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Cdc48 (p97): a 'molecular gearbox' in the ubiquitin pathway?

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Cdc48 (p97), a conserved chaperone-like ATPase of eukaryotic cells, has attracted attention recently because of its wide range of cellular functions. Cdc48 is intimately linked to the ubiquitin pathway because its primary action is to segregate ubiquitinated substrates from unmodified partners. This 'segregase' activity is crucial for certain proteasomal degradation pathways and for some nonproteolytic functions of ubiquitin. Cdc48 associates not only with different 'substrate-recruiting cofactors' but also with distinct 'substrate-processing cofactors'. The latter proteins control the degree of ubiquitination of bound substrates by shifting the polyubiquitination reaction into 'forward', 'neutral' or 'reverse'. We discuss how Cdc48 might use this 'gearbox activity' to control protein fate and propose a similar mode of action for the 19S cap of the proteasome.

Introduction

The conserved homohexameric ring-shaped AAA (ATPase associated with various activities) ATPase, called Cdc48 in budding yeast and p97 (the unfavorable name VCP, for valosin-containing protein was given after an artefact) in mammals, is a central component of the ubiquitin system. As the yeast name indicates, Cdc48 was initially identified as a protein required for progression through the cell division cycle. Studies in yeast also provided the first link between Cdc48 and the ubiquitin pathway, with the finding that the enzyme is required for the degradation of some artificial model substrates (linear ubiquitin–protein fusions) [1]. Later work showed that Cdc48 (p97) is involved in ubiquitin-dependent activation of certain transcription factors [2–4], the degradation of proteins of the endoplasmic reticulum (ER) by the ER-associated degradation (ERAD) pathway [5–9], and the control of membrane fusion [10–14]. Whether Cdc48 (p97) also functions outside the ubiquitin system is unknown but seems unlikely. Most if not all of the known Cdc48 (p97)-dependent functions seem to be directly linked to the ability of the protein to bind to (oligo)ubiquitinated proteins and to segregate them from their binding partners, or to extract them from protein complexes [3,15]. This 'segregase' function is mediated by the Cdc48 (p97) ATPase activity, which translates ATP hydrolysis into mechanical forces that move and partially rotate the outside rim of the ring-shaped enzyme [16]. Cdc48 (p97) possesses two consecutive AAA ATPase domains (called D1 and D2) and an N-terminal domain (N-domain). How Cdc48 (p97) associates with substrates is an area of active research, and two possible mechanisms have been found. First, it might bind to ubiquitinated substrates directly by its N-domain, as indicated by *in vitro* binding studies [3,17]. Second, it might bind to ubiquitinated substrates indirectly through cofactors [3,15,18–20]. Indeed, the second mechanism might be more common, because numerous putative Cdc48 (p97) 'substrate-recruiting cofactors' have been identified recently, which possess ubiquitin-binding domains and usually interact with Cdc48 (p97) by its N-domain (Box 1 and Table 1).

Remarkably, Cdc48 (p97) functions not only as a segregase; recent findings indicate that it also controls the degree of ubiquitination of the bound substrates [18,21]. This activity is brought about by so-called 'substrate-processing cofactors' of Cdc48 (p97) that either promote polyubiquitination, inhibit polyubiquitination or even deubiquitinate the bound (oligo)ubiquitinated substrate [13,18,21–24] (Box 1 and Table 1).

The diverse functions, structure and mechanistic details of the Cdc48 (p97) enzyme have been excellently reviewed previously [25–27]. Instead, here we discuss when and how Cdc48 (p97) is employed in ubiquitin-dependent pathways. Also, for reasons of simplicity, we will focus mainly on the yeast proteins and processes involved, although the principles discussed apply equally to the metazoan p97 protein (unless otherwise indicated). Specifically, we propose that Cdc48 (p97) might function similarly to a gearbox in a car and might control protein fate. We will speculate about the potential usefulness of such a 'gearbox' activity within the ubiquitin pathway and argue that it might be crucial for shifting the system from nonproteolytic to proteolytic functions of the ubiquitin system. Finally, we speculate that the 19S cap of the proteasome might have a similar mode of action.

Diverse functions reveal a common principle

Most of our current knowledge of the function of Cdc48 (p97) and its cofactors derives from studies of three different cellular pathways: the OLE pathway (see later), ERAD and the pathway for membrane fusion. Notably, several of the components involved in these pathways have been initially identified by genetic and biochemical dissection of the so-called UFD pathway ('ubiquitin-fusion degradation') that mediates the degradation of short-lived synthetic linear ubiquitin-fusion proteins [1,23,28]. Among these 'UFD proteins' are Cdc48 itself, the substrate-recruiting cofactors

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Box 1. Cofactors of Cdc48 (p97)

Cofactors of Cdc48 (p97) can be grouped into two classes (see Table 1 in main text).

Substrate-recruiting cofactors

The most prevalent substrate-recruiting cofactors are apparently the heterodimeric Ufd1–Npl4 and the protein Shp1 (p47). Substrate-recruiting cofactors possess specific ubiquitin-binding domains, for example an N-domain in Ufd1, which, interestingly, resembles the N-domain of Cdc48 (p97) [51], or a UBA (ubiquitin-associated) domain in Shp1 (p47). Ufd1–Npl4 and Shp1 (p47) form mutually exclusive complexes with Cdc48 (p97) [11,15]. Shp1 (also called Ubx1) is the founding member of the so-called Ubx proteins [52]. These proteins (including the human stress-activated kinase substrate SAKS [53]) are characterized by the presence of a so-called UBX domain, which has a three-dimensional structure similar to ubiquitin although it lacks discernible sequence similarity. Some Ubx proteins bind to ubiquitin–protein conjugates by special domains (often UBAs). Substrate-recruiting cofactors typically associate with Cdc48 (p97) by the N-domain of the AAA ATPase (see Figure 1 in main text). Not all substrate-recruiting cofactors associate with Cdc48 (p97) in a mutually exclusive manner; for example, Cdc48 can bind to Ufd1–Npl4 and Ubx2 simultaneously [35].

Substrate-processing cofactors

Some substrate-processing cofactors directly influence the degree of ubiquitination of the bound substrate, whereas others modify the bound substrates in another way. Ufd2, a U-box-containing polyubiquitination enzyme, adds further ubiquitin molecules to a Cdc48-bound mono- (oligo-)ubiquitinated substrate [23]. Interestingly, Ufd2 seems to switch the type of ubiquitin–ubiquitin linkage of the polyubiquitin chain from Lys29 to Lys48, perhaps to make the ubiquitination reaction processive [23,54]. Ufd2 does not bind to the N-domain but to the second AAA domain (D2) of Cdc48 [18,21], thereby enabling the formation of a Cdc48 complex that contains both Ufd1–Npl4 and Ufd2. Ufd3 (also known as Doa1), a WD-40 repeat-containing protein that associates with ubiquitin through a PFU (PLAA family ubiquitin-binding) domain, binds to the same D2 region and thereby blocks Ufd2-catalyzed polyubiquitination [21,55]. Ubiquitination of bound substrates is further antagonized by the deubiquitination enzymes (DUBs) of the OTU (ovarian tumor) family [12,13,21]. Notably, these proteins (Otu1 in yeast and VCIP135 in vertebrates) bind by their UBX or UBX-related UBD domains to the N-domain of Cdc48 (p97) [56] without preventing a simultaneous association of Cdc48 (p97) with the substrate-recruiting cofactors p47 (in the case of VCIP135) and Ufd1–Npl4 or Shp1 (in the case of Otu1), respectively. This arrangement also enables cooperation between Otu1 and Ufd3 in antagonizing substrate ubiquitination. Ataxin-3 (which is subject to polyglutamine expansion) is another deubiquitination enzyme and cofactor of p97 [24,57,58]. Two other Cdc48 (p97) substrate-processing cofactors do not alter the ubiquitination state of the bound substrates: peptide *N*-glycanase (PNGase) removes oligosaccharides from ERAD substrates before proteasomal degradation [59]; HDAC6, a deacetylase related to histone deacetylases, associates with ubiquitin–protein conjugates strongly and might thus also be a negative regulator of degradation [60].

Ufd1 and Npl4, and the substrate-processing cofactors Ufd2 and Ufd3 (Table 1). In the following we briefly review these pathways, mainly to compare them, and also to point out their common features.

The OLE pathway

The OLE pathway controls the synthesis of unsaturated fatty acids in yeast [4]. The key enzyme of this pathway is the Δ9-fatty acid desaturase Ole1, an integral membrane-bound protein of the ER. Ole1 provides yeast cells with unsaturated fatty acids, which are crucial for membrane fluidity and essential for viability. Transcription of *OLE1* is driven by Spt23 (and its homolog Mga2), a distant relative of the mammalian transcription factor NFκB (p50) [4,29]. Spt23 is synthesized as an inactive precursor (called p120), which is anchored to the ER by a single transmembrane span [4]. When there is a shortage of unsaturated fatty acids, Spt23 p120 homodimerizes, and one molecule of the dimer (possibly by a stochastic mechanism) becomes mono- (or oligo-)ubiquitinated [3,4]. This mono- (or oligo-)ubiquitinated p120 molecule is then processed by the proteasome to remove the transmembrane domain. Importantly, the N-terminal domain of Spt23 that is spared from degradation (the active transcription factor termed Spt23 p90) will remain bound to its unmodified p120 partner molecule until the complex is disassembled by Cdc48. This segregase reaction requires ATP and Cdc48 together with its heterodimeric substrate-recruiting cofactor Ufd1–Npl4 (i.e. the complex Cdc48–Ufd1–Npl4) [3]. Once mobilized away from the ER, Spt23 p90 can enter the nucleus and activate *OLE1* transcription. In the nucleus, p90 is degraded, most probably after it has initiated *OLE1* transcription. Interestingly, p90 degradation again involves Cdc48, plus the proteins Ufd2 and Rad23 [18]. Ufd2 is a substrate-processing cofactor that possesses E4 polyubiquitination activity – an activity that adds further ubiquitin moieties to a mono- (or oligo-)ubiquitinated substrate – in conjunction with an E3 ubiquitin ligase [18,23]. Ufd2 not only binds to Cdc48 but also to Rad23, an escort factor that guides substrates to the proteasome. Studies with UFD substrates indicate that the polyubiquitinated substrate is handed over directly from Cdc48-bound Ufd2 to Rad23 [18,30]. Importantly, Ufd2 is antagonized by two other proteins, Ufd3 (also known as Doa1) and Otu1 [21]. Whereas Ufd3 has no known activity other than its ability to displace Ufd2 from Cdc48 and thereby prevent polyubiquitination, Otu1 is a deubiquitination enzyme (DUB) of the OTU (ovarian tumor) family that deubiquitinates Cdc48-bound substrates. In this context it is noteworthy to mention that Cdc48 influences substrate ubiquitination in another way: it restricts Ufd2 (E4)-catalyzed polyubiquitination of bound substrates to yield short polyubiquitin chains (4–6 ubiquitin moieties) that are optimal for Rad23 recognition (a process termed 'size restriction') and thus proteasomal degradation [18].

Although this obviously complicated pathway has several intriguing aspects, the key conclusion relevant to our discussion is that Cdc48 functions as a 'gearbox' (see later). In the cytosol, Cdc48 acts on mono- (or oligo-)ubiquitinated Spt23 p90 to activate the transcription factor (i.e. to mobilize p90 from the ER). By contrast, later on in the pathway (apparently in the nucleus), Cdc48 in conjunction with its cofactor Ufd2 acts on the same substrate with the opposite outcome: p90 is inactivated by proteasomal degradation.

Endoplasmic reticulum-associated degradation (ERAD) pathway

ERAD is a ubiquitin-proteasome-dependent degradation pathway that is defined mainly by the initial ER localization of its protein substrates [31,32]. ERAD acts on abnormal (e.g. misfolded or misassembled) ER luminal and membrane proteins, and on normal proteins of the ER for regulatory purposes. To reach the cytosolic ubiquitin-proteasome system (which is absent from the

Table 1. Cofactors of Cdc48 (p97)

Protein	Functions	Organisms	Ubiquitin-binding domains	Cdc48 (p97) interaction domains	Interaction domains in Cdc48 (p97)	Extra domains
Substrate-recruiting cofactors						
Ufd1–Npl4	ERAD, UFD pathway, OLE pathway	Yeast, metazoans	N-terminal UT3 domain	Ufd1: BS1; Npl4: UBD (UBX related)	N-domain	Mammalian NPl4: NZF zinc finger
Shp1 (p47)	ER membrane fusion, Golgi assembly	Yeast, metazoans	UBA	UBX, BS1	N-domain	
Ubx2	ERAD	Yeast, metazoans	UBA	UBX	N-domain	
Ubx3-7[b]	Unknown	Yeast	UBX5: UBA	UBX	N-domain	
FAF1[b]	Unknown	Mammalian	Unknown	UBX	N-domain?	UAS (function unknown)
Substrate-processing cofactors						
Ufd2	E4 polyubiquitination enzyme; UFD and OLE pathways, ERAD	Yeast, metazoans (mammalian E4B)	Unknown	Unknown	D2 AAA domain	U-box, RING finger-related ubiquitin-ligase domain
Ufd3 (Doa1)	Competitor of Ufd2; UFD and OLE pathways	Yeast, metazoans (mammalian PLAP)	PFU	PUL	D2 AAA domain	WD-40
Otu1	Deubiquitinating enzyme; OLE pathway	Yeast	Unknown	UBD (UBX related)	N-domain	OTU, deubiquitinating enzyme domain
VCIP135	Deubiquitinating enzyme; Golgi complex	Mammalian	Unknown	UBX	N-domain	OTU, deubiquitinating enzyme domain
Ataxin-3	Deubiquitinating enzyme; ERAD	Mammalian	UIM	After polyQ stretch	N-domain	Josephin domain, deubiquitinating enzyme domain
PNGase	Deglycosylation enzyme; ERAD	Mammalian	Unknown	PUB	D2 AAA domain	TGc (transglutaminase domain)
HDAC6	Protein deacetylase; aggresome formation?	Mammalian	ZnF-UBP	Unknown	Unknown	HDAC-like deacetylase domain

[a]Abbreviations: BS1, binding site 1; FAF1, Fas-associated factor 1; NZF, Npl4 zinc finger; PFU, PLAA family ubiquitin-binding domain; PLAP (or PLAA), phospholipase A2-associated protein; PUB, PNGase UBA/UBX-containing domain; PUL, PLAA Ufd3 Lub1 domain; UAS, unknown domain found in FAF1 proteins; UBA, ubiquitin-associated domain; UBD, ubiquitin fold domain; UBX, ubiquitin regulatory X domain; UIM, ubiquitin-interaction motif; ZnF-UBP, ubiquitin C-terminal hydrolase-like zinc finger.
[b]Substrate-recruiting cofactor function not confirmed.

ER lumen), luminal and ER-membrane-bound substrates must be retrotranslocated to the cytosol or extracted from the ER membrane. Genetic studies showed that Cdc48–Ufd1–Npl4 is required for most ERAD substrates [5,7–9]. Although the precise function of Cdc48 in this context is not perfectly clear, the available data suggest that Cdc48 (together with the proteasome) contributes to the retrotranslocation or extraction process, or that it segregates the substrate from the proposed retrotranslocation channel [5–9,33]. ERAD also sometimes involves the polyubiquitination factor Ufd2 (E4) and apparently always the escort factor Rad23 or its homolog Dsk2 [18,34]. Notably, Cdc48–Ufd1–Npl4 is recruited to the ER through association with Ubx2, an ERAD-specific cofactor that possesses two transmembrane segments [19,20,35]. Ubx2 binds not only to ubiquitinated substrates on the cytosolic face of the membrane, but also to dedicated E3 ubiquitin ligases [35,36]. Although the precise order of events is not clear, a plausible model is that ERAD substrates when they emerge at the cytosolic face of the membrane are first ubiquitinated (which might prevent them from slipping back into the lumen [37]) and subsequently recognized by Cdc48–Ufd1–Npl4 for extraction. Analogous to the processes in the OLE and UFD pathways, proteasomal targeting might then additionally involve Ufd2 and Rad23 (or Dsk2).

Membrane fusion pathway

Homotypic membrane fusion in yeast, and the fusion of Golgi vesicles after mitosis in mammalian cells, also depends on Cdc48 (p97) [10–14]. For this function, Cdc48 (p97) associates with the substrate-recruiting cofactor called Shp1 in yeast (Cdc48–Shp1) or p47 in vertebrates (p97–p47), which similarly to the alternative Ufd1–Npl4 heterodimer possesses ubiquitin-conjugate-binding activity, which is important for this process. Remarkably, membrane fusion in mammals also requires a substrate-processing cofactor termed VCIP135, a deubiquitination enzyme of the OTU family and distant relative of yeast Otu1 [12–14]. However, whether deubiquitination serves to protect a substrate from degradation or has other functions is currently not clear, and this can only be addressed once the substrate has been identified.

A 'gearbox' could shift fates

With the discovery of substrate-processing cofactors it became clear that Cdc48 (p97) is more gifted than previously expected and that it functions not only as a chaperone-related segregase. In particular the finding that three types of cofactors can differentially influence the degree of ubiquitination of Cdc48-bound substrates [21] led us to the speculative model that Cdc48 (p97) could also function as a 'gearbox' with three positions: 'forward', for further polyubiquitination (Ufd2-catalyzed); 'neutral', for prevention of ubiquitination (Ufd3-blocked); and 'reverse', for deubiquitination (Otu1-catalyzed; this reverse state might be made even more potent through a combination of the Ufd3 and Otu1 activities) (Figure 1a). If correct, the question is what might be the *raison d'être* for this activity. A clue to this question comes from the OLE and possibly also the Golgi pathway. As noted, Cdc48 can act on Spt23 in at least two ways with a completely different net result. When in 'neutral' (i.e. in the absence of Ufd2 activity) Cdc48 mobilizes Spt23 p90 from the membrane, turning

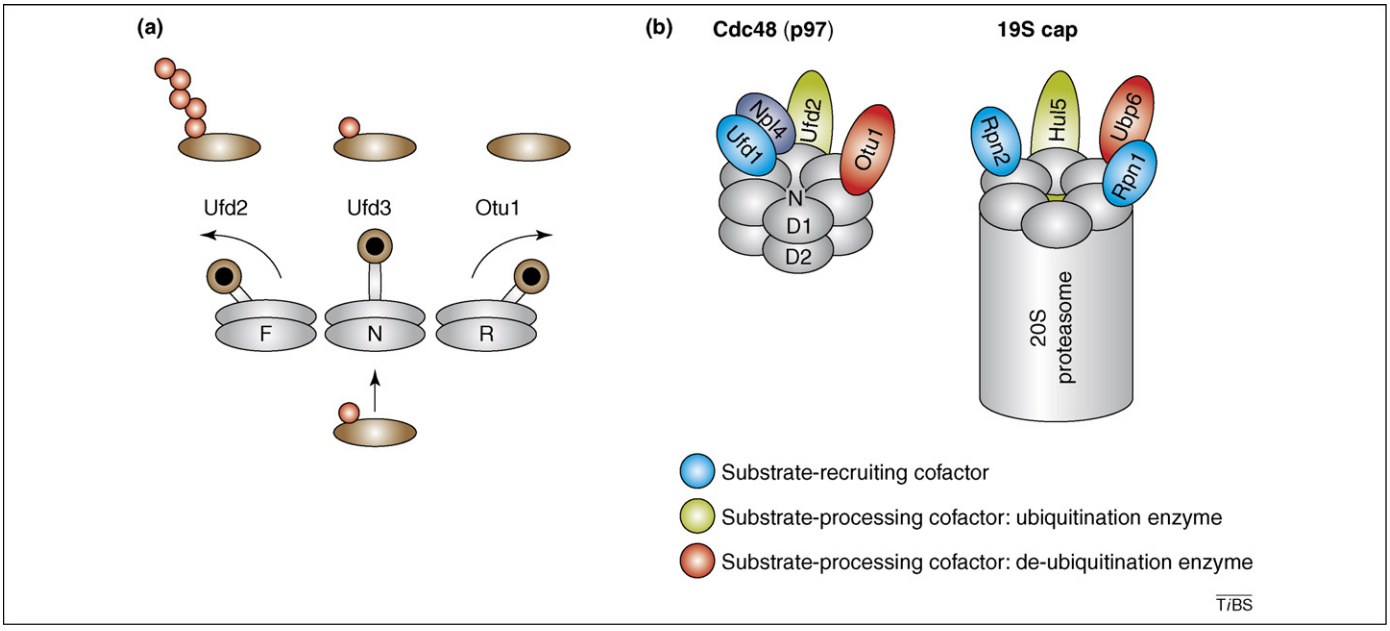

Figure 1. Putative molecular gearboxes. **(a)** Speculative model of a gearbox function of Cdc48 (p97). Mono- or oligo-ubiquitinated substrates (brown; ubiquitin, red) are recruited to the Cdc48 (p97) gearbox (gray). In the position 'forward' (F) the E4 enzyme Ufd2 polyubiquitinates the substrate, thereby promoting proteasomal degradation. In 'neutral' (N), the WD-40 protein Ufd3 competes with Ufd2 for Cdc48 binding, thereby preventing further ubiquitination of the substrate by Ufd2. In the position 'reverse' (R), the deubiquitination enzyme Otu1 removes the ubiquitin modification of the substrate. Substrates released from Cdc48 through the 'N' and 'R' positions of the gearbox are either mono- (oligo-)ubiquitinated or unmodified and thus metabolically stable. **(b)** Comparison between Cdc48 (p97) and the 19S cap of the proteasome. Cdc48 (p97) (left) is a homohexameric ring (gray). Each subunit possesses an N-domain and two consecutive AAA ATPase domains, D1 and D2. The substrate-recruiting cofactor Ufd1–Npl4 and the deubiquitination enzyme Otu1 bind to the N-domain; the polyubiquitination enzyme Ufd2 binds to the D2 domain. The Rpt1–6 ATPase of the 19S cap (right; the 19S cap can bind to only one or both ends of the 20S proteasome) is a heterohexamer (gray and dark gray). Rpn1 and Rpn2 are putative substrate-recruiting cofactors (at least through interaction by Rad23). Ubp6, a deubiquitination enzyme, and Hul5, a ubiquitin ligase, associate with the 19S cap. Because of the opposing activities of ubiquitination and deubiquitination enzymes, both Cdc48 (p97) and 19S cap can function as gearboxes, shifting the activities of the particles from a proteolytic to a nonproteolytic (chaperone-like) function.

the protein into an active transcription factor. By contrast, when the Cdc48 gearbox is shifted to 'forward' (i.e. in the presence of Ufd2 and the absence of Ufd3 and Otu1) Spt23 p90 becomes polyubiquitinated, is handed over to Rad23, and escorted to the proteasome for destruction. In the Golgi pathway, the Cdc48 gearbox might be shifted in the other direction, namely from 'neutral' to 'reverse', because the DUB VCIP135 is also required to form Golgi cisternae [14]. Because only polyubiquitinated substrates are usually degraded by the proteasome, Cdc48 (p97) through shifting the degree of ubiquitination might therefore decisively regulate the fate of the substrate. The handiness of this proposed activity is particularly evident for proteins that perform different cellular functions depending on whether they are unmodified, mono- (or oligo-)ubiquitinated, or polyubiquitinated. In addition to transcription factors (and the unknown substrate of the Golgi or membrane fusion pathway), candidate substrates could include other regulatory proteins such as proteins involved in DNA transactions (repair, recombination, replication) or chromatin function.

Is the 19S cap of the proteasome also a gearbox?

Although not suggested by sequence comparison or domain organization, Cdc48 (p97) has striking functional similarity to the AAA ATPases of the 19S cap of the proteasome [38]. This complex recruits polyubiquitinated proteins to the proteasome, which leads to their unfolding and the threading of their polypeptide chain through the narrow openings of the 20S proteasome into its proteolytic chamber. In contrast to the homohexameric Cdc48 (p97) enzyme, six different subunits (Rpt1–6) form a heterohexameric ring, which sits on top of the openings of the 20S proteasome (one on each side of the proteasome) (Figure 1b). Similarly to Cdc48, at least some of the Rpt proteins can bind to ubiquitin-conjugates directly [39]. However, ubiquitin-conjugate binding seems to be mediated by cofactors, namely Rpn1, its close homolog Rpn2, and Rpn10 [40–42]. This is analogous to the Cdc48 (p97) system and these proteins can be considered as substrate-recruiting cofactors of the Rpt ring. Because Rpn1 and Rpn2 recruit the escort factor Rad23 (loaded with ubiquitin–protein conjugates) by the single ubiquitin-like domain of Rad23, it seems possible that they could also bind to mono- or oligo-ubiquitinated proteins directly (although this has not yet been demonstrated experimentally).

Intriguingly, substrate-processing cofactors of the 19S cap seem to exist as well. Several ubiquitin ligases have been reported to function as proteasome cofactors (e.g. Ubr1, Ufd4 and Hul5) [43,44], and some of these might function similarly to Ufd2 in polyubiquitination of proteasome-bound ubiquitinated substrates. Furthermore, the 19S cap directly associates with the DUB protein Ubp6 (USP14 in vertebrates) for which the ubiquitin-like domain of Ubp6 is essential [44,45]. Interestingly, Ubp6 has the activity to remove ubiquitin moieties from the distal end of a chain [46], thereby trimming chains down to perhaps a single moiety, or it might remove ubiquitin from the substrate completely. Again, by analogy to the Cdc48 (p97) complex, Ubp6 might fulfill a similar function as Otu1 or VCIP135 in deubiquitinating proteasome-

bound substrates. At a first glance, an activity at the proteasome that blocks or even cancels out previous cellular decisions that have ordered proteins for degradation seems odd. However, it is important to note that the proteasome, in particular the 19S cap, has been functionally linked to several nonproteolytic functions as well. Examples are the suggested nonproteolytic, perhaps chaperone-related, role of the proteasome in nucleotide excision repair (NER) [47] and transcriptional control [48]. Interestingly, it seems that in these cases the substrates might also be degraded at a later point, possibly to make room for other proteins that function in the same pathway. We speculate that the 19S cap of the proteasome could also function as a gearbox by shifting the activity of the proteasome from a chaperone-related activity to a protease.

Open questions

Regarding the gearbox model, the key open question is: what shifts the lever? One obvious possibility is the availability of specific substrate-processing cofactors. Another option is that cofactor association is controlled by modification of Cdc48 (p97), the cofactors or the substrate. Indeed, Cdc48 (p97) is phosphorylated on tyrosine and serine residues upon various signals [49,50], but whether phosphorylation might influence cofactor association has not yet been experimentally addressed. Thinking of the Hsp70 chaperone system, which is crucially regulated by factors that control the ATPase cycle, one could speculate that some cofactors might control Cdc48 (p97) in an analogous way. Unfortunately, such biochemical studies are currently lacking, and it will be interesting to see whether novel types of cofactors exist that directly extend the time period of Cdc48–substrate association or facilitate substrate release. If they exist, it is plausible that they will also influence the way Cdc48 (p97) interacts with particular substrate-processing cofactors. Given the accelerated interest in this intriguing molecular machine and the pace of new discoveries, it seems likely that the Cdc48 (p97) system will continue to keep the ubiquitin field in motion.

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