The type-2 N-end rule peptide recognition activity of Ubr11 ubiquitin ligase is required for the expression of peptide transporters

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A B S T R A C T

The Ubr1-like canonical N-recognins, widely conserved ubiquitin ligases in eukaryotes, play a role in the N-end rule pathway-mediated degradation of substrates harboring basic (type-1) or bulky hydrophobic (type-2) amino acids at the N-terminus. In this study, the roles of conserved domains were studied in the Schizosaccharomyces pombe Ubr11 protein. Mutations in the UBR box and the autoinhibitory domain blocked degradation of both type-1 and type-2 substrates, expression of peptide transporter genes, and the uptake of oligopeptides. An N-domain mutant was normal for the type-1-related function, but nevertheless failed to express peptide transporters. These data suggest the importance of the type-2-related activity of Ubr11 for its in vivo function.

1. Introduction

Proteasome-dependent proteolysis of ubiquitylated substrate proteins regulates various cellular activities [1]. For ubiquitylation, ubiquitin ligase recognizes a specified motif, known as a degron, in its substrate protein. Ubr family proteins, which are found only in eukaryotes, are characterized by the presence of a distinctive zinc-finger-like UBR box domain. Seven different Ubr proteins in mouse are classified into canonical (Ubr1–Ubr3) and non-canonical groups (Ubr4–Ubr7) by their structure [2–4]. The canonical Ubr family proteins, which have the RING finger domain for ubiquitin ligase activity, together with the UBR box and autoinhibitory domain (Supplementary Fig. 1), are widely conserved from yeasts to mammals. Some, but not all of these canonical Ubr proteins (typified by Ubr1 and Ubr2 in mouse, and Ubr1 in Saccharomyces cerevisiae) are involved in the Arg/N-end rule pathway branch in the ubiquitin-dependent proteolysis. Details of the N-end rule pathway have been studied using the N-degron, ε5, a ~40-residue Escherichia coli Lac repressor-derived sequence [5]. In the Arg/N-end rule pathway, the primary determinant of an N-degron is the presence of either a basic (type-1; Arg, Lys, His) or a bulky hydrophobic (type-2; Leu, Ile, Trp, Phe, Tyr) amino acid at the N-terminus [2–6]. A Ubr1-like protein acts as an N-recognin that binds to the N-terminal residue of the N-degron and ubiquitylates the substrate protein before being degraded by the proteasome [2–4, 7, 8]. The Ubr1 family N-recognin uses distinct domains to recognize type-1 and type-2 destabilizing N-terminal amino acids. The UBR box is sufficient for recognition of type-1 residues, whereas the N-domain, which is structurally similar to the bacterial ClpS protein, is required for the recognition of type-2 residues [8, 9]. The UBR box is conserved among all UBR family proteins, but its function, especially in the non-N-recognin UBR proteins which are not involved in the N-end rule pathway, remains unknown.

We investigated the physiological role of two similar, canonical Ubr1-like proteins, Ubr1 and Ubr11, in Schizosaccharomyces pombe [10–13]. Although there are pleiotropic defects in the ubr1 mutant, Ubr1 is dispensable for the N-end rule pathway, and only Ubr11 fulfills a role as N-recognin [13, 14]. In the absence of Ubr11, cells fail to transport extracellular oligopeptides because of decreased mRNA levels for the peptide transporters, Ptr2 and Isp4 [12]. Based on our results that the C-terminal peptide of the Rec8 meiotic cohesin subunit is an endogenous substrate of Ubr11, we further confirmed the specificity of the N-terminal amino acid for the N-end rule pathway in S. pombe, in which basic and bulky hydrophobic amino acids have potent destabilizing activity, while methionine and alanine were ineffective as other organisms [2–4, 11, 13]. Since N-degron sequence was characterized in detail only in the ε5 [5, 15], we investigated the degradation-inducing sequence within the Rec8 C-terminal fragment and found that several non-overlapping regions independently fulfill the function as a part of an N-degron determinant (our unpublished results). However,
the universality of the protein’s N-degron sequence found in other organisms is still unclear. In this study, we tested the functionality of the ε8-derived N-degron, which we termed XaaNd (Supplementary Fig. 2A), in S. pombe. Further, we examined the roles of the conserved domains of N-recogin Ubr11, including UBR box, N-domain, and autoinhibitory domain whose function is not well understood. Our results suggest that all of these domains must be intact for the activation of peptide uptake. Recognition of type-1 peptides by Ubr11 is insufficient for this purpose. A key event in the expression of peptide transporter genes is the recognition of type-2 peptides and/or degradation of type-2 substrate by Ubr11.

2. Materials and methods

2.1. Yeast strains and culture conditions

The yeast strains used in this study are listed in Supplementary Table 1. To construct ubr11 mutants, the wild-type ubr11-coding region with 5’- and 3’-untranslated regions (791 and 751 bases, respectively) was obtained by PCR and cloned into the pCR4Blunt-TOPO vector (Invitrogen). One copy of the Pk epitope (GIPNPLLGLD) was appended at the N-terminus of the Ubr11 using inverse PCR, and the following five mutants (Supplementary Fig. 1) were generated with this Pk-ubr11’ template plasmid: m1 (Cys126 to Ala, within the UBR box), m2 (Asp150 to Glu, within the UBR box), m3 (Asp251 to Asn and His254 to Tyr, within the N-domain), m4 (Cys1339 to Ser, within the RING domain), and m5 (Cys1932 to Ala and Cys1935 to Ala, within the autoinhibitory domain). After sequence verification, the corresponding ubr11 gene was released from the vector and used to replace the ubr11:ura4’ allele of the host S. pombe strain. Colonies exhibiting 5-fluoroorotic acid-resistance were isolated, and correct replacement was confirmed by PCR. Expression of these mutated ubr11 genes were verified by RT-PCR and immunoprecipitation (see below).

Rich complete medium YES5, and synthetic minimal medium EMM2, were used for growth in cell culture. These media and other general yeast methods have been previously described [16]. To express proteins from the nmt promoter, cells were grown in thiamine-free EMM2 for 18–20 h at 30 °C. Stability of the N-end rule substrates (XaaNd-F-GFP) was monitored by cycloheximide chase assay. Cycloheximide was added at 100 μg/ml and used at 0.2 mM (to support cell growth) or 5 mM (to inhibit proteolysis of XaaNd-F-GFP).

2.2. Construction of plasmids

Arg–GFP and Met–GFP: The ubiquitin-Xaa–GFP unit was excised from the corresponding plasmids (Ub-R-GFP or Ub-M-GFP, obtained from Addgene) [17] by digestion using Nhel and Xbal and inserted in the Nhel site of the pDual-HFF41 vector [18]. Resultant plasmids were integrated at the leu1–32 locus of the host S. pombe strain. For S. cerevisiae, plasmids harboring the same Ub-Xaa–GFP unit in the pYES2 vector [19] were obtained from Addgene.

XaaNd-F-GFP: A variant of ε8, the N-terminal 15 amino acids of the original ε8 sequence [5], was used. The third and eighth amino acids were changed to lysines to mimic the mutated ε8-N-degron 119 [15]. The corresponding degron sequence (RHKSGAWKLPVSLVK, denoted as ArgNd, Supplementary Fig. 2A) was introduced between the ubiquitin and Flag epitope by inverse PCR to replace the ArgRec8c region with the pDual/Ub-ArgRec8c-F-GFP) template plasmid [13]. As a result, a GFP-based monitoring substrate protein, denoted as ArgNd-F-GFP, was produced from the resultant plasmid. The codon for the N-terminal Arg of this N-degron was changed to Met, Trp, or Leu by site-specific mutagenesis using inverse PCR. These plasmids were integrated at the leu1–32 locus of the host S. pombe strains.

Ubr11 and Ptr2: The ubr11+–expressing plasmid was previously described [11]. For the ptr2+ plasmid, the protein-coding region was amplified by PCR. The GFP gene was removed from the pDual-HFF41 vector [18] by cutting with NcoI and BgIII, and the ptr2+ gene was inserted.

2.3. Flow cytometry

To measure relative GFP fluorescence levels, GFP expression was induced from the nmt promoter in thiamine-free medium at least for 18 h, in order to attain steady state GFP levels for each strain. To inhibit degradation via the N-end rule pathway, cells were treated with 5 mM of dipeptides for the last 3 h. Live cells were directly analyzed by flow cytometry using FACSCalibur (Becton Dickinson).

2.4. Immunoprecipitation and immunoblotting

Preparation of total cellular protein extracts and immunoblotting were performed as described previously [11]. For primary antibodies, anti-GFP (GF200; Nacalai Tesque), anti-PSTAIRE (Cdc2, sc-53; Santa Cruz Biotechnology), and anti-α-tubulin (T5168; Sigma–Aldrich) were used. After incubation with horse-radish peroxidase-conjugated secondary antibody and its chemiluminescent substrate, signal was captured by ChemDoc XRS and quantified with Quantity One software (Bio-Rad).

To confirm expression of the Pk-tagged Ubr11 proteins from endogenous promoter, immunoprecipitation and following immunoblotting was necessary, possibly due to low expression levels of the endogenous Ubr11 protein. Cells were lysed in a buffer [50 mM NaCl, 1 mM EDTA, 50 mM HEPES, pH 7.5, and one protease inhibitor cocktail tablet/10 ml buffer (Roche Diagnostics)] with glass beads, and extracts were obtained after brief centrifugation (2300×g, 5 min). Two microsomes of the monoclonal anti-Pk antibody (MCA1360, Abd Serotec) were added to the 15 mg of protein extracts, which were then rocked for 2 h at 4 °C. Dynabeads Protein G (Invitrogen) were added, and the samples were rocked for an additional 2 h. Beads were washed three times with the same buffer, resuspended in SDS-sample buffer, and heated for 2 min. Samples were separated on a 6% polyacrylamide gel, transferred to a PVDF membrane, and probed with the anti-Pk antibody.

2.5. RNA analysis

Total RNA was prepared and analyzed by reverse transcription-coupled PCR (RT-PCR), as described previously [11]. RNA was reverse transcribed to cDNA using a random 9-mer primer, and the cDNA was then amplified by PCR with oligonucleotide primers specific for peptide transporters (ptr2 and isp4), ubr11, and GFP. The β-tubulin nda3 was used to ensure the input of equal levels of RNA for each sample.

3. Results

3.1. Universality of the N-degron sequence

The Arg–GFP (green fluorescent protein) has been used to monitor Arg/N-end rule pathway-dependent degradation in mammals and S. cerevisiae [17,19]. We compared the fluorescence levels of this Arg–GFP to that of the stable Met–GFP in S. cerevisiae and in S. pombe. However, both proteins showed equivalent fluorescence intensity (Fig. 1Aa) and also comparable GFP protein levels (Fig. 1B,
lunes 1 and 2) in S. pombe, indicating that N-degron in this GFP construct is not operative in S. pombe. To test the functionality of another N-degron sequence, a variant of the ε8 sequence (denoted here as XaaNd) (Supplementary Fig. 2A) was fused to the N-terminus of the Flag-tagged GFP (GFP), and its expression level was monitored by flow cytometry. The MetNd-GFP was highly expressed, but the fluorescence of ArgNd-GFP was greatly decreased in a wild-type strain (Fig. 1A). The same ArgNd-GFP construct was highly expressed in a ubr11Δ strain, and a comparable level of the MetNd-GFP fluorescence was detected in both wild-type and ubr11Δ strains (Supplementary Fig. 2B). We confirmed by immunoblotting that the GFP fluorescence levels were completely correlated with the GFP protein levels in each strain (Fig. 1B). When the N-terminal arginine was changed to another amino acid, TrpNd induced instability only in a wild-type strain, although its effect was modest compared to ArgNd (Fig. 1B and Supplementary Fig. 2B). In contrast, LeuNd was not effective in this context (Fig. 1B and Supplementary Fig. 2B). We confirmed by immunoblotting that the GFP fluorescence levels were completely correlated with the GFP protein levels in each strain (Fig. 1B). When the N-terminal arginine was changed to another amino acid, TrpNd induced instability only in a wild-type strain, although its effect was modest compared to ArgNd (Fig. 1B and Supplementary Fig. 2B). In contrast, LeuNd was not effective in this context (Fig. 1B and Supplementary Fig. 2B).

3.2. Role of the conserved domain in Ubr11 protein for the N-end rule pathway

Thus far, we have used only a ubr11Δ mutant that lacks the entire Ubr11 coding region [10–13]. Therefore, ubr11Δ strains, which expressed full-length Ubr11 proteins harboring missense mutation in the conserved domain, were examined. To know the function of the conserved domains, each mutant had mutation(s) within the UBR box (possible recognition site for type-1 substrate, ubr11-m1

Fig. 1. Functionality of XaaNd N-degron. (A) Arg–GFP, previously shown to be an Arg/N-end rule pathway substrate in other organisms [17,19], was expressed in yeast (S. cerevisiae and S. pombe). Its fluorescence levels, which were monitored by flow cytometry as an indicator of stability, were comparable to those of stable Met–GFP control, indicating that Arg–GFP was highly expressed in S. pombe unlike in S. cerevisiae. B. The N-degron ArgNd is functional. Steady state GFP fluorescence levels in ArgNd or MetNd-Flag-GFP (FGFP)-expressing wild-type S. pombe cells were monitored as in A. B. Expression levels of the degron-fused GFP proteins in S. pombe. Arg– and Met–GFP proteins in S. pombe (used in Aa, lanes 1 and 2) and XaaNd-GFP proteins in wild-type and ubr11Δ strains (lanes 3–10) were detected by immunoblotting. α-tubulin levels were used as a loading control. wt: wild-type strain, Δ: ubr11Δ strain (C and D) Ubr11-dependent instability of XaaNd-fused GFP. Each XaaNd-fused GFP harboring indicated amino acid at the N-terminus was induced from the thiamine-repressible strain, then synthesis of XaaNd-GFP was stopped at 0 min by adding cycloheximide and thiamine. Different amount of cell extracts were used in wild-type strain samples since expression levels of each XaaNd-GFP varied: 50 μg for ArgNd-GFP, 20 μg for TrpNd-GFP, 2 μg for MetNd- and LeuNd-GFP. For ubr11Δ strain, 2 μg of extracts were used for all samples. Cdc2 levels were monitored as a loading control using 25 μg of extracts for all samples.

Fig. 2. Degradation of type-1 and type-2 N-end rule substrates by the mutant Ubr11 protein. (A) Confirmation of the Ubr11 mutant proteins’ expression. Pk-tagged Ubr11 protein (~235 kDa) was precipitated from each strain by anti-Pk antibody and detected by immunoblotting. Precipitates from non-tagged wild-type strain were used as control (lane 1). The same extracts from the ubr11Δ-m4 strain were used for lanes 6 and 8, but the sample in lane 8 was processed without anti-Pk antibody to serve as another negative control. (B) Functionality of the mutant Ubr11 proteins. ArgNd– (type-1) or TrpNd–FGFP (type-2) was expressed in the indicated strains, and their GFP protein levels were measured by immunoblotting. (C and D) Effect of ubr11 mutations on the degradation of substrates. Instability of ArgNd– and TrpNd–FGFP in each ubr11 mutant was examined as in Fig. 1. Both type-1 and type-2 substrates were stabilized in Ubr box mutants (ubr11-m1 and -m2). Mutation of the N-domain (ubr11-m3) hampered the degradation of type-2, but not type-1, N-end rule substrate. For short-lived samples (C), 40 μg (ArgNd) or 8 μg (TrpNd) of extracts were used to detect both GFP and Cdc2. For long-lived samples (D), 2 μg and 25 μg of extracts were used to detect GFP and Cdc2, respectively.
and -m2) or N-domain (site for type-2 substrate, ubr11-m3) (Supplementary Fig. 1). All ubr11 genes (wild-type and mutants) were transcribed (Fig. 4B), and expression of the corresponding full-length protein was confirmed in all mutants (Fig. 2A). Addition of the Pk epitope did not affect the function of Ubr11 because wild-type Pk-Ubr11 could induce degradation of both ArgNd–FGFP and TrpNd–FGFP (Fig. 2B and C). In Ubr1 box mutants (ubr11-m1 and -m2) harboring a mutation in a putative type-1 amino acid-binding region, not only the type-1 substrate ArgNd–FGFP but also the type-2 substrate TrpNd–FGFP unexpectedly accumulated (Fig. 2B) because of a lack of degradation (Fig. 2D). The mRNA levels for ArgNd–FGFP were comparable in all strains (Fig. 4B), confirming that a post-transcriptional step regulated the differences in GFP protein levels. Accordingly, fluorescence intensity of the ArgNd–FGFP and TrpNd–FGFP in the UBr box mutants was comparable to that in the ubr11A mutant (Supplementary Fig. 2C), indicating that Ubr11-m1 and Ubr11-m2 mutant proteins were non-functional for both type-1 and type-2 substrates.

In contrast, a mutation in the N-domain (ubr11-m3) had differential effects on the stabilities of the type-1 and type-2 substrates. Ubr11-m3 did not affect the instability of the ArgNd–FGFP, a type-1 substrate, indicating that this mutation did not interfere with the function in destabilizing a type-1 substrate (Fig. 2B, C and Supplementary Fig. 2C). However, a type-2 substrate TrpNd–FGFP was highly expressed in the ubr11-m3 mutant because of its stabilization (Fig. 2B, D and Supplementary Fig. 2C). Therefore, ubr11-m3 mutant selectively lost type-2-specific function but could still recognize and degrade the type-1 substrate.

Proteolysis of an N-end rule substrate is inhibited by the addition of dipeptides harboring appropriate N-terminal residues because the dipeptide and N-degron of a substrate both compete for the same binding site in the Ubr protein [20]. We were able to reproduce this in our system: degradation of the ArgNd–FGFP was slowed in the presence of Lys-Leu, a type-1 dipeptide (Fig. 3A). Degradation was not completely inhibited by the dipeptide, but significant levels of ArgNd–FGFP accumulated over 3 h (Fig. 3B). Similarly, a type-2 dipeptide Tyr-Leu inhibited the degradation of TrpNd–FGFP and induced its accumulation (Fig. 3A and B). As a result, fluorescence of the ArgNd–FGFP was significantly increased after the addition of type-1 dipeptide Lys-Leu but not by type-2 dipeptide Tyr-Leu (Fig. 3C) or by non-N-end rule dipeptide Ala-Leu (data not shown). Both arginine and lysine, as an amino acid monomer, had no such an inhibitory effect (data not shown).

Similarly, TrpNd–FGFP fluorescence increased by the addition of type-2 dipeptide Tyr-Leu (Fig. 3C), but not by tryptophan or its methyl ester (data not shown). Another type-2 dipeptide Leu-Ala showed similar, but only limited activity against TrpNd–FGFP (Supplementary Fig. 2D). These dipeptides had no effect on the GFP levels in the ubr11A mutant (data not shown).

As described, ArgNd–FGFP was unstable in the type-2-specific mutant ubr11-m3 (Fig. 2B and C), indicating that type-1 N-degron normally binds to the mutant Ubr11-m3 protein. Nevertheless, fluorescence intensity of the ArgNd–FGFP in this mutant did not recovered by the addition of type-1 dipeptide Lys-Leu (Fig. 3D, vector). The defect was completely rescued by the wild-type ubr11T1-expressing plasmid. When the dipeptide transporter, Ptr2, was forcedly expressed from the heterologous nmt41 promoter, GFP protein levels increased in response to the added Lys-Leu (Fig. 3D and E) without affecting mRNA levels (Supplementary Fig. 2E). These data support the conclusion that recognition of the type-1 N-degron and Lys-Leu dipeptide by Ubr11-m3 protein was not abrogated.

3.3. Peptide uptake defect by the ubr11 mutants

To determine why the Lys-Leu dipeptide did not prevent degradation of the ubr11-m3 mutant unless Ptr2 was ectopically expressed from the heterologous nmt41 promoter. When the dipeptide transporter, Ptr2, was forcedly expressed from the heterologous nmt41 promoter, GFP protein levels increased in response to the added Lys-Leu (Fig. 3D and E) without affecting mRNA levels (Supplementary Fig. 2E). These data support the conclusion that recognition of the type-1 N-degron and Lys-Leu dipeptide by Ubr11-m3 protein was not abrogated.

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Fig. 4. Expression of peptide transporter genes is severely compromised in all ubr11 missense mutants. (A) Dipeptide utilization was abrogated by all missense ubr11 mutations. All strains were auxotrophic for leucine. The type-1 dipeptide Lys-Leu supported the leucine-dependent growth only for a wild-type strain, but a leucine monomer supported growth in all strains. Histidine and uracil were added to rescue their requirement for growth. (B) All missense ubr11 mutants were unable to induce the expression of peptide transporter genes (ptr2 and isp4). The mRNA levels were analyzed by RT-PCR. The β-tubulin nda3 gene was used as an internal control for the input RNA levels. The same mRNAs from Pk-ubr11 wild-type strain (KSP2575) were used for lanes 1 and 2, but the sample in lane 1 was processed without reverse transcriptase as a negative control. Expression of Pk-tagged ubr11 genes was detected in all strains, except ubr11Δ that lacks most of the coding region. The GFP mRNA levels were also checked for strains harboring a gene for the ArgNd–FGFP substrate (lanes 2–6). For lanes 7–9, strains lacking the GFP substrate gene were used.

Interestingly, all five ubr11 mutants failed to utilize dipeptides, as was previously reported for a ubr11A strain [12], regardless of whether the dipeptides were type-1 (Lys-Leu, Fig. 4A) or type-2 (Leu-Ala and Tyr-Leu, data not shown). It had been reported that, in the absence of low amounts of type-1 or type-2 dipeptide, the non-N-end rule dipeptide Ala-Leu was not effective in supporting the growth of leucine auxotrophic cells in a certain wild-type strain of S. cerevisiae [21,22]. In contrast, the same Ala-Leu dipeptide was effectively utilized in S. pombe [12], but none of all five mutants could use this non-N-end rule dipeptide also (data not shown).

Previously, we demonstrated that Ubr11 is required for the expression of transporter genes, ptr2 (for di/tripeptides) and isp4 (for tetra/pentapeptides) [12]. Consistent with the peptide utilization defect, the mRNA levels of both ptr2 and isp4 were greatly reduced in all ubr11 missense mutants, as well as in the ubr11A strain (Fig. 4B). The isp4 mRNA levels did not recover by the Lys-Leu dipeptide uptake in the ubr11-m3 mutant (Supplementary Fig. 2E). In conclusion, all five ubr11 mutants are unable to induce expression of peptide transporter genes, accounting for the peptide uptake defect in these strains. These data demonstrated the importance of the type-1 and type2 peptide-recognition sites, and also suggest a pivotal role of the autoinhibitory domain to positively support the transcriptional activation of peptide transporter genes.

4. Discussion

In this study, we developed an N-degron, XaaNd, and showed that XaaNd-fused GFP was a useful tool as a convenient reporter substrate for the study of N-end rule pathway. Both ArgNd and TrpNd induced degradation of inherently stable GFP, but unexpectedly, LeuNd was ineffective, unlike the active effect of an N-terminal leucine in the original ε-N-degron [5,6]. We previously showed in S. pombe that an N-terminal leucine stimulated degradation in other N-end rule substrates (Leu-DHRF and Leu-Rec8C), though its potency seemed to be weaker than that of other effective amino acids [13]. Furthermore, the inhibitory action of Leu-Ala on TrpNd–GFP degradation was much weaker than that of Tyr-Leu (Supplementary Fig. 2D). The XaaNd sequence used here lacks C-terminal 25 amino acids in the original ε-N-degron (Supplementary Fig. 2A), which overlap with the region required for the efficient degradation of substrates [5]. This truncation and weak nature of the degradation-inducing activity of leucine may additively reduce the potency of LeuNd.

By using these substrates harboring ArgNd (type-1) or TrpNd (type-2) N-degron, we investigated the roles of the domains conserved in canonical Ubr N-recogins. It is known that the UBR box is sufficient for the recognition of type-1 substrates [8]. The D176E mutation within the UBR box in S. cerevisiae Ubr1, which is equivalent to the Ubr11-m2 mutation (D150E) in this study, was identified by its type-1 substrate-specific defect [9]. Indeed, as seen by the crystal structure, this aspartic acid contacts the α-amino group of the N-terminal arginine of a substrate (Sc1 peptide) [23]. When aspartic acid in the corresponding position in the mouse Ubr1 was mutated (D150A), in vitro binding to the type-1 (Arg) peptide was completely inactivated, but the binding to the type-2 (Phe) peptide was partially affected [8]. Another UBR box mutant in the mouse Ubr1 (C127A), equivalent to the Ubr11-m1 (C126A) used in this study, also showed no in vitro binding to the type-1 peptide but residual binding to the type-2 peptide [8]. Unlike these findings in other organisms, UBR box mutants in S. pombe (Ubr11-m1 and -m2) completely prevented degradation of both type-1 and type-2 substrates in vivo (Fig. 2B and D). Further, Cys126, which is mutated in the S. pombe Ubr11-m1, is conserved in all Ubr proteins including canonical Ubr proteins not functional as an N-recogin in the Arg/N-end rule pathway (e.g., Ubr1 in S. pombe [13], Supplementary Fig. 1) and also non-canonical Ubr4–Ubr7 proteins in mammals [2]. Mutation in the corresponding cysteine residue in the Arabidopsis BIG protein (Ubr4) leads to the auxin transport defect [24]. Since this cysteine is critical for coordinating zinc ions in the zinc-finger-like UBR box in Ubr1 [23], corresponding cysteines play pivotal roles in the formation of a structural scaffold for UBR box. Also, regardless of its type-1-specific defect in the S. cerevisiae Ubr1-D176E mutant [9], the corresponding residue is conserved in all mammalian and S. pombe Ubr proteins, including those irrelevant to the N-end rule pathway [2,13] (Supplementary Fig. 1). These conserved residues among all Ubr proteins and their mutants may provide a clue for understanding the function of the UBR box other than the N-end rule pathway.

The only physiological defect in the S. pombe ubr11 mutant known so far is the unavailability of extracellular peptides [12]. All five ubr11 mutants examined in this study failed to utilize peptides due to insufficient expression of peptide transporter genes. In particular, the N-domain mutant protein (Ubr11-m3) is able to bind type-1 N-degron (ArgNd) and type-1 peptides (Lys-Leu) normally (Fig. 3D and E), but is unable to utilize dipeptides, irrespective of whether the peptide is type-1 (Lys-Leu), type-2 (Leu-Ala, Tyr-Leu), or non-N-end nature (Ala-Leu) (Fig. 4A, data not shown). It is likely that recognition of type-1 N-degron, or type-1 peptides, is insufficient for the expression of peptide transporters. Alternatively, the binding of type-1 N-end rule peptide may be uncoupled from the transcriptional activation of peptide transporter genes in the ubr11-m3 mutant. In either case, both UBR box and N-domain must be intact, and degradation of a putative type-2 substrate, or recognition of type-2 dipeptides itself is indispensable for the activation of peptide transporter genes.

In S. cerevisiae, peptides accelerate the own uptake through Ubr1-mediated degradation of Cup9, a repressor of the peptide transporter expression [21,22]. Cup9 is a substrate of Ubr1, though
it does not have an N-degron. In the absence of extracellular dipeptides, autoinhibitory domain of Ubr1 prevents its recognition of Cup9, possibly by covering the binding site for Cup9. Upon binding of dipeptides to the type-1 and type-2 recognition sites in Ubr1, conformation is switched from closed to open state, which is suitable for the recognition and ubiquitylation of Cup9. Consequently, presence of dipeptides leads to transcriptional activation of the peptide transporter gene. Interestingly, mutation in the conserved two cysteines within autoinhibitory domain allows constitutive binding of Cup9 even in the absence of dipeptides [21]. Since the role of autoinhibitory domain was evaluated only in S. cerevisiae Ubr1, we examined the in vivo effects of analogous mutation in S. pombe (Ubr11-m5), and found that these cysteines were essential to activate the expression of peptide transporter genes (Fig. 4B). Further, this mutant protein was unable to degrade both type-1 and type-2 test substrates (Supplementary Fig. 2C). In S. cerevisiae, the corresponding mutant Ubr1 protein of the autoinhibitory domain was found to be inactive for rescuing the defect in the N-end rule pathway in ubr1A cells [21]. Role of the autoinhibitory domain, other than suppression of needless degradation of substrates, remains to be determined as an important question, since the autoinhibitory domain and the corresponding cysteines are also conserved in canonical Ubr proteins including S. pombe Ubr1 which has no role for the regulation of peptide uptake or N-end rule pathway (Supplementary Fig. 1). Although Ubr ubiquitin ligase is essential for peptide transporter expression in both S. pombe and S. cerevisiae, regulation seems to be different between the two yeasts [12]. In particular, an apparent homolog of Cup9, which is a target substrate of Ubr1 for peptide utilization in S. cerevisiae, is not encoded in the S. pombe genome. It is necessary to identify a possible substrate of Ubr11 as well as a negative and positive regulatory factors for peptide transporter expression to understand how Ubr11 regulates transporter expression and how extracellular peptides affect this regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.028.

References