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Cdc48-independent proteasomal degradation coincides with a reduced need for ubiquitylation

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Ubiquitin fusion degradation (UFD) substrates are delivered at the proteasome by a handover mechanism involving the ubiquitin-selective chaperone Cdc48 and the ubiquitin shuttle factor Rad23. Here, we show that introduction of a 20 amino acid peptide extension not only rendered degradation independent of Cdc48, in line with the model that this chaperone is involved in early unfolding events of tightly folded substrates, but at the same time relieved the need for efficient polyubiquitylation and the ubiquitin shuttle factor Rad23. Removal of the ubiquitylation sites in the N-terminal UFD signal made the degradation of this substrate strictly dependent on the peptide extension and also on Cdc48 and, importantly the presence of a functional ubiquitylation machinery. This suggests that the extension in the absence of N-terminal ubiquitylation sites is not properly positioned to engage the unfoldase machinery of the proteasome. Thus the need for efficient ubiquitylation and Cdc48 in facilitating proteasomal degradation are tightly linked but can be bypassed in the context of UFD substrates by the introduction of an unstructured extension. Our data suggest that polyubiquitin-binding complexes acting upstream of the proteasome, rather than the proteasome itself, can be primary determinants for the level of ubiquitylation required for protein degradation.

Conjugation of ubiquitin chains to lysine residues is a post-translational modification that is best known for its decisive role in proteasomal degradation[1]. These chains are conjugated to proteins designated for degradation through the concerted action of ubiquitylation enzymes[2]. In sharp contrast to the topological diversity in ubiquitin chains that can target proteins for proteasomal degradation[3], a common feature appears to be the presence of ubiquitin chains that consists of at least four ubiquitin monomers. On the contrary, conjugation of a single ubiquitin, a process known as monoubiquitylation, has been implicated in a number of non-proteolytic ubiquitin-dependent processes, including membrane trafficking, DNA repair, and transcriptional regulation[4].

An attractive explanation for the dependency of proteasomal degradation of folded proteins on polyubiquitylation is the preferential binding of ubiquitin chains to the 19S regulatory particle of the proteasome[5]. The proteasome, a large proteolytic complex that is responsible for the regulated hydrolysis of proteins, contains at least two intrinsic ubiquitin binding subunits, Rpn10[6] and Rpn13[7,8]. Using a panel of synthetically ubiquitylated ubiquitin fusion degradation (UFD) substrates, it has been shown that a chain consisting of four Lys48-linked ubiquitin monomers represents the minimal signal for proteasomal degradation, which correlates with a dramatically increased affinity of the substrate for the proteasome particle[5]. However, the observations of exceptional cases in which monoubiquitylation[9] or multiple-monoubiquitylation[10] suffices for proteasomal degradation, show that ubiquitin monomers can facilitate interaction with the proteasome. Recently, it has been shown that polypeptides consisting of no more than approximately 150 amino acid residues can be targeted for proteasomal degradation by introduction of a ubiquitin monomer whereas larger proteins require polyubiquitin chains suggesting that the complexity of the substrate, and not the ubiquitin binding sites at the proteasome, may dictate the required minimal length of the polyubiquitin chain[11]. Moreover, proteasomes are able to degrade denatured proteins *in vitro* suggesting that ubiquitin as such is not a prerequisite for processing of substrates by the proteasome[12].

In addition to proteasome subunits, ubiquitin binding sites are also present in many other proteins involved in ubiquitin-dependent proteasomal degradation. For example, the ubiquitin receptors Rad23 and Dsk2, which shuttle substrates to the proteasome, contain ubiquitin binding domains by which they sequester polyubiquitylated proteins[13]. Other important proteins involved in ubiquitin-dependent degradation are the yeast Cdc48 and its metazoan ortholog p97 (also known as valosin-containing protein), which, like the proteasomal ATPases,

belong to the family of AAA-ATPases[14]. Cdc48/p97 forms a homohexameric ring that associates with several co-factors linking these ATPases to diverse cellular processes, most importantly proteasomal degradation[15], ubiquitin-dependent extraction from chromatin[16], homotypic membrane fusion[17] and macroautophagy[18,19]. It has been proposed that Cdc48, in concert with its ubiquitin-binding dimeric co-factor Ufd1/Npl4[20,21], is part of a pathway comprising a series of ubiquitin-interacting factors that, in a handover mechanism, escorts substrates to the proteasome[22].

Although $Cdc48^{Ufd1/Npl4}$ has originally been identified as a complex that is required for the degradation of cytosolic and nuclear UFD substrates[15], most studies have focused on its role in the proteasomal destruction of proteins derived from the endoplasmic reticulum (ER)[23], a process known as ER-associated degradation (ERAD)[24]. A large body of evidence suggests that Cdc48/p97 mediates the extraction of ERAD substrates from the ER membrane prior to proteasomal degradation in the cytosol[25,26]. In contrast, its functional significance in the degradation of soluble proteins is less clear. Taking into consideration the unfoldase activity of Cdc48[27], and the fact that it acts upstream of proteasomal degradation[22], it is tempting to speculate that this chaperone plays a role in initiating the unfolding of substrates prior to their interaction with the proteasome. Since proteasomes require unstructured initiation sites to engage their intrinsic unfoldase machinery[28] and stimulate the ATPase activity of the proteasomal AAA-ATPases[29], the primary role of $Cdc48/p97^{Ufd1/Npl4}$ may be the generation of loosely folded structures that can be subsequently used by the proteasome to further unravel the substrate[30].

Here we show that the need for efficient UFD-mediated polyubiquitylation and the ubiquitin-dependent chaperone Cdc48 can be simultaneously overcome by introduction of a peptide extension that is sufficiently long to act as an unstructured initiation site. Our data underscore the importance of ubiquitylation not only in properly engaging the proteasome but also in facilitating interaction with upstream factors that prepare substrates for subsequent destruction by the proteasome.

Results

To investigate the role of Cdc48 in the degradation of soluble proteasome substrates in the budding yeast *Saccharomyces cerevisiae*, we generated a set of a commonly used and well-characterized UFD substrate[31] $ubiquitin^{Gly76Val}$-green fluorescent protein (Ub-GFP) with or without a C-terminal polypeptide extension of 15 or 20 amino acids derived from the V5-His tag (Fig. 1A). Structural prediction algorithms predict the V5-His extension to be mostly random coil[30]. Furthermore, proteins whose structures have been solved containing V5-His C-terminal extensions do not show secondary structural elements or were unsolvable for the extension[32,33]. We compared the steady-state levels of these proteins in yeast strains carrying the wild-type Cdc48 allele or the mutant allele *cdc48-6*. Notably, introduction of a 20 amino acid extension resulted in reduced steady-state levels of this substrate in wild-type yeast (Fig. 1B). All three Ub-GFP fusions accumulated upon administration of proteasome inhibitor although we did not reach the same steady-state level for Ub-GFP-20aa within the 2 hr-treatment with proteasome inhibitor consistent with its half-life being shorter than those of Ub-GFP and Ub-GFP-15aa. Even more striking, we found that while each of these fusions displayed low steady-state levels in the presence of wild-type Cdc48 and accumulated upon administration of the proteasome inhibitor MG132, only the Ub-GFP fusion with a 20 amino acid-long extension was efficiently degraded by proteasomes in the *cdc48-6* strain (Fig. 1B). Analysis of the turnover confirmed that all three fusion proteins were degraded in wild-type yeast and stabilized in the presence of proteasome inhibitor (Fig. 1C), whereas Ub-GFP with a 20 amino acid extension was rapidly degraded even in the absence of functional Cdc48 (Fig. 1D). We conclude that the introduction of a 20 amino acid-long unstructured polypeptide enhances degradation and alleviates the need for Cdc48 in proteasomal degradation of a soluble UFD substrate in yeast.

It has been proposed that the ubiquitin shuttle factor Rad23 is important to facilitate the exchange of substrates from the Cdc48 complex to the proteasome[22,34]. Interestingly, we found that the Ub-GFP fusion carrying a 20 amino-acid extension was also efficiently degraded by the proteasome in yeast lacking Rad23, whereas fusions lacking the extension or containing a 15 amino acid-long extension were more stable in the *rad23Δ* strain. (Fig. 2A, B). We argued that the lack of Rad23-dependency may reflect a change in the role of ubiquitylation in the degradation of this substrate. Therefore, we next explored whether the degradation of the Ub-GFP fusion was still dependent on the ubiquitin ligase Ufd4, which is responsible for the conjugation of polyubiquitin chains to UFD substrates[35]. Whereas the degradation of Ub-GFP and Ub-GFP-15aa was strictly dependent on this ubiquitin ligase, degradation of the fusion carrying a 20 amino acid-long extension did not require the presence of Ufd4 (Fig. 2C, D). To rule out any sequence specificity given by the extension, we inserted an unrelated extensions derived from mRFP of 15 or 20 amino acids length into Ub-GFP. Only the 20-amino acid-long, but not the 15 amino acid-long, extension abrogated the need for UFD-mediated ubiquitylation (Supplementary Fig. S1).

Our observation that Cdc48/Rad23-independent degradation of UFD substrates coincides with abrogation of the need of UFD-mediated ubiquitylation does not exclude the possibility that ubiquitylation by alternative ubiquitin ligases is still required for the degradation of these substrates. To test this, we analyzed degradation of these fusions in a yeast strain carrying a mutant allele of the ubiquitin activase Uba1, which is an essential gene critical for conjugation of ubiquitin[36]. Ubiquitylation is strongly compromised in this strain as levels of total ubiquitin conjugates are drastically reduced (Supplementary Fig. S2). Interestingly, a C-terminal extension of 20 amino acids supported degradation of the UFD substrate in the *uba1-ts 26* strain (Fig. 3A). Ubiquitin pull-down experiments showed a strong reduction in Ub-GFP fusions carrying large ubiquitin conjugates in the *uba1-ts 26* strain, which are visible as a smear over the high molecular weight range of the Western blot (Fig. 3B). However, we also noted that there was little change and even a tendency to an increase in Ub-GFP species modified with one, two or three ubiquitin moieties, reflecting multiple monoubiquitylation or short ubiquitin chains that do not support proteasomal degradation of UFD substrates[5]. The observed ubiquitylation was dependent on the presence of an N-terminal ubiquitin moiety since no modified species of GFP-20aa could be detected (data not shown). These data suggest that residual ubiquitylation activity in the conditional *uba1-ts 26* mutant strain still allows the generation of low molecular weight ubiquitin conjugates of Ub-GFP. This minimal ubiquitylation activity was only sufficient to target the Cdc48-independent fusion with a 20 amino acid polypeptide extension for degradation but failed to support degradation of the Cdc48-dependent Ub-GFP and Ub-GFP-15aa. This suggests that proteasomes are able to facilitate degradation of minimal ubiquitylated Ub-GFP fusions as long as their degradation does not require Cdc48 which coincides with a requirement for more robust ubiquitylation. Consistent with the idea that some level of ubiquitylation is still required for proteasomal degradation, we found that purified proteasomes were unable to processively degrade recombinant Ub-GFP carrying a 20 amino acid-long extension *in vitro* (Supplementary Fig. S3A). Interestingly, purified proteasomes were able to process the 15 and 20 amino acid-long C-terminal extensions, albeit with low efficiency, but this was independent of the N-terminal ubiquitin moiety (Supplementary Fig. S3B). This suggests that proteasomes have access to the polypeptide extension but in the absence of ubiquitylation fail to process the GFP moiety of the fusion. A role for residual ubiquitylation in the degradation of this fusion is also in line with an earlier study demonstrating that

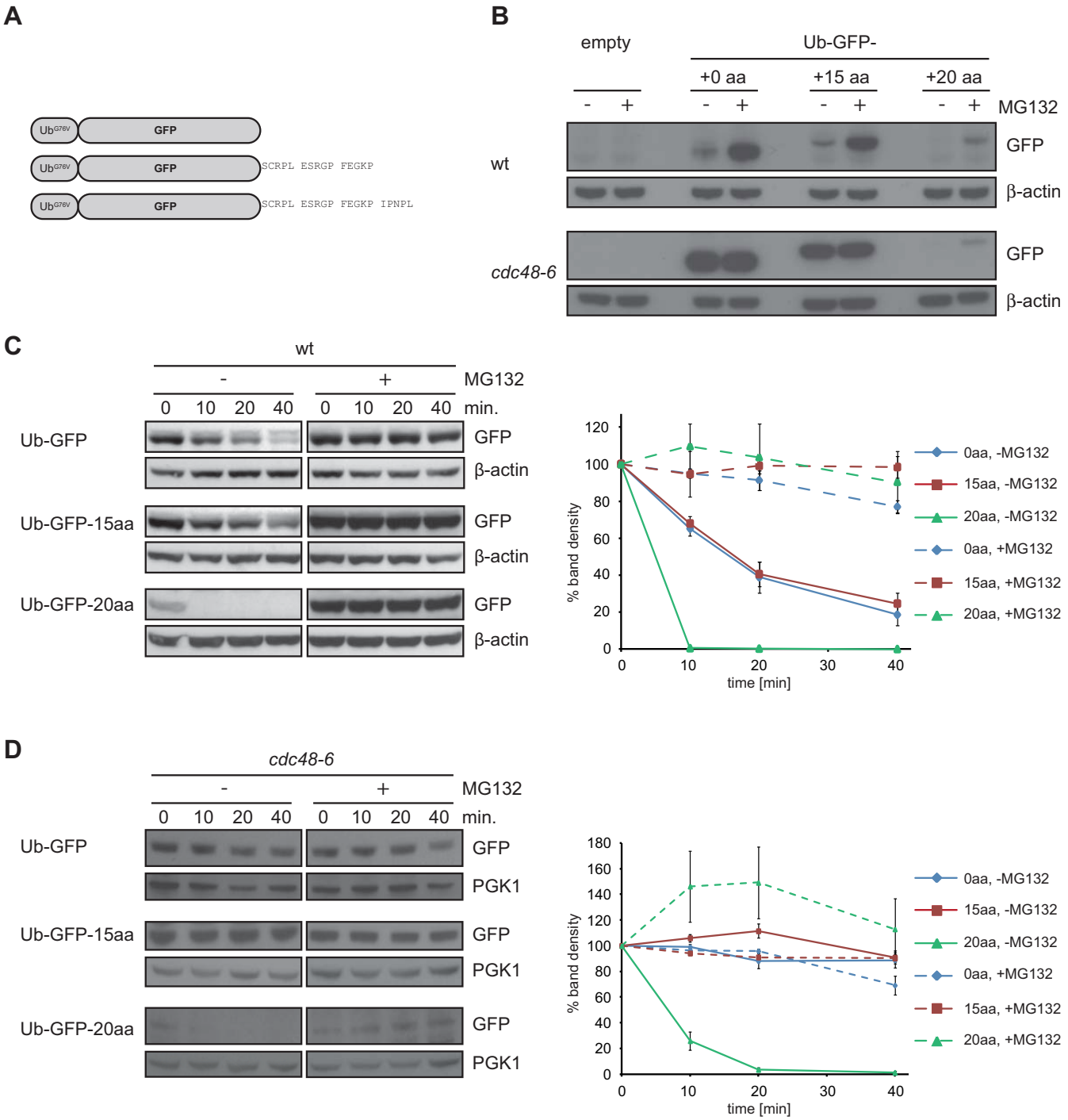

Figure 1 | C-terminal peptide extension allows proteasomal degradation of a UFD substrate independent of Cdc48. (A) Schematic representation of the different UFD substrates including the amino acid sequence of the extensions. (B) Western blot analysis showing steady-state levels of Ub-GFP, Ub-GFP-15aa and Ub-GFP-20aa in wild-type or *cdc48-6* yeast strain in the absence or presence of proteasome inhibitor MG132 using GFP antibody. β-actin was detected as loading control. (C) Cycloheximide-chase analysis of Ub-GFP, Ub-GFP-15aa and Ub-GFP-20aa turnover in wild-type yeast in the absence and presence of MG132. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. Solid lines, untreated; dashed lines, MG132-treated. (D) Same as B but in a *cdc48-6* mutant yeast strain.

only short unfolded proteins can be targeted for proteasomal degradation by monoubiquitylation[11].

To have a closer look at the role of ubiquitylation in the N-terminal UFD signal, we next investigated whether the Lys29 and Lys48 residues in the N-terminal ubiquitin moiety, which are the canonical acceptor sites for polyubiquitin chains in UFD substrates[35], were required for proteasomal degradation. Interestingly, we found that whereas Ub-GFP and Ub-GFP-15aa were stabilized when the Lys29 and Lys48 residues had been substituted for arginine residues, proteasomal degradation of the Cdc48-independent Ub-GFP-20aa substrate was not altered (Fig. 3C, D). Also an N-terminal ubiquitin in which all lysine residues had been substituted for arginine residues was still proficient to allow degradation of a GFP fusion (Ub^{K0}-GFP) but only when carrying a 20 amino acid extension (Fig. 3E). The Ub^{K0}-GFP-20aa was stabilized by administration of the inhibitor MG132 confirming that degradation of this fusion is still mediated by the proteasome. This shows that in the absence of lysine residues in the N-terminal UFD signal the degradation of the fusion becomes

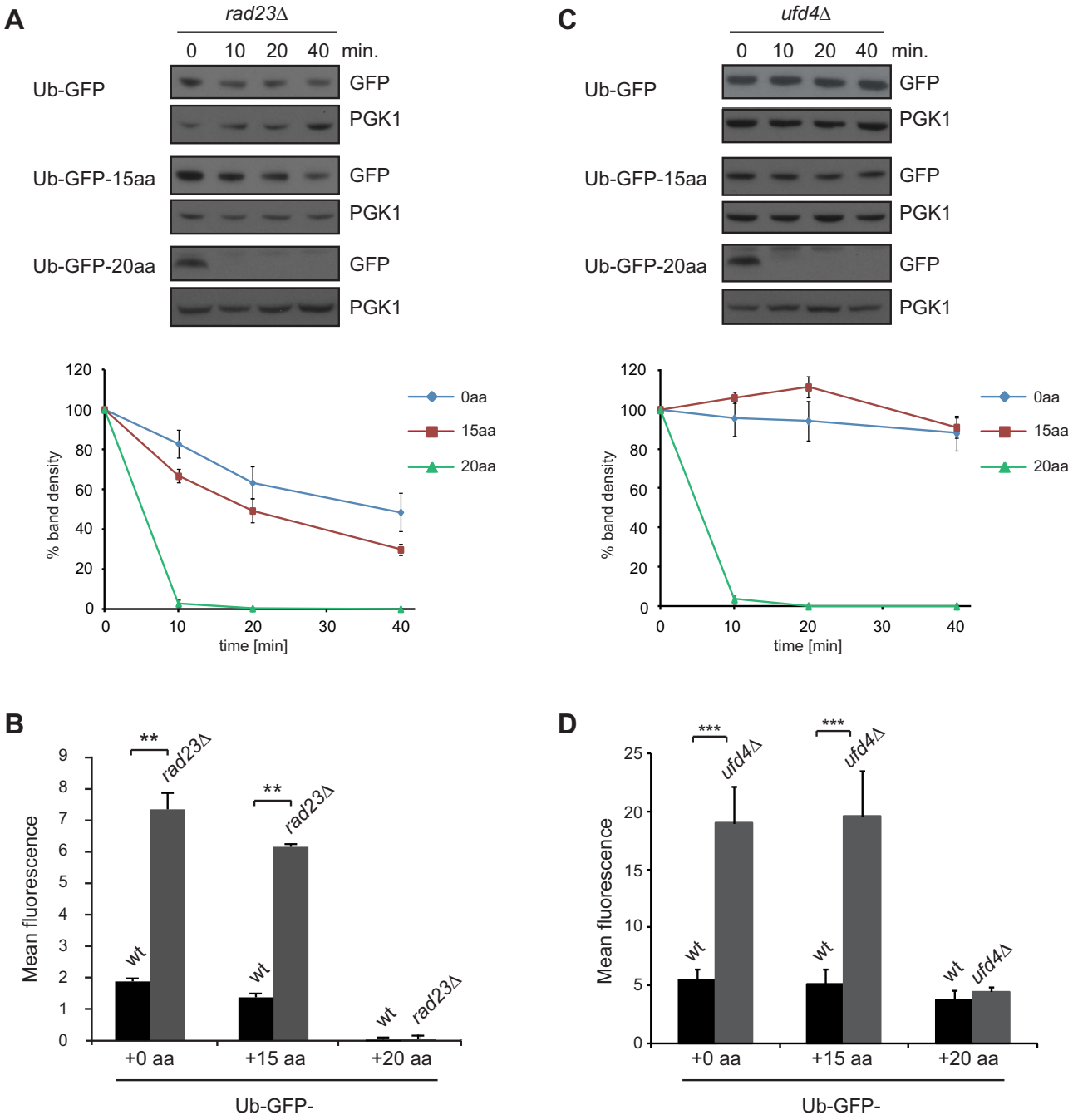

Figure 2 | C-terminal peptide extensions abrogate the need for UFD-dependent ubiquitylation. (A) Cycloheximide-chase analysis of Ub-GFP, Ub-GFP-15aa and Ub-GFP-20aa turnover in *rad23*Δ yeast strain. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. (B) FACS analysis of GFP fluorescence intensities of wild-type or *rad23*Δ yeast cells expressing Ub-GFP, Ub-GFP-15aa or Ub-GFP-20aa. For each construct, intensities of three transformants were determined. **P<0.01 (Student's *t* test). (C) Cycloheximide-chase analysis of Ub-GFP, Ub-GFP-15aa and Ub-GFP-20aa turnover in *ufd4*Δ yeast strain. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. (D) FACS analysis of GFP fluorescence intensities of wild-type and *ufd4*Δ yeast cells expressing Ub-GFP, Ub-GFP-15aa or Ub-GFP-20aa. Experiment was performed as in B. ***P<0.0001 (Student's *t* test).

strictly dependent on the C-terminal peptide extension even in wild-type yeast.

The Ub^{K0}-GFP fusion still contains a number of lysine residues in the GFP moiety of the fusion. To explore the role of ubiquitylation in the degradation of this fusion we tested the stability of the Ub^{K0}-GFP-20aa fusion in the *uba1-ts 26* strain. Unlike the Ub-GFP-20aa fusion, we found that the fusion lacking lysine residues in the N-terminal ubiquitin was stabilized in the *uba1-ts 26* strain suggesting that Ub^{K0}-GFP-20aa is still targeted for ubiquitin-dependent degradation but independent of the UFD pathway (Fig. 4A). Consistent with a possible role of UFD-independent ubiquitylation, we found that this substrate was degraded in the absence of Ufd4, but stabilized in a yeast strain lacking the ubiquitin conjugases Ubc4 and Ubc5, which cooperate with different ubiquitin ligases (Supplementary Fig. S4). The C-terminal extension is responsible for the ubiquitin-dependent degradation of the Ub^{K0}-GFP-20aa fusion since the 20 amino acid extension did indeed destabilize GFP in wild-type yeast although not to the same extent as fusions carrying a UFD signal (Fig. 4B). The

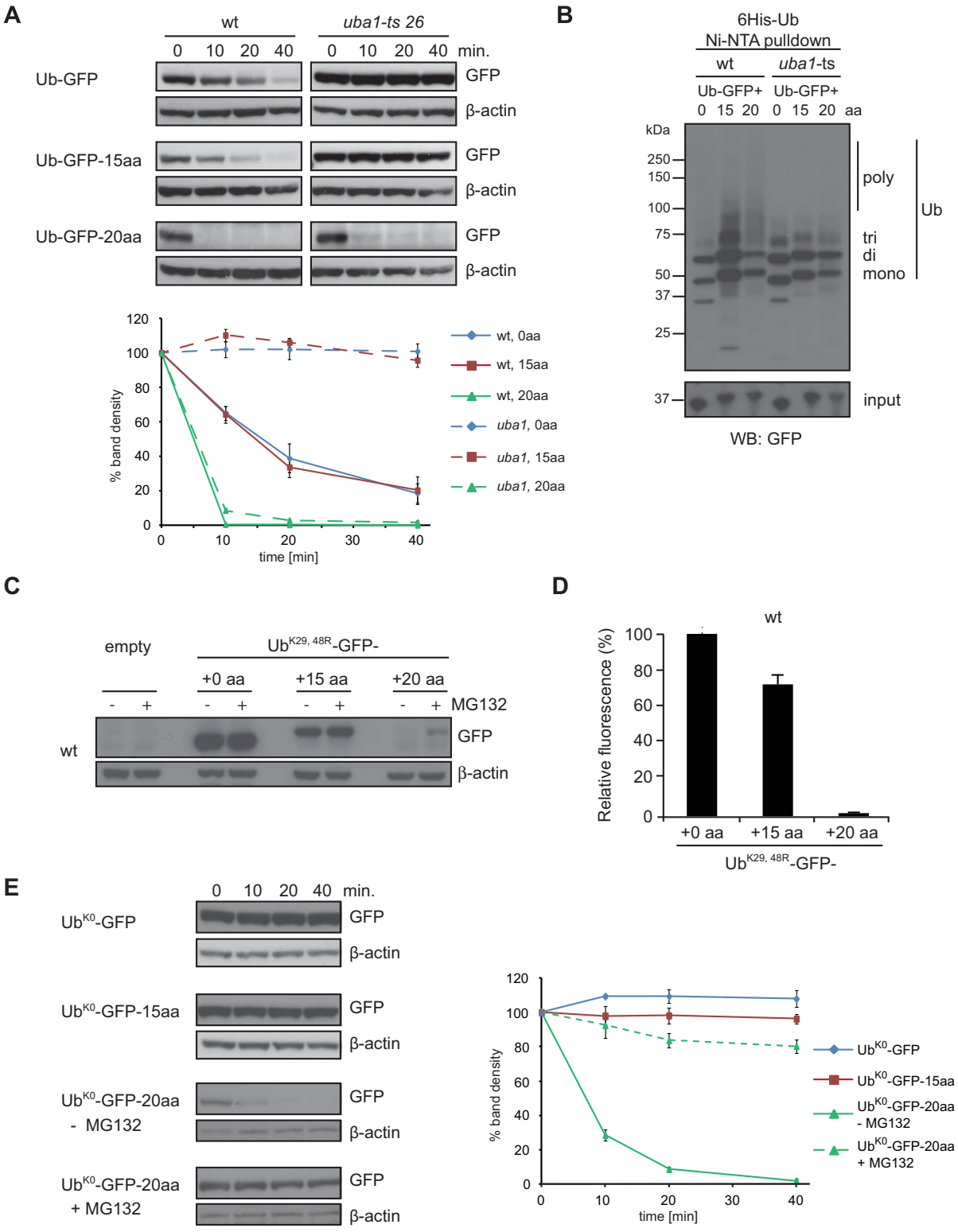

Figure 3 | A minimal level of ubiquitylation is required for Cdc48/Rad23-independent degradation of a UFD substrate with peptide extension. (A) Cycloheximide-chase analysis of Ub-GFP, Ub-GFP-15aa and Ub-GFP-20aa turnover in wild-type or in a *uba1-ts 26* mutant yeast strain. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. (B) Western blot analysis of Ni-NTA pulldown from wild-type or *uba1-ts 26* yeast cells co-expressing 6His-Ub and Ub-GFP, Ub-GFP-15aa or Ub-GFP-20aa using GFP antibody. (C) Western blot analysis showing steady-state levels of $Ub^{K29,48R}$-GFP, $Ub^{K29,48R}$-GFP-15aa and $Ub^{K29,48R}$-GFP-20aa in a wild-type yeast strain in the absence or presence of proteasome inhibitor MG132 using GFP antibody. β-actin was detected as loading control. (D) FACS analysis of GFP fluorescence intensities of wild-type yeast cells expressing $Ub^{K29,48R}$-GFP, $Ub^{K29,48R}$-GFP-15aa and $Ub^{K29,48R}$-GFP-20aa. (E) Cycloheximide-chase analysis of Ub^{K0}-GFP, Ub^{K0}-GFP-15aa and Ub^{K0}-GFP-20aa turnover in wild-type yeast. Turnover of Ub^{K0}-GFP-20aa was analyzed in the absence and presence of MG132. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. Solid lines, untreated; dashed lines, MG132-treated.

decrease in half-life of GFP was a consequence of ubiquitin-dependent degradation since GFP-20aa was stabilized in the *uba1-ts 26* strain (Fig. 4C).

Interestingly, the requirement for wild-type Uba1 to facilitate degradation of the Ub^{K0}-GFP-20aa (Fig. 4A) and GFP-20aa (Fig. 4C) coincided with a restoration of Cdc48-dependency. This supports the positive correlation between the need for efficient ubiquitylation and Cdc48-dependency, which was also observed for Ub-GFP. It also indicates that in the absence of the N-terminal ubiquitylation site (i.e. N-terminal ubiquitin without lysine residues) the 20aa extension does not suffice to bypass Cdc48. This is not unexpected as it has been shown that the functionality of unstructured initiation sites is determined not only by their length but also by their position relative to the ubiquitylation site[37]. This suggests that a C-terminal polypeptide extension is properly positioned for the proteasome when combined with an N-terminal degradation signal while in case of an internal ubiquitylation site Cdc48 may be required to generate alternative initiation sites, which re-introduces the need for efficient ubiquitylation.

Discussion

The UFD signal has been commonly used to target proteins-of-interest for ubiquitin-dependent proteasomal degradation[38]. The identification of a number of proteins that are involved in the degradation of UFD substrates, which are collectively referred to as the UFD pathway[35], has given important fundamental insights into the molecular mechanisms underlying proteasomal degradation. For example, the identification of the ubiquitin chain recognition signal[5], the discovery of the E4 multiubiquitylation enzyme[39], insights into substrate unfolding[28], the minimal length of proteasome substrates[40] and the functional significance of the proteasome binding subunit Rpn13[7,8] are all exclusively or primarily based on observations with UFD substrates. Also in the current study, it is the unique nature of the UFD substrates that allowed us to uncover a basic principle in proteasomal degradation as it enabled us to directly compare the fate of almost identical substrates carrying a single ubiquitin or, alternatively, polyubiquitin chains.

Our study provides an explanation for the central role of the UFD pathway in the degradation of substrates that are structurally different from the engineered substrates that led to the identification of this pathway[23,41,42]. We propose that a primary role of the UFD pathway is to assure that folded substrates lacking initiation sites are first delivered to Cdc48 before they are handed over to the proteasome. Although we cannot formally exclude the possibility that the 20 amino acid extension introduces conformational changes in the UFD substrate, that may affect downstream factors such as ubiquitylation, Cdc48 and Rad23 in different ways, we consider this possibility unlikely for various reasons. As pointed out above structural prediction algorithms and structural data on V5-tagged proteins support the idea that the V5 tag is largely unstructured. Moreover, we show that ubiquitylation of Ub-GFP fusions is severely impaired in the *uba1ts-26* strain regardless of the presence of the V5 tag suggesting that the difference in the stability of the Ub-GFP-20aa in this strain is unlikely to be attributed to a difference in the

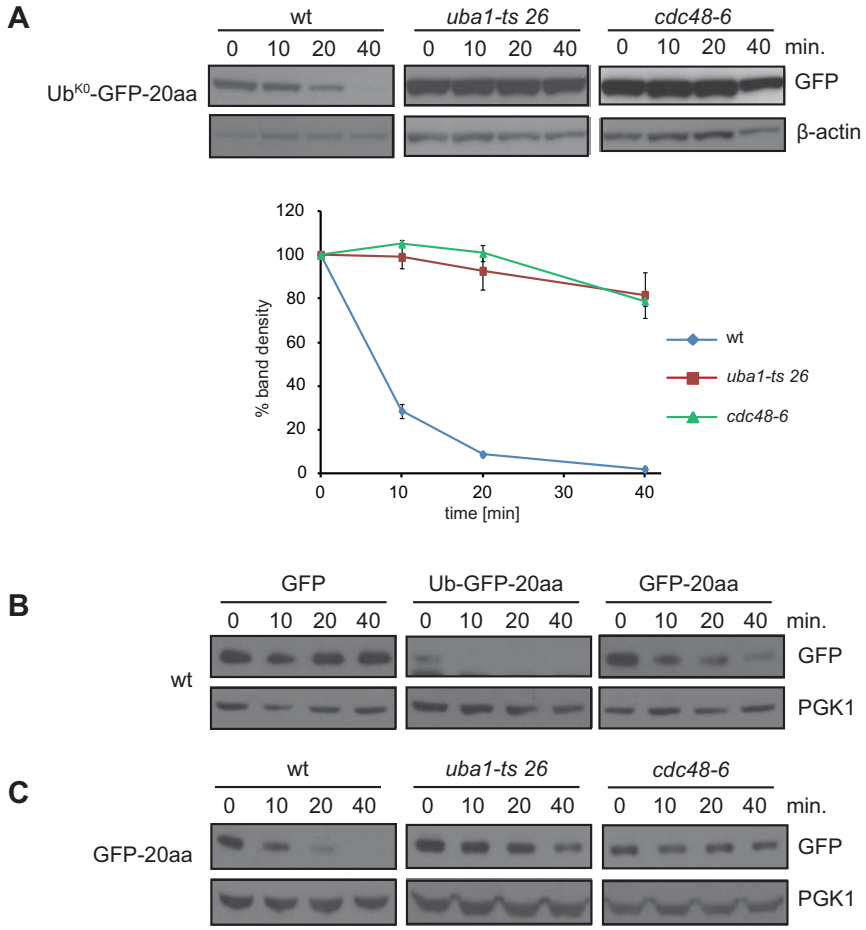

Figure 4 | Degradation of C-terminally extended substrates lacking an N-terminal ubiquitylation site requires Uba1 and Cdc48. (A) Cycloheximide-chase analysis of Ub^{K0}-GFP-20aa turnover in wild-type, *uba1ts-26* and *cdc48-6* yeast cells. Quantification of GFP levels of three independent experiments with error bars representing standard error of the mean is shown. (B) Cycloheximide-chase analysis of GFP and GFP-20aa turnover in wild-type yeast compared to Ub-GFP-20aa. (C) Cycloheximide-chase analysis of GFP-20aa turnover in wild-type, *uba1ts-26* and *cdc48-6* yeast cells.

ubiquitylation as a consequence of the V5 tag. Finally, we obtained similar results with an unrelated peptide extension consistent with the idea that the presence of a peptide extension and not an intrinsic feature specific for the V5 tag is responsible for proteasomal degradation in the absence of efficient ubiquitylation.

It remains to be seen what fraction of endogenous proteasome substrates is degraded dependent on Cdc48 and/or Rad23 but it is noteworthy that *in silico* predictions suggest that many proteins contain unstructured regions fulfilling at least the length requirement of unstructured initiation sites[43]. However, whether or not unstructured regions can function as initiation sites is not only length- but also position-dependent[28,37], which complicates the prediction of proteins that require Cdc48 for their degradation based on structural features. The N-terminal positioning of an uncleavable ubiquitin moiety in UFD substrates, which is known to dictate the preferential site of unfolding[44] and may hence confine the location for potential initiation sites, may be the main reason why this pathway has been readily and consistently identified as a common requirement for the degradation of this particular class of substrates.

It is tempting to speculate that short ubiquitin chains or monoubiquitylation may allow proteins to bypass the unfoldase action of Cdc48, which will result either in stable proteins that are not subject to proteasomal degradation (in the case of non-proteolytic ubiquitylation) or will force the proteasome to use pre-existing unfolded structures in substrates (in the case of proteolytic ubiquitylation). In this respect, it is also of interest to point out that monoubiquitylation appears to be specifically implicated in proteasomal processing of several precursor proteins, such as the membrane-tethered transcription factors Mga2 and Spt23[21], the p105 precursor of the transcription factor NF-κB[45], and the ubiquitin ligase bridging factor Def1[46]. In each of these cases it results in selective destruction of the C-terminal part of these proteins which share the presence of low complexity sequences that have been proposed to form loop-like structures from which proteasomal processing is started[47,48], in a manner that is mechanistically similar to unstructured initiation sites[28]. The fact that these processing events are targeted by means of monoubiquitylation may be of functional significance since it may bias the proteasome to pre-existing unstructured initiation sites whereas a polyubiquitin modification may allow Cdc48 to generate alternative initiation sites for degradation that will enable the proteasome to processively degrade the protein.

While our data are in line with the model that localization of a protein at the proteasome can be sufficient to facilitate proteasomal degradation[49], they suggest that this will only apply to proteins that contain unstructured initiation sites. At least one naturally occurring substrate, ornithine decarboxylase, is degraded independent of ubiquitylation in a manner that strictly depends on the presence of an unstructured region[50], similar to the substrates used in this study. Degradation of ornithine decarboxylase[51] depends not only on a domain that facilitates binding to the proteasome[52] but also on the presence of an unstructured region that has to be sufficiently long to initiate degradation[50]. The critical requirement for unstructured regions in protein degradation may also explain why some proteins can functionally operate in close proximity to the proteolytic complex without becoming subject to processive degradation[53]. Indeed, when interacting with protein complexes, proteasomes tend to preferentially degrade those proteins that carry unstructured initiation sites while leaving folded proteins unharmed[54], which has been shown to be relevant for the stable nature of ubiquitin receptors that transiently interact with the proteasome[55,56].

In conclusion, our study shows that the strict requirement for ubiquitylation in the degradation of proteasome substrates does not necessarily have to be attributed to polyubiquitin-dependent binding to the proteasome but may rather reflect involvement of polyubiquitin-specific proteins acting upstream of the proteasome.

Methods

Yeast strains and media. All experiments with wild-type and mutant strains were performed at 30°C. A detailed list of the yeast strains can be found in the supplementary information. Yeast transformed with episomal pYES2 plasmids encoding the indicated substrates were grown in synthetic medium comprising a mix of glucose, raffinose, and galactose as carbohydrate source, and then in synthetic medium containing galactose until mid-log phase. For proteasome inhibition, synthetic medium with 0.1% proline as sole nitrogen source was used and supplemented with 0.003% SDS 3 h before addition of proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132; ENZO)[57]. Cultures were incubated for 2h with 50 μM MG132.

Construction of plasmids. The plasmids were generated by introducing the Ub-GFP open reading frame into the yeast expression vector pYES2 (Invitrogen, 2μ URA3) under the regulation of the galactose-inducible GAL1 promoter[58]. The C-terminal extensions are derived from the V5 epitope tag (15aa: SCRPL ESRGP FEGKP; 20aa: SCRPL ESRGP FEGKP IPNPL) or the mRFP open reading frame (15aa: GGMAS SEDVI KEFMR, 20aa: GGMAS SEDVI KEFMR FKVRM) respectively by introducing stop codons at the indicated positions by site-directed mutagenesis (Stratagene). All constructs were verified by DNA sequencing.

Western blot analysis. Yeast expressing the indicated reporter substrate under the GAL1 promoter was grown in galactose-containing medium until mid-log phase. Protein synthesis was terminated by adding glucose (final concentration 2%) and cycloheximide (final concentration 0.1 mg/ml). One OD_{600} unit was harvested at the indicated time points and total protein extracts were prepared by lysis and precipitation in 12.5% trichloroacetic acid. Total yeast lysates were separated by SDS-PAGE (NuPAGE, Invitrogen) followed by transfer to nitrocellulose membranes (PROTRAN; Schleicher & Schuell) or PVDF membrane (Millipore). Western blot analysis was performed as described previously[56] using the following primary antibodies: a mixed monoclonal antibody specific to GFP (Roche) (1 : 2000), a polyclonal anti-GFP antibody (Ab290, Abcam) (1 : 5000), monoclonal antibodies against β-actin (Abcam) (1 : 5000) or against PGK1 (Life technologies) (1 : 5000).

Flow cytometric analysis. Yeast expressing the indicated reporter substrate was grown in synthetic medium containing galactose until mid-log phase and was subsequently subjected to flow cytometric analysis (FACScalibur, Beckton & Dickinson). CellQuest software was used to analyze the data.

Detection of ubiquitylated proteins. Yeast was co-transformed with an expression plasmid for the substrate of interest and an expression plasmid for His-tagged ubiquitin (gift from Dr. Ben Distel, AMC Amsterdam, The Netherlands). Pulldown of 6xHis-ubiquitin modified proteins was performed as described previously[56].

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Author contributions

D.G., C.H., N.P.D. designed the research; D.G., C.H., F.P.M., T.K., K.A. performed the experiments; D.G., C.H., F.P.M., T.K., K.A., N.P.D. analyzed the data; D.G., C.H., N.P.D. wrote the paper.

Additional information

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