UBIQUITIN CHAINS IN THE DSK2 UBL DOMAIN MEDIATE DSK2 STABILITY AND PROTEIN DEGRADATION IN YEAST

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Abstract

Ubiquitin-like (UBL) - ubiquitin-associated (UBA) proteins, including Dsk2 and Rad23, act as delivery factors that target polyubiquitinated substrates to the proteasome. We report here that the Dsk2 UBL domain is ubiquitinated in yeast cells and that Dsk2 ubiquitination of the UBL domain is involved in Dsk2 stability, depending on the Dsk2 UBA domain. Also, Dsk2 lacking ubiquitin chains impaired ubiquitin-dependent protein degradation and decreased the interaction of Dsk2 with polyubiquitinated proteins in cells. Moreover, Dsk2 ubiquitination affected ability to restore the temperature-sensitive growth defect of *dsk2*. These results indicate that ubiquitination in the UBL domain of Dsk2 has *in vivo* functions in the ubiquitin-proteasome pathway in yeast.

***Key words*:** UBL-UBA protein, Dsk2, UBL domain, UBA domain, ubiquitin, proteasome.

Introduction

The ubiquitin-proteasome system regulates various biologic processes by mediating the degradation of many short-lived cellular proteins. Ubiquitinated proteins are degraded in steps: ubiquitination, recognition, delivery, and degradation by the proteasome. The processes by which ubiquitinated proteins are recognized and delivered to the proteasome are regulated through ubiquitin-like (UBL)-ubiquitin-associated (UBA) proteins and ubiquitin receptors (for review, see [1,2]. UBL-UBA proteins bind polyubiquitinated proteins and deliver them to the proteasomal receptors Rpn1, Rpn10 and Rpn13 [3-8]. UBL-UBA proteins also interact and cooperate with specific E3 and E4 enzymes [9,10] and E1 activity [11]. Furthermore, Rpn10 and Dsk2 can function together as a polyubiquitin-chain length sensor [12], and several UBL domain-binding proteins interact with UBL-UBA proteins and are positively and negatively involved in the delivery pathway [6,13,14]. Together, these findings indicate that UBL-UBA

proteins have a regulatory role in the recognition and delivery of polyubiquitinated proteins.

In yeast, three UBL-UBA proteins; Rad23, Dsk2, and Ddi1, are known to be such delivery proteins. In this process, "mobile" UBL-UBA proteins interact with the proteasome together with polyubiquitinated proteins, and they are selectively degraded. UBL-UBA proteins that interact with the proteasome, however, can escape degradation. It has been demonstrated that the Rad23 UBA2 domain functions as an intrinsic stabilization signal that protects Rad23 from proteasomal degradation and the UBA2 domain deletion mutant is unstable [15]. As predicted by Walters [16], intra-molecular interactions between the UBL and UBA domains seem to contribute to the stability of Rad23/hHR23A.

Budding yeast Dsk2 is a UBL-UBA protein that helps deliver polyubiquitinated proteins to the proteasome [17-19]. To better understand the molecular mechanisms of UBL-UBA proteins in the ubiquitin-proteasome pathway, we investigated the role of Dsk2 ubiquitination using lysine mutants of the Dsk2 UBL domain. Dsk2 is quite stable in yeast [20], but we show here that Dsk2 is ubiquitinated in yeast in the UBL domain, and that ubiquitin chains of the UBL domain mediate the degradation of Dsk2 defective in the UBA domain. Dsk2 ubiquitination of UBL domains also affected ubiquitin-dependent degradation in yeast. We discuss physiological relevance of the ubiquitination of the Dsk2 UBL domain in Dsk2 stability as well as in the ubiquitin-proteasome pathway.

Materials and methods

Strains, plasmids, and media

Saccharomyces cerevisiae YPH499 (*MAT*a *ade2-101 leu2-ı trp1-63 ura3-52 lys2-801 his3-200*), used as the wild-type strain, was previously described [17]. Mutant strains (*dsk2* , *rpn1-821,* and *pre2-75, dsk2* □□□ĩ□□ı*, dsk2 rad23*), media, and growth conditions were described previously [17,21]. Plasmids carrying the glutathione S-transferase (GST)-tagged version pEG-KG-*DSK2,* were described previously [17]. Multi-copy plasmids

p*GAL1*-YEplac195 harboring *DSK2* or p*GAL1*-YEplac112 harboring His_6-T7-ubiquitin were used for the ubiquitination assay [17]. A single-copy plasmid pRS316 harboring *DSK2* and its endogenous promoter was used for complementation of *dsk2* . The growth of a *dsk2 rad23* strain transformed by this plasmid was tested on plates of minimal medium containing 2% glucose minus uracil [17]. For complementation of uracil auxotrophy, a single-copy plasmid, pRS314, harboring *URA3*-fused *DSK2* was transformed into YPH499 and these transformed strains were incubated on plates of minimal medium containing 2% glucose minus uracil and tryptophan at 30^{o}C for 3 days.

Construction of Dsk2 mutants

DSK2 deletion mutants and *Dsk2* UBA domain mutants were described previously [17,21]. The arginine-substituted mutants of the UBL domain were constructed by site-directed polymerase chain reaction (PCR) methods using pEG-KG-*DSK2* as the template. The mutated *Xba*I-*Hin*dIII fragment was subcloned into the *Xba*I-*Hin*dIII sites of pEG-KG-*DSK2*, p*GAL1*-YEplac195, and pRS316. To construct a series of UBL- and UBA-substituted mutants (UBL/UBA mutant), a *Kpn*I- *Hin*dIII fragment from UBA mutants was subcloned into the *Kpn*I-*Hin*dIII sites of a plasmid harboring mutated DSK2 in the UBL domain (pEG-KG-mutant *DSK2*). To generate *URA3*-fused *DSK2*, each DNA fragment containing Dsk2 5'UTR (*Bam*HI-*Eco*RI), *URA3* (*Eco*RI-*Eco*RI), *DSK2* (*Eco*RI-*Hin*dIII), and Dsk2 3'UTR (*Hin*dIII-*Cla*I) was constructed by PCR and these DNA fragments were fused into pBluescript-IISK+ (5'UTR-*URA3*-*DSK2*-3'UTR in pBS-IISK+). The *Bam*HI-*Xho*I fragment of this plasmid was then subcloned into the *Bam*HI/*Xho*I sites of pRS314.

Binding assay and degradation of Dsk2 in vivo

For the binding assay, His_6-T7-ubiquitin and Dsk2 were co-expressed in yeast, and cell extracts in lysis buffer containing 1 mM EGTA instead of EDTA were incubated with Talon

metal-affinity resin (CLONTECH) [17]. Talon resin or glutathione-Sepharose beads were used to pull down His_6-T7-ubiquitin, and the bound material was immunoblotted with anti-Dsk2 or anti-T7 antibodies. For detection of ubiquitinated Dsk2, Dsk2 was co-expressed with His_6-T7-ubiquitin in yeast, and the Talon resin pull-down was immunoblotted with anti-Dsk2 antibody. For the degradation assay, GST-*DSK2* in pEG-KG or DSK2 in p*GAL1*-YEplac195 was transformed into yeast. Dsk2 was overexpressed for 4 h by the addition of 2% galactose. Cell extracts were collected at 30 min intervals after the induction was shut off by adding dextrose to the medium, followed by immunoblotting with anti-Dsk2, anti-Cdc28, anti-Ddi1 (UBL-UBA protein), and anti-mAb414 (nuclear NPC) antibodies. For degradation of N-end rule substrate Leu- -gal [6], *Dsk2* or *Dsk2-KR* in YCplac22 was co-expressed with Ub-Leu- -gal in *dsk2* strain under the control of the *GAL1* promoter. After incubation at 30°C for 4 h, galactose-induction was shut off and cell samples were taken at 20 min intervals followed by immunoblotting with anti- -gal antibody.

Results

The UBL domain of Dsk2 is required for ubiquitin-dependent degradation of UBA domain-defective Dsk2.

Dsk2 is considered to be a stable protein [20], but Dsk2/Rad23 mutants that are defective in the UBA domain become unstable [15]. To investigate the role of UBL and UBA domains of Dsk2 in its own stability, we constructed a series of Dsk2-truncated mutants (Fig. 1A) and confirmed that UBA-defective Dsk2 was unstable (lanes 5, 6). This instability could be recovered by deleting the UBL domain (lane 8), indicating the requirement of the UBL domain for Dsk2 instability.

Two different types of experiments were then performed to test whether this instability depends on the ubiquitin-proteasome pathway. First, the UBA mutant (LL368AA, see Fig. 1A) was induced into proteasome mutant strains *pre2* and *rpn1* followed by immunoblotting with

anti-Dsk2 antibody (Fig. 1B). LL368AA was stabilized in *pre2* (lane 4) and *rpn1* (lane 6), whereas LL368AA was barely detectable in the wild-type strain (lane 2). In comparison, wild-type Dsk2 was stable (lanes 1, 3, 5). To confirm this result, LL368AA was expressed in a *dsk2* background (Fig 1C). Again, LL368AA expressed in *dsk2* was rapidly degraded, whereas LL368AA expressed in *dsk2 rpn1* was stable (right panel).

Second, we used a genetic method to confirm the dependence of Dsk2 instability on the ubiquitin-proteasome pathway. A series of Ura3-fused Dsk2 constructs and their UBA mutant (Ura3-1-335) were made (Fig. 1D). The stability of these Ura3-fused constructs in yeast cells was evaluated by immunoblotting. Ura3-fused Dsk2 was stable, but Ura3-1-335 was unstable in YPH499 (summarized in Fig. 1D, left panel). These constructs were then transformed into a parental strain YPH499, and we tested whether Ura3-fused Dsk2 complemented the uracil auxotrophy of YPH499 based on its growth on plated of minimal medium without uracil, the complementation of which is an indication for the stabilization of Ura3-Dsk2 protein in cells. As expected, YPH499 expressing Ura3-Dsk2 grew and YPH499 expressing Ura3-1-335 failed to grow (middle panel), because Ura3-1-335 was unstable. The proteasome mutant *pre2* strain expressing Ura3-1-335, however, did grow on plates of minimal medium without uracil (middle panel). The same result was obtained for *rpn1* (right panel). These genetic results (summarized in the left panel) indicate that Dsk2 UBA mutant 1-335 became stable in the proteasome mutant strain. Therefore, the Dsk2 UBA mutants were degraded in the ubiquitin-proteasome pathway depending on the UBL domain.

The UBL domain of Dsk2 is ubiquitinated in yeast cells.

In the course of our experiments on yeast Dsk2, we detected endogenous Dsk2 with a laddered banding pattern on the immunoblot. As this pattern was reminiscent of ubiquitinated proteins, we tested the ubiquitination status of Dsk2. A pull-down experiment in which His_6-T7-ubiquitin was precipitated with Talon resin, and the bound materials were immunoblotted with anti-Dsk2

antibody. Ubiquitin chains of Dsk2 were confirmed (Fig. 2A, lane 8).

We next determined the region of Dsk2 that is ubiquitinated. All eight lysine residues of Dsk2 lie within the UBL domain (K8, K13, K28, K33, K49, K52, K72, K76) and these residues in the UBL domain are highly conserved from yeast to humans (Fig. 2B). We constructed an arginine-substituted mutant of the Dsk2 UBL domain (Dsk2-KR) in which the eight lysine residues were replaced by arginine (Fig. 2B, bottom). To test the ubiquitination status of Dsk2-KR in cells, Dsk2-KR was expressed in *dsk2* as in Fig 2A, followed by a pull-down with Talon resin of the cell extracts and immunoblotting with anti-Dsk2 antibody (Fig. 2C). Compared with wild-type Dsk2 (lane 11), Dsk2-KR was not ubiquitinated (lanes 12, 13). Thus, the lysine residues in the UBL domain of Dsk2 are ubiquitinated in yeast.

Ubiquitin chains of the Dsk2 UBL domain mediate Dsk2 own stability.

We investigated whether ubiquitin chains are required for the instability of UBA-defective Dsk2. UBL/UBA double mutants [21] were constructed (Fig. 3A) to test for their degradation (Fig. 3B) as before. All of the double mutants; KR/GF343AA (lanes 10-12), KR/GS359AA (lanes 16-18), and KR/LL368AA (lanes 22-24), were stable in YPH499 (Fig. 3B). Degradation of Dsk2 via UBA domain, therefore, requires ubiquitin chains of Dsk2 UBL domain. Consistent with this stability, all of these mutants were not ubiquitinated in the cells (Fig. 3C, lanes 8-11). These results show clearly that Dsk2-KR recovers the instability of the Dsk2 UBA mutants. We therefore concluded that UBA-defective Dsk2 was degraded in the ubiquitin-proteasome pathway mediated by ubiquitin chains of the UBL domain. Wild-type Dsk2 was ubiquitinated, but still stable, suggesting that endogenous Dsk2 can escape from degradation even when it is fully ubiquitinated (see Discussion).

Dsk2 ubiquitination affects in vivo ubiquitin-dependent protein degradation.

UBL-UBA proteins such as Dsk2 function in the delivery of ubiquitinated substrates to the

proteasome. We investigated whether ubiquitin chains of the UBL domain affect Dsk2 function in yeast cells.

First, the effect of ubiquitin chains in the Dsk2 UBL domain on protein degradation *in vivo* was examined by using the N-end rule substrate Leu- -gal. *DSK2* disruption impairs degradation of Leu- -gal in the cells [17]. A single copy of wild-type Dsk2 or Dsk2-KR was expressed in *dsk2* (Fig. 4A). Defective degradation of Leu- -gal in *dsk2* □□□ restored by the expression of wild-type Dsk2, but was not by Dsk2-KR.

Second, the effect of ubiquitin chains on the interaction of Dsk2 with polyubiquitinated proteins in cells was examined. GST-Dsk2-KR was coexpressed with His_6-T7-ubiquitin in yeast, and the GST pull-down was immunoblotted with anti-T7 and anti-Dsk2 antibodies (Fig. 4B). Dsk2-KR decreased the ability to bind polyubiquitinated proteins in cells (lane 5), although the binding ability was not completely abolished. As a control, wild-type Dsk2 bound to polyubiquitin in cells (lane 4), but UBA deletion 78-335 did not (lane 7). Therefore, the ubiquitin chains of the UBL domain also affect the interaction between Dsk2 and polyubiquitinated proteins in yeast. Deletion of the UBL domain (78-373) bound to polyubiquitinated proteins (lane 6) (See Discussion).

Next, the complementation of *DSK2* disruption by Dsk2-KR was tested using the growth defect of *dsk2 rad23* . A strain with deletions of both *DSK2* and *RAD23* is temperature-sensitive for growth although a disruption of *DSK2* alone is viable [20]. A single copy of *Dsk2-KR* was expressed in *dsk2 rad23* (Fig. 4C, left). *Dsk2-KR* had decreased ability to complement the temperature-sensitive growth of *dsk2 rad23* , whereas wild-type *Dsk2* was able to fully complement growth.

Growth arrest of yeast and the accumulation of large amount of polyubiquitinated proteins in cells are caused by overexpression of wild-type Dsk2 in yeast [7,17], which seem to be involved in Dsk2 function in the ubiquitin-proteasome pathway. When the effect of ubiquitin

chains on the Dsk2-mediated growth arrest was examined, overexpressed Dsk2-KR failed to inhibit the growth as expected (Fig. 4C, right). Consistent with this, Dsk2-KR did not accumulate polyubiquitinated proteins in cells (data not shown). Thus, ubiquitin chains of the UBL domain are required for *in vivo* Dsk2 function.

Discussion

This paper shows that yeast Dsk2 is ubiquitinated via its UBL domain. Depending upon ubiquitin chains in the UBL domain, Dsk2 that is defective in the UBA domain were degraded by the ubiquitin-proteasome pathway. This instability was recovered by deleting ubiquitin chains in the UBL domain. The stability and function of Dsk2 are closely linked to the ubiquitin-proteasome pathway through ubiquitin chains of the UBL domain of Dsk2.

Ubiquitin chains of the Dsk2 UBL domain are a signal for Dsk2 degradation in yeast cells.

The UBL domain and the UBA domain interact with the proteasome and the polyubiquitinated proteins, respectively. UBA domain-defective Dsk2 protein, which fails to bind polyubiquitin chains of the degradation substrates, can bind to the proteasome via its UBL domain. As a result, such dysfunctional Dsk2 would occupy the proteasome receptor. In this condition, such dysfunctional Dsk2 must be degraded (eliminated) rapidly in cells. Our results show that ubiquitin chains of the Dsk2 UBL domain serve as a signal for Dsk2 degradation (Fig. 3). We suggest that the UBL domain-mediated Dsk2 degradation take part in eliminating dysfunctional Dsk2 from the receptor site of the proteasome.

The UBL domain has eight lysine residues and the UBL domain sequence of Dsk2 has high similarity to ubiquitin (Fig. 2B). When the ubiquitination status of a series of lysine mutants was tested in more detail, the K28/K33 mutant was ubiquitinated but the K28R/K33R mutant was less with different molecular weight shifts (*see Supplement data*). It is thus likely that ubiquitin chains of Dsk2 are conjugated mainly through K28/K33 of the UBL domain. In the absence of

lysines in K28/K33, however, other K sites can be used to form different chains. Based on our preliminary experiment on K28R and/or K33R mutants, Dsk2 ubiquitination seems to depend mainly on K28 rather than K33 (data not shown). We suspect that K28 of the UBL domain (equivalent to ubiquitin K29) may be a probable candidate for a major chain linkage in the UBL domain, but further analysis is required on the molecular details.

Dsk2 UBA domain may bind alternatively ubiquitin chains of the UBL domain and of polyubiquitinated substrates.

The Dsk2 UBA domain *in vitro* binds both K48- and K29-linked chains [22], and the UBA domain interacts with the UBL domain intramolecularly [23]. The Dsk2 UBA in cells binds preferentially to K48-chains of polyubiquitinated substrates [7,17]. It is reasonable that UBL-UBA proteins undergo a conformation change between 'open' and 'closed' forms [16]. The 'open' form binds to K48-linked ubiquitinated substrates via the C-terminal UBA domain and to the proteasome via the N-terminal UBL domain, which acts as a delivery factor. Alternatively, the 'closed' form intramolecularly interacts between the UBL K28/K33-linked chains and UBA domains. Ubiquitin chains of the UBL domain may contribute to a conformational change of Dsk2 protein molecules. The difference of chain preference (K48/K28) could endow Dsk2 with a distinct property in the delivery pathway.

Binding of UBL chains to its own UBA domain may affect its own stability. It is possible that the 'stockpile Dsk2' that fails to bind K48 chains of the substrates binds to the UBL K28/K33-chains through its interactions with the UBA domain, resulting in the escape of Dsk2 from degradation. This protection may explain how the wild-type ubiquitinated Dsk2 is able to escape from own degradation by the proteasome. Indeed, wild-type Dsk2 is ubiquitinated, but stable in cells [20] (Figs. 1B, C, and 3B). In relevant to this, recent works suggest that the UBA domain guard ubiquitin receptors by preventing initiation of degradation at the proteasome [24].

Our data also show that the Dsk2 UBL domain lacking ubiquitin chains impaired the

degradation of Leu- -gal (Fig. 4A) and that *in vivo* Dsk2 function in yeast required the UBL ubiquitination (Fig. 4C). Moreover, the UBL domain lacking ubiquitin chains decreased the interaction of Dsk2 with polyubiquitinated proteins in cells (Fig. 4B, lane 5), indicating that ubiquitin chains of the UBL domain can help the interaction between the Dsk2 UBA domain and polyubiquitinated substrates. Interestingly, however, deletion of the whole UBL domain did not affect the binding between the UBA domain and the polyubiquitinated substrates (Fig 4B, lane 6). Ubiquitin chains of the UBL domain might negatively mediate the interaction of putative inhibitory protein(s) with the UBA domain. In contrast, it is unlikely that the UBL ubiquitin chains affect the interaction between Dsk2 and the proteasome, because we did not detect any difference on the binding ability between the Dsk2 wild-type and KR mutant (our preliminary result).

Rad23 and Dsk2 both function in the delivery pathway. In case of Rad23, it has been already shown that the Rad23 stability is mediated by the UBA domain-dependent degradation [15]. Taken together with Dsk2 (this report), we suggest that ubiquitin chains of the UBL domain in UBL-UBA proteins play a distinct role in the ubiquitin-proteasome pathway.

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Abbreviations used are: GST, glutathione S-transferase; PCR, polymerase chain reaction; UBA, ubiquitin-associated; UBL, ubiquitin-like.

Figure legends

Fig. 1. UBA domain-defective Dsk2 is degraded through the ubiquitin-proteasome pathway.

(A) The Dsk2 UBA mutant is unstable. The GST-version of Dsk2 deletions (lanes 3-8) was expressed in YPH499 and detected by immunoblotting with anti-GST antibody (upper panel) and anti-Cdc28 antibodies (lower panel) as a loading control. The stability of the mutants is shown on the right of a diagram of the Dsk2 constructs.

(B) The UBA mutants became stable in the proteasome mutant. GST-Dsk2 and GST-LL368AA were induced by galactose in YPH499 (lanes 1, 2), *pre2-75* (lanes 3, 4), and *rpn1-821* (lanes 5, 6) strains. Dsk2 and Cdc28 were detected by immunoblotting with anti-Dsk2 (upper panel) and

anti-Cdc28 antibodies (lower panel), respectively.

(C) Instability of the UBA mutant was recovered by the proteasome mutation. Similar to (B), GST-Dsk2 and GST-LL368AA were induced by galactose in *dsk2* and *dsk2 rpn1-821*, followed by immunoblotting with anti-Dsk2 and anti-Ddi1 antibodies (a loading control). Degradation was assayed at 30-min intervals.

(D) Complementation of the uracil auxotroph by *URA3*-fused *Dsk2*. pRS314-*URA3-DSK2* or pRS314-*URA3-1-335* using the endogenous promoter was transformed into wild-type YPH499 or proteasome mutants (*pre2-75*, and *rpn1-821*), respectively. Growth was tested in a minimal medium plate without uracil. A diagram of the *URA3*-fused Dsk2 mutants is shown, summarizing both the Ura3 complement abilities and stability.

Fig. 2. The UBL domain of Dsk2 is ubiquitinated in yeast.

(A) Dsk2 is a ubiquitinated protein. Wild-type Dsk2 in YEplac195 was co-expressed with His_6-T7-ubiquitin in *dsk2* (lanes 1-4), followed by pull-down with Talon resin and immunoblotting with anti-Dsk2 antibody (lanes 5-8). Vector denotes YEplac195. The arrow indicates the non-ubiquitinated form of Dsk2 that aggregates non-covalently with the resin.

(B) Sequence alignment of the UBL domain in Dsk2 homologs. The gray regions indicate conserved lysine residues in the UBL domain. Eight lysine residues of the Dsk2 UBL domain were all replaced by arginine (Dsk2-KR), shown at the bottom.

(C) The Dsk2-KR mutant lacks ubiquitin chains. The Dsk2 (lanes 2, 5, 8, 11) or Dsk2-KR mutant (lanes 3, 6, 9, 12, 13) in pEG-KG was co-expressed with His_6-T7-ubiquitin. Cell extract (lanes1-6) and Talon resin pull-down (lanes 7-13) were immunoblotted by anti-Dsk2 antibody. Twofold-volume of Dsk2-KR sample was loaded (lane 13). Method is the same as described in (A). Vector denotes YEplac112 (control for His_6-T7-ubiquitin). This result by His pull-down was also confirmed by GST pull-down of GST-Dsk2 version of the extracts, followed by immunoblotting with anti-Dsk2 antibody (data not shown).

Fig. 3. Ubiquitin chains of the UBL domain mediate instability of the Dsk2 UBA mutant.

(A) Diagrams of the UBL and UBA double mutants. The K and R in the UBL domain indicate wild-type and the KR mutant, respectively. An asterisk in the UBA domain denotes the alanine-substituted UBA mutants (see the details at the bottom). Ubiquitination and stability of these mutants are summarized on the right.

(B) The UBL and UBA double mutant is stabilized. Degradation of the UBL and UBA double mutants was assayed in *dsk2* strain. Dsk2 and Dsk2-KR (lanes 1-6), UBA mutants (lanes 7-9, 13-15, 19-21), and UBL/UBA mutants (lanes 10-12, 16-18, 22-24) in YEplac195 were expressed by galactose induction in *dsk2* . After shut-off, the induction cell extracts were collected at 30-min intervals and immunoblotted with anti-Dsk2 and anti-mAb414 antibodies (a loading control).

(C) Ubiquitination of the UBL and UBA mutant. The ubiquitination status of Dsk2 and their UBL and UBA mutants was examined. Ubiquitination of GST-Dsk2 was tested first, and then GST version of these mutants were co-expressed with His_6-T7-ubiquitin in *pre2-75*, and analyzed as in Fig. 2. Wild-type Dsk2 (lanes 4, 7), UBA mutant (GS359AA and LL368AA) (lanes 5, 6), UBL mutant Dsk2-KR (lane 8), and the UBL/UBA double mutants (lanes 9-11).

Fig. 4. Ubiquitination of the Dsk2 UBL domain affects ubiquitin-dependent protein degradation and Dsk2 function *in vivo*.

(A) Protein degradation of N-end rule substrate. *DSK2* or *DSK2-KR* was expressed in *dsk2* from a single-copy vector YCplac22 in the presence of galactose-induced Leu- -gal. After the induction was shut-off, cell extracts were taken at 20-min intervals. Each sample was immunoblotted with anti- -gal and anti-cdc2 antibodies (a loading control, a gift from H. Nishitani).

(B) Effect of UBL ubiquitination on the interaction between Dsk2 and polyubiquitinated proteins in cells. GST-Dsk2 and its mutants were co-expressed with His_6-T7-ubiquitin in yeast. GST pull-down was immunoblotted by either anti-Dsk2 or anti-T7 antibodies.

(C) Effect of Dsk2-KR mutant on yeast growth. (Left panel); complementation test of *dsk2* . pRS316 expressing *DSK2* or DSK2-KR from the endogenous promoter was transformed to *dsk2 rad23* and their abilities to restore temperature-sensitive growth were tested by incubating in minimal medium minus uracil at 36^{o}C for 3 days. Vector denotes pRS316. Expression levels were shown on the bottom. (Right panel); Dsk2-mediated growth arrest. Both multi-copy vectors YEplac195 and YEplac112 overexpressing *DSK2* or *DSK2-KR* were transformed to YPH499 and incubated in minimal medium containing 2% glucose minus uracil and tryptophan at 30^{o}C for 2-3 days.

Research Highlights

> We show that the UBL domain of Dsk2 is ubiquitinated in yeast.

>Dsk2 that is defective in the UBA domain is unstable.

>Depending on Ub chains, the UBA-defective Dsk2 is degraded by Ub-proteasome pathway.

>Dsk2 ubiquitination affects Ub-dependent protein degradation *in vivo*.

> Ub chains of UBL domain mediate Dsk2 own stability and protein degradation in yeast.

Figure 1.

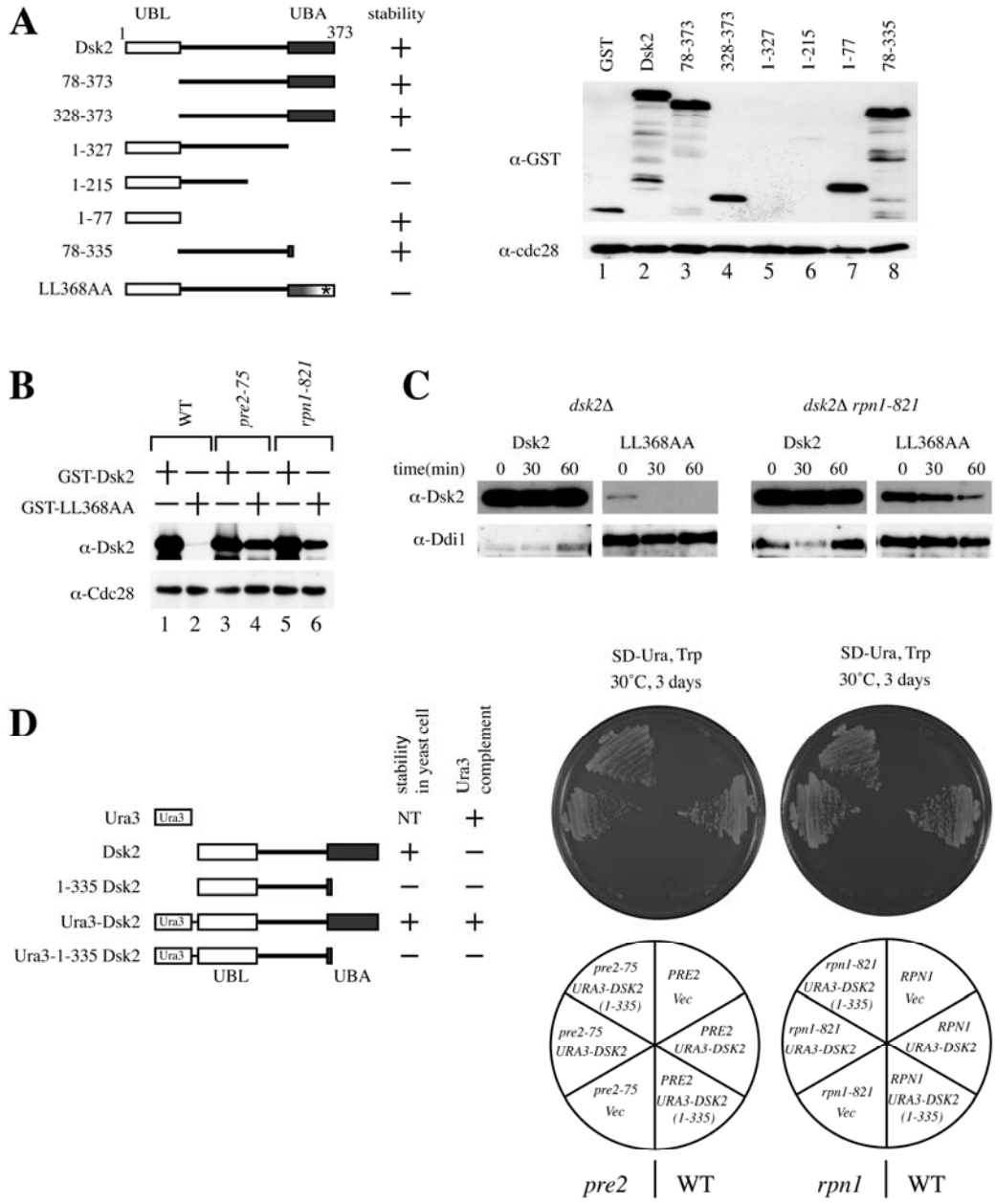

Figure 2

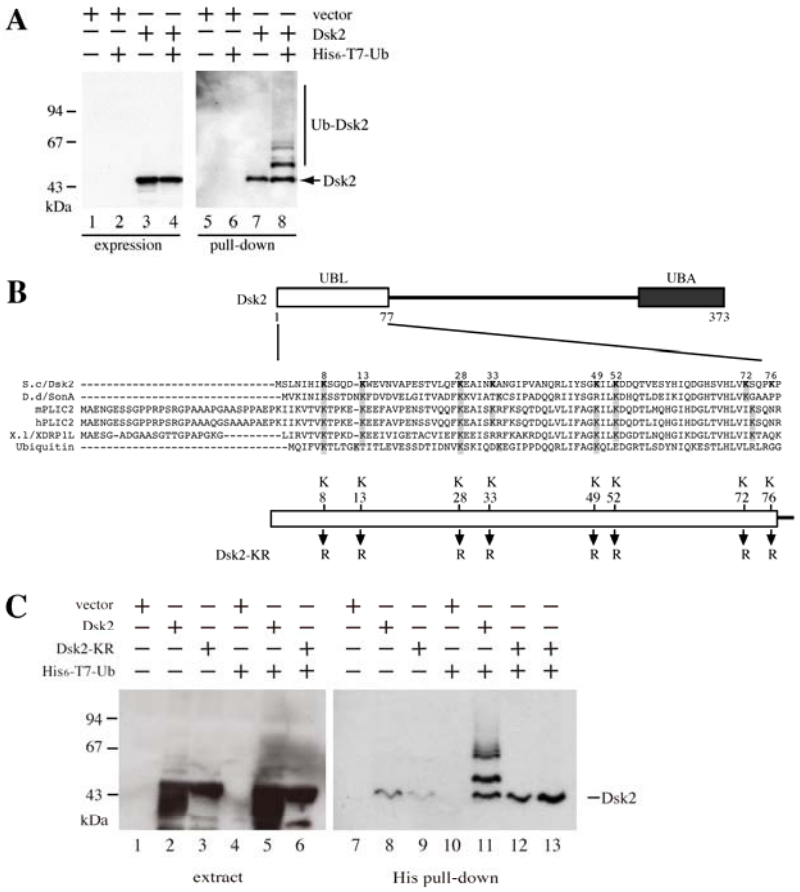

Figure 3.

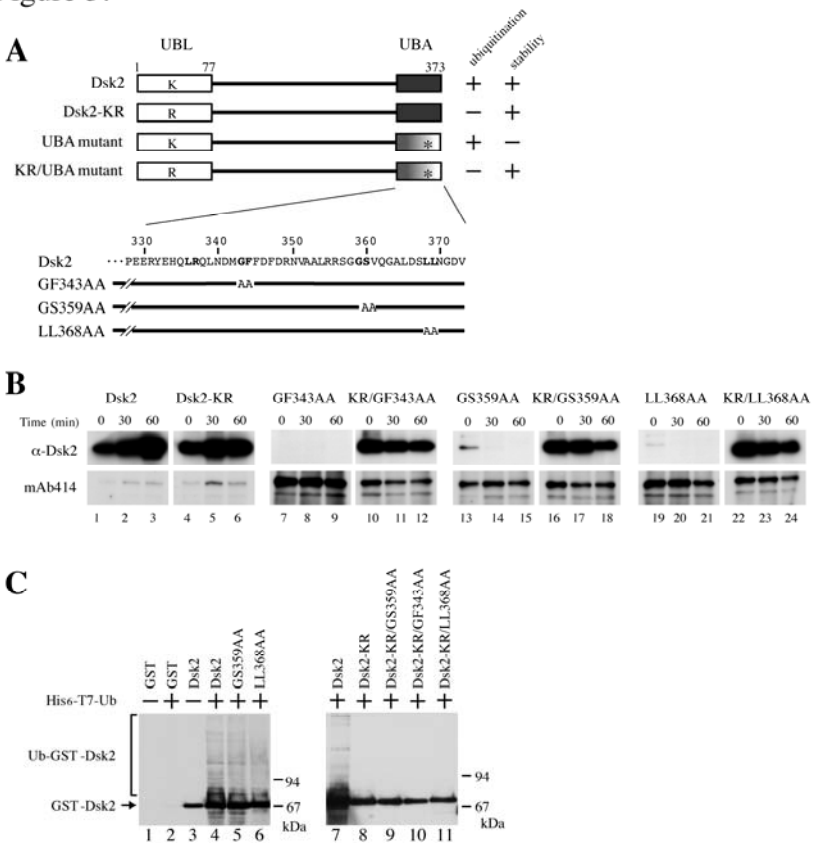

Figure 4