The Pilatus 6 M detector on the 24-ID-C beamline is funded by an NIH ORIP HEI grant (S10 RR029205). This research used resources of the Advanced Photon Source, a US Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under contract no. DE-AC02-06CH11357. Z.J. is supported as a Howard Hughes Medical Institute Fellow of the Damon Runyon Cancer Research Foundation, DRG-2315-18. This research was supported in part by a Helmsley Postdoctoral Fellowship at the Rockefeller University (to K.H.K.), funding from the Blavatnik Family Foundation (to E.N.), a research collaboration with the Waters Corporation (J.R.E.), and NIGMS grants R01 GM052586 (to T.A.R.) and T32 GM007753 (Harvard/MIT Medical Scientist Training Program). E.N. and T.A.R. are supported as Howard Hughes Medical Institute investigators. We thank P. Carvalho (Oxford University, UK) for providing reagents.

Author contributions
N.O.B. purified proteins, solved the crystal structure, and performed biochemical experiments. K.H.K. performed cryo-EM data collection and analysis. N.O.B. and K.H.K. contributed equally and appear in alphabetical order in the author list. Z.J. purified and tested Cdc48 FFF mutants and performed biochemical experiments. T.E.W. performed hydrogen/deuterium-exchange experiments with supervision from J.R.E. V.S. performed cross-linking mass spectrometry analysis with supervision from E.N. T.W. oversaw the cryo-EM experiments. N.O.B. and T.A.R. wrote the manuscript. T.A.R. supervised the project.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41594-018-0085-x.

Reprints permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to T.W. or T.A.R.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Methods

Protein expression and purification. Full-length C. thermophilum and S. cerevisiae Cdc48, Ufd1, and Npl4, as well as the Npl4 Zn finger–MPN–CTD fragment (residues 129–602) and its tagged versions, were overexpressed in E. coli BL21 DE3 (induction strain) and purified, respectively. Cdc48, Ufd1, and the Zn finger–MPN–CTD fragment were expressed with N-terminal His6 SUMO tags from the K27SUMO vector. Full-length Npl4 was expressed untagged from the pET21b vector. Buffers used for Cdc48 purifications contained 5 mM MgCl2 throughout.

Buffers used for Cdc48 purifications contained 5 mM MgCl2 throughout. For Cdc48 in complex with the UN cofactor in the presence of ADP or ATP-γS, the image stacks were collected for Cdc48–cofactor (ADP) and Cdc48–cofactor (ATP-γS), respectively. For analysis of Cdc48 alone, in the presence of ADP or ATP-γS, cryo-EM data collection was carried out on 2.951 images in boxes of 256 × 256 pixels with a dose rate of eight electrons per pixel per second. A total of 1,628 and 569 particles were subjected to reference-free 2D classification. The classes that showed strong cofactor density (classes 2, 5, and 6; 82,249 particles; ~58% of the dataset) were combined and subjected to 3D classification into ten classes. The resulting classes showed that the Cdc48–cofactor (ADP) complex exhibited excellent structural variability, most notably in the N domain and the cofactor region. Classes 2, 8, 9, and 10, showing the strongest cofactor density and relatively well-ordered N domains, were combined, and the resulting 52,178 particles were subjected to 3D refinement in RELION, thus yielding a map at 6.7-Å resolution.

For the Cdc48–cofactor (ATP-γS) complex, the 808,059 autopicked particles were extracted from 2,531 images in boxes of 256 × 256 pixels with a dose rate of ten electrons per pixel per second. A total of 141,422 particles were aligned to the Cdc48 (ADP) cryo-EM map (obtained as described above and filtered to 40 Å) with RELION 3D classification with the number of classes set to one. The resulting map showed strong density not present in the reference map. With this new map as a reference, the particles were sorted into six classes through 3D classification. The classes that showed strong cofactor density (classes 2, 5, and 6; 82,249 particles; ~58% of the dataset) were combined and subjected to 3D classification into ten classes. The resulting classes showed that the Cdc48–cofactor (ADP) complex exhibited excellent structural variability, most notably in the N domain and the cofactor region. Classes 2, 8, 9, and 10, showing the strongest cofactor density and relatively well-ordered N domains, were combined, and the resulting 52,178 particles were subjected to 3D refinement in RELION, thus yielding a map at 6.7-Å resolution.

Model building and refinement for the Cdc48–cofactor (ATP-γS) structure. The structure of human p97 bound to ATP-γS (PDB 3ETN) was used to build a homology model of C. thermophilum Cdc48 with SWISS-MODEL. The homology model of Cdc48 and the crystal structure of the Zn finger–MPN–CTD domains of Npl4 were docked into the cryo-EM density map with UCSF Chimera. The atomic model was optimized through cycles of real-space refinement with phenix. real_space_refine against half-map 1 from RELION-2 and manual rebuilding in COOT. The electron density maps and final models were deposited in the Protein Data Bank (PDB 7D7V). The atomic model was fitted against an initial density map from RELION-2, resulting in a final model with an R-factor of 0.143 (thus reducing model bias), what is the current state of the art of cryo-EM structure determination. The statistics from the data collection are detailed in Table 1.

Protease-protection experiments. Purified C. thermophilum UN was treated with increasing concentrations of trypsin (0, 2.4, 7.3, 22, and 66 μg/ml), incubated at room temperature for 30 min, and subjected to SDS–PAGE and Coomassie blue staining. Bands were subjected to mass spectrometry.
bicarbonate, reduced with 50 mM TCEP at 60 °C for 10 min, and alkylated with 0.5 μl 0.1% formic acid for MS analysis. Data were acquired over an m/z range of 50–20,000, and mass accuracy was confirmed by calibration with 500 fmol/μl of human glu-fibrinopeptide. Peptic peptides were identified with ProteinLynx Global Server (PLGS). A composite omit map with simulated annealing was generated from a single crystal at 100 K. Data were processed with XDS54 and the CCP4i and Phenix crystallographic programs Chimera, Phaser, RESOLVE, and the CCP4i and Phenix crystallographic software. A composite omit map with simulated annealing was generated with Chimera. Figures were generated with UCSF Chimera2 and PyMOL (http://www.py.mol.org/). Crystallographic software was maintained by SGRGrid. Data collection and refinement statistics are summarized in Table 2.

LC–MS/MS and cross-link mapping. Buffer components for cross-linking were 20 mM Tris (pH 7.4, Aldrich). LC–MS/MS was carried out with Thermo Scientific LC–MS-grade reagents and solvents. The cross-linker BS3 was purchased from Thermo Scientific.

- Purified protein complexes (0.5 mg/ml in 50 mM HEPES, pH 7.5, 200 mM NaCl, 5 mM MgCl₂, 0.5 mM TCEP, and 0.1 mM ATP–yS) were cross-linked with 100, 200, or 400 μM of BS3 (3.5 mM stock in water) for 30 min at room temperature. Digestion was carried out at 37 °C overnight with 0.5 μg sequencing-grade modified trypsin (Promega). An N-terminal arginine is variable modification at 10°C. Trypsin digestion with up to three missed cleavages, constant modification at 1 carbamidomethyl [C], and variable modification at 1 oxidation [M]. The cross-linker was set to BS3 (K [K 138.068 138.068 156.079 156.079]). Mass tolerances for fragments and precursors were left unaltered. mgf files were searched against a database comprising Fasta sequences of Cdc48, Ufd1, and Npl4. Cross-linking/MS data are in Supplementary Dataset 2.

- The Zn²⁺–Npl4 residues 129–602 or the full-length UN complex solutions of Chlamydomonas reinhardtii Npl4 was incubated with streptavidin agrose beads ( Pierce) in binding buffer (50 mM Tris, pH 8.0, and 150 mM NaCl) for 30 min at room temperature. The beads were washed three times with binding buffer to remove excess bait protein. Next, D LyLight 800–labeled, polyubiquitinated superfolder GFP, generated as described for the Eos substrate above and purified by gel filtration, was incubated with the beads for 0.5 h at a concentration of 50 nM in 100 μl binding buffer. The beads were again washed three times. Round material was eluted with binding buffer plus 1 mM bicine and subjected to SDS–PAGE and fluorescence scanning on an Odyssey CLX infrared scanner (Lico) followed by Coomassie blue staining.

- Yeast strains. The npl4–1 strain (Mat a npl4–1 ura3–52 leu2Δ1 trp1Δ63) was transformed with plasmids derived from pPS402 (gift from P. Carvalho, originally generated by the laboratory of P. Silver). The original plasmid encodes wild-type Npl4 under its endogenous promoter and includes a Ura3 cassette. Initial cultures were grown at room temperature, because the npl4–1 strain grows poorly at 30 °C. Yeast were spotted in tetrathionate dilution on SD–Ura plates and incubated at room temperature, 30 °C, or 37 °C for 2–3 d.

- Data availability. The atomic coordinates and structure factors for zf–Npl4–MAN–CTD have been deposited in the Protein Data Bank (PDB 5C48, ADP). The original plasmid encodes wild-type Npl4 under its endogenous promoter and includes a Ura3 cassette. Initial cultures were grown at room temperature, because the npl4–1 strain grows poorly at 30 °C. Yeast were spotted in tetrathionate dilution on SD–Ura plates and incubated at room temperature, 30 °C, or 37 °C for 2–3 d.

- Yeast experiments. The npl4–1 strain (Mat a npl4–1 ura3–52 leu2Δ1 trp1Δ63) was transformed with plasmids derived from pPS402 (gift from P. Carvalho, originally generated by the laboratory of P. Silver). The original plasmid encodes wild-type Npl4 under its endogenous promoter and includes a Ura3 cassette. Initial cultures were grown at room temperature, because the npl4–1 strain grows poorly at 30 °C. Yeast were spotted in tetrathionate dilution on SD–Ura plates and incubated at room temperature, 30 °C, or 37 °C for 2–3 d.

- Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.
Experimental design

1. Sample size
   Describe how sample size was determined.
   For cryo-EM, particles were collected and data processed until maps at the given resolutions were obtained. There were no predetermined resolution or particle number cutoffs. For crystallography, a single crystal was sufficient to obtain a dataset for structure solution. For unfolding assays, three technical replicates were considered to be representative of the experimental variation.

2. Data exclusions
   Describe any data exclusions.
   For cryo-EM data, images were manually inspected and rejected if considered of inadequate quality for further image processing (e.g., ice contamination, blurriness, bad CTF fit, etc.) During refinement, 2D and 3D classes showing poor averages were removed as described in Methods.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.
   All attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   The experiments in this study did not require randomization, as there were no groups subjected to differing treatments.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The experiments in this study did not require blinding to group allocation, as there were no groups subjected to differing treatments.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- **n/a** Confirmed
- [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [x] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [x] A statement indicating how many times each experiment was replicated
- [x] The statistical test(s) used and whether they are one- or two-sided
  - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- [x] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [x] Test values indicating whether an effect is present
  - *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
- [x] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [x] Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

### Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Rock Maker (Formulatrix, USA); Rock Imager (Formulatrix, USA); XDS (http://xds.mpimf-heidelberg.mpg.de/); Aimless (http://www.mrc-lmb.cam.ac.uk/harry/pre/aimless.html); CCP4i (http://www.ccp4.ac.uk/ccp4i-main.php); Phenix (http://www.phenix-online.org/); Coot (http://www.crgi-lmb.cam.ac.uk/personal/pemsley/); SBGrid (http://sbgrid.org/); UCSF Chimera (http://www.cgl.ucsf.edu/chimera/); CTFFIND4 (http://grigoriefflab.janelia.org/ctffind4); EMAN2 (http://blake.bcm.edu/emanwiki/EMAN2); SPARX (http://sparx-em.org/sparx/wiki/); Gautomatch (www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/); RELION and RELION2 (http://www2.mrc-lmb.cam.ac.uk/relion/index.php); Cryosparc (http://cryosparc.com/); SoftMax Pro (http://www.moleculardevices.com/systems/microplate-readers/softmax-pro-7-software); Image Studio (http://www.licor.com/bio/products/software/image_studio/); Proteome Discoverer (http://www.thermofisher.com/order/catalog/product/OPTON-30795); pLink (http://pfind.ict.ac.cn/software/pLink/); ProteinLynx Global Server (http://www.waters.com/waters/en_US/ProteinLynx-Global-SERVER-(PLGS)/nav.htm?cid=513821); DynamX 3.0 (Waters).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

### Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

- No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- No antibodies were used.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      The npl4-1 yeast strain was a gift of Dr. Pedro Carvalho.
   b. Describe the method of cell line authentication used.
      The strain was validated by complementation of the temperature sensitive phenotype with a plasmid encoding wild-type Npl4.
   c. Report whether the cell lines were tested for mycoplasma contamination.
      The strain was not tested for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
      No commonly misidentified cell lines were used.

› Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
    Provide all relevant details on animals and/or animal-derived materials used in the study.
    No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    The study did not involve human research participants.