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Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*

Michel Ghislain[1], R.Jürgen Dohmen[2], Frédéric Lévy and Alexander Varshavsky[3]

Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

[1]Present address: Unité de Biochimie Physiologique, Université catholique de Louvain, Place Croix du Sud 2-20, B-1348, Louvain-la-Neuve, Belgium
[2]Present address: Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, Geb. 26.12, D-40225, Düsseldorf, Germany

[3]Corresponding author

A library of random 10 residue peptides fused to the N-terminus of a reporter protein was screened in the yeast *Saccharomyces cerevisiae* for sequences that can target the reporter for degradation by the N-end rule pathway, a ubiquitin (Ub)-dependent proteolytic system that recognizes potential substrates through binding to their destabilizing N-terminal residues. One of the N-terminal sequences identified by this screen was used in a second screen for mutants incapable of degrading the corresponding reporter fusion. A mutant thus identified had an abnormally low content of free Ub. This mutant was found to be allelic to a previously isolated mutant in a Ub-dependent proteolytic system distinct from the N-end rule pathway. We isolated the gene involved, termed *UFD3*, which encodes an 80 kDa protein containing tandem repeats of a motif that is present in many eukaryotic proteins and called the WD repeat. Both co-immunoprecipitation and two-hybrid assays demonstrated that Ufd3p is an *in vivo* ligand of Cdc48p, an essential ATPase required for the cell cycle progression and the fusion of endoplasmic reticulum membranes. Further, we showed that, similarly to Ufd3p, Cdc48p is also required for the Ub-dependent proteolysis of test substrates. The discovery of the Ufd3p–Cdc48p complex and the finding that this complex is a part of the Ub system open up a new direction for studies of the function of Ub in the cell cycle and membrane dynamics.

Keywords: cell cycle/N-end rule/proteolysis/ubiquitin/WD repeat

Introduction

Many damaged and otherwise abnormal proteins are short-lived *in vivo*. Many regulatory proteins are metabolically unstable as well. Short lifetimes of regulatory proteins allow for rapid adjustments of their concentrations through changes in the rates of their synthesis or degradation. Features of proteins that confer metabolic instability are called degradation signals, or degrons (Varshavsky, 1991). The essential component of one degradation signal, termed the N-degron, is a destabilizing N-terminal residue of a protein (Bachmair *et al.*, 1986). A set of N-degrons containing different destabilizing residues is referred to as the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (Varshavsky, 1992, 1996). The N-end rule pathway has been found in all organisms examined, from mammals to bacteria (Bachmair and Varshavsky, 1989; Gonda *et al.*, 1989; Tobias *et al.*, 1991; Lévy *et al.*, 1996). The N-end rules of bacteria, fungi and mammals are similar but not identical (Varshavsky, 1996).

In eukaryotes, the N-degron comprises two determinants: a destabilizing N-terminal residue and a specific internal lysine (or lysines) of a protein substrate (Bachmair and Varshavsky, 1989; Johnson *et al.*, 1990; Hill *et al.*, 1993). The Lys residue is the site of formation of a substrate-linked multiubiquitin chain (Chau *et al.*, 1989), whose presence is required for the degradation of N-end rule substrates by the 26S proteasome, an ATP-dependent, multisubunit protease (Rechsteiner *et al.*, 1993; Pühler *et al.*, 1994; Hochstrasser, 1995; Jentsch and Schlenker, 1995; Hilt and Wolf, 1996). Ubiquitin (Ub) is a protein whose covalent conjugation to other proteins is involved, directly or by way of regulation, in a multitude of processes, including cell growth and differentiation, signal transduction, DNA repair and the transport of substances across membranes. In many of these processes, Ub acts through routes that involve the degradation of Ub–protein conjugates (Hershko and Ciechanover, 1992; Ciechanover, 1994; Deshaies, 1995).

The N-end rule is organized hierarchically. In eukaryotes, such as the yeast *Saccharomyces cerevisiae*, Asn and Gln are tertiary destabilizing N-terminal residues in that they function through their conversion, by the *NTA1*-encoded N-terminal amidase (Nt-amidase), into the secondary destabilizing residues Asp and Glu, whose activity requires their conjugation, by the *ATE1*-encoded Arg-tRNA–protein transferase (R-transferase), to Arg, one of the primary destabilizing N-terminal residues. The latter are bound directly by the *UBR1*-encoded 225 kDa protein called N-recognin or E3. N-recognin selects potential N-end rule substrates by binding to their primary destabilizing N-terminal residues Phe, Leu, Trp, Tyr, Ile, Arg, Lys or His (Baker and Varshavsky, 1995; Varshavsky, 1996).

The N-end rule was discovered in experiments that explored, in *S.cerevisiae*, the metabolic fate of a fusion between Ub and a reporter such as *Escherichia coli* β-galactosidase (β-gal) (Bachmair *et al.*, 1986). In this and other eukaryotes, Ub-X-β-gal is rapidly (co-translationally or nearly so) cleaved at the Ub-β-gal junction regardless of the identity of the residue X at the C-terminal side of the cleavage site, proline being the single exception. By allowing a bypass of the 'normal' N-terminal processing of a newly formed protein, this finding (Bachmair

et al., 1986) yielded an *in vivo* method for generating different residues at the N-termini of otherwise identical proteins, a technical advance that led to the N-end rule. Since nascent proteins bear N-terminal Met (a stabilizing residue), the N-degron of an N-end rule substrate must be produced from a pre-N-degron. The design of a pre-N-degron in Ub fusion-based, engineered N-end rule substrates such as Ub-X-β-gal is unlikely to be relevant to physiological N-end rule substrates, because natural Ub fusions (including the major precursors of Ub) either contain a stabilizing residue at the Ub–protein junction or bear a mutant Ub moiety that is expected to be retained *in vivo* (Varshavsky, 1996, and references therein).

Endoproteolytic cleavages of viral precursor polyproteins have been found to produce N-end rule substrates (deGroot *et al.*, 1991). However, since most non-viral proteins are not synthesized as polyproteins, the knowledge of a polyprotein-based route to an N-end rule substrate does not, by itself, address the problem of how a destabilizing residue could be generated at the N-terminus of a cytosolic protein. Most of the directly sequenced cytosolic and nuclear proteins lack destabilizing residues at their N-termini (Bachmair *et al.*, 1986). This bias stems in part from properties of the known Met-aminopeptidases, which remove N-terminal Met if, and only if, the second residue is stabilizing in the yeast N-end rule (reviewed by Varshavsky, 1996). Another likely reason for this bias is an underrepresentation of short-lived proteins in the current set of directly determined N-terminal sequences.

A destabilizing residue at the N-terminus of an N-end rule substrate must be generated through a proteolytic cleavage (or cleavages) of a pre-N-degron-containing protein by a processing protease(s) at some unspecified distance from the protein's initial N-terminus. Could this distance be just one or a few residues? If so, a short (≤10 residues) N-terminal sequence might contain both the recognition motif and the cleavage site(s) for a relevant (unknown) processing protease. On this assumption, we constructed a library of random 10 residue sequences fused to the N-terminus of Ura3p, a reporter protein, and screened for the fusions that were short lived in the presence but not in the absence of the N-end rule pathway. [A similar screen, with β-gal as a reporter, was also carried out by Sadis *et al.* (1995), see Results.] Described below is one N-terminal sequence identified by this screen. That sequence, in a fusion to a reporter protein, was used in another screen, for mutants unable to degrade the fusion. One mutant thus identified was found to be allelic to a previously isolated mutant in a Ub-dependent proteolytic system called the UFD (ubiquitin fusion degradation) pathway (Johnson *et al.*, 1995). (The UFD pathway overlaps with, but is distinct from, the N-end rule pathway.) We isolated the corresponding gene, *UFD3*, which encodes a protein containing tandem repeats of a sequence motif known as the WD repeat. We also found that Cdc48p, an essential ATPase, is an *in vivo* ligand of Ufd3p, and showed that both Ufd3p and Cdc48p play a role in the UFD pathway.

Results

N-terminal sequences that confer UBR1-dependent instability on a reporter protein

A library encoding random 10 residue sequences fused to the N-terminus of *S.cerevisiae* Ura3p was constructed,

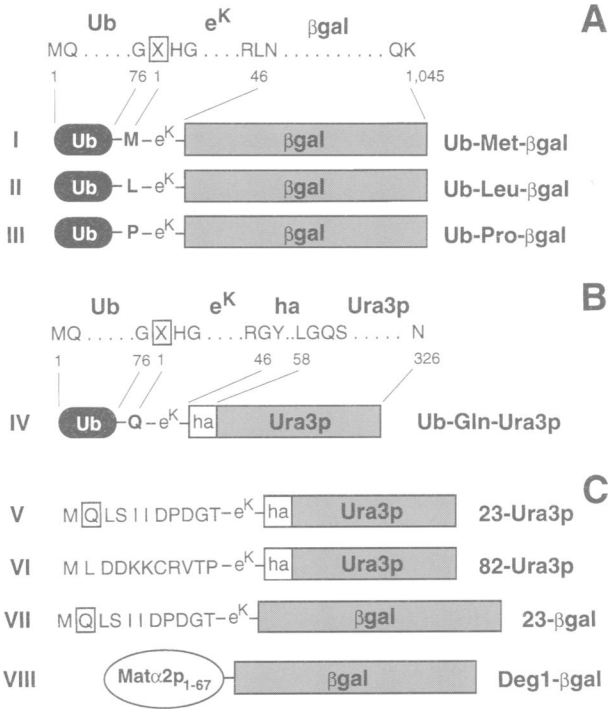


Fig. 1. Test proteins. (**A**) and (**B**) Ub-X-β-gal (constructs I–III) and Ub-Gln-Ura3p (construct IV) fusions were expressed in *S.cerevisiae* from galactose- and copper-inducible promoters, respectively (see Materials and methods). A 45 residue, *E.coli* Lac repressor-derived sequence, termed e^K [*extension containing lysines* (*K*)], between the Ub moiety and the reporter part of a fusion, bore Lys residues (Lys15 and Lys17) at least one of which is required for the degradation of e^K-based N-end rule substrates (Varshavsky, 1996). A 12 residue sequence containing an influenza hemagglutinin-derived epitope (ha) (Johnson *et al.*, 1992, 1995) was inserted between e^K and the N-terminus of Ura3p. Ub-Met-β-gal, Ub-Leu-β-gal and Ub-Gln-Ura3p were deubiquitylated rapidly *in vivo*, yielding, respectively, the long-lived Met-β-gal and the short-lived Leu-β-gal and Gln-Ura3p. By contrast, Ub-Pro-β-gal (construct III) was deubiquitylated slowly, but was also short-lived in wild-type cells because its N-terminal Ub moiety functioned as a degron recognized by the UFD pathway, a Ub-dependent system distinct from the N-end rule pathway (Johnson *et al.*, 1995). (**C**) The N-terminal Ub moiety and two abutting residues of Ub-Gln-Ura3p were replaced by random 10 residue amino acid sequences, and the resulting library of N-terminal extensions was screened for those that confer *UBR1*-dependent metabolic instability onto the Ura3p reporter (see Materials and methods). Shown are 23-Ura3p (construct V) and 82-Ura3p (construct VI), two out of 65 Ura3p fusions thus identified. In the construct VII, the 10 residue N-terminal extension of 23-Ura3p was fused to e^K-bearing β-gal. In construct VIII, the N-terminus of β-gal was fused to the 67 residue N-terminal region of Matα2p that encompasses its degron, termed Deg1 (Hochstrasser and Varshavsky, 1990).

and screened for the fusions that were short-lived in the presence but not in the absence of Ubr1p, the recognition component of the N-end rule pathway. Specifically, a pool of ~10^7 synthetic oligonucleotides encoding N-terminal Met followed by 10 random residues was fused to the 5′ end of a modified *URA3* gene that encoded a hemagglutinin epitope-tagged Ura3p (see Figure 1 and Materials and methods). Screening of this initial library for plasmids that could confer Ura$^-$ phenotype on [*ura3 ubr1*Δ] cells yielded ~10^6 clones. Previous work (Dohmen *et al.*, 1994) has shown that expression of a plasmid-borne gene encoding a Ura3p-based N-end rule substrate such as Arg-Ura3p in *ura3 S.cerevisiae* is insufficient to render cells Ura$^-$ because of rapid degradation of Arg-Ura3p. By

contrast, Arg-Ura3p is long-lived in *ubr1*Δ cells, which lack the N-end rule pathway, resulting in the Ura^+ phenotype (Dohmen *et al.*, 1994).

To enable the use of an N-end rule substrate as a conditional marker, a *S.cerevisiae* strain, termed JD54, was constructed that expressed *UBR1* from the galactose-inducible, glucose-repressible promoter P_{GAL1} (see Materials and methods). JD54 cells expressing Arg-Ura3p were Ura^+ in the presence of glucose but Ura^- in a galactose-containing medium, owing to the induction of the N-end rule pathway by galactose. Transforming into JD54 a library of plasmids that expressed Ura3p fusions bearing random-sequence N-terminal extensions, and selecting for cells that were Ura^+ on glucose but Ura^- on galactose yielded 65 distinct 10 residue N-terminal extensions. Pulse–chase analyses of Ura3p fusions bearing 10 of these extensions confirmed that the fusions were degraded rapidly in *UBR1* cells but were long-lived in *ubr1*Δ cells, and were also at least partially stabilized in *ubc2*Δ cells. [Ubc2p, one of at least 11 Ub-conjugating enzymes in *S.cerevisiae*, is bound to Ubr1p in a targeting complex of the N-end rule pathway (Madura *et al.*, 1993).] The pulse–chase data for two of the fusions, termed 23-Ura3p and 82-Ura3p (Figure 1C, constructs V and VI), are shown in Figure 2A and B, D and E. The half-lives of 23-Ura3p and 82-Ura3p were ~10 and ~12 min, respectively, in a *UBR1* strain but increased by at least 10-fold in a congenic *ubr1*Δ strain (Figure 2A and B, D and E).

The sequences of the above 65 N-terminal 10 residue extensions were largely dissimilar to each other. However, certain residues appeared to be excluded from some of the 10 positions and, in addition, 85% of these deduced sequences bore a destabilizing residue at position 2 (after the deduced N-terminal Met), while the remainder bore Val at this position (our unpublished data). In a screen similar to the one above, Sadis *et al.* (1995) have used a library of 11 residue N-terminal extensions fused to a β-gal reporter, and screened for the extensions that conferred metabolic instability on β-gal. A large fraction of the sequences thus identified targeted β-gal for degradation by the N-end rule pathway. None of the N-terminal sequences identified by Sadis *et al.* (1995) were evidently similar to the sequences identified in our screen. While this lack of sequence similarities may stem in part from differences in the screens' design [randomization immediately after N-terminal Met in our screen but after position 2 in the screen by Sadis *et al.* (1995)], it is also likely that a large number of 10 residue N-terminal sequences can produce the N-degron *in vivo*, perhaps analogously to a large number of N-terminal sequences that can function as signals for protein translocation across the endoplasmic reticulum (ER) membrane (Kaiser *et al.*, 1987). In addition, both screens explored at most 0.00001% of the *a priori* available sequence space (there are $\sim 1 \times 10^{13}$ distinct 10 residue amino acid sequences, and $\sim 1 \times 10^6$ of these were present in the library used).

The degron of 23-Ura3p

The apparent pre-N-degron of 23-Ura3p was chosen for a more detailed analysis, in part because the activity of this degron was found to require not only Ubr1p and Ubc2p but also Ate1p (R-transferase) and Nta1p (Nt-amidase) (see Introduction). These results suggested the presence of a tertiary destabilizing residue, Asn or Gln, at the processed N-terminus of 23-Ura3p. The N-terminal extension of 23-Ura3p was transplanted to the N-terminus of another reporter, *E.coli* β-gal (Figure 1C, construct VII). The resulting fusion was short-lived in *UBR1* cells ($t_{1/2}$ of ~45 min) but not in *ubr1*Δ or in *ate1*Δ and *nta1*Δ cells (Figure 2C), similarly to the effect of this extension on Ura3p (Figure 2A and D and data not shown), except that 23-Ura3p was degraded more rapidly (in *UBR1* cells) than 23-β-gal. Note also the appearance of the ~90 kDa β-gal cleavage product characteristic of short-lived β-gal-based proteins (Bachmair *et al.*, 1986) during the chase in wild-type cells but not in cells lacking either Ubr1p, Ate1p or Nta1p (Figure 2C).

Previous work (Madura *et al.*, 1993; Baker and Varshavsky, 1995) has shown that levels of β-gal activity in extracts from *S.cerevisiae* expressing β-gal-based N-end rule substrates can be used to compare relative metabolic stabilities of these substrates. In agreement with the results of pulse–chase analysis (Figure 2C), the activity of β-gal in either *ubr1*Δ or *nta1*Δ cells that expressed 23-β-gal was ~7-fold higher than the activity of β-gal in congenic wild-type cells expressing the same fusion (Figure 2F). In addition, the level of β-gal in wild-type cells expressing 23-β-gal could be increased by adding to the growth medium a dipeptide such as Arg–Ala, which bore a type 1 primary destabilizing N-terminal residue, but not by Ala–Arg or Leu–Ala, which bore, respectively, a stabilizing and a type 2 destabilizing N-terminal residue (data not shown). [Dipeptides bearing destabilizing N-terminal residues inhibit the N-end rule pathway by competing with its substrates for binding to the type 1 or type 2 binding sites of N-recognin (Baker and Varshavsky, 1991).] Taken together, these data indicated that 23-Ura3p and 23-β-gal (Figure 1C) were degraded because they acquired an N-degron that bore a tertiary destabilizing N-terminal residue, Asn or Gln.

The 23-β-gal protein was purified from *ubr1*Δ cells (in which this protein was long-lived) and subjected to microsequencing, yielding the N-terminal sequence Arg-Glu-Leu-Ser-Ile instead of the DNA-encoded sequence Met-Gln-Leu-Ser-Ile (Figure 1C, construct VII; see also Materials and methods). We concluded that the N-terminal Met of 23-β-gal was cleaved off *in vivo*. The resulting N-terminal Gln, a tertiary destabilizing residue, was deamidated by the Nta1p Nt-amidase, yielding N-terminal Glu, a secondary destabilizing residue. The N-terminal Glu was arginylated by the Ate1p R-transferase, yielding the observed sequence Arg-Glu-Leu-Ser-Ile that bore N-terminal Arg, a primary destabilizing residue (see Introduction). Map1p, an *S.cerevisiae* Met-aminopeptidase, appears to be incapable of cleaving the Met–Gln bond (Chang *et al.*, 1992). Indeed, 23-Ura3p remained short-lived in *map1*Δ cells (data not shown); this finding led us to a screen described below. A second Met-aminopeptidase, Map2p, has been identified recently in *S.cerevisiae* (Li and Chang, 1995). Purified Map2p exhibited no activity with Met–Leu–Phe or Met–His–Arg peptides *in vitro* (Li and Chang, 1995), suggesting that this enzyme is also unable to produce an N-degron *in vivo* (Moerschell *et al.*, 1990).

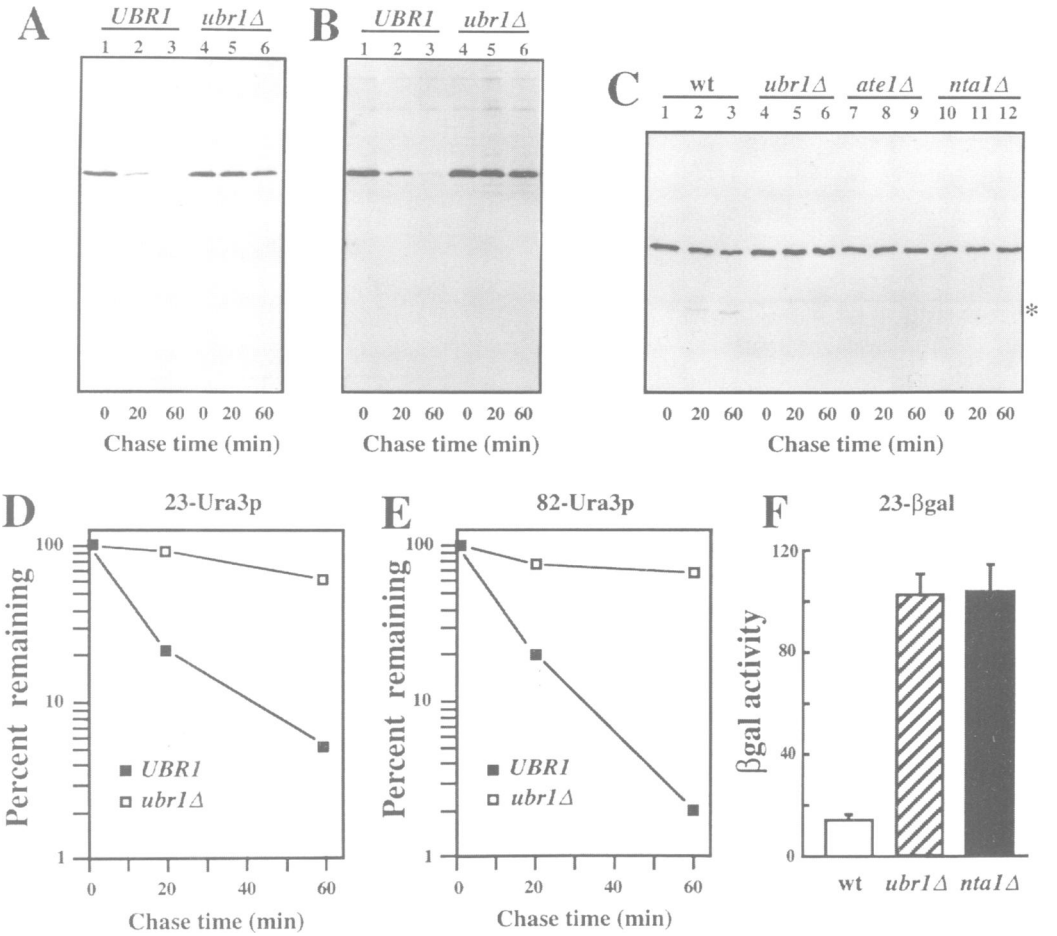


Fig. 2. Metabolic stabilities of 23-Ura3p, 82-Ura3p and 23-β-gal in *UBR1* and *ubr1*Δ strains. (**A**) Congenic *S.cerevisiae* strains *UBR1* (JD47-13C) and *ubr1*Δ*::HIS3* (JD55) expressing 23-Ura3p were labeled with [^{35}S]methionine/cysteine for 5 min at 30°C, followed by a chase for 0, 20 and 60 min, extraction, immunoprecipitation and SDS–PAGE of 23-Ura3p (see Materials and methods). (**B**) Same as (A) but with 82-Ura3p. (**C**) Same as (A) but the test protein was 23-β-gal, and two additional strains were used, SGY3 (*ate1*Δ) and SGY4 (*nta1*Δ) (Table I). The asterisk denotes an ~90 kDa β-gal cleavage product characteristic of short-lived β-gal derivatives (Bachmair *et al.*, 1986). The initial half-life of 23-β-gal (see Materials and methods) in wild-type cells (WT) was ~45 min. (**D**) Quantitation of the data in (A). (**E**) Same as (D) but with 82-Ura3p. Similar (±10%) decay curves were obtained in three independent pulse–chase experiments with all of the test proteins. (**F**) Wild-type, *ubr1*Δ and *nta1*Δ cells expressing 23-β-gal from P_{CUP1} were assayed for β-gal activity as described in Materials and methods. The values shown [in Miller units (Miller, 1972)] are the means from measurements with two independent transformants. Standard deviations are shown above the bars.

A mutant defective in degradation of 23-Ura3p is allelic to ufd3-1

To identify a protease(s) that generated the N-degron of 23-Ura3p, we carried out a screen for mutants unable to degrade 23-Ura3p but relatively unimpaired in the degradation of N-end rule substrates such as Ub-Asp-β-gal. [The pre-N-degron of Ub-Asp-β-gal is converted into the N-degron bearing N-terminal Asp through the removal of Ub by Ub-specific proteases (see Introduction).] A *ura3 S.cerevisiae* strain expressing 23-Ura3p and Asp-β-gal (Ub-Asp-β-gal) was mutagenized and screened for Ura$^+$ cells (see Materials and methods). MGY2, one of the Ura$^+$ mutants thus obtained, formed white colonies in the presence of X-Gal, a chromogenic β-gal indicator, indicating that Asp-β-gal remained short-lived in MGY2 cells.

The proteolytic defect in MGY2 was analyzed using additional test proteins, Ub-Pro-β-gal and Deg1-β-gal (Figure 1, constructs III and VIII). Ub-Pro-β-gal bears Pro at the Ub-β-gal junction, is deubiquitylated slowly (in contrast with the other 19 Ub-X-β-gals) and is short-lived *in vivo* because its N-terminal Ub moiety is recognized as a degradation signal by the UFD pathway, which is distinct from the N-end rule pathway (Johnson *et al.*, 1992, 1995; see Introduction). [To bring ubiquitin-related terms in line with the standard chemical terminology, ubiquitin whose C-terminal (Gly76) carboxyl group is covalently linked to another compound is called the *ubiquityl* moiety, with the derivative terms being *ubiquitylation* and *ubiquitylated*, instead of the earlier terms 'ubiquitination' and 'ubiquitinated'. 'Ub' refers to both free ubiquitin and the ubiquityl moiety]. In Deg1-β-gal, the N-terminus of the β-gal moiety is extended with the 67 residue N-terminal region of *S.cerevisiae* Matα2p that contains a degron of this naturally short-lived transcriptional repressor (Hochstrasser and Varshavsky, 1990). The degradation of Deg1-β-gal requires Ubc6p and Ubc7p Ub-conjugating enzymes (Chen *et al.*, 1993), whereas the degradation of Ub-Pro-β-gal requires in particular Ubc4p (Johnson *et al.*, 1992, 1995), and the degradation of N-end rule substrates requires Ubc2p (Dohmen *et al.*, 1991; Madura *et al.*, 1993), indicating that the corresponding

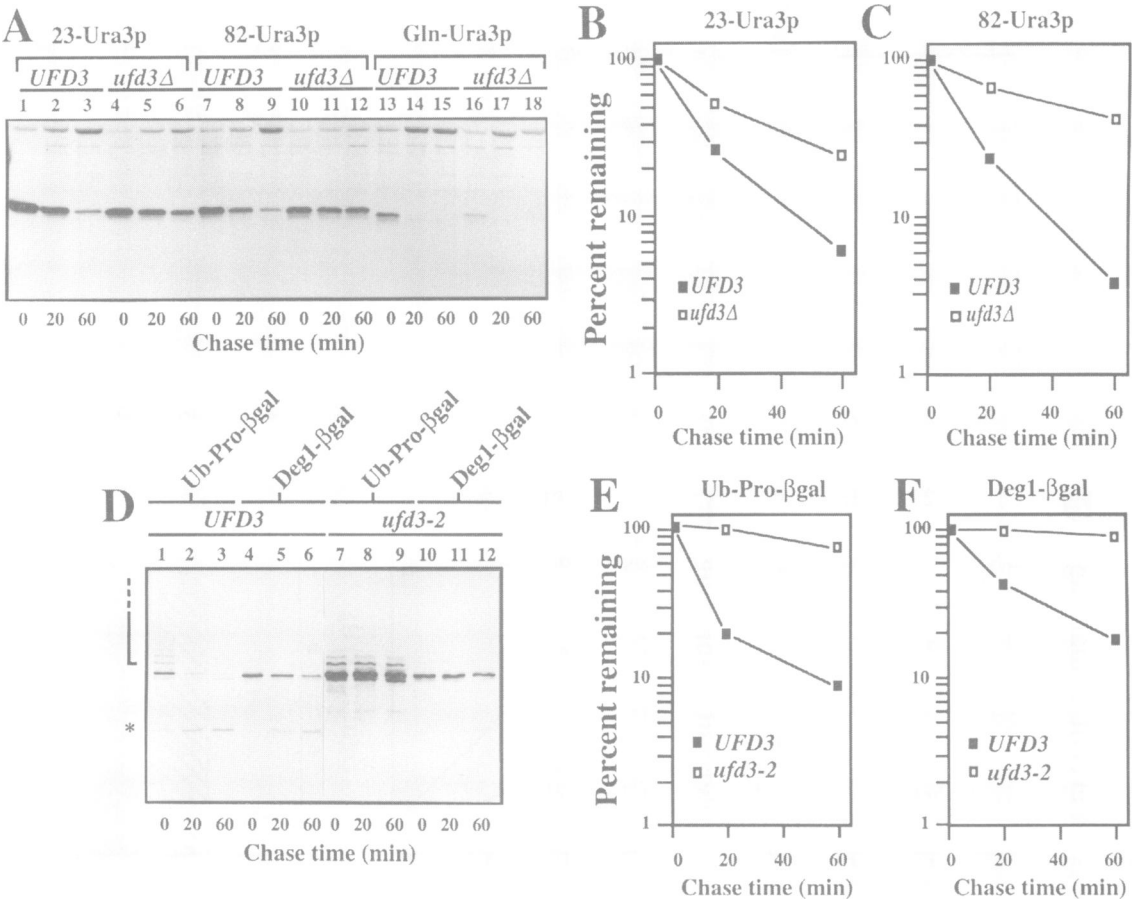


Fig. 3. *UFD3* is required for degradation of several distinct proteins. (**A**) Comparison of metabolic stabilities of 23-Ura3p, 82-Ura3p and Gln-Ura3p (Ub-Gln-Ura3p) in congenic *UFD3* (JD47-13C) and *ufd3*Δ (MGY3) strains. *S.cerevisiae* expressing either 23-Ura3p, 82-Ura3p or Ub-Gln-Ura3p (Figure 1, constructs V, VI and IV) were labeled with [^{35}S]methionine/cysteine for 5 min at 30°C, followed by a chase for 0, 20 and 60 min and processing as described in the legend to Figure 2A and Materials and methods. (**B**) Quantitation of the data in (A) for 23-Ura3p. (**C**) Same as (B) but for 82-Ura3p. (**D**) Same as (A) but the test proteins were Ub-Pro-β-gal and Deg1-β-gal (Figure 1, constructs III and VIII), and the mutant strain was MGY2 (*ufd3-2*). The asterisk denotes an ~90 kDa β-gal cleavage product characteristic of short-lived β-gal derivatives (Bachmair *et al.*, 1986). (**E**) Quantitation of the data in (D) for Ub-Pro-β-gal. (**F**) Same as (E) but for Deg1-β-gal.

Ub-dependent pathways differ at least by their targeting steps.

Surprisingly, Ub-Pro-β-gal was found to be strongly stabilized in the MGY2 mutant (Figure 3D and E). Consistent with this result, the level of the Ub-Pro-β-gal-derived ~90 kDa β-gal cleavage product characteristic of short-lived β-gal-based proteins (Bachmair *et al.*, 1986) was greatly reduced in MGY2 cells, as was the relative amount of multiubiquitylated Ub-Pro-β-gal (Figure 3D). The degradation of Deg1-β-gal was also impaired in MGY2 (Figure 3D and F). Overexpression of free Ub in MGY2 cells partially complemented their defect in degradation of Ub-Pro-β-gal (Figure 4B). This phenotype was similar to the one of a previously characterized mutant, termed *ufd3-1* (Johnson *et al.*, 1995). *ufd3-1* was isolated in a screen for mutations that perturb the UFD proteolytic pathway (see Introduction), whose substrates include Ub-Pro-β-gal (Johnson *et al.*, 1995). The *ufd3-1* mutant has reduced levels of free Ub, and can be partially complemented by overexpression of free Ub. Earlier attempts to clone *UFD3* by complementation of the *ufd3-1* mutant repeatedly yielded the poly-Ub gene *UBI4* as an extragenic suppressor (Johnson *et al.*, 1995), consistent with partial complementation of the *ufd3-1* proteolytic defect by overexpression of free Ub.

We isolated the wild-type counterpart of a mutant gene in the MGY2 strain through a complementation of its defect in degradation of Ub-Pro-β-gal, and showed that the cloned gene could also complement the Ub-Pro-β-gal degradation defect in *ufd3-1* cells (Figure 4A). The level of free Ub in MGY2 cells was lower than in congenic wild-type cells (data not shown), similarly to the phenotype of *ufd3-1* cells (Johnson *et al.*, 1995). Moreover, the original *ufd3-1* strain PM164 (Johnson *et al.*, 1995) and a strain, termed MGY3, that bore a deletion of the cloned gene (see below) failed to complement each other in a diploid background (Figure 4A). The mutation in MGY2 cells and the *ufd3-1* mutation were also shown to be meiotically linked (see Materials and methods). Thus, the mutation in MGY2 was allelic to *ufd3-1*. The cloned gene was termed *UFD3*; its mutant allele in MGY2 cells was termed *ufd3-2*.

A strong impairment of Ub-Pro-β-gal and Deg1-β-gal degradation in the recessive *ufd3-2* mutant, whose proteolytic defect was indistinguishable from that of the

*ufd3*Δ strain MGY3 (see below), indicated that Ufd3p plays a role in several Ub-dependent proteolytic pathways, perhaps through its function in regulating the level of free Ub. Thus, Ufd3p is unlikely to be involved in the production of N-degron in 23-Ura3p. A protease that generates this N-degron remains to be identified, perhaps through a further application of the screen that yielded *ufd3-2*.

A partial complementation of the proteolytic defect in *ufd3-2* cells by overexpression of free Ub (Figure 4B) explained, in retrospect, the paradoxical finding that whereas 23-Ura3p and 82-Ura3p were both stabilized in the MGY3 (*ufd3-Δ1*) strain, a Ub fusion-based N-end rule substrate such as Gln-Ura3p (Ub-Gln-Ura3p) remained short-lived in the same strain (Figure 3A–C). Indeed, deubiquitylation of the relatively highly expressed (see Materials and methods) Ub-Gln-Ura3p yields stoichiometric amounts of Gln-Ura3p and free Ub. The resulting overproduction of free Ub from Ub-Gln-Ura3p in the MGY3 (*ufd3*Δ) mutant (Figure 3A) or from Ub-Asp-β-gal in the original MGY2 (*ufd3-2*) mutant would be expected to partially complement the proteolytic defect in these strains.

UFD3 encodes a WD repeat protein

Sequencing of an ~3 kb, library-derived DNA fragment that complemented the Ub-Pro-β-gal degradation defect in *ufd3-2* cells (see Materials and methods) revealed a single open reading frame (ORF) identical to a previously identified ORF of unknown function located on chromosome XI between *SAC1* and *URA1* [this ORF was termed YKL213C by Dujon *et al.* (1994) and F715 by Tzermia *et al.* (1994)]. The *UFD3* gene encodes a 715 residue (79.5 kDa) protein containing five tandem repeats of an ~30 residue motif in its N-terminal half (Figure 5B and C). This motif, originally identified in β-subunits of heterotrimeric G proteins (Fong *et al.*, 1986), is called the WD repeat, because it often ends with the sequence Trp–Asp (WD) (Neer *et al.*, 1994). The *ufd3-2* allele of *UFD3* was found to contain a single G→A transition that resulted in a Cys→Tyr alteration at position 237, in the fifth WD repeat of Ufd3p (Figure 5C). The phenotypic effect of this single missense mutation was indistinguishable from that of the *ufd3*Δ allele (Figures 3B and 4A). The *UFD3* gene is identical to *DOA1*, which has been cloned by M.Hochstrasser (personal communication) by complementing a defect in degradation of the Matα2p transcriptional repressor in a previously isolated mutant (Hochstrasser and Varshavsky, 1990).

The five WD repeats of *S.cerevisiae* Ufd3p are highly similar (40% identical over 250 N-terminal residues) to WD repeats of the mammalian phospholipase A2-activating protein (PLAP) (Clark *et al.*, 1991) (Figure 5B), and are also similar (28% identity over 176 residues) to WD repeats of Lis1, a subunit of platelet-activating-factor acetylhydrolase (Hattori *et al.*, 1994). The 413 residue C-terminal region of Ufd3p outside of the WD repeats lacks significant similarities to sequences in databases.

Construction and analysis of a *ufd3*Δ mutant

A *LEU2*-marked *ufd3-Δ1* allele (Figure 5A) was used to replace the wild-type *UFD3* in a diploid strain (see Materials and methods). Sporulation of the resulting *ufd3-Δ1/UFD3* diploid yielded tetrads with four viable spores

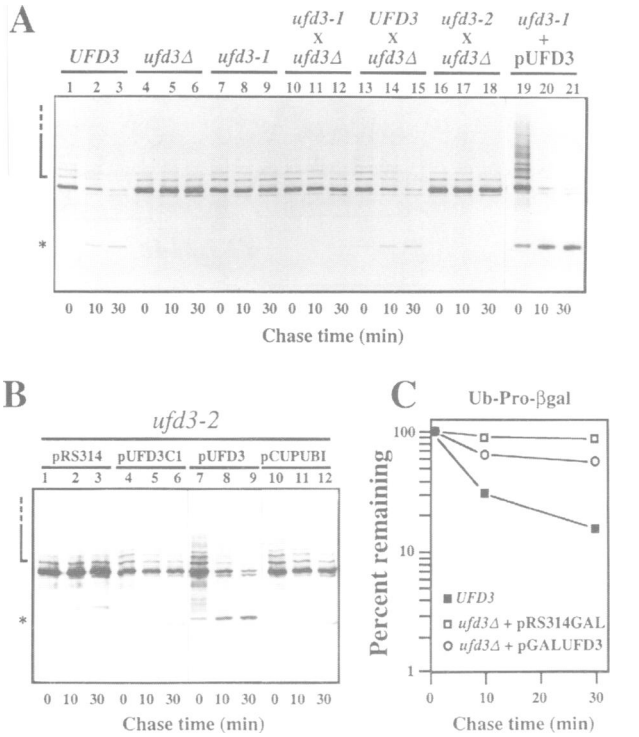
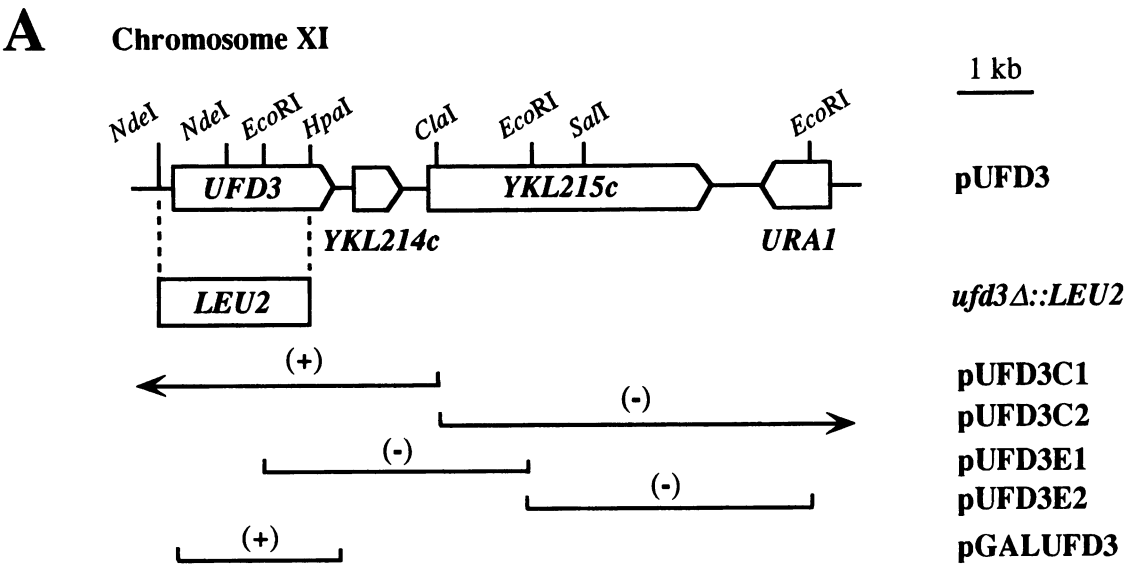


Fig. 4. Complementation analysis of *ufd3* mutants. (**A**) Degradation of Ub-Pro-β-gal in different *S.cerevisiae* strains. Lanes 1–3, *UFD3* strain JD47-13C. Lanes 4–6, *ufd3-Δ1* strain MGY3. Lanes 7–9, *ufd3-1* strain PM164. Lanes 10–12, [*ufd3-Δ1/ufd3-1*] diploid strain. Lanes 13–15, [*ufd3-Δ1/UFD3*] diploid strain. Lanes 16–18, [*ufd3-Δ1/ufd3-2*] diploid strain. Lanes 19–21, *ufd3-1* strain PM164 carrying pUFD3, a low-copy plasmid that expressed Ufd3p. Pulse–chase analyses were carried out as described in Materials and methods. (**B**) Effects of overexpression of Ufd3p and free Ub in *ufd3-2* cells on the degradation of Ub-Pro-β-gal. The *ufd3-2* strain MGY2 that expressed Ub-Pro-β-gal was transformed with the following plasmids: pRS314 (vector alone, a negative control) (lanes 1–3); pUFD3C1 (a pRS423-based high copy plasmid expressing Ufd3p) (see Figure 5A) (lanes 4–6); pUFD3 (a library-derived low copy plasmid carrying a 9.8 kb insert that contained *UFD3*) (lanes 7–9); pCUPUBI (a YEplac181-based, high copy plasmid expressing Ub from the *CUP1* promoter) (lanes 10–12). The initial half-lives of Ub-Pro-β-gal (see Materials and methods) were >60, ~5, ~40 and ~30 min for cells carrying, respectively, vector alone, pUFD3, pUFD3C1 and pCUPUBI. (**C**) Quantitation of Ub-Pro-β-gal decay in *UFD3* (JD47-13C) and *ufd3-Δ1* (MGY3) cells carrying either the vector pRS314GAL or the pRS314GAL-based pGALUFD3, which expressed *UFD3* from the $P_{GAL1/10}$ promoter.

in which the Leu$^-$ (Ufd3$^-$) phenotype segregated 2:2, indicating that *UFD3* is not essential for cell viability. However, a homozygous *ufd3-Δ1/ufd3-Δ1* diploid had a defect in sporulation: the fraction of asci with four spores was ~100-fold lower than in a congenic wild-type strain. This defect could be complemented by a low copy plasmid expressing *UFD3*. Neither *ufd3-Δ1* nor *ufd3-2* cells differed significantly from their congenic *UFD3* counterparts in their growth rate or sensitivity (measured as a decrease in plating efficiency) to acute (50°C) or chronic (38°C) heat stress, to canavanine (at 1.5 μg/ml), to caffeine (at 8 mM), to NaCl (at 0.9 M), to $CaCl_2$ (at 0.1 M), to EGTA (at 10 mM) and to nitrogen starvation (data not shown).

Pulse–chase analysis of the Ub-Pro-β-gal degradation defect in *ufd3-Δ1* cells showed it to be indistinguishable from the defect in the original (missense) *ufd3-2* mutant



B

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Ufd3p    1  MGYQLSATLKGHDQDVRDVVAVDDSKVASVSRDGTVRLWSKDDQWLGTVVYTGQGFLNSVCYDSEKELLLFG--GKDTMI   78
PLAP     1                                                    MHYMSGHSNFVSYVCIIPSSDIYPHGLIATGGND   34

Ufd3p   79  NGVPLFATSGEDPLYTLIGHQGNVCSLSFQD-GVVISGSWDKTAKVWKEGSLVYNLQAHNASVWDAKVVSFSENKFLTAS  157
PLAP    35  HNICIFSLDSPMPLYILKGHKDTVCSLSSGKFGTLLSGSWDTTAKVWLNDKCMMTLQGHTAAVWAVKILP-EQGLMLTGS  113

Ufd3p  158  ADKTIKLWQNDKVIKTFSGIHNDVVRHLAVVDDGHFISCSNDGLIKLVDMHTGDVLRTYEGHESFVYCIKLLPNG-DIVS  236
PLAP   114  ADKTIKLWKAGRCERTFLG-HEDCVRGLAILSETEFLSCANDASIRRWQI-TGECLEVYFGHTNYIYSISVFPNSKDFVT  191

Ufd3p  237  CGEDRTVRIWSKENGSLKQVITLPAISIWSVDCMSNGDIIVGSSDNLVRIFSQEKSRWASEDEINELSTQVEKSTISSKT  316
PLAP   192  TAEDRSLRIW--KHGECAQTIRLPAQSIWCCCVLENGDIVVGASDGIIRVFTESEERTASAEEIKASLSRESPLIAKVLT  270

Ufd3p  317  IEFDESKLSPYEILQSPGRKEGQIVVVKSPQGTIEAHQFSNSSWKKVGDVVGAGATGNDKKIEFEGKTYDYVFDVDIEDG  396
PLAP   271  TEPPIITPVRRTLPCRVTRSMISSCLSRLVSTSLSTSDSHLTITALHLFTTTTE                            325

Ufd3p  397  KPPLKLPINVSDNPYTAADNFLARYELPMSYRDQVVQFILKNTNGISLDQPNDNASSSAVSPSKTSVMKVLPVKQYLIME  476
       477  NYNPDTIFNGIVKINSNEKTFDDEILAQIGGALHDIDESWELLLSFANTIRSNWEIKTPAYDIVRLIVKKLPYSSDIKDY  556
       557  IEEGLGNKNITLTMLTVRILVNCFNNENWGVKLLESNQVYKSIFETIDTEPSQASAKQSQNLAIAVSTLIFNYSALVTKG  636
       637  NSDLELLPIVADAINTKYGPLEEYQECEEAAYRLTVAYGNLATVEPTLRQFANSVTWLANIKRSYGNVPRFKDIFDDLS  715
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C

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                    **     *          **    * *   *   * **  *
WD-repeat 1    11   GH  DQDVRDVVA-VDDSKVASVSR  D  GTVRL  W  S   40
WD-repeat 2    97   GH  QGNVCSLS--FQDGVVISGSW  D  KTAKV  W  K  125
WD-repeat 3   135   AH  NSVWDAKVVSFSENKFLTASA  D  KTIKL  W  Q  166
WD-repeat 4   177   IH  NDVVRHLAV-VDDGHFISCSN  D  GLIKL  V  D  206
WD-repeat 5   218   GH  ESFVYCIKL-LPNGDIVSCGE  D  RTVRI  W  S  247
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Fig. 5. The *S.cerevisiae UFD3* gene. (**A**) *UFD3* and adjacent loci on chromosome XI. ORFs are shown as arrow-shaped boxes indicating the direction of transcription. Subcloned regions are also indicated, with (+) or (–) denoting their ability to complement the proteolytic defect of *ufd3* mutants. pUFD3 is a p366-based, low-copy plasmid carrying a 9.8 kb insert that contains *UFD3*. Dashed lines delineate the region of *UFD3* that has been replaced with *LEU2* in the *ufd3-Δ1* allele. (**B**) Comparison of the amino acid sequences (in single-letter abbreviations) of Ufd3p and the mammalian PLAP protein (Clark *et al.*, 1991). Identities are highlighted by vertical bars. Dashes indicate gaps introduced to optimize the alignment. (**C**) Alignment of the five tandem repeats (WD repeats) of Ufd3p. Residues present in at least three WD repeats are marked by an asterisk. Consensus elements of WD repeats are boxed. The encircled Cys residue in the fifth WD repeat was replaced by Tyr by the *ufd3-2* mutation (see Results). Ufd3p has a sixth WD repeat (residues 251–287) that diverges from the consensus WD sequence (Neer *et al.*, 1994) at three more positions in comparison with the other five WD repeats of Ufd3p.

(Figure 4A; compare with Figure 3D and E). Strikingly, Ub-Pro-β-gal was found to be *stabilized* in the MGY2 (*ufd3-2*) strain that carried pUFD3C1, a high copy (2 μ-based) plasmid overexpressing Ufd3p, in comparison with the same strain carrying the low copy (*CEN*-based) Ufd3p-expressing plasmid pUFD3 (Figure 4B and C; compare lanes 4–6 with lanes 7–9). Similar results were obtained with the MGY3 (*ufd3-Δ1*)strain that over-expressed Ufd3p from the P_{GAL1} promoter: the half-life of Ub-Pro-β-gal in Ufd3p-overexpressing MGY3 cells was

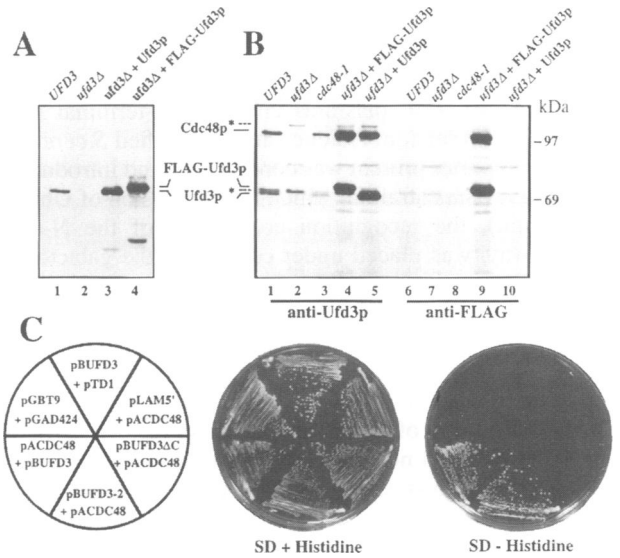

Fig. 6. Ufd3p binds to Cdc48p. (**A**) Specificity tests of anti-Ufd3p antibodies. Whole-cell extracts (10 μg of total protein) were fractionated by SDS–10% PAGE and analyzed by immunoblotting with anti-Ufd3p antibodies (see Materials and methods). Lane 1, *UFD3* strain JD47-13C. Lane 2, *ufd3*Δ strain MGY3. Lane 3, MGY3 (*ufd3-Δ1*) carrying pGALUFD3, a low-copy plasmid expressing Ufd3p from $P_{GAL1/10}$. Lane 4, MGY3 carrying pFLAGUFD3, a low-copy plasmid expressing His_6- and FLAG-tagged Ufd3p from P_{GAL1}. (**B**) Co-immunoprecipitation of Ufd3p and Cdc48p. Whole-cell extracts (175–350 μg of total protein) from [^{35}S]methionine/cysteine-labeled cells were processed for immunoprecipitation with either anti-Ufd3p or anti-FLAG antibodies, followed by SDS–8% PAGE and fluorography (see Materials and methods). Lanes 1 and 6, *UFD3* strain JD47-13C. Lanes 2 and 7, *ufd3-Δ1* strain MGY3. Lanes 3 and 8, *cdc48-1* strain DBY2030. Lanes 4 and 9, MGY3 expressing His_6-FLAG-Ufd3p from pFLAGUFD3. Lanes 5 and 10, MGY3 expressing Ufd3p from pGALUFD3. Asterisks denote non-specifically immunoprecipitated proteins. (**C**) Two-hybrid assay of interaction between Ufd3p and Cdc48p. The *S.cerevisiae* HF7c strain, which lacks *GAL4* and *GAL80*, and contains an integrated *GAL1::HIS3* reporter gene, was transformed with pBUFD3 and pACDC48, expressing, respectively, a fusion of Ufd3p to the DNA binding domain of Gal4p and a fusion of Cdc48p to the activation domain of Gal4p. In the plasmids pBUFD3-2 and pBUFD3ΔC, the DNA binding domain of Gal4p was fused, respectively, to the mutant Ufd3-C237Yp protein and to the WD repeats-containing N-terminal domain of Ufd3p. The control plasmids pGBT9, pGAD424, pTD1 and pLAM5′ expressed, respectively, the DNA binding domain of Gal4p, the activator domain of Gal4p, a fusion of the SV40 large T protein to the DNA binding domain of Gal4p and a fusion of human lamin C to the same domain of Gal4p. HF7c cells could grow in the absence of histidine in the medium if the *HIS3*-activating function of a split Gal4p protein was restored through a spatial juxtaposition of its otherwise separately expressed domains.

>30 min, in comparison with ~6 min in a congenic wild-type (*UFD3*) strain (Figure 4C).

Ufd3p is physically associated with Cdc48p

Polyclonal anti-Ufd3p antibodies were raised in rabbits against the purified His_6- and FLAG-tagged Ufd3p (His_6-FLAG-Ufd3p) that had been expressed in *E.coli*. In immunoblots of extracts from wild-type (*UFD3*) *S.cerevisiae*, these antibodies bound to a single protein with an apparent M_r of ~75 kDa, close to the predicted size of Ufd3p (79.5 kDa) (Figure 6A). This protein was absent from *ufd3-Δ1* cells (Figure 6A). In extracts from *ufd3-Δ1* cells that expressed His_6-FLAG-Ufd3p from the P_{GAL1} promoter, the same antibodies detected an abundant protein of ~77 kDa, 2 kDa larger than the untagged Ufd3p (Figure 6A). Thus, the ~75 kDa protein detected in *UFD3* cells by immunoblotting with anti-Ufd3p antibodies was in fact Ufd3p.

Anti-Ufd3p antibodies were also used in immunoprecipitation assays with extracts from [^{35}S]methionine/cysteine-labeled *S.cerevisiae*. In addition to the expected ~75 kDa Ufd3p, these antibodies precipitated an ~105 kDa protein. This species was present in immunoprecipitates from Ufd3p-containing but not Ufd3p-lacking cells (Figure 6B). Overexpression of Ufd3p (as His_6-FLAG-Ufd3p) increased the yield of immunoprecipitated ~105 kDa protein (Figure 6B). The specificity of an apparent interaction between the ~75 kDa Ufd3p and the ~105 kDa protein was probed further using a monoclonal anti-FLAG antibody. This antibody precipitated the ~105 kDa protein from extracts of *ufd3*Δ cells that expressed His_6-FLAG-Ufd3p but not from extracts of otherwise identical cells that lacked His_6-FLAG-Ufd3p (Figure 6B). To purify the ~105 kDa protein, an extract from cells expressing His_6-FLAG-Ufd3p was passed over an anti-FLAG antibody affinity column, which retained both proteins (see Materials and methods). Direct microsequencing of the eluted ~105 kDa protein yielded the N-terminal sequence GEEHKPLLDASG, identical to the deduced N-terminal sequence of the 92 kDa *S.cerevisiae* Cdc48p (minus N-terminal Met, which is expected to be removed from the nascent Cdc48p) (Fröhlich *et al.*, 1991).

To verify the *in vivo* existence of a Ufd3p–Cdc48p complex, we employed the two-hybrid assay (Phizicky and Fields, 1995). In this method, the expression of a reporter gene such as *HIS3* from the Gal4p-dependent P_{GAL1} promoter in the *S.cerevisiae* strain HF7c is designed to require the reconstitution of the Gal4p function from two unlinked domains of Gal4p that are brought together through an interaction between two proteins of interest. Specifically, the DNA binding (DB) domain of Gal4p was fused to the N-terminus of the full-length Ufd3p, yielding GBD–Ufd3p (encoded by the plasmid pBUFD3) (see Materials and methods). The full-length Cdc48p was fused to the C-terminus of the Gal4p activation domain (GAD), yielding GAD–Cdc48p (encoded by the plasmid pACDC48). Expression of either of these fusions alone in HF7c *S.cerevisiae* did not activate the expression of *HIS3* reporter. By contrast, the expression of both pBUFD3 and pACDC48 in HF7c cells resulted in the His^+ phenotype (active *HIS3* reporter), confirming the *in vivo* interaction between Ufd3p and Cdc48p (Figure 6C). Additional control experiments showed that GAD–Cdc48p and GBD–Ufd3p did not form complexes with analogous Gal4p-based fusions containing proteins such as human lamin C or SV40 large T protein, expressed, respectively, by the plasmids pLam5′ and pTD1 (Figure 6C).

Analogous two-hybrid assays with GAD–Cdc48p and GBD–Ufd3–C237Yp, the latter fusion containing the functionally inactive Cys-237→Tyr-237 *ufd3-2* allele of Ufd3p (see above), also yielded His^+ cells (Figure 6C). Thus, the defect in degradation of Ub-Pro-β-gal in *ufd3-2* cells is unlikely to result from the absence of physical interaction between Cdc48p and the mutant ufd3-2p protein.

Conditionally lethal cdc48-1 mutant is impaired in Ub-Pro-β-gal degradation

Given the requirement for Ufd3p in the Ub-dependent proteolysis and the discovery of interaction between Ufd3p

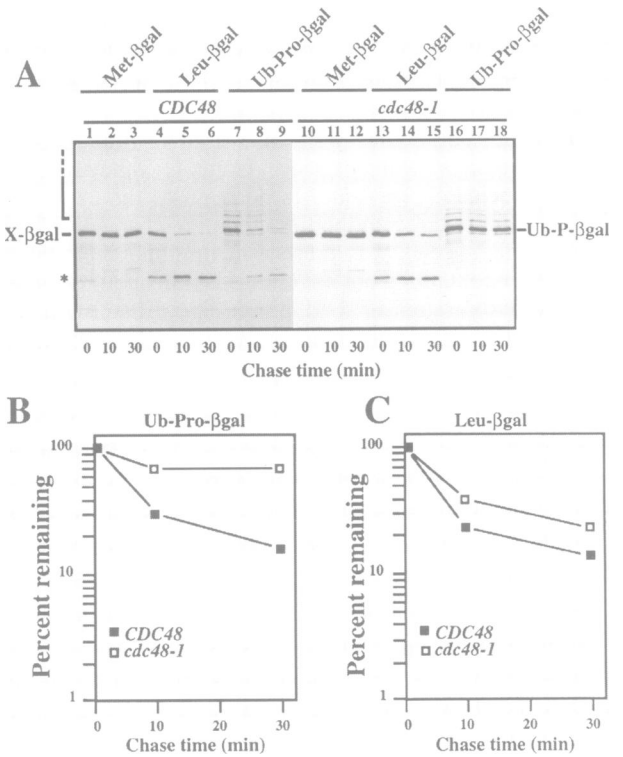

Fig. 7. Cold-sensitive *cdc48-1* mutant is impaired in the degradation of Ub-Pro-β-gal. (**A**) Pulse–chase analysis of Leu-β-gal (Ub-Leu-β-gal) and Ub-Pro-β-gal degradation. Mid-exponential cultures of congenic *CDC48* (DBY1705) and *cdc48-1* (DBY2030) strains were grown at 30°C (permissive temperature). Cells were labeled with [^{35}S]methionine/cysteine for 5 min at 30°C, followed by a chase for 0, 10 and 30 min, preparation of extracts, immunoprecipitation with a monoclonal antibody to β-gal, and SDS–PAGE (see Materials and methods). The open-ended bracket denotes the positions of multiubiquitylated Ub-Pro-β-gal species. The asterisk indicates an ~90 kDa cleavage product characteristic of short-lived β-gal derivatives (Bachmair *et al.*, 1986). (**B**) Quantitation of the data in (A) for Ub-Pro-β-gal. (**C**) Same as (B) but for Ub-Leu-β-gal. At 30°C, the initial half-life of Ub-Pro-β-gal was ~6 min in *CDC48* cells and >30 min in *cdc48-1* cells. The initial half-life of Leu-β-gal was ~5 min in *CDC48* cells and ~7 min in *cdc48-1* cells. Similar (±10%) results were obtained in two independent pulse–chase experiments.

and Cdc48p, we asked whether the Cdc48p (which, unlike Ufd3p, is essential for cell viability) is also required for Ub-dependent proteolysis. Pulse–chase experiments were carried out with Ub-Pro-β-gal and Leu-β-gal (Ub-Leu-β-gal) in congenic wild-type (*CDC48*) and *cdc48-1* strains, the latter carrying a cold-sensitive allele of *CDC48* (see Materials and methods).

Even at the permissive temperature of 30°C, the degradation of Ub-Pro-β-gal was strongly inhibited in *cdc48-1* cells relative to wild-type cells ($t_{1/2}$ of ~60 min and 6 min, respectively) (Figure 7A and B). By contrast, the degradation of Leu-β-gal (derived from Ub-Leu-β-gal) was only slightly slower in *cdc48-1* than in *CDC48* cells ($t_{1/2}$ of ~8 min and 6 min, respectively, at 30°C) (Figure 7A and C). The Ub-Pro-β-gal degradation defect of *cdc48-1* cells was even more severe (relative to wild-type cells) at the semipermissive temperature of 23°C (data not shown).

Discussion

The initial aim of this work was to identify N-terminal sequences of a reporter protein that can be proteolytically processed in the cytosol to yield any of the 12 N-terminal residues that are destabilizing in the *S.cerevisiae* N-end rule (see Introduction). We report the following results.

A library of ~10^7 plasmids encoding N-terminal Met followed by 10 random residues and a modified *S.cerevisiae* Ura3p reporter protein was constructed, and introduced into a *S.cerevisiae* strain in which the expression of Ubr1p (N-recognin), the recognition component of the N-end rule pathway, was placed under control by the galactose-inducible, glucose-repressible promoter P_{GAL1}. A screen for Ura3p fusions that were long-lived on glucose (rendering cells Ura$^+$) but short-lived on galactose (rendering cells Ura$^-$) yielded 65 fusions that differed by the sequences of their 10-residue N-terminal extensions.

The degradation of at least one of these fusions, termed 23-Ura3p, required not only Ubr1p, but also Ate1p [Arg-tRNA–protein transferase (R-transferase)] and Nta1p [N-terminal amidase (Nt-amidase)] (see Introduction), suggesting the presence of a tertiary destabilizing residue at the N-terminus of the processed 23-Ura3p protein. Sequencing of purified 23-β-gal, a counterpart of 23-Ura3p that bore the same N-terminal extension, showed that the extension's N-terminal Met was cleaved off *in vivo*. The resulting N-terminal Gln, a tertiary destabilizing residue, was deamidated and then arginylated, yielding the actually observed N-terminal sequence Arg-Glu-Leu-Ser-Ile instead of the encoded sequence Met-Gln-Leu-Ser-Ile.

Since Map1p and Map2p, the known *S.cerevisiae* Met-aminopeptidases, appear to be incapable of cleaving the Met–Gln bond (Moerschell *et al.*, 1990; Chang *et al.*, 1992; Li and Chang, 1995) (see also Results), we carried out a screen for mutants unable to degrade 23-Ura3p but relatively unimpaired in the degradation of Ub fusion-based N-end rule substrates. This screen was expected to identify mutations that perturb the production of N-degron but not the 'downstream' steps of the N-end rule pathway. However, the mutant actually isolated was found to be defective in the degradation of both 23-Ura3p and several other (normally short-lived) proteins distinct from N-end rule substrates. This and other properties of the mutant, including its abnormally low content of free Ub, suggested that it may be allelic to the previously characterized mutant *ufd3-1*, identified in a screen for mutations in the UFD proteolytic pathway (see Introduction) (Johnson *et al.*, 1995). We showed this conjecture to be correct, and isolated the corresponding gene, termed *UFD3*. (See Results for a likely explanation of how a mutation in *UFD3*, which plays a role in several proteolytic systems but appears not to be required for the production of N-degron, could be detected in the screen that utilized 23-Ura3p. This screen should still be capable of yielding mutants that are impaired in the production of N-degron; experiments to identify such mutants are under way.)

The *UFD3* gene encodes a 715 residue protein containing five tandem repeats of an ~30 residue motif (known as the WD repeat) in its N-terminal half (Figure 5B and C). The WD repeats are present in a number of eukaryotic proteins whose functions encompass cell cycle control, cell fate determination, transcription, transmembrane signaling, RNA metabolism and vesicular traffic (Neer *et al.*, 1994). WD proteins tend to occur in multiprotein complexes, in which their interactions with other proteins are

often mediated by their WD repeat regions (Neer *et al.*, 1994). The five WD repeats of *S.cerevisiae* Ufd3p are highly similar (40% identical over 250 residues) to WD repeats of the mammalian protein PLAP (Figure 5B), which has been shown to stimulate the activity of phospholipase A2 *in vitro* (Clark *et al.*, 1991). The C-terminal half of Ufd3p lacks significant similarities to sequences in databases.

An *S.cerevisiae* strain bearing a deletion/disruption allele of *UFD3* was constructed and found to be phenotypically indistinguishable from the originally isolated *ufd3-2* mutant, in which a single Cys→Tyr alteration (at position 237) has occurred within the fifth WD repeat of Ufd3p.

Antibodies to Ufd3p co-immunoprecipitated an ~105 kDa protein, which was purified, microsequenced and found to be Cdc48p, an essential ATPase that contains two ATP binding domains (Fröhlich *et al.*, 1991). The co-immunoprecipitation evidence for a Ufd3p–Cdc48p complex (Figure 6A and B) was confirmed and extended using the two-hybrid assay (Phizicky and Fields, 1995), which detected an *in vivo* interaction between Ufd3p and Cdc48p (Figure 6C). The Ufd3p–Cdc48p interaction was apparently unimpaired by a Cys→Tyr alteration within the fifth WD repeat of Ufd3p that rendered the latter functionally inactive. It remains to be determined whether the Ufd3p–Cdc48p complex is mediated by any of the other WD repeats of Ufd3p.

Saccharomyces cerevisiae Cdc48p has significant sequence similarities to the S7 subunit of the mammalian 26S proteasome (Dubiel *et al.*, 1993) and to Sug1p, an ATPase and component of the *S.cerevisiae* 26S proteasome required for Ub-dependent protein degradation and cell viability (Ghislain *et al.*, 1993; Rubin *et al.*, 1996). A conditionally lethal, cold-sensitive mutant *cdc48-1* was used to determine whether Cdc48p plays a role in degradation of a test protein such as Ub-Pro-β-gal. The results (Figure 7) showed that Cdc48p is indeed required for wild-type rates of Ub-dependent protein degradation, and suggested that the proteolysis-sustaining role of Ufd3p may be mediated through its complex with Cdc48p.

The *S.cerevisiae* Cdc48p is essential in particular for a late step in the cell division cycle: at non-permissive temperature, strains bearing temperature-sensitive *cdc48* alleles arrest as large budded cells containing an undivided nucleus in the neck between the mother and daughter cells (Fröhlich *et al.*, 1991). Recently, Cdc48p was shown to be required for the homotypic fusion of ER membranes (Latterich *et al.*, 1995). The *S.cerevisiae* Cdc48p is a member of a set of highly conserved prokaryotic and eukaryotic ATPases (Schnall *et al.*, 1994; Feiler *et al.*, 1995). The functions of this diverse family include proteasome-mediated proteolysis (Dubiel *et al.*, 1993; Rubin *et al.*, 1996), vesicular transport and fusion (Wilson *et al.*, 1989; Latterich *et al.*, 1995), biogenesis of peroxisomes (Erdmann *et al.*, 1991) and regulation of transcription (Nelböck *et al.*, 1990).

Immunolocalization studies of *S.cerevisiae* Cdc48p indicated that this apparently hydrophilic ATPase (it lacks putative membrane-spanning regions) is associated with the nuclear and ER membranes, but is also present in both the cytosol and nucleosol (Latterich *et al.*, 1995). The intracellular distribution of Ufd3p remains to be determined. One plausible (and testable) model is that Ufd3p is associated with a 'non-membrane' subpopulation of Cdc48p molecules that are present in the cytosol and/or nucleosol. Deletion of *UFD3* perturbs sporulation and Ub-dependent proteolysis but not cell viability (see Results), whereas deletion of *CDC48* is lethal (Fröhlich *et al.*, 1991). Furthermore, the *ufd3-Δ1* mutation does not result in synthetic lethality when combined with the *cdc48-1* mutation (our unpublished data). Thus, an essential function of Cdc48p must either not involve Ufd3p or involve it in a way that can be at least partially complemented by another protein. In particular, the conditional lethality of the *cdc48-1* mutant is not suppressed by overexpressing Ub (data not shown), whereas overexpression of Ub at least partially suppresses the proteolytic defect of *ufd3Δ* cells (Johnson *et al.*, 1995). If the essential function of Cdc48p involves the destruction of a short-lived inhibitor whose presence arrests cells in the late G_2 phase, Cdc48p must be sufficient for the required proteolysis even in the absence of Ufd3p.

At present, the only significant clue to the mechanics of the Ufd3p–Cdc48p function is provided by the findings that *ufd3* mutants have an abnormally low level of free Ub, and that an overexpression of Ub in *ufd3* cells at least partially complements their defect in degradation of Ub-Pro-β-gal and other test proteins (see Results, and also Johnson *et al.*, 1995). The low concentration of free Ub in *ufd3* cells is likely to account for the relative deficiency in multiubiquitylated (bearing multi-Ub chains) Ub-Pro-β-gal derivatives, and this, in turn, may account for the markedly reduced rate of Ub-Pro-β-gal degradation in *ufd3* cells. The mechanistic function of multi-Ub chains (Cook *et al.*, 1994) is unknown. Two mutually non-exclusive models for the role of a substrate-linked multi-Ub chain are first, that such a chain, through its binding to a component of the proteasome, reduces the rate of dissociation of a substrate–proteasome complex, and second, that a multi-Ub chain conformationally destabilizes a substrate to which it is covalently linked by interacting with the substrate non-covalently as well, in a manner analogous to that of chaperonins.

It is unlikely that Ufd3p regulates the level of free Ub by controlling the expression of *S.cerevisiae* Ub genes. First, the genes *UBI1–UBI3*, which produce the bulk of Ub in unstressed cells, encode Ub fusions to specific ribosomal proteins (Özkaynak *et al.*, 1987). A significant reduction in the rate of synthesis of Ub and these ribosomal proteins results in slow-growing cells (Finley *et al.*, 1989), whereas *ufd3Δ* cells are at most slightly growth impaired. Second, the expression of *UBI4*, the stress-inducible Ub gene, was found to be unperturbed in *ufd3* cells (our unpublished data). In addition, an overexpression of Ufd3p inhibits the degradation of Ub-Pro-β-gal (Figure 4B and C). Thus, an *optimal* level of Ufd3p is required for the maximal rate of Ub-Pro-β-gal degradation, which is impaired in cells that lack or overproduce Ufd3p.

One possibility is that Ufd3p, perhaps in a complex with Cdc48p, controls the amount or activity of Ub-specific proteases (isopeptidases) (Baker *et al.*, 1992; Huang *et al.*, 1995), which recycle Ub from multi-Ub chains back to the free Ub pool. Another model is suggested by the finding that p97/VCP, a mammalian homolog of Cdc48p (Koller and Brownstein, 1987; Peters

et al., 1990), binds the clathrin heavy chain *in vitro* (Pleasure *et al.*, 1993), and also by similarities between the phenotypes of *S.cerevisiae* mutants that lack, respectively, clathrin (Nelson and Lemmon, 1993) and Ufd3p. Specifically, clathrin-deficient cells have reduced levels of free Ub, and can be partially complemented by overexpression of Ub, analogously to *ufd3*Δ cells (see Results). Higher levels of free Ub are beneficial to a clathrin-lacking cell presumably because they enhance the degradation of mislocalized or misprocessed proteins that accumulate in the absence of clathrin (Nelson and Lemmon, 1993). Another possibility is suggested by the finding that Cdc48p participates in homotypic membrane fusion (Latterich *et al.*, 1995). Specifically, Ufd3p might be a functional analog of Sec17p, a *S.cerevisiae* SNAP protein that binds to Sec18p (an ATPase with significant sequence similarities to Cdc48p) and is involved in heterotypic membrane fusion (reviewed by Mellman, 1995). Although no sequence similarities were found between Ufd3p and either Sec17p or its mammalian SNAP counterpart, it should be interesting to determine whether *ufd3* mutants exhibit defects in membrane fusion similar to those of *cdc48* mutants (Latterich *et al.*, 1995). Yet another possibility is that Cdc48p (or Ufd3p) may function as a distinct E2 or E3 enzyme of the Ub system (see Introduction for the terminology).

The discovery of the Ufd3p–Cdc48p complex and the finding that this complex is a part of the Ub system open up a new direction for studies of the function of Ub in the cell cycle and membrane dynamics.

Materials and methods

Strains, media, genetic techniques and β-galactosidase assay

Saccharomyces cerevisiae was grown in rich (YPD) medium containing 1% yeast extract, 2% peptone (Difco) and 2% glucose, or in synthetic media, containing 0.67% yeast nitrogen base without amino acids (Difco), auxotrophic nutrients, and either 2% glucose (SD medium) or 2% galactose (SG medium) (Sherman *et al.*, 1986). For the induction of P_{CUP1} promoter, $CuSO_4$ was added to a final concentration of 0.1 mM. Transformation of *S.cerevisiae* was carried out using the lithium acetate method (Ausubel *et al.*, 1989). A β-gal colony overlay assay was performed using 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described by Barral *et al.* (1995). Enzymatic activity of β-gal in cell extracts from mid-exponential cultures growing on synthetic media was measured as described by Ausubel *et al.* (1989), using chlorophenol red-β-D-galactopyranoside (Eustice *et al.*, 1991). Unless stated otherwise, the *E.coli* strains used were DH5α and MC1061 (Ausubel *et al.*, 1989).

Plasmids encoding test proteins

The Ub-X-β-gal fusions (constructs I–III in Figure 1) were expressed from the galactose-inducible $P_{CYC1/GAL1}$ hybrid promoter in the high copy (2μ-based) plasmids pUB23-X (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989) and pXL (Baker and Varshavsky, 1995) that bore, respectively, *URA3* and *LEU2* markers. ('X' in these families of fusions refers to a varied amino acid residue after the last residue of Ub.) The plasmid pBAQ that expressed hemagglutinin (ha) epitope-containing Ub-Gln-Ura3p (construct IV in Figure 1) from the P_{CUP1} promoter was produced from pBAM, a pRS314-based, low copy (*CEN*-based) plasmid that encoded Ub-Met-Ura3p (Johnson *et al.*, 1995) (details are available upon request). The high copy, YEplac181-based plasmid pCUP1-UB::LEU2 (Johnson *et al.*, 1995), renamed pCUPUBI in this study, expressed ubiquitin from the P_{CUP1} promoter. YEpDeg1-URA3 is a YEp13-based plasmid that encodes the first 67 residues of Matα2p fused to β-gal (Hochstrasser and Varshavsky, 1990). All constructs were verified by nucleotide sequencing (Ausubel *et al.*, 1989).

S.cerevisiae strain in which the expression of Ubr1p is controlled by galactose

The strain JD54 (P_{GAL1}-*UBR1*) was produced from JD47-13C (Table I) (Madura *et al.*, 1993) by replacing a part of the P_{UBR1} promoter (from position –342 to –19, in the notation of Bartel *et al.*, 1990) with P_{GAL1}. Specifically, oligonucleotide-directed mutagenesis (Ausubel *et al.*, 1989) was employed to introduce a *Bam*HI site 24 bp upstream of the *UBR1* start codon in the plasmid pSOB35 (Bartel *et al.*, 1993). A 685 bp *Bam*HI fragment of pBM272 (Hovland *et al.*, 1989) that contained the $P_{GAL1/10}$ promoter was ligated to *Bam*HI-cut pSOB35. (The above *Bam*HI fragment contained a *Sma*I site, introduced by oligonucleotide-directed mutagenesis, between P_{GAL1} and P_{GAL10}. In addition, one of the two *Bam*HI sites in pBM272 was adjacent to an engineered *Eco*RI site at the 3′ end of P_{GAL10}.) The P_{GAL10} promoter was then deleted as a *Sma*I fragment, and replaced with a 589 bp *Dra*I fragment corresponding to the –834/–345 region of the *UBR1* locus (Bartel *et al.*, 1993). A *Stu*I site in this fragment and a *Pst*I site 810 bp downstream of the *UBR1* stop codon were used to produce a 7.38 kb transplacement fragment. *S.cerevisiae* JD47-13C was co-transformed with this fragment and the plasmid pCURTO314 (our unpublished data) that expressed a Ura3p-based N-end rule substrate. Transformants that were Ura^+ (long-lived Ura3p reporter) on glucose-containing medium but Ura^- (short-lived Ura3p reporter) on galactose-containing medium were selected. The transplacement was verified (Rothstein, 1991) by Southern hybridization (data not shown). JD54, one of transformants thus obtained, was cured of pCURTO314 by growing cells in YPD.

A screen for N-terminal sequences that confer UBR1-dependent metabolic instability on a reporter protein

Oligonucleotides 5′-CGCGGAATTCATG$(N)_{29}$CGGATCCG-3′ containing 29mer random sequences flanked by the *Eco*RI and *Bam*HI sites were made double-stranded by mutually primed second strand synthesis (Oliphant *et al.*, 1986), digested with *Eco*RI and *Bam*HI, and inserted into *Eco*RI–*Bam*HI-cut pBAM, immediately upstream of a sequence encoding e^K-ha-Ura3p (see above). The e^K-ha-Ura3p moiety of the resulting Met-X_{10}-e^K-ha-Ura3p fusions bore a 12 residue sequence containing the ha epitope (Field *et al.*, 1988; Johnson *et al.*, 1992, 1995), as well as e^K [extension (*e*) containing lysines (*K*)], a 45 residue sequence derived in part from *E.coli* Lac repressor (Bachmair and Varshavsky, 1989) (see also constructs V and VI in Figure 1). The pool of recombinant plasmids pCOUL was amplified in *E.coli* and transformed into the yeast *ura3* strain JD54 (Table I) in which the expression of *UBR1* had been placed under control of the P_{GAL1} promoter. Yeast transformants were first plated on SD(–Ura) medium to select for Ura^+ cells, thereby eliminating those among the pCOUL plasmids that contained stop codons within an X_{10}-coding sequence. Ura^+ transformants were then replica-plated onto SG medium containing 5-fluoroorotic acid (5-FOA). On this medium, the expression of *UBR1* (and hence also the activity of the N-end rule pathway) is induced by galactose. In the presence of FOA, which kills cells that contain a sufficiently high steady-state level of the Ura3p enzyme (Boeke *et al.*, 1984), only cells in which Met-X_{10}-e^K-ha-Ura3p became short-lived (because of the induction of the N-end rule pathway) would be expected to form colonies. Approximately 200 of the ~10^6 Ura^+ transformants initially selected on SD plates grew on SG-FOA plates. As expected, these transformants were unable to grow on SG(–Ura) plates that lacked both uracil and FOA. Plasmids isolated from 82 of the above ~200 transformants could confer a Ura^+ phenotype onto the *ura3 ubr1*Δ strain JD55 but not onto the *ura3 UBR1* strain JD47-13C (Table I). Some of the thus isolated Met-X_{10}-coding sequences were fused to a sequence encoding an e^K-bearing β-gal (see, for example, construct VII in Figure 1), by removing *URA3* as a *Bam*HI–*Sma*I fragment and replacing it with a *lacZ*-containing *Bam*HI–*Sca*I fragment of pUB23 (Bachmair *et al.*, 1986). One of these N-degron-producing N-terminal extensions of e^K-ha-Ura3p (extension #23) (Figure 1, construct V) was employed in a screen described below. Other N-terminal extensions isolated in this work will be described elsewhere.

Isolation of ufd3-2 mutant

The *ura3* strain JD47-13C (Table I) carrying the plasmid pDL that expressed Asp-β-gal (Ub-Asp-β-gal) from the $P_{GAL1/10}$ promoter was mutagenized with ethyl methanesulfonate to ~30% survival (Lawrence, 1991). Approximately 4500 mutant isolates were transformed with a plasmid encoding the short-lived 23-Ura3p test protein (Figure 1, construct V) and screened for Ura^+ cells, where 23-Ura3p was likely to be longer-lived than in parental (Ura^-) cells. The Ura^+ colonies were replica-plated on galactose-containing medium to induce the expression of Asp-β-gal (Ub-Asp-β-gal), and β-galactosidase activity was examined

Table I. *S.cerevisiae* strains used in this study

Strain	Genotype	References
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter (1989)
HF7c	*MATa ura3-52 his3-Δ200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17mer)3-CYC1-lacZ*	Feilotter *et al.* (1994)
DBY1705	*MATα leu2-3,112 ura3-52 lys2-801*	D.Botstein (Stanford University)
DBY2030	*MATa cdc48-1 ade2-1 lys2-801 ura3-52*	Fröhlich *et al.* (1991)
PM164	*MATa ufd3-1 his4-519 ura3-52 ade1-100 leu2-3,112*	Johnson *et al.* (1995)
JD47-13C	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112*	Madura *et al.* (1993)
JD51	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112*	Dohmen *et al.* (1995)
JD52	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112*	segregant of JD51, Johnson *et al.* (1995)
JD53	*MATα ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112*	segregant of JD51, Johnson *et al.* (1995)
JD55	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112 ubr1-Δ1::HIS3*	Madura and Varshavsky (1994)
JD54	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112 GAL1::UBR1*	derivative of JD47-13C
SGY3	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ate1-Δ2::LEU2*	derivative of YPH500, Grigoryev *et al.*[a]
SGY4	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 nta1-Δ2::LEU2*	Grigoryev *et al.*[a]
MGY1	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112 nta1 ufd3-2*	derivative of JD47-13C
MGY2	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112 ufd3-2*	segregant of MGY1
MGY3	*MATa ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-3,112 ufd3-Δ1::LEU2*	segregant of MGY5
MGY4	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112*	produced by mating JD52 and JD53
MGY5	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ufd3-Δ1/UFD3*	derivative of MGY4

[a]S.Grigoryev, A.E.Stewart, Y.-T.Kwon, S.M.Arfin, R.A.Bradshaw, N.A.Jenkins, N.J.Copeland and A.Varshavsky, manuscript in preparation.

using the X-Gal overlay assay. Seventeen of 38 Ura$^+$ colonies remained white on X-Gal (low levels of Asp-β-gal).

Pulse–chase analyses of 17 putative mutants identified one strain, termed MGY1, in which 23-Ura3p, but not Asp-β-gal, was significantly longer-lived than in the parental strain JD47-13C. Further pulse–chase analyses, carried out with Ub-Pro-β-gal, Asn-β-gal and Asp-β-gal in MGY1 that had been cured of the other reporter plasmids indicated that MGY1 cells were impaired in the degradation of Ub-Pro-β-gal and Asn-β-gal but not Asp-β-gal. MGY1 was crossed to the congenic wild-type strain YPH500 (Table I), and the resulting diploid was sporulated. Tetrad analyses showed that the defects in degradation of Asn-β-gal and Ub-Pro-β-gal segregated independently, suggesting the presence of two mutations in the strain MGY1. Complementation tests (Sherman *et al.*, 1986) revealed that one of these mutation affected *NTA1*, which encodes Nt-amidase (Baker and Varshavsky, 1995) (see Introduction), while the second lesion, termed *ufd3-2*, was found to be allelic to *ufd3-1*, a mutation that perturbs the degradation of Ub-Pro-β-gal (Johnson *et al.*, 1995). A meiotic segregant (termed MGY2) of the MGY1/YPH500 diploid that contained the *ufd3-2* mutation but not a mutation in *NTA1* was used to clone the wild-type *UFD3* gene.

Isolation of UFD3 and ufd3-2

The strain MGY2 (*ufd3-2*) carrying pUB23-P that expressed Ub-Pro-β-gal was transformed with a yeast genomic DNA library constructed in the *LEU2*, *CEN4*-based vector p366, also known as pBS32 (Rose and Broach, 1991) (the library was purchased from the American Type Culture Collection; ATCC #77162). Approximately 6400 Leu$^+$ transformants were plated onto SG plates and screened for white colonies (low levels of Ub-Pro-β-gal) using the X-Gal overlay assay (see above). Colonies of three (out of seven) initially picked transformants remained white upon retesting by the X-Gal assay. Pulse–chase analysis of these isolates identified one transformant in which the degradation of Ub-Pro-β-gal was restored. The library-derived plasmid, termed pUFD3, was recovered from this transformant and shown to complement the Ub-Pro-β-gal degradation defect in MGY2. Of several pUFD3 subclones (generated in the background of the vector pRS423; Christianson *et al.*, 1992), only pUFD3C1 complemented the *ufd3-2* mutation (Figure 5A). Partial sequencing from either side of the ~4.3 kb insert showed it to be identical to a region of the chromosome XI containing the ORFs YKL213c and YKL214c (Tzermia *et al.*, 1994). The YKL214c ORF was contained within a non-complementing 3.5 kb *Eco*RI fragment of pUFD3 (the plasmid pUFD3E1 in Figure 5A). By contrast, plasmids that contained the YKL213c but not the YKL214c ORF (Figure 5A) could complement the Ub-Pro-β-gal degradation defect of the *ufd3-2* strain MGY2. This and other data (see below) showed that the YKL213c ORF was the *UFD3* gene sought.

To verify that the yeast DNA insert of pUFD3 was derived from the chromosomal locus of the *ufd3-2* mutation, an ~3.6 kb *Eco*RI fragment from pUFD3E2 (Figure 5A) was inserted into the *URA3*-marked, *Eco*RI-cut integrating vector YIp5 (Struhl *et al.*, 1979), yielding pIUE2. This plasmid was linearized with *Sal*I and used to transform the *ufd3-2* strain MGY2. A Ura$^+$ transformant was selected, and mated to the *UFD3* strain JD53 that carried the *LEU2*-marked plasmid pPL expressing Ub-Pro-β-gal. The resulting diploid was sporulated, tetrads were dissected and tested for their Ufd3$^+$ (the ability to degrade Ub-Pro-β-gal) and Ura$^+$ phenotypes. A majority of tetrads were of the parental ditype class, indicating that the putative *UFD3* gene was closely linked to the chromosomal *ufd3-2* mutation.

The mutant *ufd3-2* gene of the MGY2 strain was amplified by PCR, using the AmpliTaq DNA polymerase (Perkin-Elmer) and oligonucleotide primers OX11 and OX12 (see below). The amplified DNA was cloned as a *Cla*I–*Xho*I fragment into *Cla*I–*Xho*I-cut pRS424GAL (see below). One cloned PCR product from each of the two independent assays was sequenced and found to contain the same G→A mutation.

Construction and analysis of the ufd3-Δ1 mutant

The ~4.3 kb *Cla*I fragment of pUFD3C1 (Figure 5A) was ligated into *Cla*I-cut pRS314, a *TRP1*-marked low copy vector (Sikorski and Hieter, 1989), yielding pCUC1. An ~2 kb *Nde*I–*Hpa*I fragment of pCUC1 that encompassed ~80% of the *UFD3* coding region (Figure 5A) was replaced with an ~2.2 kb *Xho*I–*Sal*I fragment of YEp13 (Broach *et al.*, 1979) containing *LEU2* (the ends of this fragment had been blunted with Klenow Pol I). An ~4.0 kb *Hin*dIII–*Pvu*I fragment of the resulting plasmid (pufd3-Δ1::LEU2) that contained the deletion/disruption *ufd3-Δ1::LEU2* allele was used to replace the wild-type *UFD3* of the diploid strain MGY4 by homologous recombination (Rothstein, 1991). MGY4 was produced by mating JD52 to JD53 (Table I). A *ufd3-Δ1/UFD3* diploid, MGY5, was sporulated and tetrads were dissected. A Leu$^+$ meiotic segregant, termed MGY3, that contained the *ufd3-Δ1::LEU2* allele was mated to the *ufd3-1* strain PM164 (Table I) (Johnson *et al.*, 1995), and also to the *ufd3-2* strain MGY2, each strain carrying pUB23-P that expressed Ub-Pro-β-gal. Pulse–chase analyses of the resulting diploid strains showed that Ub-Pro-β-gal degradation defects of the PM164 and MGY3 haploid strains failed to complement each other in a diploid. The strain produced by mating of MGY3 to PM164 was sporulated. Only six tetrads were analyzed, because of inefficient

sporulation of *ufd3* diploids (see Results). All of the examined haploid segregants were impaired in the degradation of Ub-Pro-β-gal. These data indicated that the relevant mutations in the PM164 and MGY3 strains were in the same (*UFD3*) gene.

Plasmids overexpressing Ufd3p

PCR was carried out using either the AmpliTaq (Perkin-Elmer) or Pwo (Boehringer Mannheim) DNA polymerase. Nucleotide sequencing was performed using double-stranded plasmid DNAs as templates and the Sequenase kit (US Biochemical Corp). An ~0.6 kb *Eco*RI–*Bam*HI fragment of YEplac195GAL that contained the $P_{GAL1/10}$ promoter was ligated to *Eco*RI–*Bam*HI-cut pRS314 and pRS424 (Sikorski and Hieter, 1989; Christianson *et al.*, 1992), yielding, respectively, the plasmids pRS314GAL and pRS424GAL. The *UFD3* gene was amplified by PCR, using pUFD3C1 as a DNA template and the primers OX11 (5′-CCA-ATCGATCCATGGGATATCAATTGAGT-3′) and OX12 (5′-CCACT-CGAGTTAGGAGAGATCGTCGAA-3′), which were complementary, respectively, to the 5′ and 3′ termini of *UFD3*. The PCR product was digested with *Cla*I and *Xho*I, and ligated to *Cla*I–*Xho*I-cut pRS424GAL, placing *UFD3* under control of the *S.cerevisiae* $P_{GAL1/10}$ promoter. To eliminate potential PCR-generated mutations in the *UFD3* ORF, the *Aat*II–*Bgl*II fragment of pUFD3C1 that encompassed the bulk of *UFD3* ORF was used to replace the corresponding *Aat*II–*Bgl*II fragment of the above PCR product, yielding pPCRUFD3. The identity of the *UFD3* ORF thus produced to the previously identified YKL213C and F715 ORFs (Dujon *et al.*, 1994; Tzermia *et al.*, 1994; see Results) was confirmed by nucleotide sequencing. An ~2 kb *Nco*I–*Xho*I fragment of pPCRUFD3 that contained *UFD3* was filled in with Klenow Pol I and ligated to *Bam*HI-cut pRS314GAL, yielding pGALUFD3.

The plasmid pET30f was a modified pET30a (Novagen Inc.) that allowed T7 polymerase promoter-driven expression of proteins N-terminally tagged with the His_6 (Hoffman and Roeder, 1991) and FLAG (Brizzard *et al.*, 1994) tags. FLAG is an eight residue sequence recognized by the monoclonal antibody M2 (IBI/Eastman Kodak). An *Nco*I–*Xho*I fragment of pGALUFD3 that contained *UFD3* was ligated to *Nco*I–*Xho*I-cut pET30f, yielding pETUFD3. The predicted N-terminal sequence of the resulting His_6-FLAG-Ufd3p fusion protein was **MG***H-HHHHHDYKDDDDK*AMG**YQ** [the residues of wild-type Ufd3p are in bold face, the six His residues and the FLAG epitope (DYKDDDDK) are in italics, and the three construction-derived residues are underlined]. pETUFD3 was digested with *Xba*I and *Xho*I. The resulting ~2 kb fragment encoding His_6-FLAG-Ufd3p was blunted with Klenow Pol I and ligated into *Bam*HI-cut, blunted pRS314GAL, yielding pFLAGUFD3, which overexpressed His_6-FLAG-Ufd3p from the $P_{GAL1/10}$ promoter. The epitope-tagged Ufd3p was functionally active, in that Ufd3p and His_6-FLAG-Ufd3p were indistinguishable in their ability to complement the Ub-Pro-β-gal degradation defect in *ufd3*Δ cells.

Anti-Ufd3p antibodies, immunoblotting and immunoprecipitation

Escherichia coli BL21 (DE3) (Studier *et al.*, 1990) was transformed with pETUFD3 expressing His_6-FLAG-Ufd3p from the T7 promoter. Cells were grown at 37°C to an A_{600} of ~0.5. Isopropylthiogalactoside (IPTG) was then added to a final concentration of 1 mM, and the incubation was continued for another 3 h. Cells were harvested by centrifugation and lysed by freezing–thawing in 40 ml of a lysis buffer (0.1% Triton X-100, 2 mM EDTA, 50 mM Tris–HCl, pH 8.0). Inclusion bodies containing overexpressed His_6-FLAG-Ufd3p (~30% of total protein) were resuspended in 2 ml of a binding buffer (0.1 M Na phosphate, 0.01 M Tris–HCl, pH 8.0) containing 8 M urea. Solubilized His_6-FLAG-Ufd3p was purified by Ni^{2+}-chelate affinity chromatography, using Ni^{2+}-nitrilotriacetic acid–agarose (Qiagen). Fractions containing the eluted His_6-FLAG-Ufd3p were dialyzed against a buffer (10% glycerol, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0) containing decreasing concentrations of urea to allow refolding of His_6-FLAG-Ufd3p. Polyclonal antibodies to His_6-FLAG-Ufd3p were raised in two New Zealand white rabbits using standard procedures (Harlow and Lane, 1988). The purified protein (~100 μg per rabbit) was injected with complete Freund adjuvant, followed by a boost with the same amount of antigen and incomplete Freund adjuvant 7 weeks later. Serum was collected 2 weeks after the boost, and pre-cleared by passing it through a column containing a whole-cell extract from *ufd3*Δ *S.cerevisiae* cross-linked to CNBr-activated Sepharose.

For the preparation of crude extracts, ~10^9 yeast cells were harvested by centrifugation and washed once with water. The cell pellet was resuspended in 0.5 ml of cold buffer A [1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 50 mM Na HEPES (pH 7.5), 1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin, pepstatin A, chymostatin, antipain and aprotinin, each at 2 μg/ml]. An equal volume of 0.5 mm glass beads was added, followed by vigorous vortexing for 3 min. The beads and cell debris were removed by centrifugation at 13 000 *g* for 10 min at 4°C. A 10 μl sample was withdrawn for protein determination using the Bio-Rad Protein Assay (Bio-Rad), with bovine serum albumin as a standard. Extracts containing 10 μg of total protein were fractionated by SDS–10% PAGE (Ausubel *et al.*, 1989), and then electroblotted onto a nitrocellulose membrane (Millipore) at 0.8 mA/cm^2 for 60 min, using a semidry transfer apparatus (Hoefer Scientific). For immunoblotting, the anti-Ufd3p serum (pre-cleared as described above) and secondary horseradish peroxidase-conjugated anti-rabbit antibodies were used at the dilution of 1:4000 and 1:2000, respectively. The bound secondary antibodies were detected using the ECL system (Amersham).

For immunoprecipitation assays, the cells were labeled for 1 h at 30°C with 0.15 mCi of [^{35}S]methionine/cysteine (EXPRESS; New England Nuclear), followed by preparation of extracts as described above. Ufd3p or His_6-FLAG-Ufd3p were immunoprecipitated by mixing cell extracts (175–350 μg of total protein) with, respectively, the pre-cleared anti-Ufd3p serum diluted 1:500, or the monoclonal anti-FLAG M2 antibody (3 μg/ml; IBI/Eastman Kodak) diluted 1:200, and incubating the samples with rocking for 2 h at 4°C. Protein A–Sepharose (Repligen) or immobilized protein G (Pierce) were then added, followed by rocking at 4°C for 1 h, and a 5 s centrifugation in a microcentrifuge. The pellets were washed three times with the lysis buffer, resuspended in SDS sample buffer (Ausubel *et al.*, 1989), heated at 100°C for 3 min, and subjected to SDS–10% PAGE, followed by fluorography.

Pulse–chase analysis

Saccharomyces cerevisiae cells from 10 ml cultures (A_{600} of 0.5–1) in SG or SD media containing 0.1 mM $CuSO_4$ were pelleted by a 10 s centrifugation in a microcentrifuge, and washed in the same medium. The cells were resuspended in 0.4 ml of SG or SD containing 0.1 mM $CuSO_4$, and labeled for 5 min at 30°C with 0.15 mCi of [^{35}S]methionine/cysteine (EXPRESS, New England Nuclear). The cells were harvested by centrifugation, resuspended in 0.3 ml of growth medium containing 10 mM L-methionine and 0.5 mg/ml cycloheximide, and incubated further at 30°C. Samples of 0.1 ml were withdrawn during the incubation and added to 0.7 ml of the lysis buffer (1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 50 mM Na HEPES, pH 7.5) containing also 1 mM PMSF and other protease inhibitors (see above), each at 20 μg/ml. Then 0.5 ml of 0.5 mm glass beads was added, and the cells were disrupted by vortexing three times for 1 min, with intermittent cooling on ice. The samples were centrifuged at 14 000 *g* for 15 min. The volumes of supernatants were adjusted to equalize the amounts of 10% trichloroacetic acid (TCA)-insoluble ^{35}S, and the samples were processed for immunoprecipitation and SDS–PAGE as described by Dohmen *et al.* (1995), using either the anti-β-gal (Promega) or the anti-ha (Babco Inc.) monoclonal antibody. SDS–PAGE ^{35}S patterns were detected by fluorography or autoradiography, and quantified using PhosphorImager (Molecular Dynamics). The proteins' half-life values cited herein are the 'initial' half-lives, determined by assuming first-order kinetics between the end of the pulse and the earliest chase time (Baker and Varshavsky, 1991; Lévy *et al.*, 1996).

Purification and sequencing of 23-β-gal

Extracts were prepared (using a spheroplast isolation and lysis procedure described below) from the *ubr1*Δ strain JD55 (Table I) that expressed 23-β-gal (Figure 1C, construct VII) from the P_{CUP1} promoter. 23-β-gal was isolated from the clarified lysate by affinity chromatography on a column of a monoclonal anti-β-gal antibody coupled to cross-linked agarose beads (Promega), further purified by SDS–PAGE and subjected to sequencing at the Microchemistry Facility (Harvard University) as described by Gonda *et al.* (1989). Besides the N-terminal sequence Arg-Glu-Leu-Ser (see Results), a second sequence, Ser-Leu-Val-Lys-Arg-Lys-Thr, was also detected; it matched an internal sequence of the e^K region of 23-β-gal, indicating that some molecules of 23-β-gal were cleaved 20 residues from their initial N-terminus. It is unknown whether this second cleavage was alternative to the cleavage that exposed Gln2 at the N-terminus of newly formed 23-β-gal. It is also unknown whether this second cleavage took place in wild-type (*UBR1*) cells, in which 23-β-gal was short-lived (see Results).

Purification and sequencing of a protein bound to Ufd3p

Approximately 2×10^{10} cells of the *ufd3*Δ strain MGY3 that carried the plasmid pFLAGUFD3 expressing His_6-FLAG-Ufd3p were grown to an A_{600} of ~1 in SG medium. Cells were converted to spheroplasts

using zymolyase 100T (ICN) as described by Ausubel *et al.* (1989). Spheroplasts were washed three times with 1 M sorbitol, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris–HCl (pH 7.5), and then osmotically lysed in two pellet volumes of buffer A (see above). The extract was centrifuged for 15 min at 15 000 *g*, and then for 40 min at 100 000 *g*. The supernatant (~10 ml at ~10 mg/ml of total protein) was applied to a 2 ml anti-FLAG M2 antibody affinity column (2.8 mg of the M2 monoclonal antibody per ml of gel) prepared according to the manufacturer's instructions (IBI/Eastman Kodak). The column was washed with three 10 ml samples of TBS buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.5). The bound His_6-FLAG-Ufd3p fusion protein and Cdc48p (see Results) were eluted by adding three 2 ml samples of TBS containing respectively, 50, 100 and 200 μg/ml of FLAG peptide (IBI/Eastman Kodak). The eluted proteins were fractionated by SDS–7% PAGE, and electroblotted onto a polyvinylidene difluoride membrane (Pro-Blot, Perkin Elmer/Applied Biosystems). After a brief staining with Coomassie blue, the band of 105 kDa Cdc48p protein (~12 pmol) was excised and subjected to N-terminal sequencing for 12 cycles using the Applied Biosystems 476A protein sequencer (Microchemistry Facility, Caltech).

Two-hybrid assays

The *in vivo* interaction between Ufd3p and Cdc48p was verified using the two-hybrid assay (Fields and Song, 1989; Phizicky and Fields, 1995). The pGBT9 and pGAD424 cloning vectors, the pTD1 and pLAM5′ control plasmids (Bartel *et al.*, 1993; Li and Fields, 1993) and the yeast strain HF7c (Feilotter *et al.*, 1994, see Table I) were purchased from Clontech. The *S.cerevisiae CDC48* gene was amplified from genomic DNA of JD47-13C cells (Table I) using the primers PCC1 (5′-CCACCCGGGATCCATGGGTGAAGAACATAA-3′) and PCC2 (5′-CCACTCGAGCTAACTATACAAATCATC-3′). The PCR product was digested with *Sma*I and *Xho*I, and ligated to *Sma*I–*Sal*I-cut pGAD424, yielding pACDC48 that expressed a fusion of Cdc48p to the transcription-activating domain of Gal4p. The PCR-amplified *UFD3* and *ufd3-2* genes were digested with *Nco*I, blunted with Klenow Pol I, digested with *Xho*I and then ligated to the *Sma*I–*Sal*I-cut plasmid pGBT9, yielding pBUFD3 and pBUFD3-2, which expressed fusions of, respectively, Ufd3p and Ufd3-2p to the DNA binding domain of Gal4p. The first 456 N-terminal residues of Ufd3p were also fused to the DNA binding domain of Gal4p by ligating the *Nco*I–*Pst*I fragment of pPCRUFD3 to *Nco*I–*Pst*I-cut pGBT9, yielding pBUFD3ΔC. Nucleotide sequences of the constructs were verified using the chain termination method (Ausubel *et al.*, 1989).

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