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Review

UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97

C. Schuberth[a,*] and A. Buchberger[b,*]

[a] EMBL Heidelberg, Cell Biology and Biophysics Unit, Meyerhofstraße 1, 69117 Heidelberg (Germany), e-mail: schubert@embl.de
[b] Max Planck Institute of Biochemistry, Department of Molecular Cell Biology, Am Klopferspitz 18, 82152 Martinsried (Germany), e-mail: buchberg@biochem.mpg.de

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Abstract. The highly conserved AAA ATPase Cdc48/p97 acts on ubiquitylated substrate proteins in cellular processes as diverse as the fusion of homotypic membranes and the degradation of misfolded proteins. The 'Ubiquitin regulatory X' (UBX) domain-containing proteins constitute the so far largest family of Cdc48/p97 cofactors. UBX proteins are involved in substrate recruitment to Cdc48/p97 and in the temporal and spatial regulation of its activity. In combination with UBX-like proteins and other cofactors, they can assemble into a large variety of Cdc48/p97-cofactor complexes possessing distinct cellular functions. This review gives an overview of the different subfamilies of UBX proteins and their functions, and discusses general principles of Cdc48/p97 regulation by these cofactors.

Keywords. AAA ATPase, valosin-containing protein (VCP), ubiquitin/proteasome system, protein degradation, UBA domain, ubiquitin-like fold.

Introduction

More than 10 years ago, a protein domain displaying weak amino acid sequence homology to the small protein modifier ubiquitin was identified in a number of eukaryotic proteins and named 'Ubiquitin regulatory X' (UBX) domain [1]. In a first structural study, the UBX domain was found to adopt the same three-dimensional fold as ubiquitin and was used to define a new and largely uncharacterized protein family [2]. While the UBX domain-containing protein p47 had already been described to bind to the AAA ATPase Cdc48/p97 [3–5], it became clear only recently that UBX proteins in general are cofactors for Cdc48/p97 [6–9].

Cdc48 (also known as p97 or VCP in mammals[1]) belongs to the family of AAA ATPases (ATPases associated with various cellular activities) [10]. It is a highly conserved, essential, chaperone-related protein involved in a large variety of cellular processes, including ubiquitin-dependent protein degradation and processing, fusion of homotypic membranes, nuclear envelope reassembly, cell cycle progression and others [11–13]. The underlying molecular mechanism of Cdc48/p97 action in all these processes is believed to be its "segregase" activity (term coined by

* Corresponding authors.

[1] The term 'Cdc48/p97' will be generally used throughout the text, while 'Cdc48' and 'p97' are used to indicate aspects or studies specific for the yeast and mammalian orthologue, respectively.

Jentsch and co-workers [5, 14]): Cdc48/p97 uses the energy provided by ATP hydrolysis to extract substrate proteins from protein complexes or lipid membranes. Although Cdc48/p97 is able to bind to non-ubiquitylated proteins [15–17], it appears to act primarily on ubiquitylated substrates *in vivo*. In the process of endoplasmic reticulum (ER) associated protein degradation (ERAD), Cdc48/p97 dislocates substrate proteins from the ER through a retrotranslocation pore back into the cytosol, where they are subsequently degraded by the 26S proteasome [18]. Similarly, the tetrameric ubiquitin-proline-β-galactosidase model substrate of the ubiquitin fusion degradation (UFD) pathway is believed to be processed by Cdc48 prior to its degradation [19, 20]. On the other hand, proteolysis-independent functions of Cdc48/p97 have been described. During the homotypic fusion of Golgi and ER membranes, Cdc48/p97 appears to act on elusive mono-ubiquitylated substrates during the remodelling of SNARE complexes and/or their regulators [16, 21]. In the yeast OLE (oleic acid desaturase Ole1) signal transduction pathway, the segregase activity of Cdc48 is needed to solubilize the active, processed p90 form of the transcription factor Spt23 from complexes with the unprocessed p120 precursor, which is anchored in the ER membrane [14, 22]. A similar mode of action has been described very recently in the context of nuclear envelope reformation, where p97 was found to extract Aurora B kinase from chromatin [23].

Cdc48/p97 forms a ring-shaped complex of six identical subunits that consist of two ATPase domains called D1 and D2, and an amino-terminal N domain, which is mainly responsible for cofactor and substrate binding. The ATPase domains and the N domain move relative to each other during ATP binding and hydrolysis, thereby probably providing the mechanical forces to exert the segregase function [24]. In order to provide specificity for its various cellular functions, Cdc48/p97 activity in the cell is tightly regulated in space and time by numerous different cofactors, of which UBX proteins constitute the largest known subgroup.

The UBX protein family

UBX domain structure and prevalence

The UBX domain comprises about 80 amino acid residues and was first described in the hypothetical human protein Y33K (now known as SAKS1), based on a profile in the PROSITE data base (entry 50033) [1]. While the presence of a ubiquitin-associated (UBA) domain in Y33K/SAKS1 suggested a link to the ubiquitin-proteasome system (UPS), the function of the UBX domain itself initially remained unclear. Subsequently, the structure determination of the UBX domain of human Fas-associated factor 1 (FAF1) revealed a further, unexpected link to the UPS, because the UBX domain turned out to be a close structural homologue of ubiquitin itself (Fig. 1) [2]. The absence of a carboxy-terminal di-glycine motif, however, indicated that UBX domains are not covalently attached to target proteins in a ubiquitin-like manner. Rather, they are distinct structural units defining a large family of proteins that often exhibit a modular domain architecture [2].

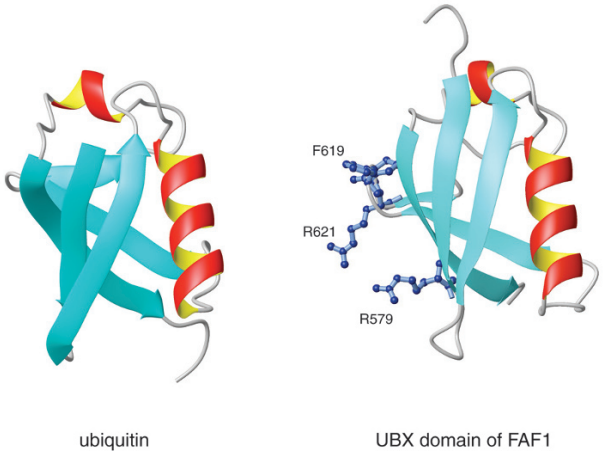

Figure 1. The UBX domain has a ubiquitin fold. Ribbon representations of the three-dimensional structures of the UBX domain from human FAF1 (right; PDB entry 1 h8c) and ubiquitin (left; PDB entry 1UBI) are shown in similar orientation. For the UBX domain, the side chains of the conserved R…FPR motif constituting the Cdc48/p97 binding site are depicted as ball-and-stick model. Ubiquitin lacks the corresponding side chains and has a shorter loop connecting strands 3 and 4.

A structure-based sequence alignment of UBX domains led to the identification of UBX domain-containing proteins in all eukaryotic species, which can readily be grouped into evolutionarily conserved subfamilies based on sequence similarities outside the UBX domain [2]. These include the widespread p47, FAF1, SAKS1, TUG and UBXD1 subfamilies, as well as the relatively small groups of Rep-8 and UBXD3 proteins (Table 1, Fig. 2). The p47 subfamily is defined by a central SEP (Shp1, eyes-closed, p47) domain involved in trimerization of p47 [25, 26] and a carboxy-terminal UBX domain. The subfamily contains *bona fide* p47 orthologues, which possess an amino-terminal UBA domain [8, 25, 27–29] and are found in most eukaryotes. Interestingly, this subfamily has diversified in vertebrates (Table 1, Fig. 2) and also comprises the close homologues p37 [30] and UBXD4, which both lack the UBA domain. In addition, the more distantly related Socius protein shares homology with

Table 1. Members of UBX domain protein subfamilies in various model organisms.

UBX subfamily	*H. sapiens*	*C. elegans*	*S. pombe*	*S. cerevisiae*
p47	p47 (NSFL1C)	UBXN-2? [a]	Ubx3 (SPAC343.09)	Shp1
	p37 (LOC137886)	UBXN-2? [a]	–	–
	UBXD4	UBXN-2? [a)]	–	–
	Socius (UBXD5)	–	–	–
FAF1	FAF1	UBXN-3	–	–
	ETEA (UBXD8, KIAA0887)	H40L08.1	Ucp10 (SPCC285.11)? [b]	Ubx2
	–	–	Ucp10 (SPCC285.11)? [b]	Ubx3
	UBXD7 (KIAA0794)	–	Ubx2 (Ucp13, SPAC2C4.15c)	Ubx5
SAKS1	SAKS1 (Y33K; LOC51035)	UBXN-1	YDFB (SPAC17C9.11c)	–
	Erasin (UBXD2, KIAA0242)	UBXN-4	–	Ubx7
	–	–	–	Ubx6
TUG	TUG (ASPL)	B0024.10	Ubx4 (YOSB, SPBC21C3.11)	Ubx4
UBXD1	UBXD1	UBXN-6	–	–
Rep-8	Rep-8 (UBXD6)	–	–	–
UBXD3	UBXD3	–	–	–
?	–	UBXN-5 [c]	–	–

–, no homologue identified.
[a] UBXN-2 has highest homology to p47, but lacks an amino-terminal UBA domain.
[b] Ucp10 has clear homology to human ETEA, but lacks, like *S. cerevisiae* Ubx3, an amino-terminal UBA domain.
[c] No UBXN-5 homologue could be identified in any organism.

other members of the p47 subfamily within the SEP and UBX domains. An interesting feature of the p47 subfamily is the presence of a second p97-binding site at the carboxy-terminal end of the SEP domain [9, 31]. This linear motif is variably called 'FxGzGQxb motif' [32], 'binding site 1' (BS1) [31] or 'SHP box' [33] and is also found in other Cdc48/p97-interacting proteins, among them the Ufd1 subunit of the heterodimeric Ufd1-Npl4 cofactor and members of the Derlin membrane protein family implicated in ER-associated protein degradation [31–35]. Intriguingly, the binding site of BS1 has been reported to overlap with the UBX domain-binding site at the N domain of Cdc48/p97 [36], even though the implications for the binding of a p47 trimer to a p97 hexamer have not yet been addressed in molecular detail.

The FAF1 subfamily is characterized by a domain architecture consisting of an amino-terminal UBA domain, a carboxy-terminal UBX domain, and a central UAS domain of unknown function exhibiting a thioredoxin-like fold (PDB entries 2ec4, 2dlx; SMART database entry sm00594). This subfamily can be further subdivided into three groups (Table 1, Fig. 2): true FAF1 homologues, which are exclusively found in insects and vertebrates, and ETEA and UBXD7 homologues, which are conserved from yeast to human. True FAF1 homologues contain two ubiquitin-like domains of unknown function in the amino-terminal third of the protein, while ETEA homologues are characterized by one or two transmembrane regions near the amino terminus. UBXD7 homologues are more distantly related to the FAF1 and ETEA subgroups. They possess neither ubiquitin-like nor transmembrane domains, but contain a ubiquitin-binding UIM (ubiquitin interaction motif) between the UAS and UBX domains (Fig. 2) [7].

The SAKS1 subfamily comprises two groups of proteins, true SAKS1 homologues and Erasin-like proteins, which are conserved from yeast to human and share a central region of homology that is not found in other UBX subfamilies. SAKS1 homologues typically contain an amino-terminal UBA domain and a carboxy-terminal UBX domain, even though some fungal homologues lack the UBA domain (Fig. 2). Erasin-like proteins exhibit a differing domain architecture: the UBX domain is not located at the extreme carboxy terminus but is followed by a membrane insertion domain [37], and the amino-terminal UBA domain is replaced by a thioredoxin-like domain weakly homologous to UAS domains of the FAF1 subfamily. While both the unique shared central region of homology as well as the overall homology to true SAKS1 proteins support the classification of Erasin-like proteins into the SAKS1 subfamily, the presence of a divergent UAS domain suggests some evolutionary relationship to the FAF1 subfamily. The analysis of UAS domain function(s) may help to clarify this point in the future.

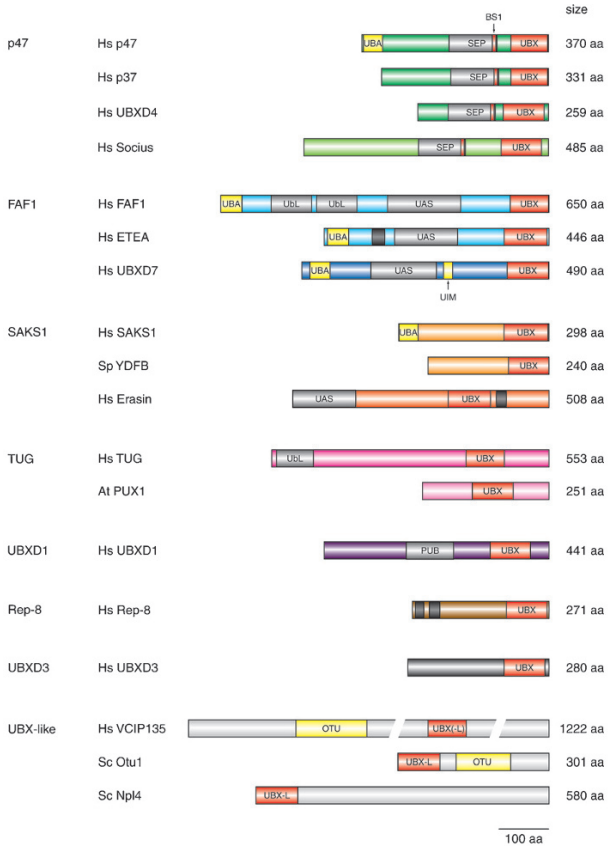

Figure 2. The UBX protein family. Subfamilies and subgroups were classified based on sequence homologies outside the UBX domain as identified by systematic PSI-BLAST searches. Shown are representative members of the subfamilies indicated at the left. Defined domains are labelled, and sequence homology outside defined domains is indicated by similar colours. In addition to the true UBX proteins, some proteins containing UBX-like domains are depicted at the bottom. Human VCIP135 possesses a recognizable UBX domain which, however, also shows significant homology to UBX-like and ubiquitin-like domains (see text). UBX and UBX-like domains are in red. The 'binding site 1' (BS1) Cdc48/p97-binding motif found in members of the p47 subfamily is indicated by a small red bar at the carboxyl-terminal end of the SEP domain. Ubiquitin-binding domains including the UBA and UIM domains and the catalytic Otubain-like ubiquitin hydrolase domain (OTU) are in yellow. Other domains discussed in the text are in grey, including SEP, ubiquitin-like (UbL), UAS and PUB domains. Transmembrane and membrane insertion regions are indicated by black, unlabelled bars. Hs, *Homo sapiens*; At, *Arabidopsis thaliana*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*.

The TUG subfamily is relatively heterogeneous, with members in all eukaryotes and a UBX domain that is localized towards the centre of the protein. Interestingly, some members of this subfamily contain a ubiquitin-like domain at their amino terminus [38]. Members of the UBXD1 subfamily, which are found in all eukaryotes except fungi, show a high degree of sequence conservation [39] and possess a central PUB domain in addition to the carboxy-terminal UBX domain [40–42]. The small vertebrate Rep8 subfamily is characterized by amino-terminal transmembrane spans and a carboxy-terminal UBX domain. Finally, UBXD3 homologues are exclusively found in mammals.

The UBX domain: a general Cdc48/p97-binding module

A structure-based sequence alignment of UBX domains revealed a highly conserved surface patch consisting of an arginine residue in strand 1 and an FPR motif in the loop connecting strands 3 and 4 (Fig. 1) [2]. This R…FPR surface patch is absent from ubiquitin and other ubiquitin-like proteins and was suggested to constitute a conserved binding site for an elusive interactor [2]. In a subsequent study presenting the solution structure of the p47 UBX domain, it was shown that this interactor is in fact the Cdc48/p97 AAA ATPase, and that the UBX domain binds to the amino-terminal N domain of p97 [3]. Molecular details of the interaction were revealed by the structure determination of a complex between the p47 UBX domain and a p97 fragment comprising the N and D1 domains [9]. The UBX domain binds to a hydrophobic pocket between the two subdomains of the p97 N domain. Intriguingly, the conserved R…FPR surface patch was found to be the major binding site of the UBX domain, and mutation of this motif greatly reduced p97 binding [9].

Mapping of the p47-p97 binding interface led to two important conclusions. First, the absence of the R…FPR motif in ubiquitin itself suggests that ubiquitin binds with significantly lower affinity to Cdc48/p97 than do UBX proteins. Even though this prediction has not been verified yet, Cdc48/p97 appears to bind more efficiently to UBX domains [8] than to ubiquitin [14, 28] in pulldown experiments *in vitro*. Second, the high conservation of the R…FPR motif within UBX domains indicates that most, if not all, UBX proteins should be able to bind Cdc48/p97 [9, 29]. Indeed, work from several laboratories studying UBX proteins in baker's and fission yeast demonstrated convincingly that the UBX domain is a general Cdc48/p97-binding module [6–8]. Subsequently, p97 binding has also been demonstrated for all UBX proteins from higher eukaryotes tested (FAF1 [43]; Erasin [37]; SAKS1 [44, 45]; AtPUX1 [46]). Together, these findings spurred the idea that UBX proteins are adaptors for Cdc48/p97 that regulate its interactions with ubiquitylated substrates [29].

In summary, proteins containing a UBX- or UBX-like (see below) domain constitute by far the largest group of *bona fide* Cdc48/p97 cofactors to date. Even though the precise cellular function of most of these proteins is still unknown, current knowledge supports the view that they form a large and diverse family of predom-

inantly adaptor proteins that recruit Cdc48/p97 to specific substrates and/or cellular locations.

Origin of the UBX domain

The close structural similarity and the functional relationship between the UBX domain and ubiquitin provokes the interesting question about the evolutionary origin of the UBX domain. Homologous recombination involving the polyubiquitin gene locus is believed to be a relatively frequent event in evolution that gave rise to, and perhaps actively maintains, ubiquitin fusions with ribosomal subunits L40 and S27 (and some other proteins) [reviewed in ref. 47]. These ubiquitin fusions appear to be advantageous for ribosomal subunit folding and/or assembly [48], and are subsequently cleaved by cellular ubiquitin hydrolases, resulting in free ubiquitin and mature ribosomal proteins. Because the free ubiquitin generated from ribosomal fusion proteins has to maintain its essential cellular functions, there has been strong selective pressure during evolution to conserve the precise wild-type ubiquitin sequence within the ribosomal fusion proteins. Interestingly, this selection pressure was apparently lost for non-cleavable ubiquitin fusions found in certain protists, leading to the evolution of divergent sequences, but not structures [49].

In addition to ribosomal ubiquitin fusion proteins, there is a large group of proteins containing ubiquitin-like domains that are more closely related to ubiquitin than to UBX domains at the sequence level [29, 50, 51]. Among them are soluble proteasomal receptor proteins like Rad23 and Dsk2/PLIC, which recruit ubiquitylated substrate proteins to the 26S proteasome, further proteasome-interacting proteins like Ubp6/USP14 and Parkin, but also proteins without detectable affinity for the 26S proteasome [51]. If these proteins containing ubiquitin-like domains also evolved by (a) recombination event(s) with the polyubiquitin gene locus, there must have been selective pressure to maintain some ubiquitin function(s) other than conjugate formation, for example recognition by ubiquitin-binding proteins at the proteasome or at other cellular locations.

It is tempting to speculate that UBX domain-containing proteins also evolved following fusion of a ubiquitin moiety to preexisting proteins, which might already have functioned as substrate receptors for an ancient Cdc48/p97-like AAA ATPase turning over ubiquitylated substrates. Subsequently, there must have been positive selection for UBX domains to bind specifically to the N domain of Cdc48/p97, and negative selection against binding to other AAA ATPases and to ubiquitin-binding domains. In support of this speculation, key residues within the Cdc48/p97 N domain for the interaction with UBX domains are not conserved in the highly homologous AAA ATPase VAT from *Thermoplasma acidophilum* [9], an archaeon lacking both ubiquitin and UBX proteins. Perhaps UBX proteins and Cdc48/p97 coevolved in order to allow eukaryotic cells to deal efficiently with specific substrates requiring segregase activity for turnover, while bulk degradation of ubiquitylated proteins occurs in a Cdc48/p97-independent manner. Of note, some Cdc48/p97 cofactors, including the Npl4 subunit of the Ufd1-Npl4 heterodimer and the deubiquitylating enzyme Otu1 (Fig. 2), possess amino-terminal domains that interact with the N domain of Cdc48/p97 [31, 36, 52] in a manner resembling that of UBX domains [36]. Like UBX domains, these domains exhibit the β-grasp fold of the ubiquitin superfamily, and even though they lack the R…FPR motif found in true UBX domains, mutational and structural analyses demonstrated that residues at or close to the corresponding positions make equivalent contacts to Cdc48/p97 [31, 36]. As these domains show higher sequence similarity to each other than to UBX domains, ubiquitin-like domains, or ubiquitin itself, we suggest to classify them as 'UBX-like'. It is unclear whether UBX-like domains diverged from true UBX domains, or whether a second, independent ubiquitin fusion event may have given rise to this relatively small group of Cdc48/p97 cofactors. In favour of the first possibility, the human deubiquitylating enzyme VCIP135 harbours a domain with clear homology to UBX domains that may represent a 'missing link' between UBX, UBX-like and ubiquitin-like domains. PSI-BLAST homology searches using residues 770 to 860 of human VCIP135 as query sequence generate hits with representatives from all three domain families (data not shown). In contrast to ubiquitin-like domains, however, the UBX(-like) domain of VCIP135 possesses a true R…FPR motif, consistent with its ability to interact with p97 [53].

Regulation of Cdc48/p97 function by UBX proteins and other cofactors

A regulation system based on combinatorial cofactor interactions

The large number and diversity of Cdc48/p97 functions in the cell requires the precise regulation of its activity not only to keep the right balance between different simultaneous tasks, but also to turn on or off specific functions during the cell cycle. This spatial and temporal regulation is mediated by different cofactors, which assemble in certain combinations into distinct Cdc48/p97 complexes possessing specific functions. Cofactors can occupy two partially over-

Table 2. Classification of Cdc48/p97 cofactors.

Type of Cofactor	Identified Proteins[a]	Features	Functions
Substrate-recruiting major	Ufd1-**Npl4**, **p47/Shp1, p37, UBXD4**[b) c)], **Socius**[b) c)]	UBX(-like) domain AND BS1 motif Mutually exclusive binding to Cdc48/p97	Decision between major cellular pathways: membrane fusion *versus* protein degradation (and others)
Substrate-recruiting additional	**Ubx2(/ETEA**[c]**), Ubx5**[d]**, Erasin**[c) d)], VIMP, Derlins, **SAKS1**[c) d)], **FAF1**[c) d)]	UBX(-like) domain OR BS1 motif Binding to Cdc48/p97 is not mutually exclusive Join major substrate-recruiting cofactor	Co-adaptors for specific pathways: improve substrate binding and/or provide additional spatial regulation
Substrate-processing	**VCIP135, Otu1**, Ufd3, Ufd2, PNGase[d], Ataxin3[d]	Any Cdc48/p97 interaction motif Combinatorial binding within one Cdc48/p97 complex possible Join distinct substrate-recruiting complexes	Additional enzymatic activities Regulation of substrate fate: stabilization *versus* degradation
Miscellaneous	**AtPUX1,** SVIP	Any Cdc48/p97 interaction motif No cross-talk with other cofactors?	Regulation of oligomeric state or localization

[a)] UBX and UBX-like proteins are depicted in bold;
[b)] assignment to the major substrate-recruiting category is solely based on homology to p47/Shp1 and p37 and thus speculative;
[c)] adaptor function inferred;
[d)] involvement of a major substrate-recruiting cofactor inferred.

lapping binding sites in the Cdc48/p97 N domain [9, 36] and at least one additional interaction site in the D2 domain [42, 52, 54]. As the Cdc48/p97 homohexamer offers six times these binding sites, the potential to interact with different cofactor combinations is enormous. However, not all combinations are possible because cofactors apparently bind in a hierarchical manner. From the current state of knowledge, four groups of Cdc48/p97 cofactors can be distinguished: major versus additional substrate-recruiting cofactors, substrate-processing cofactors and miscellaneous interactors (Table 2). The major and additional substrate-recruiting cofactors connect Cdc48/p97 to specific cellular pathways. Cofactors of the substrate-processing class, in contrast, accelerate and/or regulate the fate of substrates after recruitment, often by exerting additional enzymatic activities downstream of substrate turnover by Cdc48/p97. The last category contains cofactors that are believed to regulate Cdc48/p97 activity by various other mechanisms. Notably, UBX and UBX-like proteins occupy the most central positions in this regulation system.

Substrate-recruiting cofactors

Although Cdc48/p97 can directly interact with unfolded proteins [15, 55] and with ubiquitin [14] *in vitro*, it is believed that Cdc48/p97 requires cofactors of the major substrate-recruiting subgroup to exert its functions *in vivo*. Substrate-recruiting cofactors act as adaptor proteins for Cdc48/p97, allowing a stable association of the Cdc48/p97 complex with typically ubiquitylated substrate proteins [14, 16, 28, 29, 56]. The recruitment function is often highlighted by the presence of ubiquitin-binding domains (Fig. 2). For example, proteins with a UBA/UBX domain architecture like Shp1/p47, yeast Ubx2 and Ubx5 and the mammalian proteins FAF1 and SAKS1 (Y33K), are able to bind to ubiquitylated substrates with their amino-terminal UBA domain while interacting with Cdc48/p97 via their carboxy-terminal UBX domain [8, 28, 43, 45].

Substrate-recruiting cofactors can be subdivided into major substrate-recruiting cofactors like p47/Shp1 or the heterodimer Ufd1-Npl4, and proteins acting as coadaptors like Ubx2 or VIMP (Table 2). The importance of the major substrate-recruiting cofactors lies in the separation of fundamentally distinct cellular functions of Cdc48/p97. The Cdc48/p97$^{Shp1/p47}$ and p97^{p37} complexes control the fusion of homotypic membranes [4, 30], while ubiquitin-dependent protein degradation pathways require the Cdc48/p97$^{Ufd1\text{-}Npl4}$ complex [5, 14, 19, 56–61]. The crucial role of major substrate-recruiting cofactors in these processes is reflected by the strong phenotypes of mutants in the corresponding genes in yeast: *UFD1* and *NPL4* are essential, and null mutants of the yeast p47 homologue *SHP1* display a severe growth phenotype [7, 8, 59, 62, 63]. Surprisingly, however, *shp1* mutants have also been found to exhibit defects in certain ubiquitin-dependent degradation pathways [7, 8]. Conversely, the p97$^{Ufd1\text{-}Npl4}$ complex is

also involved in the reformation of the nuclear envelope double-membrane structure after mitosis [64, 65]. Even though the mechanism underlying the latter process has recently been shown to be the $p97^{Ufd1\text{-}Npl4}$-mediated extraction of Aurora B from chromatin [23], an activity resembling the mobilization function of the $Cdc48/p97^{Ufd1\text{-}Npl4}$ complex in the ERAD and OLE pathways, the possibility exists that even the major substrate-recruiting cofactors possess partially overlapping functions. Clarification of this important point has, however, to await the identification of additional cellular pathways and substrates involving $Cdc48/p97^{Ufd1\text{-}Npl4}$ *versus* $Cdc48/p97^{Shp1/p47}$, and a more detailed understanding of the differences between both complexes at the molecular level.

In contrast to *UFD1*, *NPL4* and *SHP1*, deletions of other yeast *UBX* genes give rise to mild, if any, phenotypes [6–8]. Together with the unexpected finding that the yeast Ubx2 protein acts as coadaptor for the $Cdc48^{Ufd1\text{-}Npl4}$ complex in ERAD [66, 67], the classification of these UBX proteins in a separate category of additional substrate-recruiting cofactors appears appropriate (Table 2). It is likely that several mammalian UBX proteins with demonstrated or assumed substrate-recruiting function also belong to this latter category. SAKS1 (Y33K) and Erasin are both involved in ERAD [37, 44, 45], while FAF1 appears to possess a role in the degradation of a mammalian UFD model substrate [43]. The role of these UBX proteins in well-characterized Ufd1-Npl4-dependent pathways suggests that they, like Ubx2, also function as additional substrate-recruiting cofactors of the $p97^{Ufd1\text{-}Npl4}$ complex, even though this has not yet been addressed experimentally. In a more general sense, non-UBX cofactors like the mammalian VIMP protein and members of the Derlin family also fit into this group. Even though they do not appear to bind ERAD substrates directly, they recruit Cdc48/p97 to sites of ERAD at the ER membrane [68, 69], thereby probably improving the efficiency of substrate turnover.

Mechanism of separating different Cdc48/p97 functions

Specific discrimination between different basic Cdc48/p97 functions in the cell is achieved by the mutually exclusive binding of the major substrate-recruiting cofactors to Cdc48/p97, resulting in the formation of distinct $Cdc48/p97^{Ufd1\text{-}Npl4}$, $Cdc48/p97^{p47}$ and $Cdc48/p97^{p37}$ complexes [30, 56, 70]. While the molecular basis underlying this exclusivity is not entirely clear, it appears to rely on the simultaneous presence of a UBX(-like) domain and the BS1-binding motif in Ufd1-Npl4, p47/Shp1 and p37. Because the binding sites for UBX(-like) domain and BS1 on the N domain of Cdc48/p97 overlap [9, 31, 36], it is unlikely that both binding modules interact with the same N domain of the Cdc48/p97 hexamer. If binding to two adjacent N domains is assumed, then the stable p47/Shp1 trimer (and probably also the *bona fide* trimers p37, UBXD4 and Socius) would occupy all six N domains of the Cdc48/p97 hexamer, thus precluding any Ufd1-Npl4 interaction. Conversely, stable binding of Ufd1-Npl4 to any two N domains would leave no space for the binding of a symmetric p47/Shp1 trimer. In addition to the steric hindrance, Ufd1-Npl4 and p47/Shp1 seem to induce different conformational changes in the Cdc48/p97 hexamer [26, 36, 71] that might prevent binding of the respective other major substrate-recruiting cofactor. In contrast, Cdc48/p97 cofactors harbouring only a single UBX(-like) or a BS1-binding module, e.g. Ubx2, the mammalian Derlins and VCIP135, are apparently still able to dock onto unoccupied N domains of $Cdc48/p97^{Ufd1\text{-}Npl4}$ or $Cdc48/p97^{p47}$ complexes (see below).

Shp1/p47 regulates Cdc48/p97 activity in time and space

The UBX protein p47 was the first cofactor of mammalian p97 to be identified [4]. $p97^{p47}$ is required for the fusion of homotypic membranes of the nuclear envelope, the ER and the Golgi apparatus [4, 53, 64]. The reassembly of the Golgi complex and the reformation of the nuclear envelope are membrane fusion events that occur only once during the cell cycle, at the end of mitosis. Thus, an additional layer of regulation for the $p97^{p47}$ complex is required, which is achieved by the temporal and spatial regulation of the adaptor itself. During interphase, $p97^{p47}$-mediated Golgi membrane fusion is prevented by the strictly nuclear localization of p47 mediated by its nuclear localization signal [72]. In addition, p47 is phosphorylated by Cdk1 and thereby kept inactive after nuclear envelope breakdown in order to prevent an immediate reversion of Golgi fragmentation prior to and during mitosis [72]. At the end of mitosis, the inhibitory phosphorylation is removed and the active $p97^{p47}$ complex can act on its substrates until p47 is sequestered again in the newly formed nucleus. This strict spatial and temporal regulation of p47 poses the question how other homotypic fusion processes are mediated during interphase. Recently, the p47 paralogue p37 was identified and shown to regulate p97 in the maintenance of ER and Golgi during interphase [30]. Interestingly, the activity of the $p97^{p37}$ complex during interphase appears to be independent of substrate ubiquitylation, in contrast to $p97^{p47}$ activity [30]. Whether the other p47 subfamily members, UBXD4 and Socius, are also involved in homotypic

membrane fusion events, perhaps in a cell cycle- or tissue-specific manner, remains to be addressed.

The Cdc48/p97$^{Ufd1\text{-}Npl4}$ complex is fine-tuned by Ubx2 in space

The notion that major substrate-recruiting cofactors are necessary, but not always sufficient to direct Cdc48/p97 to specific cellular pathways was supported by the unexpected finding that the yeast Ubx2 protein further regulates the function of Cdc48/p97$^{Ufd1\text{-}Npl4}$ [66, 67]. Cdc48/p97$^{Ufd1\text{-}Npl4}$ has been found to be involved in several different ubiquitin-dependent protein degradation pathways [5, 14, 57–61], in the mobilization of the Spt23 transcription factor [14] and in nuclear envelope reformation after mitosis [23, 64, 65]. In addition, p97$^{Ufd1\text{-}Npl4}$ has been linked to mitotic spindle disassembly and chromosome segregation [73, 74], although this has been disputed recently [75]. These diverse functions raise the question how substrate specificity of the Cdc48/p97$^{Ufd1\text{-}Npl4}$ complex is achieved.

At least as part of an answer, additional substrate-recruiting cofactors for Cdc48/p97$^{Ufd1\text{-}Npl4}$ have been identified in the ERAD pathway. Among them, Ubx2 was the first UBX protein shown to be able to form a stable complex with Cdc48$^{Ufd1\text{-}Npl4}$ [67]. As an integral ER membrane protein, Ubx2 provides a means of spatial regulation of Cdc48$^{Ufd1\text{-}Npl4}$ by recruiting the complex to the ER [67, 76]. In the ERAD process itself, Ubx2 plays a central role as a coadaptor for Cdc48$^{Ufd1\text{-}Npl4}$ that is required for the stable binding of Cdc48$^{Ufd1\text{-}Npl4}$ to ERAD substrates and ubiquitin ligases [66, 67]. Ubx2 thus improves the efficiency of ERAD by coordinating the ubiquitylation and retrotranslocation activities necessary for substrate turnover [66, 67, 76].

In the mammalian system, the regulation of p97$^{Ufd1\text{-}Npl4}$ in ERAD appears to be even more complex. Besides the putative Ubx2 orthologue ETEA (Fig. 2), the UBX proteins Erasin and SAKS1 (Y33K) and the non-UBX p97 cofactor VIMP may act as additional recruitment factors [34, 37, 44, 45]. On top of this, mammalian members of the Derlin family, which possess Cdc48/p97-binding sites of the BS1 type, also contribute to the recruitment of Cdc48/p97 to the ER membrane in ERAD [34, 35]. The direct interaction of p97 with the mammalian ERAD ubiquitin ligases Hrd1 and gp78 provides yet another means of coupling p97 to the ERAD process [68, 69, 77, 78]. The molecular interplay between all these cofactors and Ufd1-Npl4 in regulating p97 activity in ERAD remains an interesting topic for future studies.

Turnover of recruited substrates: substrate-processing cofactors

In contrast to the substrate-recruiting cofactors, substrate-processing factors of Cdc48/p97 are a heterogeneous group of proteins that often possess enzymatic activity themselves (Table 2). They are thought to act downstream of the substrate-recruiting cofactors and to expedite, and sometimes control, the fate of substrates after their recruitment to Cdc48/p97. Only few known substrate-processing cofactors possess UBX(-like) domains, including VCIP135 and Otu1 (see below). The best-characterized non-UBX substrate-processing factors are Ufd2 and Ufd3 from budding yeast. Ufd2 and Ufd3 compete for binding to the D1/D2 domains of Cdc48 [52] and are thereby able to determine the fate of substrate proteins [reviewed in ref. 79]. Ufd2 promotes degradation of oligoubiquitylated Cdc48$^{Ufd1\text{-}Npl4}$ substrates by catalyzing ubiquitin chain elongation [20], thus marking them for recognition by the 26S proteasome. In addition, Ufd2 also provides a physical link to the 26S proteasome *via* the proteasomal receptors Rad23 and Dsk2 [80]. Ufd3, in contrast, stabilizes substrates due to its antagonistic binding to Cdc48 [52]. In higher eukaryotes, Ufd2 controls myosin assembly and myofibril organization by catalyzing the degradation of the myosin-specific chaperone, UNC-45 [81, 82].

While Ufd2 and Ufd3 provide an additional layer of control in certain Cdc48-dependent degradation pathways, other substrate-processing cofactors appear to execute their (enzymatic) function on predetermined substrates without further regulatory impact. For example, peptide:N-glycanase (PNGase) removes sugar moieties from glycosylated ERAD substrates before they are degraded by the 26S proteasome [40, 41, 44, 83]. The deubiquitylating enzyme (DUB) Ataxin-3 is another substrate-processing factor involved in mammalian ERAD [84, 85], even though the physiological significance of removing ubiquitin moieties from ERAD substrates is still unclear. Remarkably, and in analogy to Ufd2, both PNGase and Ataxin-3 link p97 with the 26S proteasome by means of interacting with the human Rad23 homologues HHR23A and B [41, 83, 86, 87]. This tight coupling of Cdc48/p97 segregase activity with the 26S proteasome is likely to ensure an efficient downstream turnover of Cdc48/p97 substrates destined for degradation.

The UBX(-like) proteins VCIP135 and Otu1: substrate-processing DUBs

The human UBX(-like) protein VCIP135 is a DUB of the Otubain family and so far the only cofactor identified to cooperate with the p97^{p47} and p97^{p37} complexes in membrane fusion events [53, 88]. The

deubiquitylation of a presumably oligo-ubiquitylated, unknown $p97^{p47}$ substrate by VCIP135 is required for the reassembly of mitotic Golgi fragments *in vitro* [21, 53]. VCIP135 has also been shown to cooperate with the $p97^{p37}$ complex in maintaining Golgi and ER membranes during interphase [30]. Intriguingly, the DUB activity of VCIP135 is dispensable in the context of $p97^{p37}$ function, consistent with the ubiquitylation-independent activity of $p97^{p37}$ in membrane fusion processes. This raises the interesting possibility that VCIP135 combines two distinct substrate-processing activities: a general, still uncharacterized function required for $p97^{p47}$- and $p97^{p37}$-dependent processes, and the DUB activity specifically required for deubiquitylation of $p97^{p47}$ substrates.

In contrast to VCIP135, the yeast Otubain family member Otu1 has been characterized in the context of $Cdc48^{Ufd1\text{-}Npl4}$-dependent degradation processes [52], where it participates in the aforementioned antagonistic interplay of Ufd2 and Ufd3. Otu1 can bind to Cdc48 simultaneously with Ufd3 and remove preexisting ubiquitin tags from substrates stabilized by Ufd3 [52], thereby preventing their degradation. Obviously, this stabilizing effect of Otu1 and Ufd3 only makes sense for substrates that are still functional, as is the case for the transcription factor Spt23 in the OLE pathway studied by Rumpf and Jentsch [52]. In ERAD, in contrast, Cdc48/p97 substrates have already been identified as misfolded by the ER quality control system and are destined for degradation. This would make any antagonistic function of Otu1 and/or Ufd3 dispensable, if not undesirable. In accordance with this consideration, Ufd2 is involved in the degradation of ERAD substrates [80, 89] and is found in one complex with $Cdc48^{Ufd1\text{-}Npl4}$ and Ubx2, while Ufd3 is not [our unpublished data].

Other means of regulation

Although most Cdc48/p97 cofactors can be classified according to the categories substrate-recruiting and –processing, additional mechanisms to regulate Cdc48/p97 activities exist. One possibility is to control the oligomeric state of Cdc48/p97. Such a function has been described for the TUG homolog Pux1 from *Arabidopsis thaliana*, which disassembles hexameric AtCdc48 complexes into smaller units [46, 90]. *PUX1* mutants display accelerated plant growth, but specific cellular pathways regulated by AtPux1 have not yet been identified.

The rat protein SVIP (small VCP-interacting protein) is a non-UBX p97/Cdc48 interactor anchored to microsomal membranes, which has been shown to be mutually exclusive with p47 and Ufd1-Npl4 in p97 binding [70]. As SVIP comprises only 76 amino acids, it is unlikely to be a classical substrate-recruiting cofactor. Rather, it appears to act as a negative regulator of p97 in the ERAD pathway [91], perhaps by interfering with the formation of a stable $p97^{Ufd1\text{-}Npl4}$ complex at the ER.

Concluding remarks

Many members of the UBX protein family are still uncharacterized. As regulatory cofactors, their functional analysis provides an excellent starting point for the elucidation of novel cellular functions of Cdc48/p97. In particular, studying UBX proteins which are only present in higher eukaryotes, like Rep-8 and UBXD3, will help to reveal interesting new Cdc48/p97-dependent processes specific to multicellular organisms.

For some mammalian UBX proteins, cellular functions have already been described, yet the involvement of Cdc48/p97 has not been addressed. The putative mammalian homologue of Pux1, TUG, binds to the GLUT4 glucose transporter and regulates its plasma membrane localization [38, 92], suggesting a role of Cdc48/p97 in vesicular transport to the plasma membrane. Besides its characterization as substrate recruitment factor in proteolysis pathways [43], the human FAF1 protein has been implicated in apoptosis and the NFκB pathway [93–98]. Furthermore, the rat UBX protein Socius has been found to interact with Rho GTPases and was suggested to be involved in the organization of the actin cytoskeleton [99].

In conclusion, even though some principles governing the regulation of Cdc48/p97 activities by UBX and non-UBX cofactors have emerged, much remains to be learned about the cellular pathways controlled by specific cofactors and about the interplay of different cofactors at the molecular level. And how many Cdc48/p97 cofactors still await identification?

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