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Placing a Disrupted Degradation Motif at the C Terminus of Proteasome Substrates Attenuates Degradation without Impairing Ubiquitylation*

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Background: Initiation of protein degradation by the proteasome requires the presence of an unstructured motif, usually located at the termini of the substrate.
Results: Disruption of a hydrophobic sequence within such an unstructured motif abolishes proteasomal degradation.
Conclusion: The hydrophobicity requirement reveals a novel feature of degradation initiation sites.
Significance: Diverse proteasome substrates can be selectively stabilized by fusing a disrupted degradation initiation site to their termini.

Protein elimination by the ubiquitin-proteasome system requires the presence of a *cis*-acting degradation signal. Efforts to discern degradation signals of misfolded proteasome substrates thus far revealed a general mechanism whereby the exposure of cryptic hydrophobic motifs provides a degradation determinant.

We have previously characterized such a determinant, employing the yeast kinetochore protein Ndc10 as a model substrate. Ndc10 is essentially a stable protein that is rapidly degraded upon exposure of a hydrophobic motif located at the C-terminal region. The degradation motif comprises two distinct and essential elements: *DegA*, encompassing two amphipathic helices, and *DegB*, a hydrophobic sequence within the loosely structured C-terminal tail of Ndc10. Here we show that the hydrophobic nature of *DegB* is irrelevant for the ubiquitylation of substrates containing the Ndc10 degradation motif, but is essential for proteasomal degradation. Mutant *DegB*, in which the hydrophobic sequence was disrupted, acted as a dominant degradation inhibitory element when expressed at the C-terminal regions of ubiquitin-dependent and -independent substrates of the 26S proteasome. This mutant stabilized substrates in both yeast and mammalian cells, indicative of a modular recognition moiety. The dominant function of the mutant *DegB* provides a powerful experimental tool for evaluating the physiological implications of stabilization of specific proteasome substrates in intact cells and for studying the associated pathological effects.

Degradation signals, or degrons, are usually defined as minimal elements within proteins that are essential for the degradation of substrates by a proteolytic apparatus (1). An important property of autonomous degrons is that they are interchangeable; and once genetically fused to an otherwise long lived protein, they confer metabolic instability (2). Uncontrolled protein degradation might confer deleterious consequences at the cellular and the whole organism levels. Consequently, degron exposure is tightly regulated (2).

Protein degradation by the ubiquitin (Ub)[2]-proteasome system can be classified into two major categories: regulated degradation of short lived proteins and protein quality control associated degradation (PQCD). Although the enzymatic machinery that targets protein ubiquitylation is well characterized, our knowledge of the mechanism(s) by which the ubiquitylation machinery recognizes degrons is still limited (2). Understanding these mechanisms is particularly challenging in PQCD pathways, where a diverse repertoire of substrates is ubiquitylated by a limited number of ubiquitylation E3 ligase complexes, thus necessitating recognition of multiple degrons by a single E3 ligase complex.

Studies of PQCD degrons have thus far revealed a general paradigm whereby exposure of hydrophobic sequences, generally buried within the protein core, provides a degradation signal (3–7). We have previously characterized a PQCD degron in detail, using the yeast kinetochore protein Ndc10 as a model substrate (5). Ndc10, normally a stable protein, is rapidly degraded upon exposure of hydrophobic motifs localized to its C-terminal region. The degradation is dependent on the endoplasmic reticulum membrane-embedded Ub E3 ligase, Doa10. Notably, placing the degron at the C terminus of otherwise stable proteins triggers Doa10-dependent protein degradation, demonstrating its autonomous nature.

Further characterization of the Ndc10 degron revealed that it comprises two distinct and essential elements: *DegA*, consisting

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[2] The abbreviations used are: Ub, ubiquitin; aa, amino acid(s); Can, canavanine; cODC, C-terminal ODC; mODC, mouse ODC; ODC, ornithine decarboxylase; polyUb, polyubiquitin; PQCD, protein quality control-associated degradation.

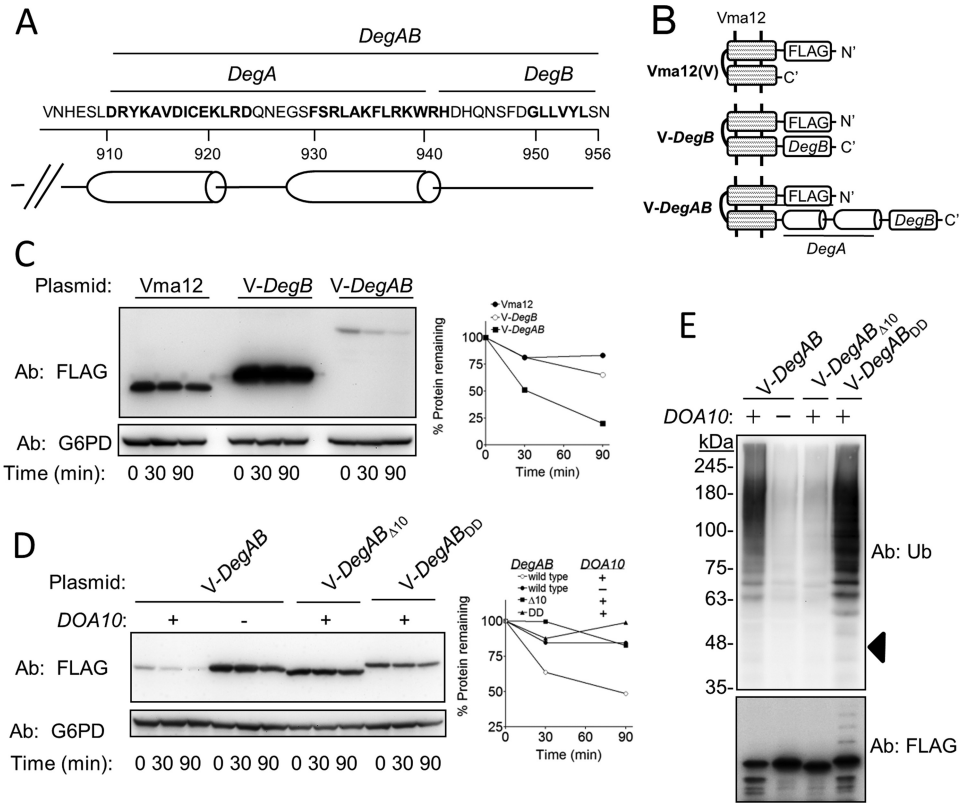

FIGURE 1. **The hydrophobic core of *DegB* of the Ndc10 degron is dispensable for ubiquitylation but essential for proteasomal degradation.** *A*, amino acid sequence and structure prediction of the Ndc10 degron are shown. *B*, schematic presentation of Vma12-degron fusions used in *C–E*. V-*DegB*: Vma12-*DegB*; V-*DegAB*: Vma12-*DegAB*. *C*, *DegB* is insufficient to confer degradation to Vma12. Cells expressing the indicated proteins were subjected to cycloheximide-chase degradation assay, as described under "Experimental Procedures." *D*, intact *DegB* is required for the degradation of Vma12-*DegAB*. Wild type and *doa10*Δ cells, expressing intact Vma12-*DegAB* or a truncated protein in which the last 10 aa were removed (Δ10), or a mutant protein in which two Leu residues at the hydrophobic core of *DegB* were replaced with Asp (DD), were subjected to cycloheximide degradation assay. *E*, the DD mutation within *DegB* did not impair the ubiquitylation of Vma12-*DegAB*. The various Vma12-*DegAB* proteins described in *D* were purified from cells using anti FLAG-agarose affinity gel. PolyUb chains conjugated to V12-*DegAB* were detected after separation on 5–15% gradient SDS-PAGE. *Triangle* indicates the estimated molecular mass of unconjugated V-*DegAB*.

of a hydrophobic surface buried between two amphipathic helices, and *DegB*, a hydrophobic sequence confined to the Ndc10 loosely structured C-terminal tail (Fig. 1*A*). Consistent with the general PQCD degron paradigm, exposure of an intact amphipathic helix within *DegA* is obligatory for ubiquitylation (5). The underlying role of *DegB* in protein degradation has remained obscure.

We hereby report a novel role for *DegB* as a degradation initiation determinant. We found that in contrast to *DegA*, the hydrophobicity of *DegB* was irrelevant for ubiquitylation but was essential for degradation by the 26S proteasome. Moreover, a mutant *DegB*, in which the hydrophobic sequence was disrupted, acted as a dominant degradation inhibitory element when expressed at the C terminus of Ub-dependent and -independent substrates. Importantly, the inhibition of degradation by mutant *DegB* was not specific to yeast proteasomes, as it similarly stabilized the transcription factor XBP1, an established proteasome substrate (8, 9) expressed in the human embryonic kidney (HEK) 293T cell line.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains

Details are provided in Tables 1 and 2.

Yeast, HEK293T Cells, and Bacterial Media

Yeast-rich media, minimal media, and bacterial media were prepared according to standard protocols. HEK293T cells were maintained in DMEM (10% FCS, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin).

Site-directed Mutagenesis

Site-directed mutagenesis was performed using *Pfu* Ultra polymerase, according to the manufacturer's instructions (Stratagene). All products were verified by sequencing.

Plasmid Construction

C-terminal Tagging of Mouse Ornithine Decarboxylase (mODC) and RPN10-GFP with DegB—DNA fragments containing the last C-terminal 15 amino acids (aa) of Ndc10 (*DegB*) were amplified with 3′- and 5′-flanking sequences complemented to regions within the C terminus of the target genes *RPN10-GFP* and *mODC*. The DNA fragments were integrated into their corresponding targets, after linearization of the backbone plasmids, by yeast recombination techniques. The presence of the 15-aa insertions was verified by sequencing.

TABLE 1
Yeast strains

Yeast	Genotype	Source
TRy108	*MATα his3-Δ200, leu2–3112, ura3–52, lys2–801, trp1–1, gal2*	Ref. 32
TRy171	*MATα his3-Δ200 leu2–3112, ura3–52, lys2–801, trp1–1, gal2, doa10-Δ1::HIS3*	Ref. 33
TRy647	*MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, rpn10Δ::kanMX*	Open Biosystem
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Open Biosystem

TABLE 2
Plasmids

Plasmid	Relevant markers	Source
pTR425	pFa6a-GFP-KanMX6	Ref. 34
pTR809	pOC9	Ref. 6
pTR883	pRS414 GPDp-FLAG-Vma12–6HIS-*DegAB*$_{\Delta 10}$-CyC1t	This work
pTR891	pRS416 GPDp-Rpn10-GFP-CyC1t	This work
pTR892	pRS416 GPDp-Rpn10-GFP-cODC (C441A)-CyC1t	This work
pTR990	Ubiquitin CUP1 promoter (CEN/LYS2)	M. Hochstrasser
pTR913	pRS414 GPDp-FLAG-Vma12-His$_6$-*DegAB*-Cyc1t	This work
pTR1075	pRS416 GPDp-Rpn10-GFP-*DegB*-CyC1t	This work
pTR1085	pRS416 GPDp-Rpn10-GFP-*DegB*$_{DD}$-CyC1t	This work
pTR1114	pRS414 GPDp-FLAG-Vma12-His$_6$-*DegAB*$_{DD}$-Cyc1t	This work
pTR1155	pEFIRES-P FLAG-ATZ-mODC	Ref. 35
pTR1157	pRS414 GPD-FLAG-mODC	This work
pTR1266	YCplac111 RFP-PUS1	M. Hochstrasser
pTR1274	pRS416 GPDp-Rpn10-GFP-*DegB*$_{DD}$-CyC1t	This work
pTR1291	pRS414 GPD-FLAG-mODC (Δ37)-*DegB*	This work
pTR1316	pUC57-*DegAB-DegB*$_{DD}$	This work
pTR1317	pUC57-*DegAB*$_{DD}$-*DegB*	This work
pTR1333	pRS414 GPDp-FLAG-mODC (Δ15)-*DegB*-CyC1t	This work
pTR1335	pRS414 GPDp-FLAG-mODC (Δ15)-*DegB*$_{DD}$-CyC1t	This work
pTR1338	pRS414 GPDp-FLAG-Vma12-His$_6$-*DegAB*$_{DD}$-*DegB*-CyC1t	This work
pTR1339	pRS414 GPDp-FLAG-Vma12-His$_6$-*DegAB-DegB*$_{DD}$-CyC1t	This work
pTR 1370	pEFIRES-P FLAG-ATZ-mODC	This work
pTR 1371	pEFIRES-P FLAG-ATZ-mODC (Δ37)-*Deg*$_B$	This work
pTR1372	pEFIRES-P FLAG-ATZ-mODC (Δ15)-*DegB*$_{DD}$	This work
pTR1373	pEFIRES-P FLAG-ATZ-mODC (Δ15)-*DegB*	This work
BT1	pcDNA3-XBP1s	Ref. 9
BT2	pcDNA3-XBP1u	Ref. 9
BT3	pcDNA3-XBP1s-*DegB*$_{DD}$	This work
BT4	pcDNA3-XBP1u-*DegB*$_{DD}$	This work

Construction of C-terminal DegAB with Tandem Repeats of DegB—The genes encoding for *DegAB-DegB*$_{DD}$ or *DegAB*$_{DD}$-*DegB* were synthesized *de novo* and cloned into pUC57 vector by GeneScript. To generate plasmids pTR1338 and pTR1339, the relevant DNA fragments were digested from pUC57 vector using the restriction enzymes AgeI and SalI and cloned into pTR913 predigested with the same restriction enzymes.

Construction of URA3-HA-GFP-DegB—To obtain plasmids containing Ura3-HA-GFP, the GFP-encoding gene fragment from plasmid pFa6a-GFP (pTR425) was PCR-amplified and cloned into the pOC9 plasmid (pTR801) at AatII and BamHI sites, creating plasmid pTR1269. The last 100 aa of Ndc10, with or without the L950D and L951D mutations, were amplified from pTR913 and pTR1114, respectively, and cloned into the pTR1269 at BamHI and SalI restriction sites to obtain pTR1270 and pTR1272, respectively.

Construction of XBP1s-DegB$_{DD}$ *and XBP1u-DegB*$_{DD}$—*DegB*$_{DD}$ was fused to X*BP1s* or X*BP1u* on pcDNA3.1 plasmids by PCR through the addition of 45 bp of the *DegB*$_{DD}$ sequence to the reverse primers. The PCR products were cloned into NotI and ApaI sites of pcDNA3.1.

Determination of Canavanine Sensitivity

Yeast cells were grown to late logarithmic phase, serially diluted 10-fold, and spotted on SD-Arg-Ura plates with 1.5 mg/ml of the Arg analog L-canavanine (Can) or on SD-Ura plates and incubated at 30 °C for 5 or 2 days, respectively. Cells were spotted at 1 A_{600}/spot and 10-fold serial dilutions thereof.

Cycloheximide-chase

Cycloheximide-chase was performed essentially as described previously (5). Briefly, logarithmically growing cells were treated with 0.5 mg/ml cycloheximide, and culture aliquots were removed at the designated time points. After incubating the cells in 0.1 M NaOH for 5 min at room temperature, cells were resuspended in SDS gel-loading buffer and heated at 95 °C for 5 min. Proteins were then separated on 5–15% SDS-PAGE followed by immunoblotting. Antibodies used for immunoblotting were anti-FLAG mAb (Sigma-Aldrich); anti-GFP mAb and anti-hemagglutinin (HA) mAb (Roche Applied Science); anti-glucose-6-phosphate dehydrogenase polyclonal antibodies (Sigma-Aldrich). Experiments were repeated two to four times and quantified using a G-Box chemi XR imaging system and GeneTools software (Syngene, UK). Representative results are shown.

Metabolic Labeling, Pulse-Chase Analysis, and Immunoprecipitation

After starvation in methionine/cysteine-free Dulbecco's modified Eagle's medium for 45 min, HEK-293T cells, transfected with plasmids pcDNA3-XBPu-*DegB*$_{DD}$ and pcDNA3-XBPs-*DegB*$_{DD}$ were metabolically labeled with 500

μCi/ml [^{35}S]methionine/cysteine (1,200 Ci/mmol) at 37 °C for 10 min. Pulse-chase experiments, cell lysis, and immunoprecipitation was performed as described previously (9). Purified proteins were analyzed by SDS-PAGE followed by film exposure. The experiment was repeated twice, and densitometry was performed using GeneTools software. Antibodies against XBP1 were from Santa Cruz Biotechnology. Representative results are shown.

Ubiquitylation Assay

Ubiquitylation assays were performed according to Loayza and Michaelis (10). Cells co-expressing the indicated FLAG-Vma12-*DegAB* plasmids, together with a plasmid containing copper-induced Ub, were incubated in selective media containing 100 μM $CuSO_4$ until late logarithmic phase. Approximately 25 A_{600} cell units were harvested and then lysed by addition of 1.5 ml of 2 N NaOH, 1 M β-mercaptoethanol. The lysate was incubated on ice with 5% trichloroacetic acid. Proteins were separated by centrifugation at 17,000 $\times$ *g* for 10 min at 4 °C, and the pellet was resuspended in 100 μl of sample buffer. Cell extracts were diluted 30-fold with buffer supplemented with protease inhibitors (Sigma) and 5 mM *N*-ethylmaleimide. Extracted proteins were pulled down by incubation with anti-FLAG M2 affinity gel (Sigma) at 4 °C for 3 h. Bead complexes were washed three times and then released from the gel by incubation with 3×FLAG peptides at 4 °C for several hours while shaking. Eluted proteins were separated by 5–15% SDS-polyacrylamide gradient gel and visualized by immunoblotting, using anti-FLAG and anti-Ub antibodies.

RESULTS

The Hydrophobic Core of DegB of the Ndc10 Degron Is Dispensable for Ubiquitylation but Essential for Proteasome-mediated Degradation—To investigate the precise role of *DegB* in protein degradation, we constructed reporter substrates composed of the stable endoplasmic reticulum protein Vma12 (11), fused to either *DegB* or to *DegAB* which accounts for the full-length of the Ndc10 degron (Fig. 1, *A* and *B*). Cycloheximide-chase degradation experiments in yeast revealed that Vma12-*DegB* remained stable, whereas Vma12-*DegAB* was rapidly degraded in a *DOA10*-dependent manner (Fig. 1, *C* and *D*). Thus, *DegB* is essential but insufficient for triggering Vma12 degradation.

DegB comprises a hydrophobic core composed of residues Gly-Leu-Leu-Val-Tyr-Leu (Fig. 1*A*). To examine whether the hydrophobicity of *DegB* is required for degradation, we compared the stability of Vma12-*DegAB* with that of Vma12-*DegAB*$_{\Delta 10}$, where the last 10 residues of Ndc10 were deleted, and with that of Vma12-*DegAB*$_{DD}$, in which the two Leu residues at positions 950 and 951 were substituted with negatively charged Asp residues, thereby disrupting the hydrophobic core. As shown in Fig. 1*D*, both the truncation and missense mutations substantially stabilized Vma12-*DegAB*, further emphasizing the fundamental role of the intact *DegB* in Vma12 degradation.

To examine the role of *DegB* in Ub conjugation, Vma12-*DegAB* fusion proteins were isolated by immunoprecipitation from the respective cell extracts using anti-FLAG Abs. PolyUb

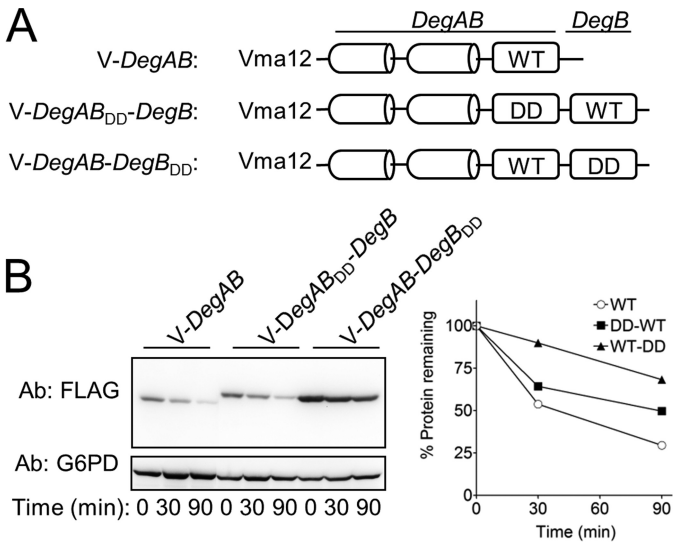

FIGURE 2. **DegB$_{DD}$ abolishes degradation when positioned at the C-terminal region of a proteasome substrate.** *A*, schematic presentation of *DegB* tandem sequences used in *B*. *B*, cycloheximide-chase degradation analysis reveals that fusing *DegB*$_{DD}$ to Vma12-*DegAB* abolished degradation whereas fusing intact *DegB* to Vma12-*DegAB*$_{DD}$ hardly affected degradation.

conjugates in the immune complexes were subsequently detected by immunoblotting analysis with anti-Ub. As anticipated, high molecular mass polyUb conjugates were observed in wild type cells but were hardly detected in *doa10*Δ cells (Fig. 1*E*). Vma12-*DegAB*$_{\Delta 10}$ ubiquitylation was also barely visible, demonstrating the importance of intact *DegB* for ubiquitylation. Unexpectedly, ubiquitylation was substantially augmented in Vma12-*DegAB*$_{DD}$, suggesting that the hydrophobicity of *DegB* is required for degradation, but irrelevant for ubiquitylation. We therefore hypothesized that the hydrophobic segment is required for processes downstream to ubiquitylation.

DegB$_{DD}$ Abolishes Degradation if Positioned at the C-terminal Region of a Proteasome Substrate—Because *DegB* is localized at the C terminus of Ndc10, we next investigated whether its localization is a critical determinant for degradation. To this end, we generated recombinant proteins, expressing wild type and mutant *DegAB* and *DegB* in tandem. As shown in Fig. 2*B*, wild type *DegB* restored protein degradation when fused C-terminally to the mutant degron (*DegAB*$_{DD}$-*DegB*), as also indicated by the similarity to the degradation kinetics of the wild type Vma12-*DegAB*. Thus, once proteolysis was initiated, the presence of *DegB*$_{DD}$ per *se* did not impede degradation. In the reciprocal experiment, when the mutant *DegB*$_{DD}$ was fused C-terminally to the wild type degron, protein degradation was substantially attenuated. The capacity of *DegB*$_{DD}$ to reverse the destabilizing properties of *DegB* when expressed C-terminally to it confirmed that the position of *DegB* at the extreme C terminus was an essential feature of the Ndc10 degron and that *DegB*$_{DD}$ operated as a dominant inhibitor of proteasomal degradation.

Impairment of Hydrophobicity Does Not Affect the Intracellular Localization of GFP-DegAB—A possible explanation for the observed stabilization conferred by *DegB*$_{DD}$ was that it redirected the proteins to alternative intracellular compartments, such as inclusion bodies, where they were no longer accessible

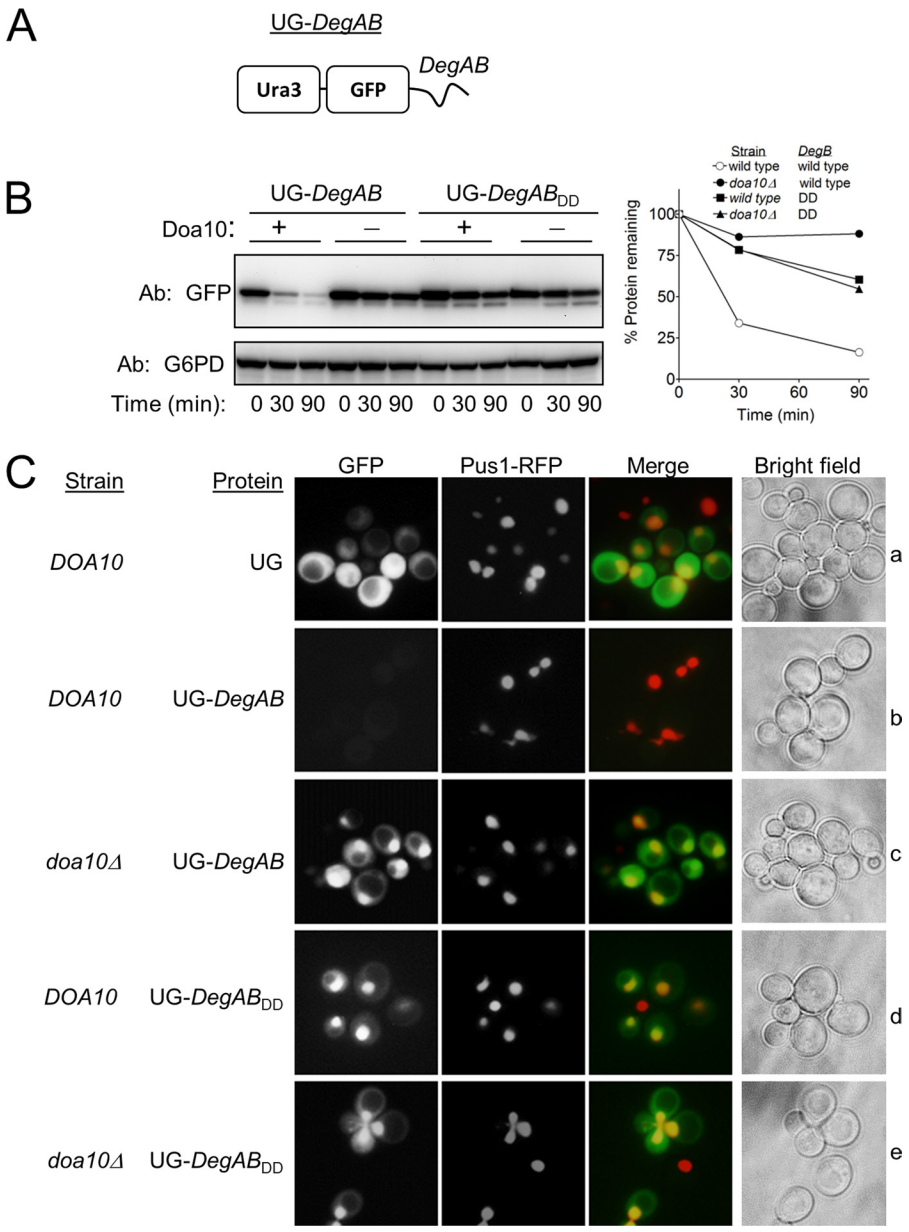

FIGURE 3. **DegB$_{DD}$ did not alter the nuclear localization of GFP-*DegAB*.** *A*, schematic presentation of Ura3-GFP-*DegAB* (UG-*DegAB*). *B*, degradation kinetics of UG-*DegAB* and UG-*DegAB*$_{DD}$ in wild type and *doa10*Δ cells are shown. Proteins levels were measured using anti GFP antibodies. *C*, the cellular localization of the GFP fusion proteins was determined by fluorescence microscopy. Nuclear staining of the accumulated proteins was indicated by their co-localization with Pus1-RFP, a nuclear protein. No changes in the cellular localization of the various stabilized proteins were indicated.

to the 26S proteasome (hence stabilized). To examine whether *DegB*$_{DD}$ influenced the cellular distribution of proteins, a reporter was constructed where the *URA3* gene product orotidine-5′-phosphate decarboxylase was fused to GFP, followed by *DegAB* to form Ura3-GFP-*DegAB* (Fig. 3*A*). *DegAB* positioned C-terminally to Ura3-GFP induced Doa10-dependent degradation of the fusion protein, whereas the addition of *DegAB*$_{DD}$ did not (Fig. 3*B*). Next, the cellular distribution of the fusion proteins in wild type and in *doa10*Δ cells was determined by fluorescence microscopy and compared with that of the nuclear-localized red fluorescence protein (RFP)-Pus1(12). Notably, whereas Ura3-GFP was localized in the cytosol, Ura3-GFP-*DegAB*, expressed in *doa10*Δ cells, was found mainly in the nucleus, suggesting that *DegAB* contained a nuclear localization signal (Fig. 3*C*, *c* and *e*). Ura3-GFP-*DegAB*$_{DD}$ was similarly localized in the nucleus in both wild type and *doa10*Δ cells (Fig. 3*C*, *d* and *e*). Taken together, these findings indicate that substrate stabilization via *DegB*$_{DD}$ was not due to altered intracellular localization.

DegB Degron Function Is Independent of Ubiquitylation—Degrons are generally defined as structural features within proteins that determine ubiquitylation, thus serving as recognition signals for E3 Ub ligases (2). We were intrigued by the finding that the disruption of *DegB* impaired degradation rather than ubiquitylation and postulated that *DegB* functions as an authentic degradation initiation site and thus, may also influence the degradation of Ub-independent 26S proteasome substrates. To test this hypothesis, we employed a yeast reporter

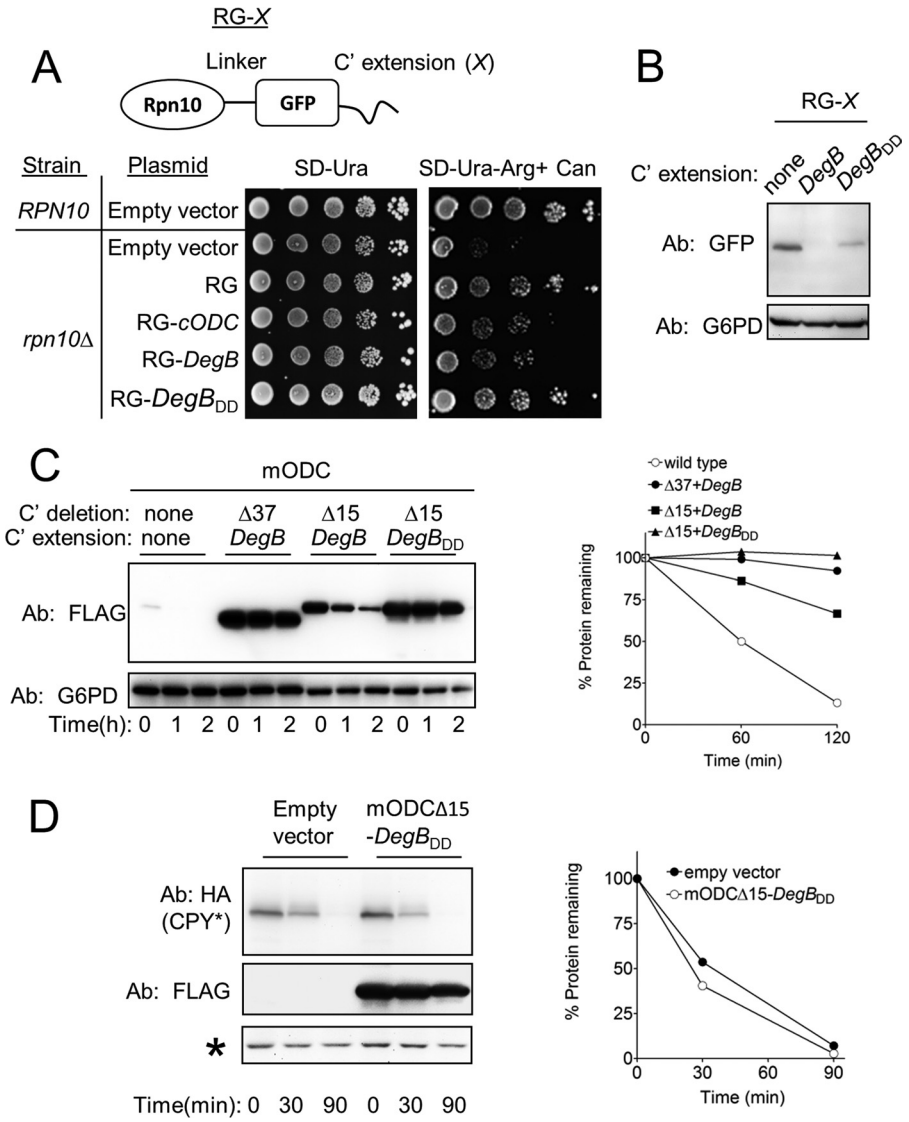

FIGURE 4. **DegB degron function is independent of ubiquitylation.** *A*, *upper panel*, schematic presentation of Rpn10-GFP fusion (RG-*X*), where *X*- is the various C-terminal extensions. *A*, *lower panel*, growth sensitivity to amino acid analog of *rpn10*Δ cells strains expressing RG-*X* is determined. Cells growing to late logarithmic phase were spotted as 10-fold serial dilutions onto SD-Ura or onto SD-Ura-Arg medium containing 1.5 μg/ml Can. Colony growth was determined after incubation at 30 °C for 4 days. *B*, disruption of the hydrophobicity of *DegB* increased the steady-state levels of RG-*DegB* fusion protein. *C*, $DegB_{DD}$ stabilized mODC. Degradation of mODC that was subjected to the indicated C-terminal truncations and replacements was measured in yeast by cycloheximide-chase degradation assay. *D*, degradation of CPY* remained intact in cells co-expressing mODCΔ15-*DegAB*. *Asterisk*, a crossreacting protein that served as a loading control. CPY*, mutant carboxypeptidase Y.

system for Ub-independent proteasomal degradation that relies on the capacity of cells to sustain growth in the presence of Can (13), a property that is strictly dependent on active 26S proteasomes. Thus, yeast cells lacking Rpn10, an integral component of the 19S regulatory complex, are viable, but grow poorly on plates containing Can (14, 15). Expression of Rpn10-GFP that integrates into the 26S proteasome in *rpn10*Δ cells, reestablishes Can resistance (13). Using Can resistance as a measure of Rpn10 stability it was found that fusion of Rpn10-GFP to a C-terminal unstructured sequence, such as the 37-residue-long C terminus of ODC (cODC), destabilized Rpn10-GFP, and consequently decreased Can resistance (13). It is therefore postulated that cODC functions as a degradation initiation cue (13).

To test whether *DegB* can functionally substitute for cODC, the stability of Rpn10-GFP proteins, fused to various degrons, was similarly investigated by means of their ability to confer Can resistance to *rpn10*Δ cells (Fig. 4*A*). In agreement with previous findings (13), growth of *rpn10*Δ cells was considerably reduced in the presence of Can and was restored upon the expression of Rpn10-GFP, but not of Rpn10-GFP-cODC. Consistent with results of the Vma12-*DegB* stability experiments, the growth of *rpn10*Δ cells in the presence of Can was facilitated by expression of Rpn10-GFP-$DegB_{DD}$ but not by expression of Rpn10-GFP-*DegB*, likely indicating that the reporter protein carrying the mutant *DegB* was stable. This possibility was confirmed by measurement of Rpn10-GFP derivatives. Both Rpn10-GFP and Rpn10-GFP-$DegB_{DD}$ were readily detected whereas Rpn10-GFP-*DegB* was undetectable (Fig. 4*B*). Taken together, we concluded that *DegB* functioned as a degradation initiation determinant in the context of the Rpn10-GFP reporter and that this feature was absent in $DegB_{DD}$.

Although Rpn10-GFP-*DegB* is likely an Ub-independent degradation substrate, Rpn10 potentially can undergo ubiquitylation when bound to the proteasome (16–18). To further validate the Ub-independent function of *DegB*, we next examined its function in an Ub-independent degradation system, utilizing ODC (19, 20), which is targeted for degradation through binding to the polyamine-induced protein, antizyme (21). In yeast, the degradation of heterologously expressed mODC by the 26S proteasome is dependent on its 37-aa cODC degron (22). Hence we tested whether *DegB* is capable of replacing cODC in targeting mODC for degradation.

As reported previously (23), mODC was rapidly degraded in yeast (Fig. 4*C*). Yet, we found that replacing the 37-aa-long cODC degron with *DegB* stabilized the protein, most likely due to the elimination of Cys residue at position 441, which is required for recognition by the 26S proteasome (22). Indeed, when only the extreme 15 aa of mODC were replaced with *DegB*, thus maintaining the essential Cys-441, the mODC-*DegB* protein was degraded, albeit with slower kinetics than the wild type mODC. Conversely, replacing the extreme 15 aa of mODC with $DegB_{DD}$ considerably attenuated degradation of the protein. These results established a critical requirement for the hydrophobic region within *DegB* for degradation by the 26S proteasome.

Next, we considered the possibility that fusion proteins containing $DegB_{DD}$ bind to the proteasome, but cannot be degraded, thereby obstructing entry of other proteasome substrates to the proteasome 20S catalytic core. The data shown in Fig. 4*A* argue against this mode of action because $DegB_{DD}$, when fused to Rpn10-GFP, reduced Can sensitivity, indicative of increased proteasome activity. To verify that indeed the proteasome remained active when expressing $DegB_{DD}$, we examined the degradation of mutant carboxypeptidase Y, a PQCD substrate of the Hrd1 pathway (24). As shown in Fig. 4*D*, when co-expressed with mODC-$DegB_{DD}$, mutant carboxypeptidase Y was degraded with the same kinetics as in cells expressing an empty vector or mODC-$DegB_{DD}$. Thus, the expression of mODC-$DegB_{DD}$ did not significantly inhibit the proteasome activity.

$DegB_{DD}$ Stabilized XBP1u and XBP1s Expressed in HEK293T Cells—We next tested whether $DegB_{DD}$ is also effective in stabilizing substrates of mammalian proteasomes, by fusing it to XBP1, a key transcription factor of the endoplasmic reticulum unfolded protein response. XBP1 exists in two forms: XBP1u, an unspliced form, and XBP1s, a spliced form produced upon noncanonical mRNA splicing (25, 26). XBP1s is an active transcription factor (27), whereas biological functions have not been ascribed to XBP1u, other than inhibiting XBP1s (28). Importantly, XBP1u is an extremely short lived protein that utilizes Ub-dependent and Ub-independent mechanisms of proteasomal degradation. XBP1s, on the other hand, is degraded with half-life of 30–60 min and most likely requires ubiquitylation for its destruction (8, 9). XBP1, in its two forms, thus provides an excellent model substrate to test the effect of $DegB_{DD}$ on proteasomal degradation in mammals. The degradation kinetics of XBP1u and XBP1s $DegB_{DD}$ fusion proteins were followed by pulse-chase analysis in HEK293T cells, transiently expressing the respective proteins. Similar to the results

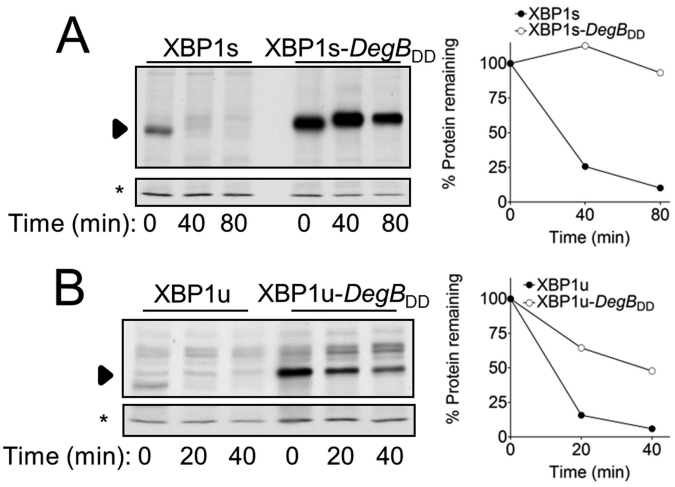

FIGURE 5. **$DegB_{DD}$ stabilized XBP1u and XBP1s expressed in HEK293T cells.** Degradation of the indicated proteins, expressed from a pcDNA3 vector, was determined by pulse-chase analysis. Cells metabolically labeled with [^{35}S]methionine/cysteine were sampled at the indicated time periods, followed by lysis, and immunoprecipitation, as described under "Experimental Procedures."

obtained in yeast, $DegB_{DD}$ expressed at the C terminus stabilized both XBP1u and XBP1s; albeit, the degradation of XBP1u was not completely abolished (Fig. 5*B*). These findings suggest a general function of $DegB_{DD}$ as an inhibitor of degradation of Ub-dependent and -independent proteasome substrates.

DISCUSSION

In this work we have investigated the function of *DegB*, one of two essential elements of the Ndc10 degron. We found that an intact C-terminal *DegB* is required for the initiation of degradation by the 26S proteasome and that the replacement of two Leu residues with negatively charged Asp residues within the hydrophobic core of *DegB* inhibited proteasomal degradation of various substrates. The inhibition by mutant *DegB* required that it was located at the C terminus. This inhibitory function was conserved from yeast to mammals. Thus, $DegB_{DD}$ can operate as a universal *cis*-acting sequence that inhibits proteasomal degradation.

Degradation of the 26S proteasome substrates requires the presence of two essential elements: *trans*-acting factors that enhance substrate binding to subunits of the lid complex of the 26S proteasome and *cis*-elements that initiate degradation (29). The latter comprise an unstructured region that can be inserted into the catalytic cavity of the proteasome, where proteolysis occurs (13, 30). The length of the unstructured region is an important feature of the degradation initiation signal because short unstructured elements cannot trigger degradation (11). Further requirement of unstructured degradation initiation sites is a certain minimal length that separates them from the proteasome-*trans*-acting determinant (11).

The results presented in this study are consistent with an auxiliary role for the loosely structured *DegB* determinant at the Ndc10 extreme C terminus because its deletion prevented Ndc10 degradation (Fig. 1*D*). However, a further C-terminal truncation inhibited substrate ubiquitylation. Therefore, we have previously speculated that in the Ndc10 degron, *DegB* is required for both ubiquitylation and proteasomal degradation

(5). Selective mutation analysis of *DegB* has now led us to revise this initial hypothesis and assign a degradation initiation function to *DegB*. This novel function is supported by the following findings: (i) disruption of the 6-aa hydrophobic core of *DegB* stabilized Vma12-*DegAB* without impairing ubiquitylation (Fig. 1*E*). (ii) *DegB* enhanced the proteolysis of Ub-independent proteasome substrates such as Rpn10-GFP (Fig. 3, *A* and *B*) and mODC (Fig. 3*C*) whereas $DegB_{DD}$ inhibited proteolysis (Figs. 1*D*, 2*B*, 3, *B* and *C*, 4*B*, and 5). (iii) For *DegB* to function, it must be localized at the C terminus where, presumably, insertion into the proteasome catalytic core of proteins containing the Ndc10 degron is initiated (Fig. 2).

As indicated above, the finding that DegBDD stabilized Vma12-DegAB without impairing ubiquitylation challenged our previous findings that removal of 10 aa from the 100-aa Ndc10 degron abolished ubiquitylation (Ref. 5 and Fig. 1*E*). It is possible that, unlike the selective replacement of hydrophobic aa within DegB, the removal of 10 aa disrupted the integrity of the preceding amphipathic helices within DegA and hence abolished this ubiquitylation determinant (5).

The *cis*-acting *DegB* determines degradation, provided that it is expressed at the C terminus of the protein (Fig. 2). Furthermore, when wild type and mutant *DegB* were expressed in tandem, the C-terminal *DegB* sequence element was always the dominant. This finding led us to conclude that: (i) *DegB* is primarily required for the initiation of proteolysis from the C terminus and (ii) $DegB_{DD}$ *per se* does not hinder degradation. It functions as a stabilizing factor only when expressed at the C terminus; but once proteolysis is initiated, $DegB_{DD}$ can no longer stop it. (iii) The distance between the substrate recognition motif and the initiation site is not restricted to a minimal length because extending Vma12-$DegAB_{DD}$ by 15 aa of *DegB* did not alter the degradation kinetics.

It is well documented that the degradation of Ub-independent proteasome substrates such as Rpn10-GFP-cODC (Fig. 3, *A* and *B*) and mODC (Fig. 3*C*) requires an unstructured C-terminal region with a minimal length requirement for degradation (13, 22). Here we demonstrate that the hydrophobic aa property of the unstructured region is an additional critical feature of the degron. Our findings are in agreement with previous *in vitro* studies of 20S proteasome activity that indicated the existence of hydrophobic interactions between synthetic peptides and several noncatalytic proteasome sites (31). These interactions induced opening of the 20S channel via the α-rings *in vitro* and the release of proteolytic peptide products from the proteasome core during proteolysis (31). Notably, studies of the ATP-dependent Lon protease from *Escherichia coli* demonstrated a prerequisite for the presence and positioning of hydrophobic core residues within the C terminus of its substrates (32). Interestingly, Asp substitutions that disrupt hydrophobicity inhibit Lon-substrate binding and substrate degradation (31). This similarity may indicate an evolutionary conservation of elements of the proteolytic machinery, from bacteria to mammals.

Based on our findings, we propose that $DegB_{DD}$ can function as a dominant element for the stabilization of various short lived proteins by means of C-terminal fusion. Traditionally, to investigate the functional consequences of inhibition of proteasome-mediated degradation, proteins are stabilized either through the disruption of degrons or through the inhibition of the catalytic activity of components of the Ub conjugation machinery, mainly the proteasome. These methods are problematic in that degrons of many UPS substrates are either unknown or poorly defined and that inhibition of UPS components frequently causes pleiotropic off-target effects. The use of $DegB_{DD}$ offers a simple solution that circumvents these two drawbacks by enabling selective stabilization of a target protein. Our findings, that fusing $DegB_{DD}$ to target proteins did not affect proteasome activity (Figs. 3*A* and 4*D*), validate this experimental approach. Thus, the dominant function of mutant *DegB* provides a powerful experimental tool to evaluate the physiological and pathological implications of stabilizing specific proteins in intact cells.

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REFERENCES

1. Varshavsky, A. (1991) Naming a targeting signal. *Cell* **64,** 13–15
2. Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.* **9,** 679–690
3. Johnson, P. R., Swanson, R., Rakhilina, L., and Hochstrasser, M. (1998) Degradation signal masking by heterodimerization of MATα2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell* **94,** 217–227
4. Fredrickson, E. K., Rosenbaum, J. C., Locke, M. N., Milac, T. I., and Gardner, R. G. (2011) Exposed hydrophobicity is a key determinant of nuclear quality control degradation. *Mol. Biol. Cell* **22,** 2384–2395
5. Furth, N., Gertman, O., Shiber, A., Alfassy, O. S., Cohen, I., Rosenberg, M. M., Doron, N. K., Friedler, A., and Ravid, T. (2011) Exposure of bipartite hydrophobic signal triggers nuclear quality control of Ndc10 at the endoplasmic reticulum/nuclear envelope. *Mol. Biol. Cell* **22,** 4726–4739
6. Gilon, T., Chomsky, O., and Kulka, R. G. (1998) Degradation signals for ubiquitin system proteolysis in *Saccharomyces cerevisiae*. *EMBO J.* **17,** 2759–2766
7. Arteaga, M. F., Wang, L., Ravid, T., Hochstrasser, M., and Canessa, C. M. (2006) An amphipathic helix targets serum and glucocorticoid-induced kinase 1 to the endoplasmic reticulum-associated ubiquitin-conjugation machinery. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 11178–11183
8. Navon, A., Gatushkin, A., Zelcbuch, L., Shteingart, S., Farago, M., Hadar, R., and Tirosh, B. (2010) Direct proteasome binding and subsequent degradation of unspliced XBP-1 prevent its intracellular aggregation. *FEBS Lett.* **584,** 67–73
9. Tirosh, B., Iwakoshi, N. N., Glimcher, L. H., and Ploegh, H. L. (2006) Rapid turnover of unspliced Xbp-1 as a factor that modulates the unfolded protein response. *J. Biol. Chem.* **281,** 5852–5860
10. Loayza, D., and Michaelis, S. (1998) Role for the ubiquitin-proteasome system in the vacuolar degradation of Ste6p, the α-factor transporter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18,** 779–789
11. Inobe, T., Fishbain, S., Prakash, S., and Matouschek, A. (2011) Defining the geometry of the two-component proteasome degron. *Nat. Chem. Biol.* **7,** 161–167
12. O'Hara, L., Han, G. S., Peak-Chew, S., Grimsey, N., Carman, G. M., and Siniossoglou, S. (2006) Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg^{2+}-dependent phosphatidate phosphatase. *J. Biol. Chem.* **281,** 34537–34548
13. Takeuchi, J., Chen, H., and Coffino, P. (2007) Proteasome substrate degradation requires association plus extended peptide. *EMBO J.* **26,** 123–131
14. Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998) The regulatory particle of the *Saccharomyces cerevisiae* proteasome. *Mol. Cell. Biol.* **18,** 3149–3162
15. Lambertson, D., Chen, L., and Madura, K. (1999) Pleiotropic defects

caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*. *Genetics* **153,** 69–79
16. Isasa, M., Katz, E. J., Kim, W., Yugo, V., González, S., Kirkpatrick, D. S., Thomson, T. M., Finley, D., Gygi, S. P., and Crosas, B. (2010) Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. *Mol. Cell* **38,** 733–745
17. Lipinszki, Z., Kovács, L., Deák, P., and Udvardy, A. (2012) Ubiquitylation of *Drosophila* p54/Rpn10/S5a regulates its interaction with the UBA-UBL polyubiquitin receptors. *Biochemistry* **51,** 2461–2470
18. Kim, H. T., and Goldberg, A. L. (2012) S5a/Rpn10, a UIM-protein, as a universal substrate for ubiquitination. *Methods Mol. Biol.* **832,** 653–660
19. Kahana, C., and Reiss, Y. (2005) Cell-free assay for ubiquitin-independent proteasomal protein degradation. *Methods Mol. Biol.* **301,** 83–96
20. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360,** 597–599
21. Murakami, Y., Tanaka, K., Matsufuji, S., Miyazaki, Y., and Hayashi, S. (1992) Antizyme, a protein induced by polyamines, accelerates the degradation of ornithine decarboxylase in Chinese hamster ovary cell extracts. *Biochem. J.* **283,** 661–664
22. Takeuchi, J., Chen, H., Hoyt, M. A., and Coffino, P. (2008) Structural elements of the ubiquitin-independent proteasome degron of ornithine decarboxylase. *Biochem. J.* **410,** 401–407
23. Mamroud-Kidron, E., and Kahana, C. (1994) The 26S proteasome degrades mouse and yeast ornithine decarboxylase in yeast cells. *FEBS Lett.* **356,** 162–164
24. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273,** 1725–1728
25. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107,** 881–891
26. Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415,** 92–96
27. Lee, A. H., Iwakoshi, N. N., and Glimcher, L. H. (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* **23,** 7448–7459
28. Lee, A. H., Iwakoshi, N. N., Anderson, K. C., and Glimcher, L. H. (2003) Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 9946–9951
29. Schrader, E. K., Harstad, K. G., and Matouschek, A. (2009) Targeting proteins for degradation. *Nat. Chem. Biol.* **5,** 815–822
30. Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E., and Matouschek, A. (2004) An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat. Struct. Mol. Biol.* **11,** 830–837
31. Gur, E., and Sauer, R. T. (2008) Recognition of misfolded proteins by Lon, a AAA^+ protease. *Genes Dev.* **22,** 2267–2277
32. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MATα2 repressor. *Cell* **74,** 357–369
33. Swanson, R., Locher, M., and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. *Genes Dev.* **15,** 2660–2674
34. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14,** 953–961
35. Bercovich, Z., and Kahana, C. (2004) Degradation of antizyme inhibitor, an ornithine decarboxylase homologous protein, is ubiquitin-dependent and is inhibited by antizyme. *J. Biol. Chem.* **279,** 54097–54102