Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation

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Known activities of the ubiquitin-selective AAA ATPase Cdc48 (p97) require one of the mutually exclusive cofactors Ufd1/Npl4 and Shp1 (p47). Whereas Ufd1/Npl4 recruits Cdc48 to ubiquitylated proteins destined for degradation by the 26S proteasome, the UBX domain protein p47 has so far been linked exclusively to nondegradative Cdc48 functions in membrane fusion processes. Here, we show that all seven UBX domain proteins of *Saccharomyces cerevisiae* bind to Cdc48, thus constituting an entire new family of Cdc48 cofactors. The two major yeast UBX domain proteins, Shp1 and Ubx2, possess a ubiquitin-binding UBA domain and interact with ubiquitylated proteins *in vivo*. Δ shp1 and Δ ubx2 strains display defects in the degradation of a ubiquitylated model substrate, are sensitive to various stress conditions and are genetically linked to the 26S proteasome. Our data suggest that Shp1 and Ubx2 are adaptors for Cdc48-dependent protein degradation through the ubiquitin/proteasome pathway.

Keywords: VCP; Sel1; Rpn10; VCIP135; UFD

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INTRODUCTION

The abundant, highly conserved AAA ATPase Cdc48 (p97 in mammals) is involved in a variety of important cellular processes including membrane fusion, ubiquitin-mediated protein degradation and mitotic spindle disassembly (Woodman, 2003; Cheeseman & Desai, 2004). The common function of Cdc48 underlying these processes is believed to be the ATP-dependent disassembly of oligomeric substrate proteins (Zhang *et al*, 2002), whereas the specificity of this 'segregase' activity is determined by distinct, mutually exclusive cofactors (Meyer *et al*, 2000). The heterodimeric Ufd1/Npl4 cofactor is required for Cdc48 functions related to the ubiquitin/proteasome pathway, including endoplasmic reticulum (ER)-associated degradation (ERAD; Hampton, 2002), mobilization of the processed transcription factor SPT23 (Rape *et al*, 2001) and protein degradation through the ubiquitin fusion degradation (UFD) pathway (Johnson *et al*, 1995; Rape *et al*,

2001). The alternative cofactor p47 recruits Cdc48 to the postmitotic fusion of Golgi and ER membranes (Kondo *et al*, 1997; Roy *et al*, 2000). Interestingly, nuclear envelope assembly in *Xenopus* egg extracts requires p97^{p47} and p97^{Ufd1/Npl4} activities (Hetzer *et al*, 2001). Recently, p47 was shown to bind ubiquitylated proteins through an N-terminal ubiquitin-associated (UBA) domain (Meyer *et al*, 2002), a ubiquitin-binding module also present in proteins involved in the ubiquitin/proteasome pathway, including Rad23 and Dsk2 (Hofmann & Bucher, 1996; Buchberger, 2002). The UBA domain of p47 is required for efficient p97^{p47}-mediated Golgi membrane fusion *in vitro* (Meyer *et al*, 2002), suggesting the existence of a critical, ubiquitylated substrate(s) in that assay. It was therefore postulated that Ufd1/Npl4 targets to Cdc48 multiubiquitylated substrates destined for proteasomal degradation, whereas p47 recruits monoubiquitylated substrates that are not to be degraded, perhaps even protecting them from multiubiquitylation (Meyer *et al*, 2002; Wang *et al*, 2004).

p47 and its yeast homologue Shp1 possess a 'ubiquitin regulatory X' (UBX) domain. The UBX domain displays high structural similarity with ubiquitin and defines a large, evolutionarily conserved protein family (Buchberger *et al*, 2001). Despite their wide distribution, functional data on UBX proteins are very limited. In particular, a potential involvement of UBX proteins other than Shp1/p47 in Cdc48-mediated processes has not been addressed systematically.

In an effort to elucidate cellular functions of UBX proteins, we started to characterize the seven family members of *Saccharomyces cerevisiae*, which we propose to name as Shp1 (Ubx1) and Ubx2 through Ubx7 (Fig 1). Here, we establish that all seven UBX proteins bind Cdc48, suggesting that the UBX domain is a general Cdc48-binding module. Moreover, a subset of UBX proteins possessing an amino-terminal UBA domain binds ubiquitylated proteins *in vivo*. Importantly, we show that Shp1 and Ubx2 are involved in proteasomal protein degradation, thus demonstrating for the first time that they are adaptors for degradative Cdc48 activities.

RESULTS

All yeast members of the UBX family interact with Cdc48

Shp1 has been shown to bind Cdc48 (Braun *et al*, 2002). To test whether Cdc48 binding is a general property of UBX domain

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proteins, we performed two-hybrid assays with all seven yeast UBX proteins. Indeed, all seven proteins interacted with Cdc48, as indicated by the ability of the tester yeast strain to grow on selective medium lacking histidine (Fig 2A). To confirm the interaction with Cdc48 at endogenous expression levels, we performed immunoprecipitation experiments with strains expressing chromosomally epitope-tagged versions of UBX proteins under control of their own promoters. Consistent with the two-hybrid data, Cdc48 was co-precipitated with all seven UBX proteins (Fig 2B). Taken together, our data from two different experimental approaches indicate that Cdc48 binding is a common feature of UBX proteins.

The UBX domain is a key determinant of Cdc48 binding

The UBX domain is the only sequence motif shared by all seven UBX proteins, and thus a likely general Cdc48-binding module. To test this hypothesis, carboxy-terminally truncated mutants of Shp1, Ubx2, Ubx3 and Ubx5 lacking the UBX domain were analysed for Cdc48 interaction in two-hybrid assays (Fig 3A). In comparison with the full-length protein, Shp1^{ΔUBX} showed significantly weaker but detectable binding to Cdc48, as indicated by growth on medium lacking histidine, but not under more stringent selection on medium lacking adenine. In contrast, the truncated versions of the other three UBX proteins completely lost their ability to bind Cdc48. Next, we tested the ability of isolated UBX domains to interact directly with Cdc48 in GST pulldown assays using recombinant proteins (Fig 3B). The UBX domains of Shp1, Ubx5 and Ubx7 bound Cdc48 very strongly, whereas that of Ubx4 showed only a weak interaction (Fig 3B, right panel). Unfortunately, we could not test the UBX domains of the other three UBX proteins in this assay because we were unable to express them in a soluble form. In summary, we show that the UBX domain is sufficient for binding of Shp1, Ubx4, Ubx5 and Ubx7 to Cdc48, and necessary in the case of Ubx2, Ubx3 and Ubx5, suggesting that the UBX domain is a general binding module for Cdc48.

Fig 1 | UBX proteins of *S. cerevisiae*. UBX (red) and UBA (yellow) domains are labelled. Significant homology outside these domains (Buchberger *et al*, 2001) is indicated by similar colours. The corresponding open reading frame (ORF) names are: Shp1 (alias Ubx1; YBL058W), Ubx2 (alias Sel1; YML013W), Ubx3 (YDL091C), Ubx4 (YMR067C), Ubx5 (YDR330W), Ubx6 (YJL048C) and Ubx7 (YBR273C).

UBA/UBX proteins bind ubiquitylated proteins *in vivo*

Shp1, Ubx2 and Ubx5 have an N-terminal, variant UBA domain that is functional in ubiquitin binding *in vivo* and *in vitro*, with an apparent preference for oligo- and multiubiquitin chains (supplementary Fig S1 online). We tested the ability of full-length Shp1, Ubx2 and Ubx5 at endogenous expression levels to interact with ubiquitylated proteins *in vivo*. Upon immunoprecipitation of the three UBA/UBX proteins, a high-molecular-weight ubiquitin-reactive smear indicative of ubiquitylated proteins was co-precipitated with Shp1, Ubx2 and Ubx5 (Fig 4A). In a reciprocal experiment, we used strains expressing a myc-tagged version of ubiquitin. Again, Ubx2, Shp1 and Ubx5 co-precipitated with ubiquitylated proteins (Fig 4B).

To extend our analysis to a defined ubiquitylated substrate, we performed immunoprecipitation experiments in strains expressing ubiquitin–proline–β-galactosidase (Ub–P–βGal), a well-established model substrate of the UFD degradation pathway (Johnson *et al*, 1995). Shp1 and Ubx2 were co-precipitated with Ub–P–βGal and vice versa (Fig 4C,D), whereas no interaction could be detected between Ubx5 and Ub–P–βGal (data not shown). Together, these results establish that the UBA/UBX proteins Shp1 and Ubx2 bind ubiquitylated proteins *in vivo*.

Fig 2 | All yeast members of the UBX family interact with Cdc48.

(A) Two-hybrid analysis. Combinations of fusion proteins of the GAL4 activation domain (AD) and DNA-binding domain (BD) with Cdc48 and the indicated UBX proteins were expressed in PJ69-4a. Growth on SC-Leu-Trp-His plates (-His) indicates two-hybrid interaction.

(B) Coimmunoprecipitation. Lysates of yeast strains expressing 3HA-epitope-tagged UBX proteins were subjected to anti-HA immunoprecipitation (IP), followed by immunoblotting (WB) using anti-HA and anti-Cdc48 antibodies as indicated. The positions of UBX proteins (asterisks) and Ig heavy chains (HC) are indicated. The parental YPH499 strain served as negative control (WT).

Fig 3 | The UBX domain is a key determinant of Cdc48 binding. (A) Two-hybrid interactions between Cdc48 and full-length or C-terminally truncated UBX proteins were analysed as described for Fig 2A. Growth on SC-Leu-Trp-His plates (–His) indicates two-hybrid interaction; and growth on SC-Leu-Trp-Ade plates (–Ade) indicates strong interaction. The C-terminally truncated UBX proteins span the following amino-acid residues: Shp1^{ΔUBX}, 1–348; Ubx2^{ΔUBX}, 1–430; Ubx3^{ΔUBX}, 1–359; Ubx5^{ΔUBX}, 1–419. (B) Direct binding of isolated UBX domains to Cdc48. *In vitro* binding of recombinant Cdc48 to beads loaded with GST alone (–) or the indicated GST-UBX domain fusion proteins was detected after SDS-PAGE by staining with Coomassie brilliant blue (CBB; left panel) or anti-Cdc48 western blot (WB; right panel). Equal loading of the beads with GST proteins was confirmed by Coomassie staining (CBB) of the gel (left panel). For the western blot, only 5% of GST–Shp1^{UBX}, GST–Ubx5^{UBX} and GST–Ubx7^{UBX} as compared to GST–Ubx4^{UBX} and the GST control were loaded to avoid excessive signal strength (right panels). The isolated UBX domains comprise the following residues: Shp1^{UBX}, 341–423; Ubx4^{UBX}, 270–358; Ubx5^{UBX}, 410–500; Ubx7^{UBX}, 207–295.

Shp1 and Ubx2 are involved in protein degradation

The interaction of Shp1 and Ubx2 with bona fide multiubiquitylated proteins and with the UFD substrate Ub–P–βGal suggests a role for these UBA/UBX proteins in protein degradation through the ubiquitin/proteasome pathway. To directly test this hypothesis *in vivo*, we performed pulse-chase experiments to compare the half-life of Ub–P–βGal in wild-type, *Δshp1*, *Δubx2* and *ufd1-2* strains (Fig 5A). In the wild type, Ub–P–βGal was short-lived with

an apparent half-life of 9 min. In contrast, in cells harbouring a classical mutation in the UFD pathway, that is, *ufd1-2*, Ub–P–βGal was strongly stabilized with an apparent half-life of more than 100 min even at permissive temperature. Importantly, deletion of *SHP1* or *UBX2* led to a significant stabilization of Ub–P–βGal (apparent half-life 20 and 25 min, respectively). To exclude the possibility that this stabilization is caused by a general proteolysis defect of *Δshp1* and *Δubx2* cells, we analysed the degradation of R–βGal, a short-lived substrate of the N-end rule pathway (Bachmair *et al*, 1986) that does not require Cdc48 (Ghislain *et al*, 1996). R–βGal showed identical degradation kinetics in wild-type, *Δshp1* and *Δubx2* cells (Fig 5B). In summary, Shp1 and Ubx2 are involved in the degradation of a Cdc48-dependent, but not of a Cdc48-independent model substrate, consistent with a role as cofactors of Cdc48 in ubiquitin-dependent protein degradation.

Links to stress tolerance and proteasomal degradation

The role of Shp1 and Ubx2 in Ub–P–βGal degradation prompted us to investigate whether *Δshp1* and *Δubx2* mutants possess defects under stress conditions generating elevated levels of aberrant proteins. Indeed, *Δshp1* cells were hypersensitive towards elevated temperature, cadmium and the amino-acid analogue fluoro-phenylalanine, whereas *Δubx2* cells exhibited a less pronounced but significant sensitivity towards cadmium and fluoro-phenylalanine (Fig 6A). In order to genetically link *SHP1* and *UBX2* to the 26S proteasome, we constructed double mutants lacking the nonessential proteasomal subunit Rpn10 and Shp1 or Ubx2. Whereas *Δrpn10* cells grew like wild type, *Δshp1Δrpn10* and *Δubx2Δrpn10* double mutants exhibited strong synthetic phenotypes under normal and stress conditions (Fig 6A). Furthermore, *Δshp1Δubx2* double mutants displayed synthetic lethality (Fig 6B). Together, our phenotypic characterization shows that Shp1 and Ubx2 possess important, overlapping functions in cellular stress tolerance that are linked to proteasomal protein degradation.

DISCUSSION

In this study, we identify UBX domain-containing proteins as a new family of Cdc48 interactors. On the basis of our data, we postulate that all UBX proteins are specificity factors recruiting Cdc48 to (as yet unknown) cellular targets. UBX proteins could confer specificity by recognition sites outside the UBX domain, and/or by distinct temporal and spatial distribution patterns inside the cell. Besides p47, VCIP135 and the yeast UBX proteins investigated in this study, further examples of UBX proteins with at least partly characterized function are the Fas-associated factor FAF-1, possessing as yet unclear roles in apoptosis and NF-κB activation (Chu *et al*, 1995; Park *et al*, 2004), and TUG, a factor regulating transport of the GLUT4 glucose transporter to the plasma membrane by an unknown mechanism (Bogan *et al*, 2003). Our data suggest that p97 might also be involved in these processes, thus further broadening the spectrum of p97 functions.

A particularly interesting subset of UBX proteins, including Shp1 (p47), Ubx2 and Ubx5, possesses an N-terminal UBA domain. We show for the first time that these UBA/UBX proteins bind ubiquitylated proteins *in vivo*. Importantly, we demonstrate a role for Shp1 and Ubx2 in protein degradation, as *Δshp1* and *Δubx2* mutants are partly defective in Ub–P–βGal degradation,

Fig 4 | UBA/UBX proteins bind ubiquitylated proteins *in vivo*. Lysates of yeast strains expressing 3HA-epitope-tagged UBA/UBX proteins alone (A), together with a myc-tagged version of ubiquitin (B), or with Ub-P-βGal under the control of a galactose-inducible promoter (C,D), were subjected to immunoprecipitation (IP). (A) Anti-HA IP, followed by WB against ubiquitin (Ub) or HA as indicated. The parental YPH499 strain served as negative control (WT). (B) Anti-myc IP of ubiquitylated proteins from cells expressing myc-ubiquitin (+ Ub^{myc}), followed by WB against myc and HA epitopes. Lysates from strains not expressing myc-ubiquitin (- Ub^{myc}) served as negative control. The positions of HA-tagged UBX proteins, ubiquitylated proteins (Ub_n) and Ig HCs are indicated. (C) Anti-βGal IP after galactose-induced (Gal) expression of Ub-P-βGal, followed by WB against βGal, Shp1 or HA as indicated. Lysates from glucose-grown cells served as negative control (Glc). (D) Anti-HA IP (HA) after galactose-induced expression of Ub-P-βGal, followed by WB against βGal or HA as indicated. Unspecific immunoglobulins served as negative control (Ig). The positions of HA-tagged UBX proteins, Ub-P-βGal, ubiquitylated Ub-P-βGal (Ub_n-P-βGal), the deubiquitylation product P-βGal and Ig HCs are indicated. A prominent 90 kDa degradation product of Ub-P-βGal is marked by an asterisk. Bands crossreacting with the HA antiserum are marked by closed arrowheads.

sensitive to stress conditions generating aberrant proteins and synthetic defective with *Δrpn10*. Our data for Shp1 appear to contradict conclusions from previous work claiming that rat p47 binds and acts on monoubiquitylated proteins (Meyer *et al*, 2002; Wang *et al*, 2004). However, Meyer *et al* did not directly compare binding of p47 to proteins carrying ubiquitin chains of varying length. In fact, they show that immobilized p47 binds multi-ubiquitylated proteins from HeLa cell extracts, albeit less efficiently than Ufd1/Npl4 (Meyer *et al*, 2002). Furthermore, a recent study showed that downregulation of p47 in mammalian cells results in slightly elevated levels of ubiquitylated proteins (Wojcik *et al*, 2004). Finally, while this manuscript was under revision, an involvement of the fission yeast Shp1 homologue in ubiquitin-dependent protein degradation was reported (Hartmann-Petersen *et al*, 2004). Taken together, it appears that Shp1/p47 can recruit ubiquitylated proteins to degradative as well as nondegradative activities of Cdc48.

Whereas our work concentrated on the two major UBA/UBX proteins in yeast, Shp1 and Ubx2, a recent study by Decottignies

et al (2004) on Ubx4, Ubx6 and Ubx7 showed, in agreement with our results, that all three UBX proteins bind Cdc48, and that the UBX domain of Ubx7 is sufficient for Cdc48 binding. Whereas single and double mutants in these UBX proteins did not exhibit significant phenotypes, a *Δubx4Δubx6Δubx7* triple knockout strain showed a pronounced sporulation defect. Interestingly, the *Δubx4Δubx6Δubx7* mutant was also found to stabilize Ub-P-βGal, suggesting some redundant role of Ubx4, Ubx6 and Ubx7 in proteasomal degradation. As none of the three UBX proteins possesses a known ubiquitin-binding motif, their role in Ub-P-βGal degradation remains unclear. It is possible that these UBX proteins recruit further ubiquitin-binding proteins to Cdc48. However, indirect effects of the triple mutation on proteasomal degradation cannot currently be excluded.

Given the accumulated evidence for the involvement of UBX proteins in the ubiquitin/proteasome pathway, Ufd1/Npl4 can no longer be considered the only adaptor for Cdc48 activities involving protein degradation. Nevertheless, a principal role of Ufd1/Npl4 is suggested by the fact that *UFD1* and *NPL4* are

Fig 5 | Shp1 and Ubx2 are involved in the degradation of Ub-P-βGal. DF5 wild-type (WT) and $\Delta shp1$, $\Delta ubx2$ and $ufd1-2$ mutant cells expressing Ub-P-βGal (A) or R-βGal (B) were pulse-labelled with [35 S]methionine, followed by a chase with excess unlabelled methionine and cycloheximide. After the indicated chase times, βGal was immunoprecipitated and analysed by SDS-PAGE followed by autoradiography. A characteristic degradation product is marked (asterisk). The bottom panels show the quantification by PhosphorImager analysis. In (A), the mean values with standard deviation from three independent experiments are shown.

essential genes in *S. cerevisiae*, and that much higher amounts of multiubiquitylated proteins are bound to Ufd1/Npl4 as compared to p47 (Meyer et al, 2002). Also, the defect in Ub-P-βGal degradation is much stronger in $ufd1-2$ cells than in $\Delta shp1$ or $\Delta ubx2$ cells (Fig 5A). We interpret this fact to indicate that Ufd1/Npl4 is an essential component of the UFD pathway, whereas the

two UBX proteins play auxiliary roles by recruiting excess Ub-P-βGal to Cdc48 in an experimental setting that likely saturates the Cdc48^{Ufd1/Npl4} complex. Specific cellular targets of UBX proteins remain to be identified.

Given the large number of UBX proteins and the existence of additional, unrelated cofactors of Cdc48 such as UFD2, UFD3

Fig 6 | Shp1 and Ubx2 are linked to stress tolerance and proteasomal degradation. (A) Shp1 and Ubx2 are required for growth under stress conditions. Serial dilutions of WT, $\Delta shp1$, $\Delta ubx2$ and $\Delta rpn10$ single mutants, and $\Delta shp1\Delta rpn10$ and $\Delta ubx2\Delta rpn10$ double mutants were grown on YPD or SD agar plates containing the indicated additions. (B) Synthetic lethality of $\Delta shp1\Delta ubx2$. Upon tetrad dissection, viable $\Delta shp1\Delta ubx2$ cells were only obtained if Shp1 (YC-SHP1) or Ubx2 (YC-UBX2) were provided from a plasmid carrying a URA marker (SC-Ura). Forced loss of the plasmid on 5-FOA plates rendered the $\Delta shp1\Delta ubx2$ cells inviable.

and SVIP, a highly variable regulatory network for degradative as well as nondegradative Cdc48 functions must be expected to be operative *in vivo*. The identification of regulatory mechanisms and further substrates will be the next step towards a better understanding of Cdc48 functions in the cell.

METHODS

Cloning and yeast techniques. Standard protocols were followed for general yeast techniques, two-hybrid assays and strain constructions. Yeast and *Escherichia coli* expression plasmids were constructed using standard cloning techniques; details are available upon request from the authors. Strains and plasmids used in this study are listed in supplementary Tables 1 and 2 online, respectively.

Immunoprecipitations. Yeast cells (100 ml, $OD_{600} = 0.8$) expressing 3HA-tagged UBX proteins and/or myc-tagged ubiquitin were harvested and lysed using glass beads in buffer A (50 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamide, 20 mM N-ethyl-maleimide (NEM) and 20 μM MG132). Lysates were incubated for 3 h in buffer A containing 0.3% NP-40 with 2 μg monoclonal anti-HA antibody or polyclonal anti-myc antibody (Santa Cruz) coupled to protein A Sepharose beads (Amersham). For immunoprecipitations with strains expressing Ub-P- β Gal, lysates were prepared in buffer B (50 mM Tris (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.1% NP-40, 2 mM PMSF, 2 mM benzamide, 20 mM NEM and 20 μM MG132), precleared for 1 h with protein A sepharose beads, and incubated overnight with 4 μg anti- β Gal antibody (Promega) coupled to protein A Sepharose beads. Bound proteins were analysed by immunoblotting against Cdc48 (rabbit anti-Cdc48, gift from S. Jentsch), ubiquitin (monoclonal anti-Ub, Santa Cruz), β Gal, or the respective epitope tag used for precipitation.

In vitro Cdc48-binding assay. Cdc48 and GST fusion proteins of UBX domains were purified from *E. coli* according to standard protocols. Glutathione beads (10 μl) (Amersham) in buffer C (1 $\times$ TBS, 0.1% Triton X-100) were saturated with GST-UBX domains at 4 °C and washed with buffer C. Cdc48 (10 μg) was incubated with the loaded beads for 1 h at 4 °C. The beads were extensively washed with buffer C, and bound Cdc48 was detected after SDS-PAGE by Coomassie staining or anti-Cdc48 immunoblot.

Pulse-chase experiments. To determine the *in vivo* half-life of β Gal at 30 °C, cells (15 ml, $OD_{600} = 1.0$) were harvested, resuspended in 1 ml SCGal-Met-Ura medium and labelled with

150 μCi [^{35}S]methionine for 4 min. Cells were washed twice with 1 ml SCGal medium and resuspended in 5 ml SCGal containing 0.5 mg/ml cycloheximide. Aliquots of 1 ml were harvested after chase times of 0, 10, 20 and 30 min, and subjected to immunoprecipitation with monoclonal β Gal antibodies. After SDS-PAGE, [^{35}S] β Gal was detected by autoradiography and quantified on a PhosphorImager.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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