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Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome

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Recruitment of substrates to the 26S proteasome usually requires covalent attachment of the Lys48-linked polyubiquitin chain. In contrast, modifications with the Lys63-linked polyubiquitin chain and/or monomeric ubiquitin are generally thought to function in proteasome-independent cellular processes. Nevertheless, the ubiquitin chain-type specificity for the proteasomal targeting is still poorly understood, especially *in vivo*. Using mass spectrometry, we found that Rsp5, a ubiquitin-ligase in budding yeast, catalyzes the formation of Lys63-linked ubiquitin chains *in vitro*. Interestingly, the 26S proteasome degraded well the Lys63-linked ubiquitinated substrate *in vitro*. To examine whether Lys63-linked ubiquitination serves in degradation *in vivo*, we investigated the ubiquitination of Mga2-p120, a substrate of Rsp5. The polyubiquitinated p120 contained relatively high levels of Lys63-linkages, and the Lys63-linked chains were sufficient for the proteasome-binding and subsequent p120-processing. In addition, Lys63-linked chains as well as Lys48-linked chains were detected in the 26S proteasome-bound polyubiquitinated proteins. These results raise the possibility that Lys63-linked ubiquitin chain also serves as a targeting signal for the 26S proteaseome *in vivo*.

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Introduction

Ubiquitin (Ub) is an evolutionarily conserved protein responsible for numerous biologically important processes through its covalent conjugation to client proteins in all eukaryotes (Hershko and Ciechanover, 1998). Protein ubiquitination is regulated by the E1 (Ub-activating enzyme)-E2 (Ub-conjugating enzyme)-E3 (Ub-ligase) cascade reaction. Ub conjugation to lysine residues in a substrate-attached Ub leads to the formation of polymeric Ub chains. In yeast, all seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) of Ub can be used for chain formation, resulting in Ub chains of different topologies (Peng *et al*, 2003). Of these chains, the best understood type is the polyubiquitin chain linked through K48 of Ubs. K48-linked Ub chains with a length of four or more Ubs serve as the predominant proteasome-targeting signal (Thrower *et al*, 2000; Pickart and Fushman, 2004). K11- and K29-linked chains are also involved in proteasome-dependent protein degradation (Baboshina and Haas, 1996; Koegl *et al*, 1999; Jin *et al*, 2008). In contrast, K63-linked Ub chains and mono-ubiquitination are generally thought to function in proteasome-independent processes such as DNA repair, signal transduction and receptor endocytosis *in vivo* (Hicke, 2001; Pickart and Fushman, 2004), whereas emerging *in vitro* studies imply K63-linked chains support the proteasomal degradation (Hofmann and Pickart, 2001; Kim *et al*, 2007). Much less is known about the functions of chains with other topologies.

Rsp5, an essential HECT-type E3 in *Saccharomyces cerevisiae*, is involved in various biological processes through both proteasome-dependent and proteasome-independent pathways (Horak, 2003). In the former case, Rsp5 ubiquitinates the largest subunit Rpb1 of RNA polymerase II upon DNA damage and the mRNA export factor Hpr1, leading to their proteasomal degradation (Beaudenon *et al*, 1999; Gwizdek *et al*, 2005). Rsp5 also ubiquitinates the membrane-anchored transcriptional factors Spt23 and Mga2 leading to proteasomal processing (Hoppe *et al*, 2000). In the latter cases, Rsp5 ubiquitinates several plasma membrane proteins for their Ub-dependent endocytosis (Hein *et al*, 1995; Galan and Haguenauer-Tsapis, 1997; Dunn and Hicke, 2001).

Although Rsp5 participates in multiple cellular processes, the most important function is in the OLE pathway (Hoppe *et al*, 2000; Jentsch and Rumpf, 2007). In this pathway, the expression of Ole1, a Δ9 fatty acid desaturase, is tightly regulated by two related transcription factors, Spt23 and Mga2. Both proteins are synthesized as inactive precursors (p120s), which are anchored to the endoplasmic reticulum (ER) membrane by their single C-terminal transmembrane domains. Upon unsaturated fatty acid restriction, p120 is ubiquitinated by Rsp5 and subsequently processed by the 26S proteasome to remove the transmembrane domain. The processed N-terminal 90 kDa protein (called p90) is segregated by the Cdc48-Ufd1-Npl4 complex. This allows p90 to enter the nucleus and to activate transcription of target genes (Rape *et al*, 2001). Importantly, the lethality of Δ*rsp5* mutations can be suppressed by the addition of oleic acid to the growth media or by overproduction of Spt23-p90 or Mga2-p90, suggesting that the essential role of Rsp5 is ubiquitination of p120 for proteasomal processing (Hoppe *et al*, 2000).

A major unsolved question in Rsp5 biology is the topology of the Ub chains on its substrate *in vivo*. Earlier and recent

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studies suggest that Rsp5 assembles the K63-linked chains on its substrates, which function in proteasome-independent pathways (Galan and Haguenauer-Tsapis, 1997; Kee *et al*, 2006). As the function of Rsp5 lies on both the proteasome-dependent and -independent pathways, it is possible that Rsp5 attaches the different types of Ub chains to different substrates. In this view, Rsp5 may attach the K48-linked Ub chains to a subset of the substrate to be targeted by the 26S proteasome such as Rpb1, Hpr1, Spt23-p120 and Mga2-p120. Alternatively, it is possible that there are little or no specific functions of each type of Ub chains. Recent studies showed that the ubiquitinated cyclin B with heterogeneous short chains is degraded by the 26S proteasome *in vitro* (Hanna *et al*, 2006; Kirkpatrick *et al*, 2006). Considering this view, it is plausible that Rsp5 may attach exclusively K63-linked Ub chains to the substrate for proteasomal degradation.

In this study, we analysed the Ub chain topologies of several Rsp5 substrates by mass spectrometry (MS) and found that Rsp5 attaches entirely K63-linked Ub chains to its substrates *in vitro*. Unexpectedly, the 26S proteasome was able to bind and degrade efficiently the substrates with K63-linked chains *in vitro*. To investigate whether K63-linked ubiquitination is involved in the proteasomal targeting *in vivo*, we next dissected the ubiquitination of Mga2-p120 by quantitative MS. We found that the ubiquitinated p120 contains relatively high levels of K63-linked chains and Lys48-linkages to a lesser extent, and the K63-linked chains are sufficient for the proteasome-binding and p120-processing. Furthermore, we detected K63-linked chains within the proteasome-bound polyubiquitinated proteins. These results suggest that K63-linked polyubiquitin chains can serve as the proteasomal targeting as well as K48-linked chains.

Results

Rsp5 assembles K63-linked ubiquitin chains in vitro

We reported earliera convenient method for preparing polyubiquitinated substrates for the 26S proteasome using Rsp5 *in vitro* (Saeki *et al*, 2005). For this device, the PY motif, a Rsp5-recognition site, was introduced to a natural proteasome substrate Sic1, a CDK inhibitor, termed 'Sic1^{PY}'. The Sic1^{PY} was efficiently ubiquitinated by Rsp5 and degraded rapidly by the purified 26S proteasome *in vitro* (Saeki *et al*, 2005). Considering the present scenario, it is critically important to know the topology of the Ub chains that are formed by Rsp5. To this end, we first performed the Sic1^{PY} ubiquitination assay using a series of Ub mutants. A Sic1^{PY} mutant in which all the lysine residues except for K36 were replaced to arginine (Sic1K36^{PY}) was also tested to monitor the number of attached Ub (Figure 1A). Unexpectedly, both Sic1^{PY} and Sic1K36^{PY} were attached with K11-, K33-, K48- and K63-linked Ub chains as judged by their gel mobilities compared with lysine-less Ub (UbK0). Two UbK0 molecules were attached to Sic1K36^{PY}, suggesting that the N terminus, in addition to the K36 residue, is utilized for ubiquitination. On the basis of this consideration, the average length of each Ub chain on Sic1^{PY} was estimated as follows K11- (3 to 4 Ubs), K33- (3 to 4 Ubs), K48- (2 or 3 Ubs) and K63-linked Ub chain (3 or more Ubs).

To determine more accurately the Ub chain-type specificity of Rsp5, we analysed the ubiquitinated Sic1^{PY} by MS. The method is based on the detection of specific linkages in the tryptic digests of Ub chains (Peng *et al*, 2003). We found that the peptide mass fingerprinting by MALDI-TOF-MS can be applied to determine the relative abundance of Ub-linkages (Supplementary Figures S1–S3). To analyse the ubiquitinated Sic1^{PY} by MS, the ubiquitinated Sic1^{PY} was isolated from the reaction mixtures by using the hexahistidine-tag of Sic1^{PY} in denatured condition (Figure 1B). The gel portion containing the ubiquitinated Sic1^{PY} was exercised and subjected to in gel digestion with trypsin. The following MALDI-MS analysis revealed that the ubiquitinated Sic1^{PY} with wild-type Ub contains only K63-linkage. In contrast, only K48-linkage was detected in the preparation using UbK63R (Figure 1C). These results indicate that Rsp5 preferentially assembles the K63-linked Ub chains on Sic1^{PY} under normal conditions without forming other types of linkages. It is worth noting that when K63 of Ub is mutated, Rsp5 catalyzes K48-linked chain synthesis, indicating that Rsp5 uses other lysine residues for the chain formation when the preferred site is missing. In this study, we used Ubc4 as E2 because Ubc1, Ubc4 and Ubc5 were found to work equivalently with Rsp5 (Supplementary Figures S4–S6). We further determined the topology of Ub chains of the self-ubiquitinated Rsp5 and two native Rsp5 substrates, Mga2 and Rpb1, *in vitro*. MS analysis revealed that these ubiquitinated substrates contained only K63-linkages (Supplementary Figures S1, S6 and S7). These results suggest that Rsp5 ubiquitinates its substrates exclusively with K63-linked chains *in vitro*.

26S proteasome efficiently degrades the ubiquitinated Sic1^{PY} with K63-linked chains

We have showed previously that the ubiquitinated Sic1^{PY} is degraded by the yeast 26S proteasome, and this system was utilized for evaluating the activity of mutant proteasomes (Sone *et al*, 2004; Isono *et al*, 2005; Saeki *et al*, 2005). In this study, we found that this ubiquitinated Sic1^{PY} contains only K63-linked chains (Figure 1). To confirm this unexpected result, we employed the following systematic degradation assays. The wild-type 26S proteasome was affinity purified from the *RPN11-3xFLAG* cells, in which the *RPN11* gene was tagged with three tandem Flag epitopes (Saeki *et al*, 2005). As a control for degradation assay, we also prepared the 26S proteasome lacking Rpn10, an intrinsic Ub receptor, from the *RPN11-3xFLAG Δrpn10* mutant cells (Figure 2A).

To optimize our degradation assay system, the wild-type 26S proteasome was titrated in the degradation assay with 200 nM of substrates. Strikingly, low concentrations (25 nM) of the 26S proteasome were sufficient to degrade the K63-linked ubiquitinated Sic1^{PY} with wild-type Ub (Figure 2B). In contrast, the *Δrpn10* 26S proteasome cannot degrade the K63-linked ubiquitinated Sic1^{PY} (Supplementary Figure S8) as observed for the K48-linked ubiquitinated Sic1 (Verma *et al*, 2004).

To exclude the possibility that the ubiquitinated Sic1^{PY} contains undetectable levels of K48-linkages, which may stimulate proteasomal degradation, we performed the degradation assay using the ubiquitinated Sic1^{PY} with UbK48R, which contains only K63-linkages (Figure 2C). The Sic1^{PY} ubiquitinated with UbK48R was degraded at a comparable dose and rate with wild-type Ub (Figure 2B and C). Thus, the results suggested that K63-linked Ub chains promote the degradation by the 26S proteasome. We believe that the concentration of the 26 proteasome is biologically

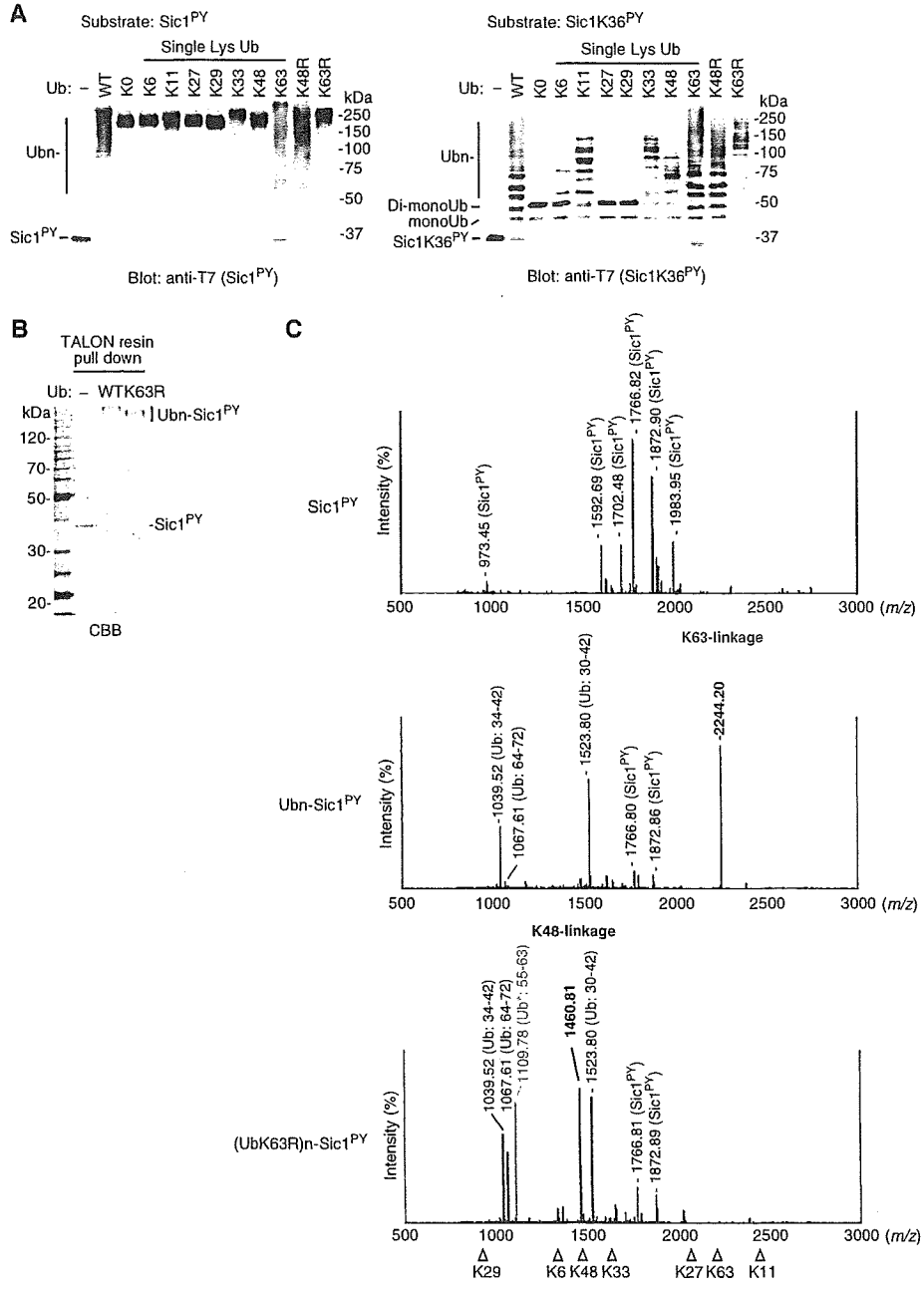


Figure 1 Rsp5 assembles K63-linked ubiquitin chains on Sic1^{PY}. (**A**) Multiple types of Ub chains can be formed on Sic1^{PY} when using Ub mutants. T7-Sic1^{PY}-His$_6$ (Sic1^{PY}, left) or its single lysine construct (Sic1K36^{PY}, right) was ubiquitinated by E1, E2 and Rsp5, with the indicated Ub mutants and analysed by western blotting with anti-T7 antibody. (**B**) Purification of the ubiquitinated-Sic1^{PY}. After ubiquitination as in (A), the samples were denatured with 6 M urea and the Sic1^{PY} Ub conjugates were pulled down with TALON resin. As a control, Ub-omitted reaction was conducted. Gels were stained with Coomassie brilliant blue (CBB). (**C**) MS analysis of the purified ubiquitinated-Sic1^{PY}. Gel regions of the ubiquitinated-Sic1^{PY} (Ubn-Sic1^{PY} in A) were excised and subjected to in gel-digestion with trypsin. The resulting peptides were analysed by MALDI-TOF mass spectrometry (MS). The major and specific peaks are labeled. The peak corresponding to K63- and K48-linkages are indicated in blue and red, respectively. The Ub fragments derived from the mutants are indicated by Ub* in green. The ideal masses (*m/z*) of all seven specific ubiquitin linkages are indicated by the triangles.

relevant: The concentration of the 26S proteasome in yeast cells is estimated to be ~60 nM, if the proteasomes are distributed evenly throughout cells and to be ~8.6 μM, if all the proteasomes are present in nucleus (Russell *et al*, 1999; Ghaemmaghami *et al*, 2003; Jorgensen *et al*, 2007).

To investigate the types of ubiquitination that undergo degradation, we prepared the ubiquitinated Sic1^{PY} with different topologies by a series of mutant Ubs; K48-linked chains with UbK63R (K63R), short Ub chains possibly containing K33-linkage with UbK48R K63R (K48R K63R), and multiple mono-ubiquitination with methylated Ub (m) and lysine-less

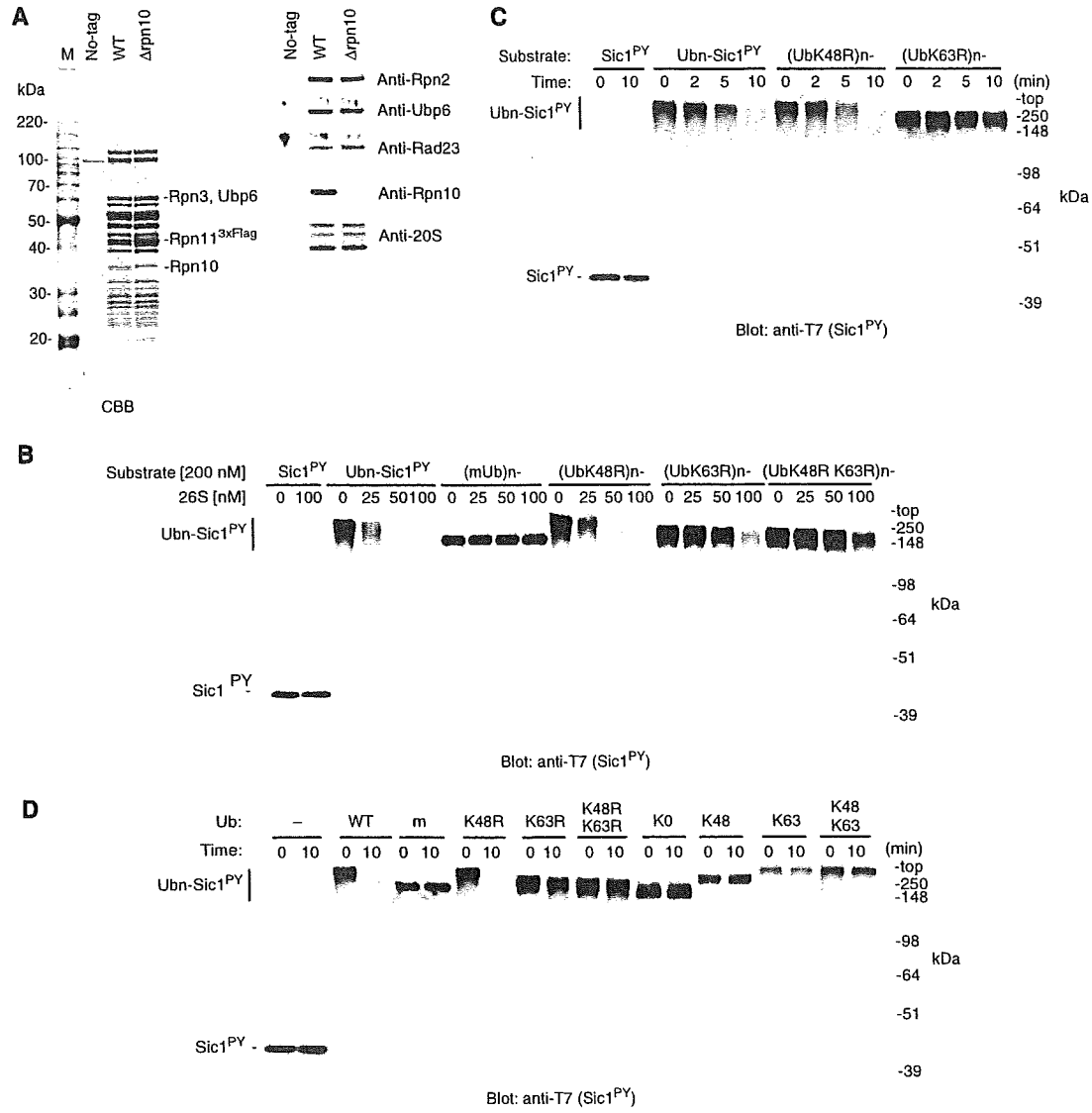


Figure 2 The 26S proteasome degrades K63-linked polyubiquitinated-Sic1^{PY}. (**A**) The purified 26S proteasomes from *RPN11-3xFLAG* strain (WT) or Δ*rpn10 RPN11-3xFLAG* strain (Δrpn10) and the mock purified materials from the parent wild-type strain (no-tag) were subjected to SDS–PAGE followed by staining with CBB (left) or analysed by western blot with the indicated antibodies (right). (**B**) Degradation of the ubiquitinated-Sic1^{PY} by the wild-type 26S proteasome. Sic1^{PY} was ubiquitinated by Rsp5 with wild-type Ub (Ubn-Sic1^{PY}, resulting K63-linked polyUb chain), with methylated Ub (mUb, multiple mono-ubiquitination), or with Ub mutants; UbK48R (K63-linked polyUb chains), UbK63R (K48-linked polyUb chains), or UbK48R K63R (short Ub chains, probably contains K33-linkage). The Ub-omitted reaction was also tested (Sic1^{PY}) as a control. Each substrate (200 nM) was incubated with 25 to 100 nM of the wild-type 26S proteasome at 25°C for 10 min. The reaction was terminated by the addition of SDS-loading buffer and was analysed by western blotting with T7-antibody. (**C**) Kinetic analysis of Sic1^{PY} degradation. Each substrate (200 nM) was incubated with the wild-type 26S proteasome (50 nM) at 25°C. The reaction was terminated at the indicated time points and analysed as in (B). (**D**) Degradation assay of Sic1^{PY} Ub conjugates with lysine-less (K0), single-lysine only (K48 or K63) and double-lysines (K48 + K63) Ub mutants. Each substrate (200 nM) was incubated with the wild-type 26S proteasome (50 nM) at 25°C for 10 min and analysed as in (B).

Ub (K0). In comparison to the K63-linked ubiquitinated Sic1^{PY}, higher concentrations (100 nM) of the 26S proteasome were required for the degradation of the K48-linked ubiquitinated Sic1^{PY}. On the other hand, the ubiquitinated Sic1^{PY} with short chains were only slightly degraded (Figure 2B), indicating that the short Ub chains are a less favorable signal for proteasomal degradation. As expected, the 26S proteasome failed to degrade the multiply mono-ubiquitinated Sic1^{PY} with methylated Ub (mUb) and UbK0 (Figure 2B and D). Thus, the K63-linked Ub chains are efficient signal for degradation, whereas multiple mono-Ubs and short chains are not. To simplify this, we further prepared the ubiquitinated Sic1^{PY} with single lysine Ub mutants. Although a single- or double-lysine Ub mutants (e.g., K48, K63, K48, K63) were polymerized efficiently on Sic1^{PY} by Rsp5, the Sic1^{PY} Ub conjugates were incompetent to proteasomal degradation (Figure 2D), which is apparently consistent with the lack of their interaction with the 26S proteasome as described below.

As $Sic1^{PY}$ is an artificially devised substrate for Rsp5, we also examined the degradation of the Rpb1, a subunit of the RNA polymerase II complex, as a native Rsp5 substrate (Beaudenon *et al*, 1999; Somesh *et al*, 2005). Rpb1 was efficiently polyubiquitinated by Rsp5 and was degraded by the 26S proteasome *in vitro* (Supplementary Figure S7). MS analysis showed that the ubiquitinated Rpb1 in the reaction contained only K63-linkage (data not shown).

26S proteasome and its ubiquitin receptors bind K63-linked ubiquitin chains

We further investigated whether K63-linked Ub chains are suitable for the targeting signal of the 26S proteasome. We noticed that the self-ubiquitinated Rsp5 is neither degraded nor deubiquitinated by the 26S proteasome (data not shown), like the self-ubiquitinated Cdc34 (Elsasser *et al*, 2002). By using this property, we analysed the interaction between the purified 26S proteasome and the Ub chains on GST-Rsp5 by native-PAGE (Figure 3A and B). In the presence of the ubiquitinated Rsp5 with wild-type Ub, the migration of the 26S proteasome was retarded due to their binding, whereas Rsp5- or Ub-omitted reactions had no effects (Figure 3B). These data indicate that the 26S proteasome binds directly with the K63-linked Ub chains on GST-Rsp5. Expectedly, the 26S proteasome also binds efficiently the ubiquitinated Rsp5 with UbK63R, which contains K48-linkages. In contrast, the proteasome binds only weakly with multiple mono-ubiquitinated and short polyubiquitinated Rsp5, which were generated by methylated Ub (mUb) or UbK48R K63R (K48R K63R), respectively (Figure 3B). The gel shift by the respective Ub conjugates occurred in a dose-dependent manner (Supplementary Figure S9), suggesting that the 26S proteasome binds with high affinity to both K48- and K63-linked Ub chains almost equivalently, but with low affinity to the multiple mono-Ubs or short Ub chains (Figure 3B, left). Although polyubiquitination occurred with a single- or double-lysine Ub mutants (UbK48, UbK63 or UbK48 + UbK63) on Rsp5 (Figure 3A), the assembled chains were incompetent for proteasome-binding irrespective of K48- and/or K63-linked chains (Figure 3B, right). These results clearly indicate that certain lysine residue(s) other than the remaining lysine residue is required for their proper conformations. Consequently, the 26S proteasome was not able to degrade the polyubiquitinated $Sic1^{PY}$ with single-lysine Ub mutants (Figure 2D).

The 26S proteasome utilizes multiple Ub receptors such as Rpn10, Rpn13, Rad23 and Dsk2 (Elsasser *et al*, 2002; Verma *et al*, 2004; Richly *et al*, 2005; Husnjak *et al*, 2008; Schreiner *et al*, 2008). To investigate the binding properties of the Ub receptors against K63-linked Ub chains, we performed a GST pull-down assay with a mixture of free K63-linked chains. We found that both Rpn10 and Rad23 have markedly high affinity with long chains, more than seven Ubs, but low affinity with short chains. This property was also observed in a comparative analysis using free K48-linked chains (Figure 3D). The results are consistent with a previous study, which showed that the maximum affinity of human Rad23 for K48-linked Ub chains is reached with a chain length of six or more Ubs (Raasi *et al*, 2004).

Mga2-p120 is modified mainly with K63-linked ubiquitin chains in vivo

Is the K63-linked Ub chain recognized and targeted by the 26S proteasome *in vivo*? One way to show this is to isolate and dissect Ub conjugates of Rsp5 substrate. Among the known substrates of Rsp5, we selected Mga2 because the essential role of Rsp5 is the ubiquitination of Spt23-p120 and Mga2-p120 and their regulations have been characterized extensively (Hoppe *et al*, 2000; Rape *et al*, 2001; Shcherbik *et al*, 2003; Shcherbik and Haines, 2007).

To purify the Mga2-p120 Ub conjugates in enough amounts for MS analysis, we investigated optimal conditions, in which the ubiquitination levels were enhanced and the ubiquitinated proteins were stabilized *in vivo*. Because Mga2 is a protein of a very low abundance, ~300 copies per cell (Ghaemmaghami *et al*, 2003), we produced N-terminally Flag-tagged Mga2 under the *GAL1* promoter. The ubiquitination and the processing of overproduced Mga2 is governed by Rsp5, Ubp2, Cdc48 and the proteasome (Supplementary Figure S10), indicating that the overexpressed Mga2 seems to be functionally equivalent to endogenous one. In addition, the *PDR (pleiotropic drug registance) 5* gene was deleted to increase sensitivity to the proteasome inhibitor MG132 (Fleming *et al*, 2002). To enhance the ubiquitination levels of Mga2, we used a plasmid that constitutively expresses wild-type Ub (Supplementary Figure S12).

Under these conditions, we successfully isolated the Mga2-p120 Ub conjugates to a detectable level in a CBB stained gel (Figure 4A). The gel portion of the Mga2-p120 Ub conjugates was excised and subjected to MS analysis (Figure 4B). Within the multiple ion peaks corresponding to Mga2 and Ub peptides, a strong peak of K63-linkage and a relatively weak peak of K48-linkage were detected.

To quantify the Ub linkages by MS, we next used SILAC (stable isotope labeling by amino acids in cell culture): the Δ*lys2* background strains were grown in SILAC medium supplemented with 'heavy' lysine ($^{13}C_6$-Lys) (de Godoy *et al*, 2006). To exclude the possibility that the Ub-linkages detected in MS is due to contaminants, we first grew the cells with a control plasmid in 'light' medium, whereas the cells carrying the P_{GAL1}-*MGA2* plasmid in heavy medium. After incubation with 1% galactose and 100 μM MG132 for 2 h, equal amounts of the cells from the two cultures were mixed together. The Mga2 Ub conjugates were then purified and subjected to MS analysis. As the incorporation of heavy lysine results in a mass shift of 6 Da, K-containing peptides are detected as heavy ions by MS. As shown in Figure 4C, the heavy lysine-labeled Ub linkages were only detected as heavy ions. The absence of light ions indicates that the detected Ub chains were not contaminant.

Next, the yeast cells carrying the P_{GAL1}-*MGA2* plasmid grown in heavy medium were treated with MG132, whereas the same cells grown in light medium were treated with DMSO. Then, the p120 Ub conjugates were purified and subjected to MS analysis. Comparing the intensities of SILAC ion pairs, the relative amounts of Ub-linkages were determined both the K63- and K48-linkages were increased to 1.75- and 3.84-fold, respectively, by inhibition of the proteasome (Figure 4D). To quantify the absolute amount of the Ub chains on Mga2-p120, we prepared a control Ub peptides that consist of a 1:1 mixture of K48- and K63-linked di-ubiquitins. The tryptic digests of the Mga2 Ub conjugates from the

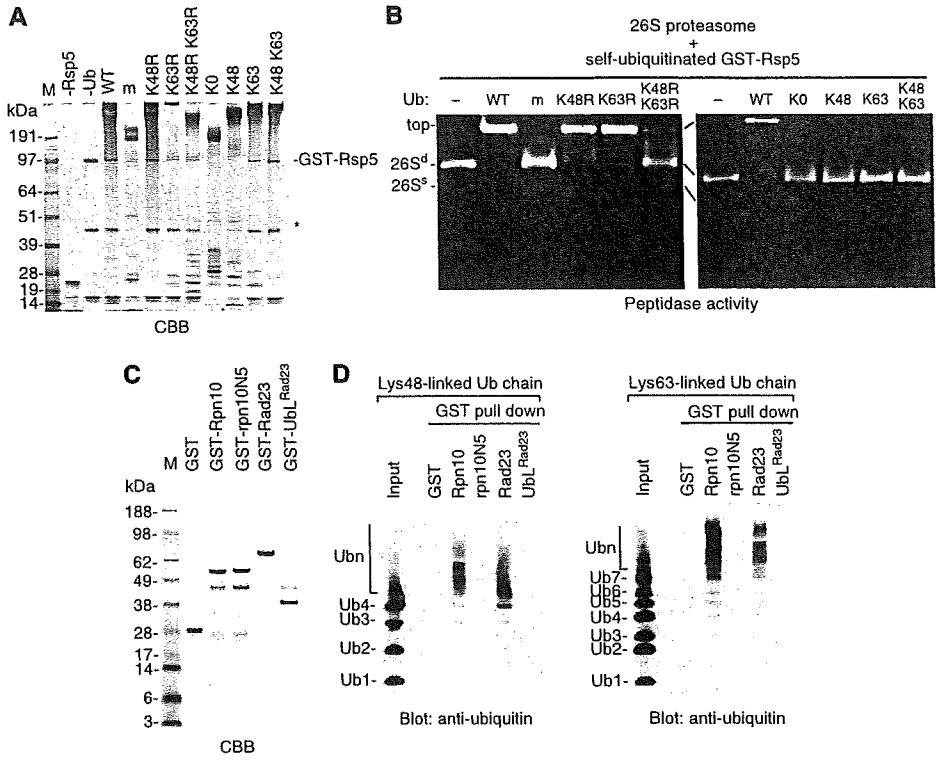


Figure 3 The 26S proteasome and its ubiquitin receptors bind K63-linked polyubiquitin chains. (**A**) Self-ubiquitination of GST-Rsp5 with Ub mutants. GST-Rsp5 was self-ubiquitinated with a series of Ub mutants. After the reactions, a portion was analysed by SDS–PAGE. The topologies of ubiquitination on Rsp5 are as follow K63-linked chains with wild-type Ub (WT), with UbK48R (K48R), with UbK63 (K63) or with UbK48 + K63 (K48 K63); K48-linked Ub chains with UbK63R (K63R) or with UbK48 (K48); short Ub chains that probably contain K33-linkages with UbK48R K63R (K48R K63R); multiple mono-Ub with methylated-Ub (m) or with lysine-less Ub (K0). The degradation products of GST-Rsp5 are indicated by the asterisk. (**B**) Association of the 26S proteasome and self-ubiquitinated Rsp5 monitored by gel shift assay. The 26S proteasome was preincubated with the self-ubiquitinated GST-Rsp5 produced as in (A), then, the mixtures were subjected to 3.5% native-PAGE followed by in-gel activity assay using suc-LLVY-amc. As a control, Ub-omitted reaction (−) was also tested. Doubly capped and singly capped species of the 26S proteasomes are indicated by $26S^d$ and $26S^s$, respectively. (**C**) SDS–PAGE analysis of GST-fusion proteins. GST-fusion proteins (1 μg) were subjected to SDS–PAGE followed by CBB-staining. GST-rpn10N5 is an Ub-interacting motif mutant, in which LAMAL sequence was mutated to NNNNN. GST-UbL^{Rad23} is a deletion mutant of C-terminal Ub-associated domains. (**D**) GST pull-down assays of free K48- and K63-linked Ub chains with the proteasomal Ub receptor proteins. Free K48-linked (left) or K63-linked (right) Ub chains were preincubated with the indicated GST-fusion proteins, then, GST-fusion proteins were pulled down with glutathione-immobilized agarose. The bound materials were eluted with SDS-loading buffer and analysed by western blot with anti-Ub antibody. GST alone, GST-rpn10N5 and GST-UbL^{Rad23} were used as the control.

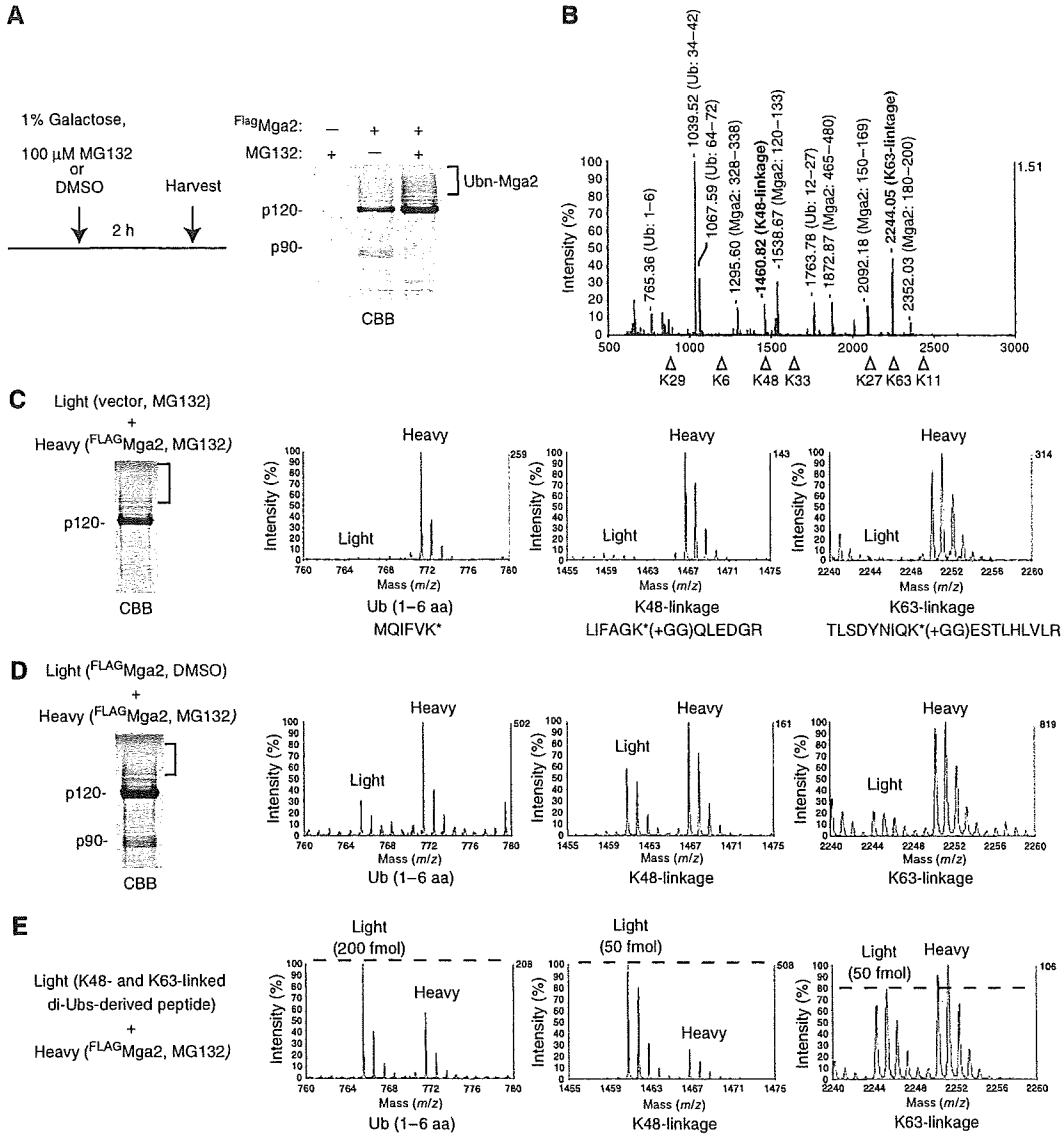
MG132-treated cells (heavy) were mixed with the control Ub peptides (light), then, analysed by MS (Figure 4E and Supplementary Figure S13). The absolute amounts of total Ub and the linkages were determined by respective ion pairs, and the percentages of chains were then calculated (Figure 4F). Strikingly, the K63-linked chains occupied ~57% of total Ubs and existed in 4.7-fold larger amount than K48-linkaged chains.

K63-linked ubiquitin chains are sufficient for the p120-processing in vivo

To investigate the functional significance of the K63-linked Ub chains, we first analysed steady-state levels of Mga2 in mutant Ub-expressing cells. In the wild-type Ub-overexpressing cells, surprisingly, the p120 levels were greatly decreased to ~38% as compared with that in the cells carrying a control plasmid (Figure 5A). The decrease was abrogated by MG132, suggesting that the overproduced Ub stimulates the degradation of Mga2. Importantly, the Mga2 degradation was also observed in both the UbK48R- and the UbK63R-expressing cells, ~58% decrease and ~32% decrease, respectively (Figure 5A). In contrast, the p120 protein levels remained unchanged in the UbK48K63R-expressing cells.

Next, we performed a chase experiment to monitor the rate of p120-processing (Figure 5B). In the wild-type Ub-overexpressing cells, the half-life of the p120 was determined to ~30 min. Concomitant with decrease of the p120 levels, the p90 levels were increased and subsequently degraded after 60 min (data not shown). Similarly, in both the UbK48R- and UbK63R-expressing cells, the p120-processing also occurred as fast as in the wild-type Ub-expressing cells. In contrast, the p120-processing only slightly occurred in the cells expressing the UbK48R K63R mutant or in the MG132-treated cells.

Because each Ub mutants were expressed at levels 25- to 50-fold higher than endogenous Ub (Figure 5D), the mutants should inhibit the formation of chains. To confirm this, we quantified the Ub linkages in the ubiquitinated-Mga2 from the mutant Ub-expressing cells by MS. As expected, only K63-linkage was detected in the ubiquitinated Mga2 from the UbK48R-expressing cells, whereas only K48-linkage was



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	Total Ub	K48-linkage	K63-linkage
DMSO	53.9 fmol	8.6 fmol (15.9%)	18.2 fmol (33.8%)
MG132	123.2 fmol	15.0 fmol (12.1%)	70.0 fmol (56.8%)
MG132/DMSO	x 2.29	x 1.75	x 3.84

Figure 4 Mga2-p120 is modified with mainly K63-linked ubiquitin chains *in vivo*. (**A**) SDS–PAGE analysis of the Mga2 Ub conjugates from MG132-treated cells. Wild-type cells (YYS1246, *Δpdr5 Δlys2* background) carrying P_{GAL1}-^{FLAG}*MGA2* (^{Flag}Mga2) and P_{TDH3}-*Ub* plasmids were cultured in SRaf medium. Mga2 expression was induced by 1% galactose for 2 h in the presence of 100 μM MG132 or DMSO. Mga2 and its Ub conjugates were affinity-purified by anti-Flag M2 agarose, and subjected to SDS–PAGE analysis followed by CBB staining. (**B**) MS spectrum of the ubiquitinated-Mga2. The gel portion of the Mga2-p120 Ub conjugates from MG132-treated cells, indicated by a blanket in (A), was excised and subjected to in-gel digestion with trypsin. The resulting peptides were analysed by MALDI-TOF MS. Peaks corresponding to K48- and K63-linkages are indicated in red and blue text, respectively. (**C**) Detection of the heavy isotope-labeled Ub chains of the Mga2 Ub conjugates using SILAC. The cells (YYS1301) carrying a control plasmid grew in light medium and the cells (YYS1303) carrying the P_{GAL1}-^{FLAG}*MGA2* (^{Flag}Mga2) grew in heavy medium. After the addition of 1% galactose for 2 h in the presence of 100 μM MG132, the two cultures were mixed and analysed as in (A). MS spectra of the SILAC ion pairs are presented: a linear Ub peptide (1–6 amino acids), Ub K48-, and K63-linkages. (**D**) Stabilizations of both the K48- and K63-linked Ub chains on the Mga2 by proteasome-inhibition. The cells (YYS1303) were grown in heavy or light medium. Mga2 expression was induced by 1% galactose for 2 h in the presence of 100 μM MG132 for heavy culture or DMSO for light culture. Then, the two cultures were mixed and analysed as in (C). (**E**, **F**) Absolute quantitation of the Ub chains on the Mga2. The tryptic digests of light Ub chains (a 1:1 mixture of K48- and K63-linked di-Ubs) were used as internal standards. Note that the mixture of di-Ubs generates a Ub (1–6 amino acid) and each linkages at a 4:1 ratio. The heavy lysine-labeled Mga2 Ub conjugates were prepared as in (C). Then, the peptides were mixed and the relative ion intensities were analysed by MS. The amount of the total Ub, K48-linkage and K63-linkage were calculated. See also, Supplementary Figure S13.

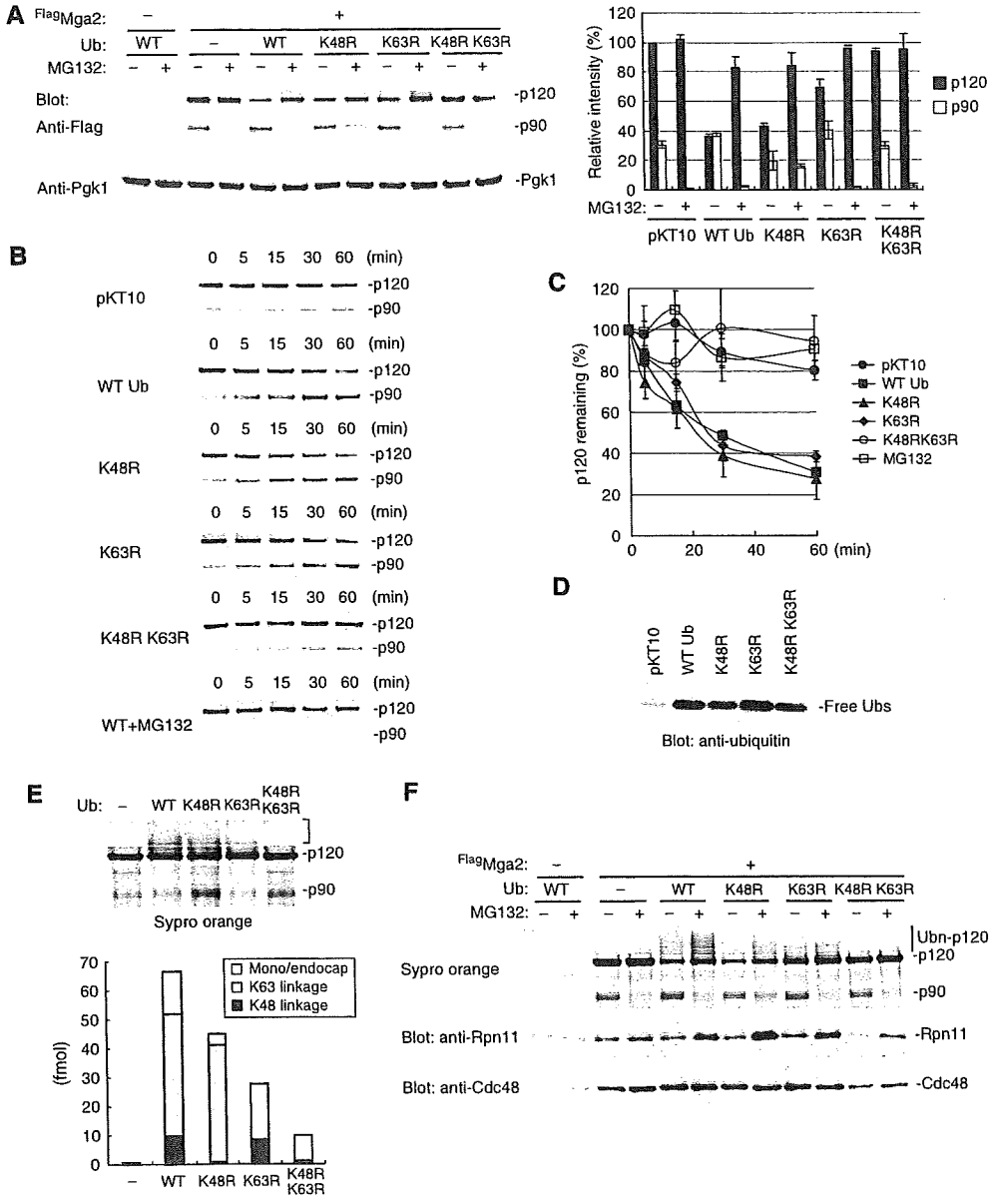


Figure 5 K63-linked Ub chains promote Mga2-p120 processing *in vivo*. (**A**) Steady-state levels of Mga2 in the Ub mutant-expressed cells. The cells expressing ^{Flag}Mga2 and respective Ubs (YYS1301-1306) were cultured in SRaf medium. Mga2 was expressed by 1% galactose for 3 h in the presence or in the absence of 100 μM MG132. The total cell extracts were analysed by western blot with anti-Flag antibody for the detection of both Mga2-p120 and Mga2-p90 (upper) and anti-Pgk1 antibody as a loading control (lower). The protein levels of p120 and p90 are shown with mean ± s.d. values of three experiments in the right graph. (**B, C**) Cycloheximide-chase analysis of Mga2. The respective cells as in (A) were cultured in SRaf medium. After Mga2 expression by 1% galactose for 1 h, translation was inhibited by cycloheximide at a final concentration of 0.4 mg/ml. Aliquots were taken at the indicated time points after cycloheximide addition. The total cell extracts were analysed by western blot with anti-Flag antibody for the detection of both Mga2-p120 and Mga2-p90 (upper) and anti-Pgk1 antibody as a loading control (lower). To inhibit proteasome activity, cells were treated with MG132 (100 μM) upon the Mga2 expression. The protein levels of p120 are shown with mean + SD values of three experiments in the graph. (**D**) Expression levels of wild type and mutant Ubs. Cell extracts were analysed by western blot with anti-Ub. (**E**) Absolute quantitation of the Ub chains on the Mga2 from the mutant Ub-expressed cells. The Mga2 Ub conjugates were purified from the MG132-treated cells cultured in SILAC medium. The ubiquitination of Mga2 was confirmed by Sypro Orange-staining and was quantified by MS as in Figure 4E. See also Supplementary Figure S14. (**F**) The 26S proteasome binds the ubiquitinated Mga2 *in vivo*. Mga2 and its Ub conjugates were immunoprecipitated under a mild condition and were analysed by western blot analysis with anti-Rpn11 and anti-Cdc48 antibodies. The ubiquitination levels of Mga2 were monitored by Sypro Orange-staining.

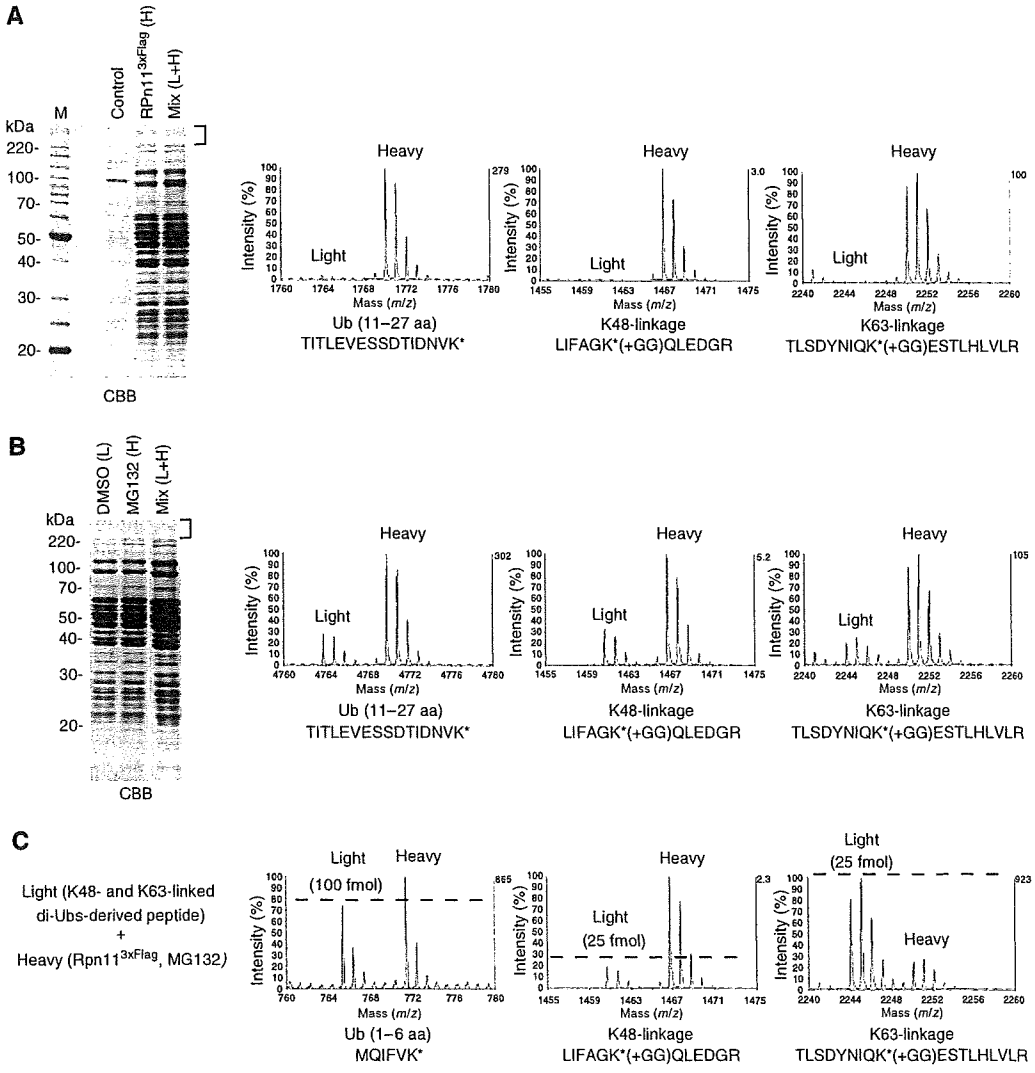
detected from the UbK63R-expressing cells (Figure 5E and Supplementary Figure S14). These results indicate that each Ub chains can be formed on p120 independently and are functionally equivalent in the p120-processing.

The 26S proteasome binds K63-linked ubiquitin chains in vivo

If K63-linked Ub chains serve as a targeting signal for the 26S proteasome, the interaction between the 26S proteasome and

the p120 Ub conjugates should be observed *in vivo*. To test this, we performed the Mga2 immunoprecipitation assay under mild conditions, without high salt wash. As shown in Figure 5F, the 26S proteasome was co-precipitated with K63-linked ubiquitinated Mga2 from the UbK48R-expressed cells in which the proteasome activity was inhibited by MG132. The interaction was detected at background levels from DMSO (mock)-treated cells, suggesting that the 26S proteasome continuously processes the K63-linked ubiquitinated p120. In contrast, Cdc48, the Ub-dependent segregase for p90, was also detected in the Mga2 precipitates, but the binding was apparently not correlated with the ubiquitination levels of p120 and was not affected by MG132. Possibly, Cdc48 may recognize only a subset of the ubiquitinated Mga2 and/or may be resting until the p90 generation. Nonetheless, the result suggests that the K63-linked Ub chains are sufficient to the proteasomal targeting.

A recent study showed that total levels of K63-linked Ub chains as well as K48- and K11-linked chains were elevated by the treatment of MG132 in mammalian cells (Bennett *et al*,



D

	Total Ub	K48-linkage	K63-linkage
DMSO	44.7 fmol	39.7 fmol (88.8%)	2.17 fmol (4.8%)
MG132	152.3 fmol	134.0 fmol (88.0%)	8.63 fmol (5.6%)
MG132/DMSO	x 3.40	x 3.37	x 3.96

Figure 6 Quantitation of the proteasome-bound Ub chains. (**A**) Identification of the K63-linked Ub chains from the purified 26S proteasome. The *RPN11-3xFLAG* cells (YYS1320) grew in heavy medium and the control cells (YYS1314) grew in light medium were treated with 100 μM MG132 for 2.5 h. Then, the 26S proteasome was purified under a mild condition and analysed as in Figure 4C. MS spectra of SILAC pair ions are presented: a Ub peptide (11-27 amino acid), Ub K48-, and K63-linkages. (**B**) Both the K48- and K63-linkages were accumulated by proteasome-inhibition. The *RPN11-3xFLAG* cells (YYS1320) were grown in heavy or light medium. The heavy culture was treated with 100 μM MG132 for 3 h, whereas the light culture was treated with DMSO for 2.5 h. The proteasomes were purified and relative ion intensities were measured as in Figure 4D. (**C, D**) Absolute quantitation of the Ub chains of the proteasome-bound ubiquitinated proteins. Tryptic digests of the heavy lysine-labeled Ub conjugates in (A) were mixed with the internal standard Ub peptides and analysed by MS. The amount of the total Ub, K48-linkage, and K63-linkage were calculated. See also, Supplementary Figure S15 and S16.

2007). Their observation and our results raise the possibility that K63-linked Ub chains are widely utilized for the proteasome-dependent protein degradation. To investigate this, we analysed the proteasome-bound ubiquitinated proteins by MS using SILAC. Under a mild condition, the polyubiquitinated proteins were co-purified with the 26S proteasome from the Ub-overexpressed cells (Supplementary Figure S15). By using MS, we found that K63-linkages in addition to K48-linkages within the ubiquitinated proteins (Figure 6A and Supplementary Figure S15). The other types of Ub linkages including K11-linkage were not detected even when analysed by using a nano LC-coupled MALDI-MS/MS (data not shown). Importantly, the levels of both the K48- and K63-linkages were increased to 3~4-fold by MG132-treatment (Figure 6B and Supplementary Figure S15). By using the control Ub peptides, the absolute amount of the K63-linkages was calculated as 4~5% of total Ub, and the K48-linkages were predominant, ~88%, as expected (Figure 6C, D and Supplementary Figure S16). It is known that Hul5, a proteasome-bound E4, assembles K63-linked chains onto the proteasome-bound ubiquitinated substrate *in vitro* (Crosas *et al*, 2006), suggesting the possibility that the presence of the K63-linkages is due to the Hul5 activity. However, K63-linkage was still detected in the proteasome-bound Ub conjugates from Δ*hul5* cells (Supplementary Figure S17), indicating that the K63-linked chains are formed by an E3(s) other than Hul5. Collectively, these results suggest that K63-linked Ub chains may be widely utilized for the proteasome-dependent degradation.

Discussion

Rsp5-mediated ubiquitination results in K63-linked polyubiquitin chains

We started this study by investigating the chain topology of the ubiquitinated $Sic1^{PY}$, which was used to evaluate the proteasome activity *in vitro* (Sone *et al*, 2004; Isono *et al*, 2005; Saeki *et al*, 2005; Kriegenburg *et al*, 2008). Although four different types of Ub chains can be assembled by Rsp5 using Ub mutants (Figure 1 and Supplementary Figure S5), direct analysis by MS revealed that the ubiquitinated $Sic1^{PY}$ with wild-type Ub contained only K63-linked chains (Figure 1). In addition, we found that Rsp5 itself and two native Rsp5 substrates were also modified with K63-linked Ub chains *in vitro* (Supplementary Figures S1, S6 and S7). Thus, Rsp5 seems to assemble exclusively the K63-linked Ub chains on substrate, as reported previously (Galan and Haguenauer-Tsapis, 1997; Kee *et al*, 2006).

In our *in vivo* analysis, we used Mga2-p120 processing in an experimental setting because this process has been proven to be solely dependent on ubiquitination by Rsp5 followed by cleavage by the 26S proteasome. Relatively low levels of K48-linked Ub chains in addition to K63-linked chains were detected in the ubiquitinated Mga2-p120 (Figure 4). How K48-linked chains were introduced remains unclear. After the processing, the released p90 (active form) is subsequently ubiquitinated by Ufd2, an E4 that can extend K48-linked chains on ubiquitinated substrates (Johnson *et al*, 1995; Saeki *et al*, 2004), and is degraded by the 26S proteasome in the nucleus (Richly *et al*, 2005). As both Mga2 and Ub were overexpressed in our experimental setting, Ufd2 could target the Mga2-p120 under such nonphysiological conditions. However, the Mga2-p120 Ub conjugates from Δ*ufd2* cells still contained the K48-linked chains (Supplementary Figure S11), indicating that Ufd2 is not responsible for the K48-linked chain formation. Another possibility is that Ubc1 could preassemble the K48-linked chains prior to Rsp5-mediated ubiquitination of Mga2-p120 because Ubc1 itself can form K48-linked Ub chains (Hodgins *et al*, 1996; Rodrigo-Brenni and Morgan, 2007). However, only K63-linkage was detected in the Mga2-p120 Ub conjugates made by Rsp5 even if Ubc1 was used as E2 *in vitro* (Supplementary Figure S6). Ubp2 is known as a deubiquitinating enzyme that regulates Rsp5-mediated ubiquitination (Kee *et al*, 2005, 2006). We found that the ubiquitination levels of Mga2-p120 were markedly increased in Δ*ubp2* cells (Supplementary Figure S11). Therefore, there is no doubt that Rsp5 ubiquitinates the overexpressed Mga2 used in this study, but it is plausible that there is an additional E3 for Mga2-p120 in the cells as proposed previously (Shcherbik *et al*, 2003).

Why are not all Rsp5 substrates degraded by the 26S proteasome in vivo?

Although there are many Rsp5 substrates in the cells, only a subset of substrates are degraded by the 26S proteasome (Horak, 2003). One possibility is that the length of K63-linked Ub chains is also a key determinant for proteasomal degradation. We showed that the proteasomal Ub receptors efficiently bound long K63-linked Ub chains (Figure 3D). Most plasma membrane proteins are attached by Rsp5 with multiple mono-Ubs or a short chain with up to four Ubs (Rotin *et al*, 2000), in which Ub lengths might simply be enough to escape proteasomal targeting. Ubp2 might maintain short Ub chains on such Rsp5 substrates (Kee *et al*, 2005, 2006). Another possibility is the structural property of the substrate itself. It has been proposed that a loosely folded region is required for efficient proteasomal degradation (Prakash *et al*, 2004; Piwko and Jentsch, 2006). Some degradable Rsp5 substrates may contain this 'engagement' site. Our results support this notion, judging from the findings that physiological substrates (e.g., Sic1 and Rpb1) were readily degraded by the 26S proteasome, whereas nonphysiological substrates (e.g., Rsp5 and GFP) were not (data not shown).

Ubiquitin chain topologies for proteasomal targeting signal

It is widely accepted that K48-linked Ub chains play a central role in the proteasome-dependent proteolysis, whereas K63-linked chains function in proteasome-independent processes (Chau *et al*, 1989; Finley *et al*, 1994; Hershko and Ciechanover, 1998; Pickart and Fushman, 2004). However, previous and recent studies have raised possibilities that K11- and/or K63-linked Ub chains also serve as proteolytic signals (Baboshina and Haas, 1996; Hofmann and Pickart, 2001; Kirkpatrick *et al*, 2006; Kim *et al*, 2007; Jin *et al*, 2008). In this study, we showed that homogeneous K63-linked chains with sufficient length served as the proteasomal targeting signal *in vitro* (Figures 2, 3 and Supplementary Figures S7–S9). Moreover, our experiments, although carried out under a nonphysiological condition in which Ub was overexpressed, suggested that K63-linked Ub chains can be utilized to the proteasome *in vivo* (Figures 5, 6 and Supplementary Figures S13–S17). Direct analysis of the proteasome-bound ubiquitinated proteins suggests that

K63-linked Ub chains, even at a small fraction, are certainly utilized in the proteasome-dependent protein degradation (Figure 6). Curiously, we failed to detect K11-linked chains in this assay, possibly, this type of Ub conjugates may be delivered to Cdc48/p97 *via* UBX-UBA proteins as recently reported (Alexandru *et al*, 2008). In contrast to previous reports (Guterman and Glickman, 2004; Kirkpatrick *et al*, 2006; Boutet *et al*, 2007), multiple-mono Ubs and short Ub chains are unlikely to be signals for the proteasomal targeting (Figures 2 and 3).

Our work is reminiscent of the earlier study in yeast; overexpression of UbK63R in Δ*ubi4* cells caused hypersensitivity to various stresses including heat, amino acid analogues and UV (Arnason and Ellison, 1994). It is known that the ubiquitin-proteasome pathway degrades damaged cellular proteins by heat stress and amino acid analogues (Ciechanover *et al*, 1984). It is likely that K63-linked Ub chains are involved in protein quality control in cells. In this context, it should be noted that Rsp5 was shown to be also involved in degradation of stress-induced abnormal proteins (Hoshikawa *et al*, 2003). Our results are consistent with these observations in the sense that K63-linked Ub chains would serve as the proteasomal degradation signal. Further studies are needed to solve more precisely this long-standing and fundamental question.

Materials and methods

Plasmids, strains, and protein purifications

The plasmids, yeast strains and protein purification methods are described in the Supplementary data.

In vitro degradation assay

The ubiquitination of $Sic1^{PY}$ was performed as described previously (Saeki *et al*, 2005).

The degradation was initiated by adding the polyubiquitinated substrate to the purified 26S proteasome in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10% glycerol) containing 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$ at 25°C. The reaction was terminated by adding SDS sample buffer and analysed by western blotting with anti-T7 antibody.

Mass spectrometry

Protein samples were analysed by MALDI-TOF mass spectrometry (Voyager DE-PRO or 4800 MALDI TOF/TOF, Applied Biosystems) as described previously (Saeki *et al*, 2004; Tanaka *et al*, 2008) with minor modifications. Briefly, CBB-stained protein bands were excised from the SDS–polyacrylamide gel, destained and in-gel digested with 10 μg/ml of modified trypsin (Trypsin Gold, Mass Spec Grade, Promega). For SILAC analysis, the excised gel was treated with 0.5% RapiGest (Waters) for improvement of tryptic digestion. To prepare the Ub internal standard peptides, a 1:1 mixture of K48-linked and K63-linked di-ubiquitins (Boston Biochem) was subjected to in gel-digestion with trypsin. MS and MS/MS data were obtained according to the instructions provided by the manufacturer and then analysed by ProteinPilot software 2.0 (Applied Biosystems) or manually.

Gel shift assay

The self-ubiquitinated GST-Rsp5 (2 pmol) was incubated with a purified 26S proteasome (2 pmol) in 5 μl total volume of buffer A plus 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$ for 5 min on ice, mixed with dye and then subjected to 4% native-PAGE (Elsasser *et al*, 2002). The gel was incubated with 0.1 mM succinyl-Leu-Leu-Val-Tyr-7-amide-4-methyl-coumarin (suc-LLVY-amc) in buffer A plus 2 mM ATP and 5 mM $MgCl_2$ for 10 min at 25°C. The proteasome bands were visualized under UV light (360 nm) and analysed by the gel documentation system (UVP Inc.) equipped with the UV cut-off filter (410 nm cut off, Kenko, Japan).

Purification of the ubiquitinated Mga2-p120 from yeast cells

Yeast cells (YYS1246; Δ*pdr5* Δ*lys2*) were transformed with P_{GAL1}-^{FLAG}*MGA2* (pOKA606), which expresses Flag-tagged Mga2 under the galactose-inducible promoter, and *pKT10-Ub* plasmid (pOKA601), which constitutively express wild-type Ub. The transformants were cultured to an OD_{600} between 0.6 and 0.8 in SRaf-Ura-Trp medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% raffinose, 400 mg/l adenine, 10 mM phosphate buffer, pH 7.5). For stable isotope labeling experiments, the cells were grown in SILAC medium (0.67% yeast nitrogen base without amino acids, 2% raffinose, 20 mg/ml ^{13}C-lysine, amino acid mixtures omitting appropriate nutrients, 400 mg/l adenine, 10 mM phosphate buffer, pH 7.5). Then, ^{Flag}Mga2 was produced by the addition of galactose (1% final) for 2 h in the presence of 100 μM MG132 (20 mM stock in DMSO, Peptide Institute, Japan). The cells (corresponding to 100 OD_{600}) were lysed by glass beads in lysis buffer, 50 mM HEPES-Na, pH 7.5, 100 mM NaCl, 10% glycerol, 10 mM iodoacetamide, 1 mM 1,10-Phenanthroline, 100 μM MG132, and 2 × concentration of protease complete inhibitor cocktail (-EDTA, Roche). After removal of the glass beads, Triton-X100 (1% final) was added and incubated for 30 min at 0°C. The extracts were cleared by centrifugation and incubated with anti-FLAG M2 agarose beads (Sigma) for 1.5 h at 4°C. Beads were washed twice with lysis buffer containing 1% Triton-X100 and twice with lysis buffer containing 1 M NaCl, then, twice with lysis buffer containing 0.2% Triton-X100. The Mga2 and its ubiquitinated species were eluted with 400 μg/ml Flag peptide (Sigma) in the same buffer. For immunoprecipitation experiments, the washing step with high salt buffer was omitted.

Purification of the proteasome-bound ubiquitinated proteins

The *RPN11-3xFLAG* tagged cells (YYS1338) were cultured an OD_{600} between 0.6 and 0.8 in SILAC medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 20 mg/ml ^{13}C-lysine, amino acid mixtures omitting appropriate nutrients, 400 mg/l adenine, 10 mM phosphate buffer, pH 7.5). Then, the cells were treated with 100 μM MG132 or DMSO for 2.5 h, and lysed by glass beads in lysis buffer, 50 mM HEPES-Na, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM iodoacetamide, 1 mM 1,10-Phenanthroline, 100 μM MG132 and 2 × concentration of protease complete inhibitor cocktail (-EDTA, Roche). After removal of the glass beads, the extracts were cleared by centrifugation and incubated with anti-FLAG M2 agarose beads (Sigma) for 1.5 h at 4°C. Beads were washed three-times with same buffer and the proteasomes were eluted with 400 μg/ml 3 × Flag peptide (Sigma) in the same buffer.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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An Inhibitor of a Deubiquitinating Enzyme Regulates Ubiquitin Homeostasis

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SUMMARY

The dynamic and reversible process of ubiquitin modification controls various cellular activities. Ubiquitin exists as monomers, unanchored chains, or protein-conjugated forms, but the regulation of these interconversions remains largely unknown. Here, we identified a protein designated Rfu1 (*r*egulator of *f*ree *u*biquitin chains 1), which regulates intracellular concentrations of monomeric ubiquitins and free ubiquitin chains in *Saccharomyces cerevisiae*. Rfu1 functions as an inhibitor of Doa4, a deubiquitinating enzyme. Rapid loss of free ubiquitin chains upon heat shock, a condition in which more proteins require ubiquitin conjugation, was mediated in part by Doa4 and Rfu1. Thus, regulation of ubiquitin homeostasis is controlled by a balance between a deubiquitinating enzyme and its inhibitor. We propose that free ubiquitin chains function as a ubiquitin reservoir that allows maintenance of monomeric ubiquitins at adequate levels under normal conditions and rapid supply for substrate conjugation under stress conditions.

INTRODUCTION

Ubiquitination is a reversible posttranslational modification of cellular proteins that plays important roles in the regulation of several cellular processes, such as protein quality control, protein trafficking, cell-cycle regulation, DNA repair, apoptosis, and signal transduction (Hershko and Ciechanover, 1998; Mukhopadhyay and Riezman, 2007). Ubiquitin (Ub) is a highly conserved 76 amino acid protein that covalently attaches to the lysine residue(s) of target proteins via its carboxy-terminal glycine residue. Since Ub itself contains seven lysines, it can attach to several other Ubs, allowing the formation of polyubiquitin chains. Thus, Ub exists intracellularly as either a monomer, a substrate-conjugated polyubiquitin or monoubiquitin, or unanchored Ub chains.

One of the well-characterized functions of Ub is serving as a tag for selective proteolysis by the 26S proteasome, which is a large multisubunit protease complex (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). In the ubiquitin-proteasome system (UPS), ubiquitination of a substrate is catalyzed by a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-ligase (E3). Multiple Ubs are covalently added to a substrate successively by these enzymes, thus producing a substrate conjugated with polyubiquitin. The 26S proteasome recognizes the polyubiquitinated substrate and degrades the substrate after the polyubiquitin chain is cleaved off by deubiquitinating enzymes (DUBs) (Amerik and Hochstrasser, 2004; Ventii and Wilkinson, 2008). The released polyubiquitins or free Ub chains are further disassembled to monomeric Ubs by DUBs, and the resulting Ubs are reutilized.

Ubiquitination also plays a role in vacuolar sorting of both endocytic and biosynthetic membrane proteins (Mukhopadhyay and Riezman, 2007; Schnell and Hicke, 2003). At the endosome, Ub serves as a signal to sort cargo proteins into the multivesicular body (MVB), which is a critical step to their transport to lysosomes. Ub is removed from the cargo before entry into the internal vesicles of the MVB. In yeast, Doa4, a DUB, is responsible for deubiquitination of cargo proteins at the endosome (Katzmann et al., 2001; Nikko and Andre, 2007). Doa4 is recruited to the endosome and its activity is stimulated by Bro1, a class E Vps protein (Richter et al., 2007).

There seems to be a need for maintaining adequate intracellular levels of Ub, particularly the level of monomeric Ub, and indeed more Ubs are required under stress conditions (Finley et al., 1987). Yeast cells with insufficient amounts of monomeric Ub caused by mutation of DUB genes, such as *DOA4* or *UBP6*, are sensitive to an amino acid analog, and the expression of excess Ub compensates for this defect (Chernova et al., 2003; Papa and Hochstrasser, 1993; Swaminathan et al., 1999). Similarly, yeast cells become stress sensitive when *UBI4*, a heat shock gene encoding polyubiquitin, is deleted (Finley et al., 1987). In mice, a mutation in *Uch-L1* leads to gracile axonal dystrophy (*gad*) (Osaka et al., 2003). Moreover, the deletion of polyubiquitin gene *Ubc* and that of *Ubb* result in embryonic lethality and hypothalamic neurodegeneration, respectively (Ryu et al., 2007, 2008). However, an excess amount of Ub is also not beneficial to cells. In yeast, overexpression of Ub makes cells sensitive to stressful insults such as treatment with cadmium, arsenite, and paroromycin (Chen and Piper, 1995). To circumvent these situations, cells appear to possess several systems to regulate the level of monomeric Ub. One such regulatory system appears to operate at the level of transcription of

Ub-encoding genes. In yeast, among the four Ub-encoding genes *UBI1–4,* transcription of the *UBI4,* a polyubiquitin gene, is heat inducible (Finley et al., 1987). Another mechanism responsible for maintaining stable levels of intracellular Ub is an increase in Ubp6-associated proteasome in response to Ub deficiency, which efficiently retrieves Ub from Ub-conjugated substrate (Hanna et al., 2007).

Various eukaryotic organisms including mouse, rat, fly, nematoda, plants, and yeasts have significant intracellular levels of unanchored Ub chains, indicating the ubiquitous presence of such chains (van Nocker and Vierstra, 1993). However, the physiological significance of these chains remains largely elusive. The chains could be generated through release from polyubiquitinated substrates or by Ub-ligating enzymes from monomeric Ub. Several DUB mutants in yeast, including *doa4, ubp6, ubp8, ubp10,* and *ubp14*, show changes in monomeric Ub level and/or unanchored Ub chains or small Ub species (Amerik et al., 2000a). It was proposed that one function of unanchored Ub chains is competitive inhibition of polyubiquitin-substrate binding to the 26S proteasome (Amerik et al., 1997), but the exact roles of free Ub chains are poorly understood to date.

In the present study, we report the isolation of Rfu1. Rfu1 was found to regulate the cellular levels of monomeric Ub and free Ub chains and to inhibit Doa4. In addition, we found that heat shock rapidly and significantly decreased free Ub chains, and this effect was in part dependent on a balance between Doa4 and Rfu1. Based on our results, we propose that unanchored Ub chains serve as a Ub reservoir preventing the supply of excess amounts of monomeric Ubs under normal states but can supply monomeric Ubs rapidly when Ub is urgently required.

RESULTS

Isolation of Rfu1

To identify new cofactors of Cdc48, a protein involved in various cellular processes such as UPS-mediated protein degradation, membrane fusions, cell-cycle progression, and apoptosis (Woodman, 2003), we screened for multicopy suppressors of the *cdc48-3* temperature-sensitive mutant. In addition to *CDC48*-containing plasmids, we obtained several plasmids with an overlapping region (Figure 1A). Introduction of these plasmids suppressed the temperature-sensitive growth of *cdc48-3* at 34.5°C but not at 37°C, suggesting that their suppressing activities are partial. Deletion analysis identified the suppression activity in a fragment containing the entire YLR073c open reading frame (ORF) and its flanking regions (Figures 1A and 1B). We provisionally named this gene *RFU1* (regulator of *f*ree *u*biquitin chains 1, for the reason described below). *RFU1* encodes a protein of 200 amino acids and its function is unknown. Rfu1 displays marginal homology to two very different mammalian DUBs: AMSH and UBPY (alias UBP8) (Figure S1 available online) (McCullough et al., 2004; Naviglio et al., 1998; Row et al., 2007). The homologous regions are largely outside of the catalytic domain of these DUBs. Disruption of *RFU1* did not alter yeast cell growth at normal growth temperatures or at 37°C (Figures 1C and 1D), indicating that *RFU1* is a nonessential gene.

Genetic Interactions of *RFU1* with *cdc48-3* and Proteasome Genes

To examine whether Rfu1 and Cdc48 are involved in related functions, the *cdc48-3Δrfu1* double mutant was created. Indeed, we found that the *cdc48-3Δrfu1* mutant was more sensitive to elevated temperatures than the *cdc48-3* mutant (Figures 1C and 1D). In the next step, we examined the physical interaction between Cdc48 and Rfu1 by immunoprecipitation of 3×HA-tagged Rfu1, but we could not detect any apparent interaction (data not shown). These results suggest that *RFU1* is genetically related to *CDC48* but does not interact physically with Cdc48. Since one function of Cdc48 is to promote the degradation of proteins in the UPS (Ghislain et al., 1996), we examined whether UPS phenotypes would be enhanced by combining the *Δrfu1* mutation with a defect in the UPS machinery. We checked the temperature sensitivities of double mutants involving *Δrfu1* and two different proteasome mutants, *ΔN rpn2* and *rpt1/cim5-1*. Rpn2 and Rpt1 are a non-ATPase subunit and an ATPase subunit of the 26S proteasome, respectively (Ghislain et al., 1993; Isono et al., 2007). Both *Δrfu1ΔN rpn2* and *Δrfu1rpt1* mutants exhibited profound growth defects at elevated temperatures compared to *ΔN rpn2* and *rpt1* mutants, respectively (Figure 1E). The results led us to speculate that although Rfu1 may not be a Cdc48 cofactor, it appears to play an important role in the UPS in general. We therefore further investigated the function of Rfu1.

RFU1 Disruption and Overexpression Change Ub Profile

In spite of the genetic interactions of *RFU1* with various UPS genes, the degradation machinery in the UPS did not appear to be defective in the *Δrfu1* mutant. We examined the β-galactosidase activity of an exogenously expressed UFD (Ub fusion degradation) substrate, Ub-Pro-β gal (Figure S2) (Johnson et al., 1995). As expected, *cdc48-3* cells showed a significant increase of β-gal activity. In contrast, the *Δrfu1* mutant did not show a noticeable increase compared with the wild-type, indicating that Rfu1 is not involved in UPS-mediated protein degradation. In addition, we confirmed that *RFU1* deletion did not affect the mRNA levels of *UBI1–4* genes (Figure S3).

Since Rfu1 has a weak homology to two mammalian DUBs, and mutations of several yeast DUBs show characteristic changes in the bulk profile of cellular Ubs (Amerik et al., 2000a), we examined the Ub profiles of the *Δrfu1* mutant by using anti-Ub blot analysis. In our assay system, wild-type cells in early growth phase had a bulk Ub profile with monomeric Ub, unanchored Ub chains, as well as slowly migrated high-molecular-weight (HMW) forms, which presumably correspond to various Ub-conjugated proteins (Figure 2A). Surprisingly, the amount of free Ub chains was clearly decreased in *Δrfu1* cells, while the level of monomeric Ub was increased compared with the wild-type. In *cdc48-3* cells, HMW ubiquitinated proteins were increased and free Ub chains were decreased compared with wild-type cells. The *cdc48-3Δrfu1* cells showed further increase of HMW ubiquitinated protein levels relative to those of *cdc48-3* cells and marked reduction of free Ub chains (Figure 2A). Furthermore, cells carrying *Δrfu1ΔN rpn2* or *Δrfu1rpt1* mutations showed higher levels of HMW ubiquitinated proteins compared with cells harboring a single mutation of *ΔN rpn2* or *rpt1*, respectively

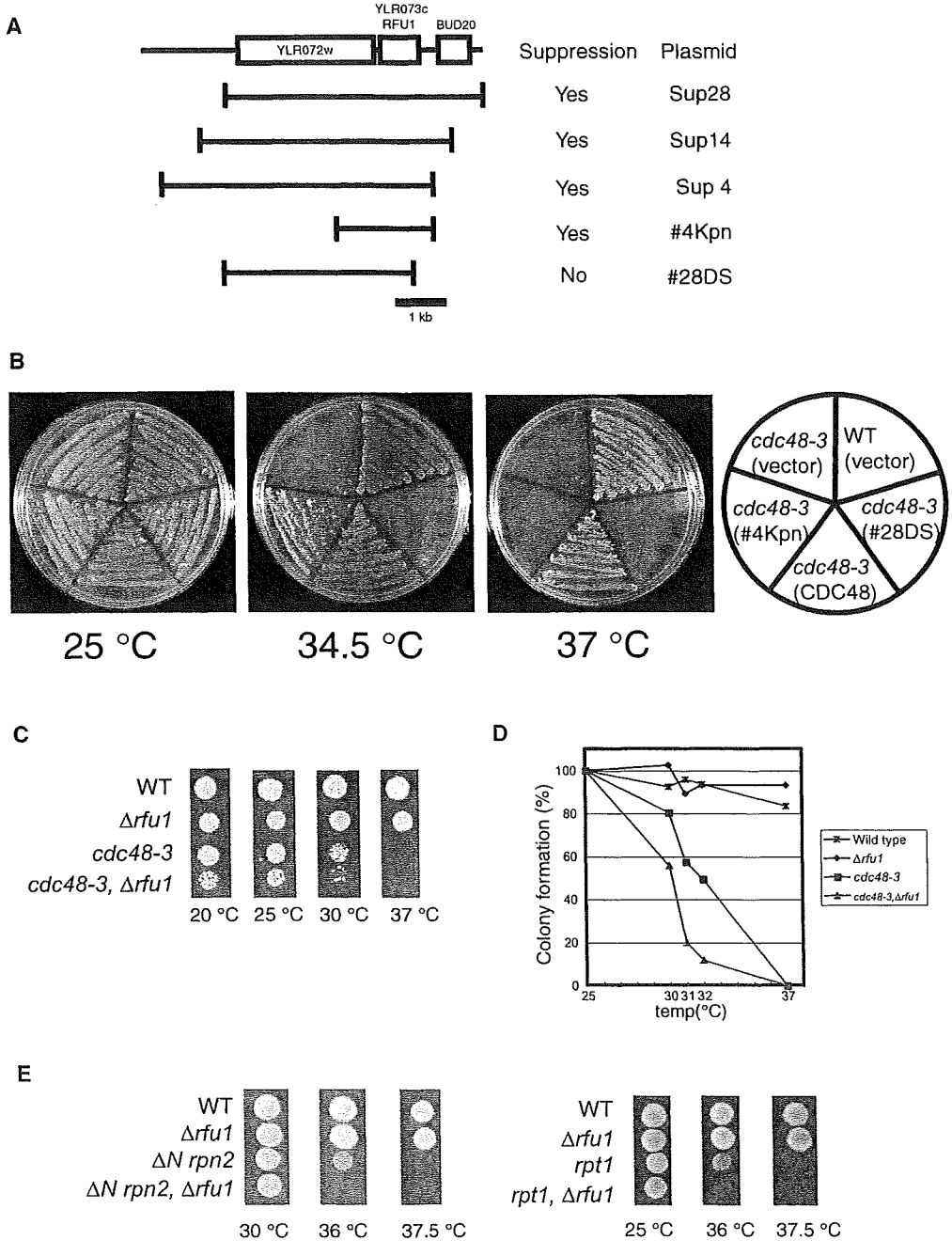

Figure 1. Isolation of *RFU1*

(A) DNA regions involved in suppressing the temperature-sensitive growth of *cdc48-3* and plasmids tested for their suppressor activity. Plasmid #28DS lacks the first 112 nucleotides of the *RFU1* gene.

(B) Effect of *RFU1* expression on the growth of *cdc48-3*. Wild-type cells with a vector, *cdc48-3* cells with a vector, and *cdc48-3* cells expressing the plasmid (*CDC48* or #4Kpn or #28DS) were streaked on SC-Trp plates and incubated at 25°C, 34.5°C, or 37°C for 3 days.

(C) Synthetic enhancements of growth sensitivity of *Δrfu1* with *cdc48-3*. Cells grown in early log phase were spotted on YPAD medium and grown at the indicated temperatures for 3 days.

(D) Colony-forming ability of wild-type, *Δrfu1*, *cdc48-3*, and *Δrfu1cdc48-3* cells at the indicated temperatures. Data are average of two experiments.

(E) Synthetic enhancements of growth sensitivity of *Δrfu1* with *ΔN rpn2* or *rpt1/cim5-1* mutation. Experiments were conducted as in (C).

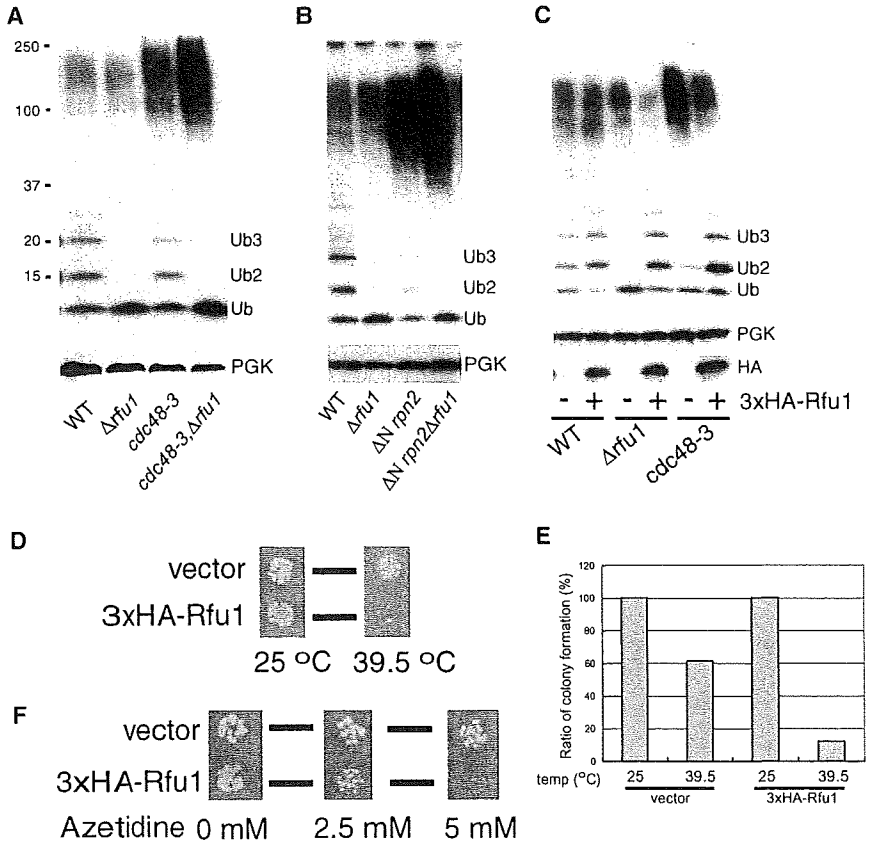

Figure 2. Changes in Accumulation of Different Ub Forms in *Δrfu1* and Other Mutants and Stress Sensitivity by Overexpression of Rfu1

(A) Disappearance of free Ub chains and high level of monomeric Ub in *Δrfu1* mutants. *Top*, anti-Ub immunoblot analysis. The position of size standard in kDa is indicated on the left. Monomeric Ub and Ub chain positions are marked. *Bottom*, anti-phosphoglycerate kinase (PGK) immunoblot, a control for protein loading.

(B) Accumulation of high-molecular-weight (HMW) ubiquitinated proteins in *ΔN rpn2Δrfu1* cells. Anti-Ub and anti-PGK immunoblot analyses were performed using wild-type, *Δrfu1*, *ΔN rpn2*, and *ΔN rpn2Δrfu1* cells.

(C) Effects of 3×HA-Rfu1 overexpression on Ub profiles. Immunoblot analyses using anti-Ub, anti-PGK, and anti-HA antibodies of wild-type, *Δrfu1*, or *cdc48-3* cells containing a vector or a plasmid that expresses 3×HA-Rfu1 under the GPD promoter.

(D) Sensitive phenotypes of 3×HA-Rfu1-overexpressing cells to sustained heat treatment. Wild-type cells containing a vector or a plasmid overexpressing 3×HA-Rfu1 were grown in early log phase at 25°C in SC-Ura culture. Cells were diluted and spotted on SC-Ura plates. Plates were placed at 39.5°C or 25°C for 17 hr and returned to 25°C, and viable cells were allowed to grow for 2 days.

(E) Quantitative analysis of (D). Data are average of two experiments.

(F) Sensitive phenotype of 3×HA-Rfu1-overexpressing cells to AZC (azetidine-2-carboxylic acid). Cells were spotted on SC-Ura plates with the indicated concentrations of AZC and incubated at 25°C for 3 days.

(Figures 2B and S4). Therefore, disruption of *RFU1*, which causes increased production of monomeric Ub, appears to enhance substrate ubiquitination, resulting in marked accumulation of HMW ubiquitinated proteins when combined with a mutated component of the UPS, although yet unidentified events could also enhance ubiquitination in the double mutant.

Next, we examined whether overexpression of Rfu1 has the opposite effect of that observed in *Δrfu1* cells on the Ub profile. Indeed, overexpression of 3×HA-tagged Rfu1 in wild-type cells resulted in reduction of monomeric Ub and increase of free Ub chains (Figure 2C). In addition, overexpression of 3×HA-Rfu1 in *Δrfu1* cells restored free Ub chain formation.

The growth of yeast cells lacking the polyubiquitin gene (*UBI4*) is similar to that of wild-type cells over the normal range of growth temperatures (Finley et al., 1987). However, they are hypersensitive to certain stresses probably because *Δubi4* cells cannot maintain the required levels of Ub during stress. If Rfu1 inhibits the supply of monomeric Ubs, overexpression of Rfu1 should have similar effects to those observed in *Δubi4* cells. To test this, wild-type cells overexpressing 3×HA-Rfu1 or harboring an empty vector were spotted on a plate, incubated at 39.5°C for 17 hr, and then placed at 25°C. Cells overexpressing 3×HA-Rfu1 were more sensitive to the sustained heat treatment than control cells (Figures 2D and 2E). Moreover, cells overexpressing 3×HA-Rfu1 were more sensitive to azetidine-2-carboxylic acid (AZC), a toxic analog of proline, than control cells (Figure 2F). Based on these results, we speculated that Rfu1 regulates Ub homeostasis by inhibiting the production of monomeric Ub and by promoting the formation of free Ub chains.

During the course of these experiments, we noticed that 3×HA-Rfu1 overexpression also caused less accumulation of HMW ubiquitinated proteins in *cdc48-3* than vector expression (Figure 2C). Together with the findings of profound growth defect and high levels of HMW ubiquitinated proteins in the double mutants, an excess amount of ubiquitinated proteins may have toxic effects, and this may explain the isolation of *RFU1* in our screening. Consistent with this notion, overexpression of Ub caused growth retardation in *cdc48-3* and in *cdc48-3Δrfu1* cells (Figure S5).

Interaction of Rfu1 with Doa4

What is the molecular mechanism of Rfu1-mediated modulation of the levels of monomeric Ub and of free Ub chains? First, we tested the possibility that Rfu1 directly binds to free Ub chains. Recombinant proteins including MBP, MBP-Rfu1, MBP-Rpn10, and MBP-UQ1(UBA), a MBP fusion with the UBA domain of human ubiquilin 1, were prepared (Figure S6), and they were incubated with K48-linked or K63-linked Ub chains (Figure S7). An MBP pull-down assay showed MBP-Rpn10 binding to

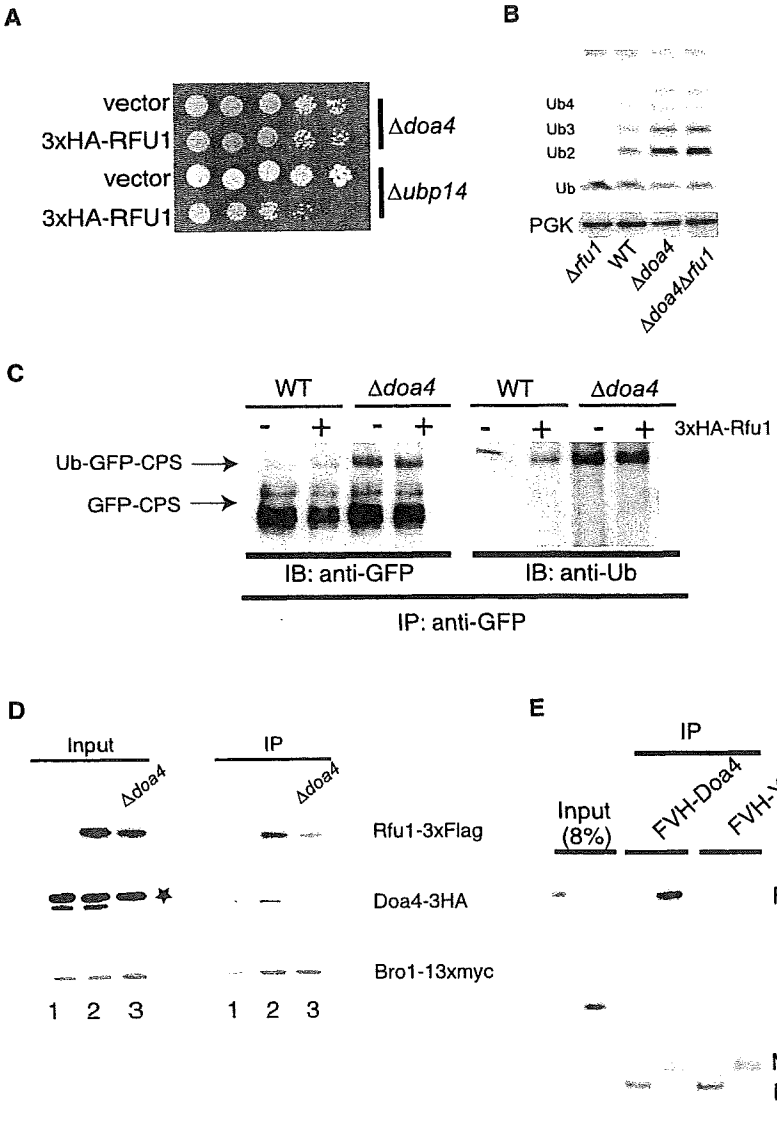

Figure 3. Rfu1 Interactions with Doa4

(A) Overexpression of 3×HA-Rfu1 inhibits the growth of *Δubp14* cells but not of *Δdoa4* cells.

(B) Resemblance of *Δdoa4Δrfu1* and *Δdoa4* mutants with regard to the level of monomeric Ub and Ub chains. *Top*, anti-Ub; *bottom*, anti-PGK immunoblot.

(C) Detection of ubiquitinated GFP-carboxypeptidase S (CPS) by overexpression of 3×HA-Rfu1 in control cells. Immunoprecipitation was performed using anti-GFP from control cells or *Δdoa4* cells expressing GFP-CPS with or without 3×HA-Rfu1. Immunocomplex was analyzed using anti-GFP and anti-Ub. Strains: Y703 and Y704 with plasmids pKU90 and E405 or vector.

(D) Association of Rfu1-3×Flag with Doa4-3×HA and Bro1-13×myc in vivo. Immunoprecipitation was performed using anti-Flag and the resulting immunocomplexes were analyzed by immunoblot using anti-Flag, anti-HA, and anti-myc. Lane 1: cells expressing nontagged Rfu1, Doa4-3×HA, and Bro1-13×myc (Y845); lane 2: cells expressing Rfu1-3×Flag, Doa4-3×HA, and Bro1-13×myc (Y860); lane 3: *Δdoa4* cells expressing Rfu1-3×Flag and Bro1-13×myc (Y862). Asterisk indicates nonspecific band for HA.

(E) In vitro binding between recombinant MBP or MBP-Rfu1 and FVH-Doa4 or FVH-Yuh1. MBP or MBP-Rfu1 was mixed with FVH-Doa4 or FVH-Yuh1, and the proteins were isolated with amylose resin. Samples were analyzed by immunoblot using anti-Flag and anti-MBP.

K48-linked Ub chains and MBP-UQ1(UBA) binding to K63-linked Ub chains as positive controls. However, MBP-Rfu1 bound to neither K48-linked nor K63-linked Ub chains (Figure S7).

In the next step, we speculated that Rfu1 could be related to DUBs based on the partial sequence similarity of Rfu1 and the two different DUBs. We then postulated that Rfu1 negatively regulates DUBs that catalyze the reaction of free Ub chains to monomeric Ub. A genome-wide analysis of GFP-fusion proteins of yeast has shown localization of Rfu1-GFP in the endosome (Huh et al., 2003). Among 19 DUBs in yeast including 16 UBP, 1 UCH, and 2 OTU, only Doa4, the yeast counterpart of human UBPY, is localized in the endosomes, in addition to the cytoplasm (Amerik et al., 2000b; Huh et al., 2003). Moreover, the *Δdoa4* mutant showed reduced levels of monomeric Ub and accumulation of small Ub species, (Dupre and Haguenauer-Tsapis, 2001; Nikko and Andre, 2007; Papa and Hochstrasser, 1993), which seems to be the opposite pattern of the Ub profile in *Δrfu1* cells. Interestingly, we found that the Ub profile of wild-type cells overexpressing an epitope-tagged Doa4 is similar to that of *Δrfu1* cells: high levels of monomeric Ub and massive reduction of free Ub chains (Figure S8). Based on these findings, we decided to test the possibility that Rfu1 is an inhibitor of Doa4.

We tried to obtain genetic evidence for the negative effect of Rfu1 on Doa4. Ubp14 is another DUB, which also plays a role in the control of free Ub chains, and the *Δubp14Δdoa4* double mutant was reported to show a synthetic growth defect (Amerik et al., 1997). We overexpressed 3×HA-Rfu1 in *Δubp14* and *Δdoa4* cells. If Rfu1 inhibits Doa4, Rfu1 overexpression would cause a growth defect in *Δubp14* cells, like that observed in *Δubp14Δdoa4*. Indeed, overexpression of 3×HA-Rfu1 caused a growth defect in *Δubp14* cells but not in *Δdoa4* cells (Figure 3A).

We also speculated that the altered Ub profile observed in *Δrfu1* mutant was due to elimination of Rfu1-mediated inhibition of Doa4. In this case, the Ub profile of *Δrfu1Δdoa4* cells should look like that of *Δdoa4* cells. Therefore, we compared the Ub profiles of *Δrfu1Δdoa4* and *Δdoa4* cells. As expected, in *Δrfu1Δdoa4* cells, the characteristic Ub pattern of *Δrfu1* was no longer observed; instead, its Ub pattern was quite similar to that of *Δdoa4* cells (Figure 3B). In contrast, the Ub profile of *Δrfu1Δubp14* showed a combined pattern of the two single mutants (Figure S9). These results indicate that Doa4 and Rfu1 control free Ub chains in the same pathway, whereas Ubp14 and Rfu1 regulate free Ub chains independently.

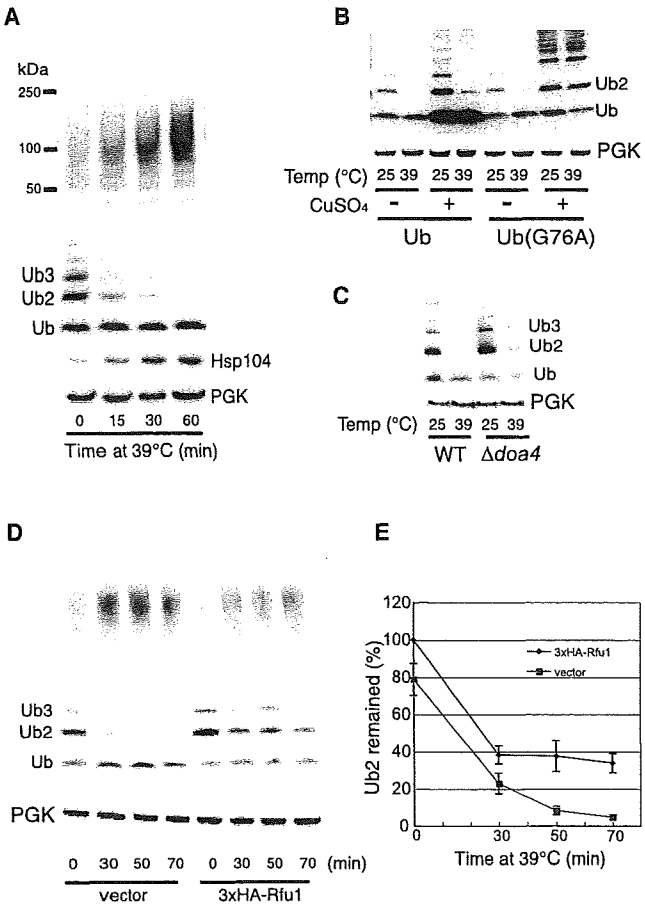

Figure 4. Free Ub Chains and Heat Shock Response

(A) Heat shock causes marked reduction in free Ub chains. Cells in early log phase at 25°C were heat shocked at 39°C at the indicated times. *Top*, anti-Ub; *middle*, anti-Hsp104; *bottom*, anti-PGK immunoblot.

(B) Resistance of Ub(G76A) chains to heat shock. Wild-type cells expressing CUP1-regulated monomeric Ub or Ub(G76A) were treated or not treated with 1 mM $CuSO_4$ for 4 hr and heat shocked at 39°C for 1 hr.

(C) Partial resistance of free Ub chains in Δ*doa4* cells to heat shock. Anti-Ub and anti-PGK immunoblot analyses were conducted for Δ*doa4* and wild-type cells. Cells were heat shocked at 39°C for 1 hr.

(D) Partial prevention of decrease of free Ub chains by overexpression of 3×HA-Rfu1. Cells harboring a vector or expressing 3×HA-Rfu1 were heat shocked at 39°C for 30, 50, and 70 min. *Top*, anti-Ub; *bottom*, anti-PGK.

(E) Relative Ub2 level observed in (D). Ub2 level in cells expressing 3×HA-Rfu1 is normalized to 100%. Data are mean ± SEM values of three independent experiments.

We then tested the involvement of Rfu1 in Doa4-mediated processes in vivo. Doa4 functions in a variety of cellular processes including the removal of polyubiquitin chain from Ub-conjugated proteins, which are targeted to proteasomes for degradation (Papa et al., 1999). Doa4 also controls DNA replication and protects cells against DNA damage (Fiorani et al., 2004; Singer et al., 1996). Moreover, *Δdoa4* mutant cells contain lower levels of monomeric Ub than the wild-type and exhibit accelerated degradation of monomeric Ub (Swaminathan et al., 1999). However, the major function of Doa4 is deubiquitination of ubiquitinated cargos sorted into luminal vesicles of late-endosomal multivesicular bodies (MVBs) (Amerik and Hochstrasser, 2004). In *Δdoa4* cells, cargo proteins such as carboxypeptidase S (CPS) accumulate in ubiquitinated forms (Dupre and Haguenauer-Tsapis, 2001; Katzmann et al., 2001). If Rfu1 inhibits the Doa4 activity, Rfu1 overexpression should result in phenocopying Δ*doa4* mutant on such proteins. Control and *Δdoa4* cells were transformed with two plasmids: a plasmid expressing GFP-CPS and another overexpressing 3×HA-Rfu1 or an empty vector. GFP-CPS was immunoprecipitated with anti-GFP antibody, and GFP-CPS and ubiquitinated GFP-CPS were detected by immunoblotting using anti-GFP and anti-Ub (Figure 3C). We used vacuolar proteases-deficient *Δpep4Δprb1* cells to facilitate the detection of the ubiquitinated form of GFP-CPS (Amerik et al., 2006). Consistent with previous results (Katzmann et al., 2001; Amerik et al., 2006), the ubiquitinated form of GFP-CPS was observed in *Δdoa4* cells. In addition, the ubiquitinated form of GFP-CPS was clearly observed in cells overexpressing 3×HA-Rfu1 but not in cells harboring the empty vector. These results added further support for the role of Rfu1 as an inhibitor of Doa4.

Next, we investigated whether Rfu1 physically interacts with Doa4 by immunoprecipitating the Rfu1-3×Flag protein from yeast cell lysates (Figure 3D). For this purpose, we created cells in which endogenous *RFU1* and *DOA4* were replaced with *RFU1-3×FLAG* and *DOA4-3×HA*, respectively. In addition, we replaced *BRO1* with *BRO1-13×MYC*. Bro1 is an endosome recruiting factor for Doa4: in the absence of Bro1, Doa4 localization to endosomes is abolished (Luhtala and Odorizzi, 2004). Bro1 also stimulates the Dub activity of Doa4 by direct binding (Richter et al., 2007). As shown in Figure 3D, Rfu1-3×Flag coimmunoprecipitated Doa4-3×HA as well as Bro1-13×myc (lane 2). However, Rfu1-3×Flag did not bind with Doa4-3×HA in Δ*bro1* cell (data not shown), perhaps due to a lower level of Rfu1-3×Flag in Δ*bro1* cells (data not shown), although other explanations are possible. Surprisingly, Bro1-13×myc was coimmunoprecipitated with Rfu1-3×Flag in the *Δdoa4* mutant, suggesting that Rfu1 binds Bro1 directly (Figure 3D, lane 3). These results indicate that Rfu1 forms a complex with Doa4 and Bro1.

We also investigated whether Rfu1 binds to Doa4 directly in vitro. N-terminal Flag-tagged and C-terminal V5- and His-tagged full-length Doa4 (FVH-Doa4) and Yuh1 (FVH-Yuh1) were expressed and purified from yeast (Figure S6). Yuh1 is another yeast DUB. FVH-Doa4 and FVH-Yuh1 were mixed with recombinant MBP or MBP-Rfu1 to investigate whether these tagged proteins bind to MBP or MBP-Rfu1. MBP-Rfu1, but not MBP, specifically bound to FVH-Doa4 but not FVH-Yuh1 (Figure 3E), indicating that Rfu1 physically interacts with Doa4.

Stress Response, Free Ub Chains, Doa4, and Rfu1

The Ub profile varies with the growth phase of yeasts. Free Ub chains are abundantly observed in log phase but not in stationary phase (Amerik et al., 1997 and data not shown). We speculated that the formation of free Ub chains depends on cellular conditions. To test this, we examined the effects of environmental stresses such as heat shock on the Ub profile. Wild-type cells were grown at 25°C to an early log phase and then heated at 39°C (Figure 4A). The heat treatment increased HMW ubiquitinated proteins, consistent with the previous findings in

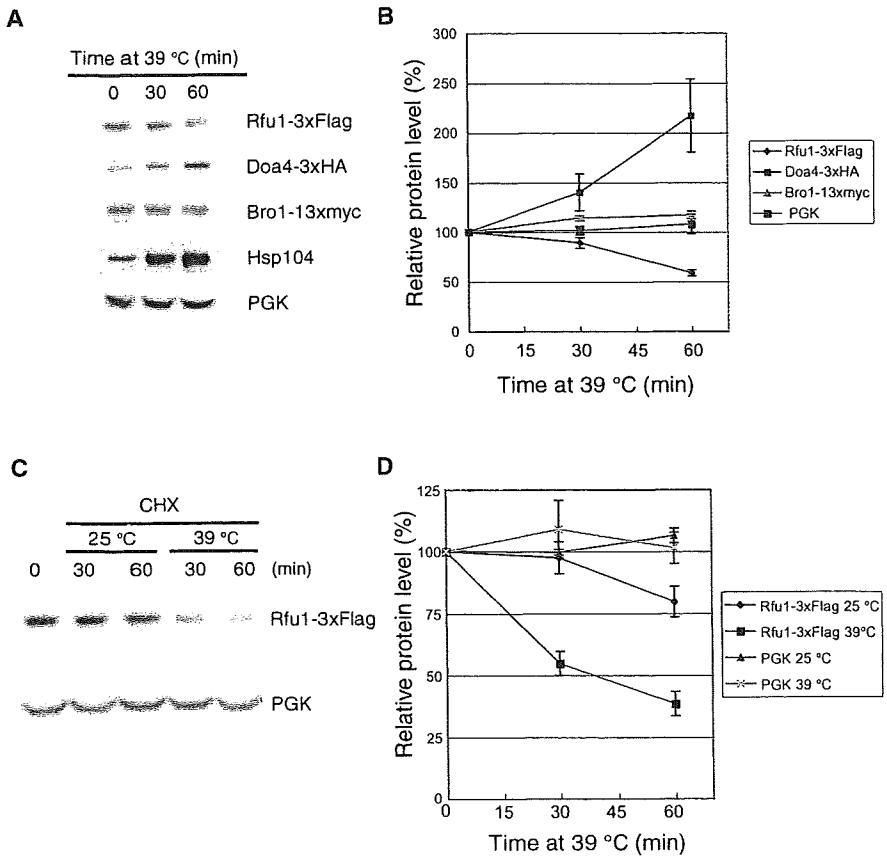

Figure 5. Rfu1-3×Flag, Doa4-3×HA, and Bro1-13×myc Protein Levels in Response to Heat Shock

(A) Rfu1-3×Flag, Doa4-3×HA, Bro1-13×myc, and PGK protein levels under heat shock at 39°C. Total cell lysates obtained at the indicated time points were analyzed by immunoblotting with anti-Flag, anti-HA, anti-myc, anti-Hsp104, and anti-PGK.

(B) Quantification of protein levels. Protein levels were quantified from the bands shown in (A). Data are mean ± SEM values of three independent experiments.

(C) Rfu1-3×flag and PGK protein levels under heat shock in the presence of cycloheximide.

(D) Quantification of protein levels. Protein levels were quantified from the bands shown in (C). Data are mean ± SEM values of three independent experiments.

mammalian cultured cells (Carlson et al., 1987). The heat treatment also resulted in marked increase in Hsp104 protein, one of the major heat-inducible proteins. Surprisingly, heat treatment resulted in disappearance of free Ub chains; their level decreased after 15 min of such treatment.

We speculated that one reason for the marked decrease in free Ub chains upon heat treatment was the disassembling of free Ub chains by certain DUBs to produce more monomeric Ub, which can be used for ubiquitination reactions. Accordingly, we examined the involvement of DUB activity in stress-induced disappearance of free Ub chains. Ub(G76A), in which the last amino acid of glycine in Ub is replaced with alanine, is reported to form a Ub chain, but it is resistant to the action of deubiquitinating enzymes (Hodgins et al., 1992). Plasmids expressing G76A monomeric Ub or wild-type monomeric Ub under the regulation of Cup1 promoter were introduced into wild-type cells (Figure 4B). In the absence of copper, when essentially only endogenous Ub-encoding genes were expressed, heat treatment markedly decreased the level of free Ub chains. Copper-induced exogenous wild-type Ub expression increased free Ub chains but decreased the chains upon heat shock. On the other hand, in cells expressing Ub(G76A), heat shock did not reduce the amount of small Ub oligomer-like species. Although we cannot exclude the possibility that the observed results are due to reasons other than that UbG76A is a poor substrate for DUBs, the above findings emphasize the potential involvement of DUB(s) in the disappearance of free Ub chains.

We further investigated whether Doa4 mediates the heat shock-induced decrease in free Ub chains. To test this, *Δdoa4* cells were heat shocked and the Ub profiles of the cells were examined. Compared with wild-type cells, substantial amounts of free Ub chains remained in *Δdoa4* cells even after 1 hr of heat shock (Figure 4C), suggesting the involvement of Doa4 in this process. Next, we examined the effect of 3×HA-Rfu1 overexpression. If Rfu1 inhibits Doa4, overexpression of 3×HA-Rfu1 should phenocopy *Δdoa4* mutant with respect to heat shock-induced disappearance of free Ub chains. Wild-type cells overexpressing 3×HA-Rfu1 were heat shocked, and the change in free Ub chains was examined. We found that overexpression of 3×HA-Rfu1 under the regulation of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter significantly inhibited the disappearance of free Ub chains (Figures 4D and 4E). These results indicate that disappearance of free Ub chains is at least partly mediated by a balance of the actions of Doa4 and Rfu1.

Since the mRNA levels of *RFU1* and *DOA4* decrease and increase upon heat shock, respectively (Gasch et al., 2000), we decided to examine the effects of heat shock on the levels of their proteins. We found that the changes in Rfu1-3×Flag and Doa4-3×HA levels paralleled the reported changes in their mRNA levels upon heat treatment (Figures 5A and 5B). The amount of Rfu1-3×Flag decreased by 40%–50% after 60 min of heat shock. In contrast, heat shock increased the Doa4-3×HA level, though the increase was modest compared with the marked increase in the heat shock protein Hsp104. The level of Bro1-13×myc remained unchanged upon heat shock. These results were consistent with the notion that heat shock produces more Doa4 that is uninhibited by Rfu1and can thus contribute to disassembling free Ub chains.

We also tested whether decrease of Rfu1-3×Flag was also mediated at the protein level and thus examined the stability of Rfu-3×Flag. In cells treated with cycloheximide followed