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The Ubiquitin-associated (UBA) 1 Domain of *Schizosaccharomyces pombe* Rhp23 Is Essential for the Recognition of Ubiquitin-proteasome System Substrates Both *in Vitro* and *in Vivo*[*] [S]

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Background: The structure of Rhp23 is unusual in that it contains two ubiquitin binding domains.
Results: Only the internal domain of Rhp23 is required for it to act as a shuttle factor.
Conclusion: The C-terminal ubiquitin binding domain is redundant for substrate recognition.
Significance: This is the first time that the functions of ubiquitin binding domains have been tested *in vivo* in *S. pombe*.

The ubiquitin-proteasome system is essential for maintaining a functional cell. Not only does it remove incorrectly folded proteins, it also regulates protein levels to ensure their appropriate spatial and temporal distribution. Proteins marked for degradation by the addition of Lys[48]-linked ubiquitin (Ub) chains are recognized by shuttle factors and transported to the 26 S proteasome. One of these shuttle factors, *Schizosaccharomyces pombe* Rhp23, has an unusual domain architecture. It comprises an N-terminal ubiquitin-like domain that can recognize the proteasome followed by two ubiquitin-associated (UBA) domains, termed UBA1 and UBA2, which can bind Ub. This architecture is conserved up to humans, suggesting that both domains are important for Rhp23 function. Such an extent of conservation raises the question as to why, in contrast to all other shuttle proteins, does Rhp23 require two UBA domains? We performed *in vitro* Ub binding assays using domain swap chimeric proteins and mutated domains in isolation as well as in the context of the full-length protein to reveal that the Ub binding properties of the UBA domains are context-dependent. *In vivo*, the internal Rhp23 UBA1 domain provides sufficient Ub recognition for the protein to function without UBA2.

Ubiquitin (Ub)[4] is a conserved 76-amino acid protein that is utilized by eukaryotic cells as a dynamic signaling molecule to regulate many intracellular pathways. However, by far the most studied Ub-dependent process is its use as a signal to target proteins for degradation by the 26 S proteasome, a multisubunit protease. An enzyme cascade involving activating, conjugating, and ligase enzymes transfers Ub to a lysine residue of a specific target protein (1–4). Ub itself has seven lysine residues, all of which can nucleate chain formation. However, biochemical and genetic studies have implicated only Lys[29]-, Lys[48]-, and Lys[63]-linked chains as having a role in protein degradation with Lys[48] chains being by far the most important (5, 6). A Ub chain of at least four molecules in length is required for the protein substrate to be efficiently recognized by the 26 S proteasome (5, 7). In addition, Lys[11]-linked chains have recently been shown to be specifically targeted by the cell cycle regulator E3 ligase anaphase-promoting complex/cyclosome (8).

Protein substrates are recognized at the proteasome by multiubiquitin receptors. Two subunits of the proteasome, Rpn10 (also called Pus1 in fission yeast) and Rpn13, have the ability to recognize multiubiquitin chains via their C-terminal ubiquitin-interacting motif (UIM) and N-terminal Pru domain (9–11), respectively. Members of another class of Ub receptors only interact transiently with the proteasome and are not subunits of the multiprotein protease. This class of receptors has been termed "shuttle proteins" as they are thought to transport ubiquitinated substrates destined for degradation from the E3 ligase to the proteasome. Functionally, each of the shuttle proteins shares similar biochemical properties. They each have a multiubiquitin binding domain to recognize UPS substrates and a proteasome-interacting domain to transiently interact with the proteasome (12, 13).

The best characterized of these receptors in *Schizosaccharomyces pombe* are Dph1 (equivalent to Dsk2 in budding yeast), Rhp23 (Rad23), and Pus1 (Rpn10). Pus1 uniquely appears to

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[4] The abbreviations used are: Ub, ubiquitin; *cs*, cold-sensitive; Fw, forward primer; Rev, reverse primer; *ts*, temperature-sensitive; UBA, ubiquitin-associated domain; UPS, ubiquitin-proteasome system; UIM, ubiquitin-interacting motif; PMG, *Pombe* minimal glutamate; NAT, nourseothricin.

function both as a subunit of the proteasome and as a shuttle protein (10, 14). It contains an N-terminal von Willebrand factor type A domain that recognizes the proteasome (12) and a C-terminal UIM (10, 15). Dph1 and Rhp23 both contain a ubiquitin-like domain at the N terminus to dock onto the proteasome (16–18) and a ubiquitin-associated (UBA) domain at the C terminus that binds Ub (19). Unusually, Rhp23 contains an additional internal UBA domain that is conserved in all of its eukaryotic orthologues and therefore must be important for the *in vivo* function of the Rhp23 protein (20, 21). We call the internal Rhp23 UBA domain UBA1 and the C-terminal domain UBA2.

In fission yeast, single deletion of the *pus1*, *dph1*, or *rhp23* genes results in viable cells with a modest proteolytic phenotype. In addition, *dph1*Δ*rhp23*Δ and *dph1*Δ*pus1*Δ double mutants display a mild synthetic phenotype for growth. In contrast, the *rhp23*Δ*pus1*Δ double mutant shows a dramatic synthetic growth phenotype. At 25 °C, the double mutant growth rate is severely compromised compared with wild type, whereas at 36 °C, the double mutant strain is not viable. The triple mutant *dph1*Δ*rhp23*Δ*pus1*Δ cannot be constructed, demonstrating that shuttle factors are essential (13).

Surface plasmon resonance experiments have shown that the isolated human hRAD23A UBA1 domain binds preferentially to Lys^{63} chains over Lys^{48}, whereas the UBA2 domain binds Lys^{48}-linked chains better than those linked through Lys^{63} (22, 23). This result suggests a model in which the two domains may allow Rhp23 to recognize a greater variety of substrates because Lys^{63} can also signal for degradation.

However, work carried out by Heessen *et al.* (24) has shown that a single point mutation in the UBA2 domain of Rad23 in *Saccharomyces cerevisiae* results in a 75% decrease in its half-life. Subsequent removal of the ubiquitin-like domain to prohibit Rad23 from binding to the proteasome restabilized the level of the protein, leading the authors to conclude that the UBA2 domain protects Rad23 from being degraded by the proteasome during substrate transport (24). More recent work by the same group using domain swap experiments in which UBA1 was replaced with UBA2 and vice versa demonstrated that only UBA2 at the C terminus had a protective effect (25).

Rhp23 is also involved in nucleotide excision repair where it forms a complex with Rhp41 (Rad4) to recognize photolesions and help initiate DNA repair. Within this complex, the role of Rhp23 again seems to be to confer stability because a lack of the homologue Rad23 causes degradation of Rad4 and Xeroderma pigmentosum Group C protein. However, neither UBA domain seems to be involved in the nucleotide excision repair pathway (26–28).

In this study, we prepared a series of Rhp23 mutants in which either (*a*) point mutations were introduced in both UBA domains to disrupt Ub binding or (*b*) the UBA domains were interchanged. Chimeric proteins were also constructed to test whether the Pus1 UIM could be replaced with a UBA. Using a combination of *in vitro* biochemical and biophysical binding assays as well as phenotypic characterization of the *in vivo* *rhp23*Δ*pus1*Δ phenotype, we demonstrate that the Rhp23 UBA domains differ in their affinities for Ub chains when in isolation compared with the context of the full-length Rhp23 or Pus1 proteins. We also show that the UBA1 domain is primarily responsible for binding Ub conjugates for the UPS.

EXPERIMENTAL PROCEDURES

Strains and Media—This study used strains derived from $972h^+$ and $975h^-$ using the standard background *leu1-32, ura4-D18, and ade6-M216*. For the *in vivo* assay, we used *pus1*::*NatRh*$^-$ (this study) *rhp23*::*ura4*$^+$*h*$^+$ (13).

All strains were grown in either liquid or solid yeast extract with supplements or PMG with the appropriate antibiotic or nutritional selection. Crosses and lithium acetate transformations with pREP81 plasmid were performed via standard protocols (29).

General DNA Methods—Primers used for site-directed mutagenesis of *rhp23*$^+$ in pBS were as follows: Rhp23 M157A: Fw 5′-GTC GAA ATA TGG TAG AAG CGG ATA CGA ACG CAG CG; Rev 5′-CGC TGC GTT CGT ATC CCG CTT CTA CCA ATT TTC AC; Rhp23 L183A: Fw 5′-GGC AGT GGA ATA CTT AGC AAC TGG TAT TCC CGA AG; Rev 5′-CTT CG G GAA TAG TTG CTA AGT ATT CCA CTG CC; Rhp23 L332A: Fw 5′-TAG ATT ATG TCA AGC TG G CTT CGA CAG AAA; Rev 5′-TTT CTG TCG AAG CCA GCT TGA CAT AAT CTA; and Rhp23 F358A: Fw 5′-GCT GCT AAT ACC TTG CCG AGC ATG GAC ATG; Rev 5′-CAT GTC CAT GCT CGG CAA GGT AAT TAG CAG C.

Mutations were made using the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutated and authentic *rhp23*$^+$ were cloned into the pREP81 and pGEX6P1 plasmids in the SalI and BamHI sites using Fw 5′-ACG CGT CGA CGT ATG AAT TTG ACA TTC AAA AAT CTA CAG CAG and Rev 5′-CGC GGA TCC GCT TAAG GTT CAT CCT CAG ATT CAT GT and Fw 5′-CGC GGA TCC ATG AAT TTG ACA TTC AAA ATC TAC and Rev 5′-ACG CGT CGA CGT TAA GGT TCA TCC TCA GAT TCA TGT CC, respectively. Mutated and authentic individual UBA domains were also cloned into the BamHI and SalI sites of pGEX6P1 using Rhp23 Fw and Rev 5′-ACG CGT CGA CGT TAA ATG TCT TCG GGA ATA CCA GTT AAT for UBA1. UBA2 was amplified using Fw 5′-CGC GGA TCC ATT CAA ATT ACT CAA GAA GAA TCT G and Rhp23 FL Rev.

Construction of Chimeric Domain Swap Constructs—Chimeric proteins of Pus1ΔUIM (amino acids 1–195) fused to either Rhp23 UBA1 (amino acids 146–190) or UBA2 (amino acids 314–368) domains were created by homologous PCR. In the case of Pus1ΔUIM+UBA1, Pus1ΔUIM was amplified using Fw 5′-ACG CGT CGA CAT GGT GTT AGA AGC AAC GAT GA and Rev 5′-TCG ACA GCA ACA TTT CGT GTT GAG AAG CAA CTA CAC CTT GTC CAA, whereas the UBA1 domain was amplified by Fw 5′-TTG GAC AAG GTG TAG TTG CTT CTC AAC AAC GAA ATG TTG CTG TCG A and Rev 5′-CGG GAT CCT CAA ATG TCT TCG GGA ATA CC. For Pus1ΔUIM+UBA2, Pus1ΔUIM was amplified using the same Fw primer and Rev 5′-CAG ATT CTT CTT GAG TAA TTT GAA TTT GAG AAG CAA CTA CAC CTT GTC CAA, whereas the UBA2 domain was amplified by Fw 5′-TTG GAC AAG GTG TAG TTG CTT CTC AAA TTC AAA TTA CTC AAG AAG AAT CTG and Rev 5′-CGG GAT CCT TAA GGT TCA TCC TCA GAT TCA TGC C. PCR products were

purified by gel extraction, and both Pus1ΔUIM and UBA PCR products were used as template DNA to amplify the new domain swap construct. For Pus1ΔUIM+UBA1, the Pus1ΔUIM Fw primer was used with the UBA1 Rev primer. Pus1ΔUIM+UBA2 was amplified using the Pus1ΔUIM Fw and UBA2 Rev primers. Products were then inserted into pGEX6P1. Rhp23UBA1mutΔUBA2+UBA1 was made in the same way using Fw 5′-ACG CGT CGA CAT GGT GTT AGA AGC AAC GTG A and Rev 5′-TCG ACA GCA ACA TTTC GTT G CT GAA TTC CAC CAG AAG GTA to amplify Rhp23M157A/L183AΔUBA2, whereas Fw 5′-TGC CTT ACC TTC TGG TGG AAT TCA GCA ACG AAA TGT TGC TGT and Rev 5′-ACG CGT CGA CTT AAA TGT CTT CGG GAA TAC CAG were used to amplify UBA1. Rhp23UBA1mutΔUBA2 Fw and UBA1 Rev primers were then used to produce the chimeric construct in pGEX6P1.

In Vitro Binding Assay—GST fusion proteins were produced from recombinant *Escherichia coli* BL21 (DE3) pLysS cells containing the various pGEX6P1 constructs grown at 37 °C to an OD of 0.4–0.8. Isopropyl 1-thio-β-D-galactopyranoside was added to 0.1 mM, and cells were incubated at 25 °C for 4 h. Cells were lysed by sonication in a GST binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.1% Triton X-100 supplemented with 1 mM PMSF and one Complete protease inhibitor tablet (Roche Applied Science)/50 ml of buffer). The fusion proteins were then purified on glutathione-Sepharose 4B beads (GE Healthcare) following the manufacturer's protocol. 5–30 μl of beads were incubated with 100 μl of either Lys^{48}- or Lys^{63}-Ub chains (final concentrations of 6.25 ng/μl for Lys^{48}-linked Ub_{2-7} and 12.5 ng/μl for Lys^{63}-linked Ub_{3-7} chains supplied by Boston Biochem) in TBS buffer supplemented with one complete EDTA-free inhibitor tablet (Roche Applied Science)/50 ml. Mixtures were incubated for at least 2 h at 4 °C, and beads were washed five times with TBS buffer to remove unbound chains. After the final wash, the beads were boiled in SDS-PAGE gel loading buffer for 2 min to release bound proteins, which were then separated by SDS-PAGE and visualized by Coomassie staining and Western blot analysis using anti-Ub antibody (Dako) at 1:1000 in 5% BSA.

Protein Purification and Identification—Recombinant full-length Rhp23 protein with and without mutations as well as the isolated domains were purified from crude extracts of recombinant *E. coli* using glutathione-Sepharose 4B beads as described above. The samples used for fluorescence anisotropy measurements were further purified by size exclusion chromatography using a Sephadex S-200 column (GE Healthcare) equilibrated in 50 mM Tris, pH 7.5, 100 mM NaCl, and 0.5 mM EDTA. Prior to this step, GST was removed by incubating the protein samples with Precission protease 3C at a 1:200 molar ratio for at least 6 h at 4 °C. The purity of the samples was confirmed by SDS-PAGE. Circular dichroism (CD) spectroscopy was performed (see supplemental Experimental Procedures) to confirm the structural integrity of the full-length Rhp23 mutants. The Rhp23 molar extinction coefficient was calculated experimentally by amino acid analysis.

Fluorescence Anisotropy Assay—The K63C mutation was introduced into Ub by site-directed mutagenesis. Lys^{48}- and Lys^{63}-linked Ub_2 were prepared as described (30, 31). Mono-Ub, Lys^{48}-, and Lys^{63}-linked Ub_2 were fluorescently labeled according to the manufacturer's instructions with the thiol-reactive maleimide derivative of Oregon Green 488 (Invitrogen). The reaction mixture contained a 100 nM concentration of fluorescent probe (mono-Ub, Lys^{48}-, or Lys^{63}-Ub_2) and 0–375 μM concentrations of various Rhp23 constructs in a total volume of 20 μl. The reaction mixtures were prepared in triplicate, and fluorescence polarization was recorded in black low protein-binding 384-well plates (Corning) using the PHERAstar FS plate reader (BMG Labtech) equipped with a fluorescein polarization module. The binding curves were analyzed in Prism 5 (GraphPad) using a one-site binding model with nonspecific binding (NS), starting anisotropy level (BG), and ligand depletion by binding to the fluorescent probe (*P*) calculated according to the following equation.

$$Y = \frac{B_{max} \times (P + x + K_d) - \sqrt{(P + x + K_d)^2 - (4P \times x)}}{2P} + NS \times x + BG \quad \text{(Eq. 1)}$$

In Vivo Assay—pREP81 plasmids were stably integrated into *rhp23*::*ura* and crossed to produce an *rhp23*:*ura pus1*::*natR* double mutant using standard protocols. For each cross, 1 ml of water was mixed with 5 μl of β-glucuronidase (Sigma, reference number G0876) before sterilizing through a filter and aliquoting. A loop full of the crossed cells taken from the middle of the cross was suspended in the β-glucuronidase solution and incubated overnight at 25 °C. The produced spores were washed twice in 1 ml of water, and 5000 spores were plated on PMG-Ura-Leu+NAT and incubated at 36 °C to detect rescue.

RESULTS

Mutation of Rhp23 UBA Domains—To dissect the role of the Rhp23 UBA1 and UBA2 domains in the UPS, we constructed mutant versions of each domain that had lost the ability to interact with Ub chains. Care was taken in the design of these mutant versions to avoid causing a gross loss of structural integrity. The authenticity of the mutant folds was verified by circular dichroism (supplemental Fig. S1). To predict key residues important for Ub interaction, we used the data reported by Ryu *et al.* (17) that characterized the interaction between human RAD23B and Ub. Using these structural findings and a sequence comparison of the *S. cerevisiae* and *S. pombe* homologues, we identified two residues in each UBA domain that are predicted to be important for Ub binding (17). These residues are Met^{157} and Leu^{183} within UBA1 and Leu^{332} and Phe^{358} within the UBA2 domain. All of these residues were mutated to alanine to disrupt the binding of the UBAs to Ub. These mutant versions of Rhp23 were cloned into the *S. pombe* expression vector pREP81 and the bacterial expression vector pGEX6P1 to produce WT Rhp23, Rhp23 M157A/L183A (Rhp23UBA1mut), Rhp23 L332A/F358A (Rhp23UBA2mut), and Rhp23 M157A/L183A/L332A/F358A (Rhp23UBA1&UBA2mut). In addition, individual pGEX6P1 UBA1 (residues 146–190), UBA1 M157A/L183A (UBA1mut), UBA2 (residues 314–368), and UBA2 L332A/F358A (UBA2mut) were constructed (Fig. 1).

Ubiquitin Binding Assays of Isolated UBA Domains—Recombinant GST fusion proteins of the individual authentic and

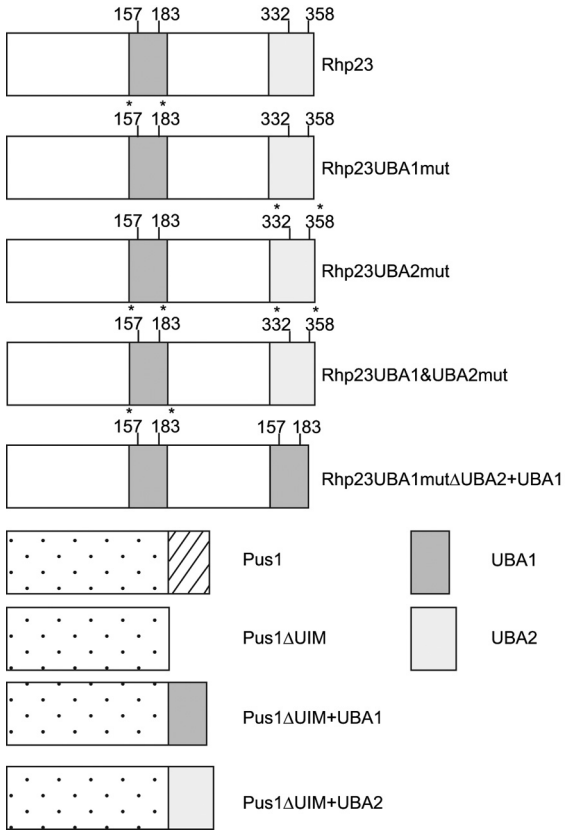

FIGURE 1. **Proteins and point mutations of Rhp23 and Pus1**. The UBA1 domain of Rhp23 was mutated, and residues 157 (methionine) and 183 (leucine) were replaced with alanine. Corresponding mutations were made in the UBA2 domain at residues 332 (leucine) and 358 (phenylalanine). The UBA1 and UBA2 domains were taken to be residues 146–190 and 314–368, respectively. These domain boundaries were used to produce domains and chimeric proteins. The UIM domain of Pus1 was taken to be residues 196–243. *, Residues mutated to alanine.

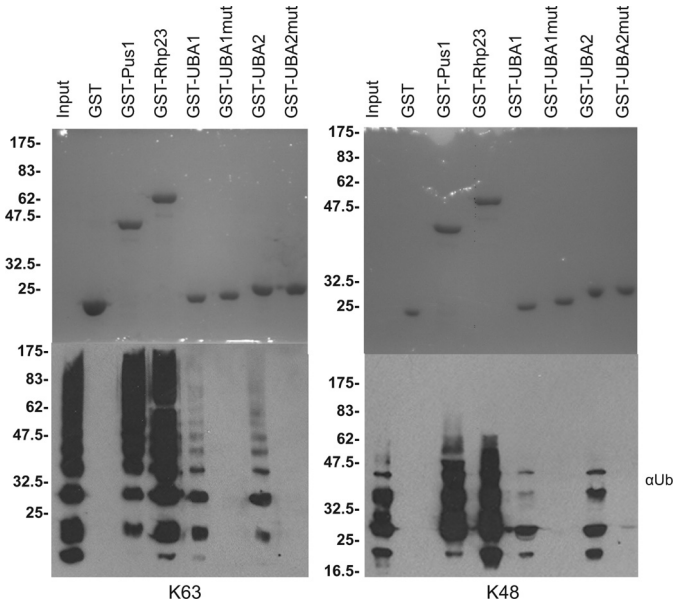

FIGURE 2. **Binding of full-length Rhp23 constructs and UBA domains to Lys^{48}- and Lys^{63}-linked Ub chains.** GST fusion proteins of full-length Pus1 and Rhp23 and the isolated authentic and mutant UBA domains were incubated with Lys^{48}-linked Ub$_{2-7}$ and Lys^{63}-linked Ub$_{3-7}$. Proteins were visualized by separating by 10% SDS-PAGE (*top panel*), and blots were probed with a Ub antibody (*bottom panel*). The authentic UBA domains can bind Ub chains of both linkage type; however, in each case, introduction of the two point mutations disrupts the interaction.

mutant UBA1 and UBA2 domains were bound to glutathione-Sepharose 4B beads. Visualization by Coomassie staining following SDS-PAGE confirmed the presence of a single species of the expected size for all four proteins. These fusion proteins were tested for their ability to bind Lys^{48}- and Lys^{63}-linked Ub chains. WT full-length Rhp23 and Pus1 as GST fusions and GST were used as positive and negative controls, respectively, on a Western blot with anti-Ub antibodies (Fig. 2). The results showed that both UBA domains bind comparably to Lys^{48}- and Lys^{63}-Ub chains but with a lower affinity when compared with full-length Rhp23 and Pus1. Under these experimental conditions, the subsequent introduction of the point mutations M157A and L183A into UBA1 and L332A and F358A into UBA2 expressed as isolated domains resulted in no detectable binding to either Lys^{48} or Lys^{63} chains.

To quantify these results and to avoid potential artifacts arising from the GST tag, the binding of untagged and fluorescently labeled mono-Ub, Lys^{48}-Ub$_2$, and Lys^{63}-Ub$_2$ to full-length Rhp23 and to the individual authentic and mutated UBA domains was measured using fluorescence anisotropy (Fig. 3). Under these experimental conditions, full-length Rhp23 showed an approximately 15-fold preference for binding to Lys^{48}-Ub$_2$ compared with Lys^{63}-Ub$_2$ or mono-Ub and produced affinities of 7.0 ± 0.8, 103 ± 36, and 138 ± 47 μM, respectively (Fig. 3*A* and Table 1). Authentic UBA1 also showed a preference for Lys^{48}-Ub$_2$ (Fig. 3*B* and Table 1). Binding of Ub to UBA2 was significantly weaker, displaying non-appreciable affinities (>200 μM) for mono-Ub and Lys^{63}-Ub$_2$ but significant binding to Lys^{48}-Ub$_2$ (48 ± 13 μM; Fig. 3*C* and Table 1). As expected, the introduction of the M157A and L183A mutations into the UBA1 domain and the L332A and F358A mutations into the UBA2 domain resulted in a considerable loss of binding to Lys^{48}-Ub$_2$ in both cases (Fig. 3*E*).

Ubiquitin Binding Assays of Rhp23 with Mutated UBA Domains—GST fusion Rhp23UBA1mut, Rhp23UBA2mut, and Rhp23UBA1&UBA2mut were also tested for their ability to bind to Ub chains by Western blot analysis. Rhp23UBA1mut could not bind Lys^{63} chains and had only a limited residual ability to bind Lys^{48} chains (Fig. 4). However, mutations in the UBA2 domain (Rhp23UBA2mut) gave rise to a protein that could bind to both Lys^{48} and Lys^{63} chains at levels comparable with WT Rhp23. Rhp23UBA1&UBA2mut was also unable to bind to either type of chain.

Fluorescence polarization measurements were also used to determine the affinities of the full-length Rhp23 constructs for Ub. As WT Rhp23 displayed a strong preference for binding to Lys^{48}-linked chains, only Lys^{48}-Ub$_2$ was tested. Rhp23, Rhp23UBA1mut, Rhp23UBA2mut, and Rhp23UBA1&UBA2mut yielded affinities for Lys^{48}-Ub$_2$ of 7.0 ± 0.8, 34 ± 7, 11.9 ± 0.7, and >200 μM, respectively (Fig. 3, *A* and *D*, and Table 1). The affinity of Rhp23UBA1mut for Lys^{48}-Ub$_2$ (34 ± 7 μM) is similar to that of the isolated UBA2 domain (48 ± 13 μM) and is consistent with the binding of Lys^{48}-linked chains to this domain determined by Western blot analysis. The Rhp23UBA2mut and authentic protein displayed similar affinities for Lys^{48}-Ub$_2$,

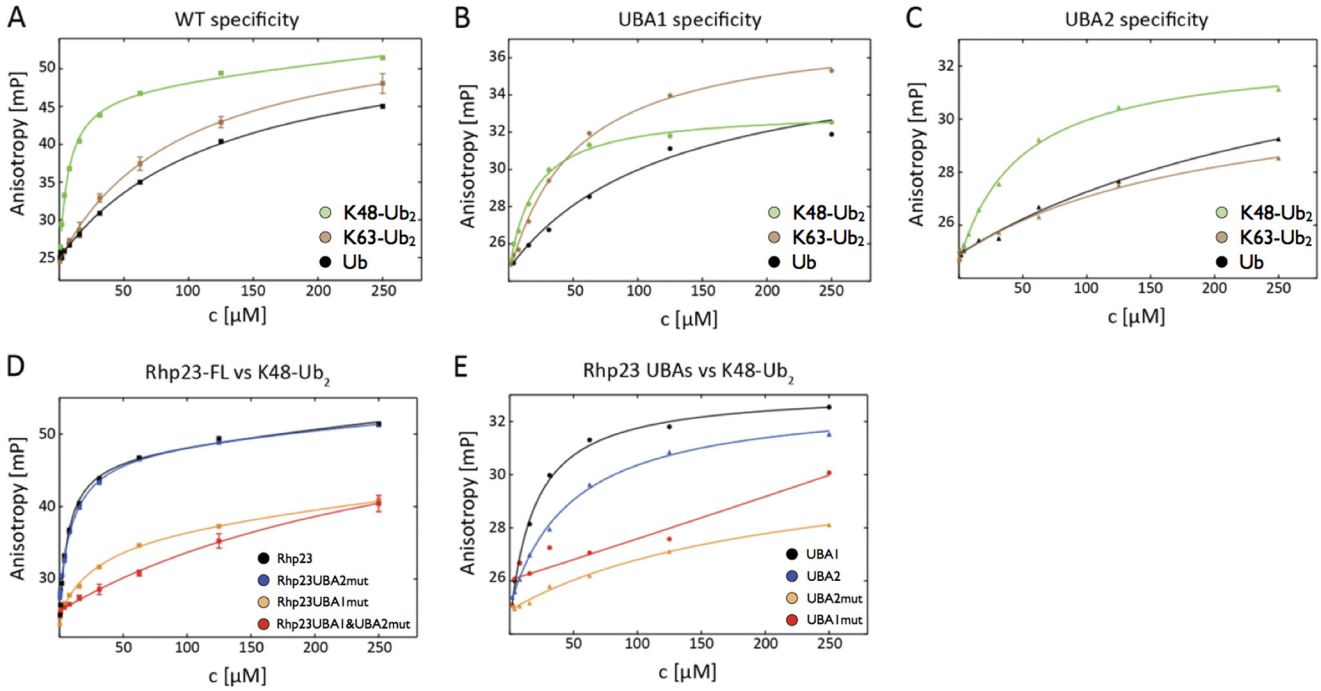

FIGURE 3. **Interaction of Rhp23 with mono- and di-Ub.** The binding of mono-Ub, Lys^{63}-, and Lys^{48}-linked Ub$_2$ to full-length Rhp23 and to the isolated UBA1 and UBA2 domains was determined using fluorescence polarization. *A*, full-length authentic Rhp23 binding to Lys^{48}-Ub$_2$ (*green*), mono-Ub (*black*), and Lys^{63}-Ub$_2$ (*brown*). Rhp23 bound Lys^{48}-Ub$_2$ with 7.0 μM affinity but shows a more than 10-fold lower affinity (138 and 103 μM, respectively) for both mono-Ub and Lys^{63}-Ub$_2$. *B* and *C*, UBA1 (*B*) and UBA2 (*C*) binding to mono-Ub and Lys^{48}- and Lys^{63}-Ub$_2$. Binding curves for the association of the isolated authentic UBA1 and UBA2 domains with mono-Ub (*black*), Lys^{48}-Ub$_2$ (*green*), and Lys^{63}-Ub$_2$ (*brown*) are shown. UBA1 has an ~6-fold lower affinity for mono-Ub compared with Lys^{48}-Ub$_2$ (18 *versus* 126 μM) but shows less ability to discriminate Lys^{63}-Ub$_2$ (51 μM). UBA2 is a weaker binder of Ub. Its measured affinities for both mono-Ub and Lys^{63}-Ub$_2$ were >200 μM. Lys^{48}-Ub$_2$ bound more strongly (48 μM) but much weaker than UBA1. *D*, binding of full-length authentic and mutant Rhp23 to Lys^{48}-Ub$_2$. In the context of full-length Rhp23 (authentic Rhp23; *black*), mutation of UBA2 (*blue*) had almost no effect, reducing the affinity for Lys^{48}-Ub$_2$ from 7.0 to 11.9 μM. However, when UBA1 was mutated (*orange*), the affinity decreased about 4.5-fold to 34 μM. Mutation of both UBAs (*red*) resulted in almost complete loss of binding with an affinity of >200 μM. *E*, binding of the isolated mutant UBA1 and UBA2 domains to Lys^{48}-Ub$_2$. Mutation of UBA1 (*red*) results in the loss of Lys^{48}-Ub$_2$ binding, but the mutated UBA2 domain (*orange*) retains some residual affinity (>200 μM). Authentic unmutated UBA1 (*black*) and UBA2 (*blue*) are the same as in *B* and *C*. *mP*, millipolarization units. Error bars represent standard deviation of multiple measurements.

TABLE 1

Binding affinities of wild-type and mutant Rhp23 for mono-Ub, Lys^{48}-, and Lys^{63}-linked Ub$_2$

ND, not detectable; —, not determined.

	K_d		
	Mono-Ub	Lys^{48}-Ub$_2$	Lys^{63}-Ub$_2$
		μM	
Rhp23 WT	138 ± 47	7.0 ± 0.8	103 ± 36
Rhp23UBA1mut	—	34 ± 7	—
Rhp23UBA2mut	—	11.9 ± 0.7	—
Rhp23UBA1&UBA2mut	—	>200	—
UBA1 WT	126 ± 80	18.8 ± 4.7	51 ± 16
UBA1mut	—	ND	—
UBA2 WT	>200	48 ± 13	>200
UBA2mut	—	>200	—

again consistent with the results of the Western blot analysis. Taken together, these results suggest that the UBA1 domain is responsible for the majority of the interaction of Rhp23 with Lys^{48}-linked Ub.

Ubiquitin Binding Assays of Pus1 and Rhp23 Chimeric Proteins—To test the ability of each Rhp23 UBA domain to recognize Ub chains independently of the protein context, Pus1ΔUIM, Pus1ΔUIM+UBA1, and Pus1ΔUIM+UBA2 were produced. In the Pus1ΔUIM+UBA1 and Pus1ΔUIM+UBA2 constructs, the Pus1 UIM was replaced with either the Rhp23 UBA1 or the UBA2 domain. In this context, only UBA1 restored the Pus1 function lost by the removal of the UIM motif (Fig. 5). Unlike Pus1ΔUIM+UBA1, the chimeric protein containing the UBA2 domain was unable to recognize either Lys^{48} or Lys^{63} chains. This finding further highlights the role of UBA1 as the major Ub binding unit of Rhp23 (Fig. 5). To explore further whether it is an intrinsic property of the UBA1 domain or its position within the Rhp23 protein that confers its Ub binding properties, another chimeric version of Rhp23 was constructed in which the authentic UBA2 domain of the Rhp23UBA1mut construct, which shows reduced binding to Ub chains (see Fig. 4), was replaced by the UBA1 domain (see Fig. 1). When tested as a GST fusion, the Rhp23UBA1mutΔUBA2+UBA1 mutant protein bound both Lys^{48} and Lys^{63} chains to levels comparable with WT Rhp23 (Fig. 6). This result clearly demonstrated that UBA1 function is independent of the position of the domain within the protein.

In Vivo Function of the Different Rhp23 Mutant Constructs—Previous work has established that the *ts* phenotype of the *S. pombe rhp23Δpus1Δ* mutant results from a failure to deliver substrates to the proteasome (13). Therefore, the rescue of this strain at a restrictive temperature by expression of an Rhp23 mutant would indicate the presence of an intact UBA domain able to mediate this role of Rhp23 in the UPS. The fission yeast expression vector pREP81 containing either authentic or mutated *rhp23* was stably integrated into an *rhp23*-null strain carrying a *ura* selectable marker. These strains were then crossed to a *pus1*-null strain containing the *natR* marker. Asci were digested with β-glucuronidase, and 5000 spores were

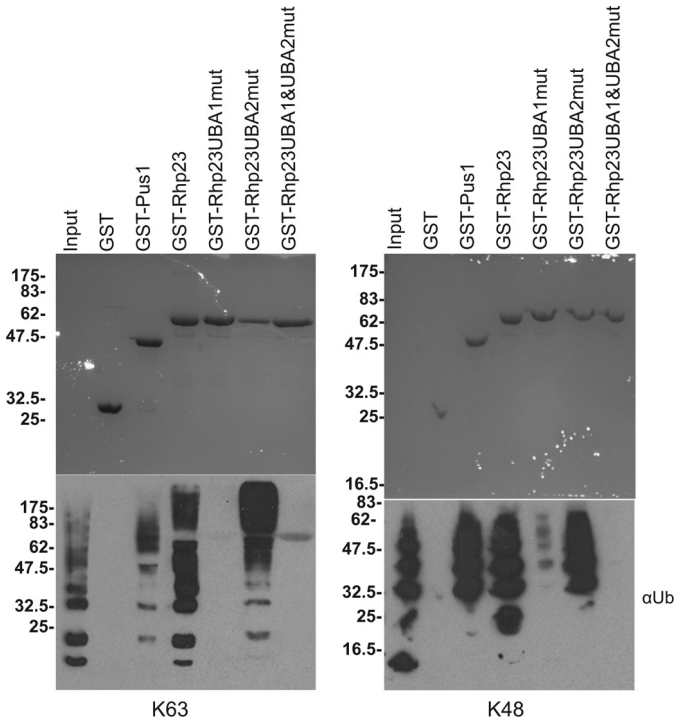

FIGURE 4. **Point mutations in full-length Rhp23 disrupt Lys^{48}- and Lys^{63}-linked Ub chain binding to the UBA1 domain.** GST fusion proteins of full-length Pus1, WT Rhp23, and mutated Rhp23 were incubated with Lys^{48}-linked Ub_{2-7} and Lys^{63}-linked Ub_{3-7}. Proteins separated by 10% SDS-PAGE were visualized (*top panel*), and blots were probed with Ub antibody (*bottom panel*). WT Rhp23 and Rhp23 containing point mutations in the UBA2 domain (Rhp23UBA2mut) can bind Ub chains of both linkage types. Point mutations in the UBA1 domain (Rhp23UBA1mut) render Rhp23 unable to bind Lys^{63} chains, and its ability to bind Lys^{48}-linked Ub is dramatically reduced. Rhp23 carrying mutations in both domains (Rhp23UBA1&UBA2mut) can no longer bind either chain type.

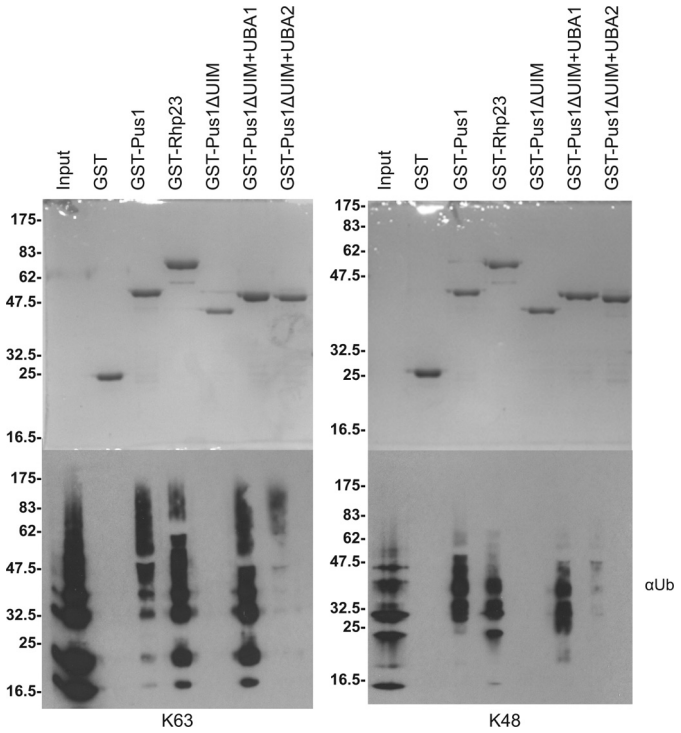

FIGURE 5. **In the context of Pus1/Rhp23 chimeric proteins, only the UBA1 domain binds Lys^{48}- and Lys^{63}-linked Ub chains.** GST fusion proteins of full-length Rhp23, Pus1, Pus1ΔUIM, and chimeric proteins in which the UBA domains of Rhp23 had individually replaced the UIM domain of Pus1 were incubated with Lys^{48}-linked Ub_{2-7} and Lys^{63}-linked Ub_{3-7}. Proteins were separated by 10% SDS-PAGE (*top panel*), and blots were probed with Ub antibody (*bottom panel*). Pus1ΔUIM cannot bind either chain type, but the addition of the UBA1 domain (in construct Pus1ΔUIM+UBA1) restored the Ub binding. Replacement of the Pus1 UIM with the Rhp23 UBA2 domain generated a chimeric protein (Pus1ΔUIM+UBA2) that did not bind either chain type.

plated onto PMG-Leu-Ura+NAT to select for double mutants that carry the plasmid and grow at 36 °C. The results shown in Fig. 7 reveal that, as expected, the empty pREP81 vector did not rescue the phenotype, whereas expression of WT Rhp23 produced viable cells. Expression of both the Rhp23UBA1mut and the Rhp23UBA1&UBA2mut constructs was unable to rescue the double mutant lethal phenotype. In contrast, the Rhp23UBA2mut construct rescued the lethal phenotype as did the WT Rhp23. Although differences in the levels of expression of the authentic and mutant Rhp23 proteins from the pREP81 promoter might obscure subtle differences in the efficiencies with which they can rescue the *rhp23*Δ*pus1*Δ mutant strain at a restrictive temperature, these results support the *in vitro* studies and indicate that the UBA1 domain of Rhp23 is sufficient for the recognition of ubiquitinated substrates *in vivo* for the UPS.

DISCUSSION

It is now recognized that Ub has important roles in many aspects of cellular regulation (32, 33) and in disease (34–37). A more complete understanding of Ub signaling will require determination of how mono-Ub and the various Ub chains are recognized by different domains that differ not only in sequence but also in architecture. In this regard, the two UBA domains of Rhp23 that can be distinguished by their Ub binding properties are an example. These domains differ in sequence but adopt a very similar three-dimensional structure composed of three consecutive α-helices and are both able to bind Lys^{48} and Lys^{63} chains (21).

Although extensive work has been carried out to dissect the roles of the UBA domains, this is the first study where the function of these domains has been directly compared both in isolation and in the context of full-length protein. Our results show that the Ub binding properties of the individual domains and full-length Rhp23 differ. The isolated Rhp23 UBA1 and UBA2 both bind to Lys^{48} and Lys^{63} chains albeit with different affinities. However, in the context of the full-length protein, both *in vitro* and *in vivo*, Lys^{48} and Lys^{63} chain binding is primarily a function of UBA1.

We have demonstrated that a functional UBA1 domain is sufficient to rescue the *ts* phenotype of the *rhp23*Δ*pus1*Δ *S. pombe* strain, suggesting that the UBA2 domain is redundant. This result was unexpected given that previous studies had reported that UBA2 displays a preference for Lys^{48} chains, whereas UBA1 better recognizes Lys^{63}-linked chains (22). However, our results do concur with similar studies in *S. cerevisiae* that concluded that expression of Rad23 with a mutated UBA1 could not fully rescue the *cs* phenotype of a *rad23*Δ*rpn10*Δ strain (38). In this context, it should be noted that the *cs* phenotype is not as severe as the *ts* phenotype of *rhp23*Δ*pus1*Δ. The authors of this work also showed that

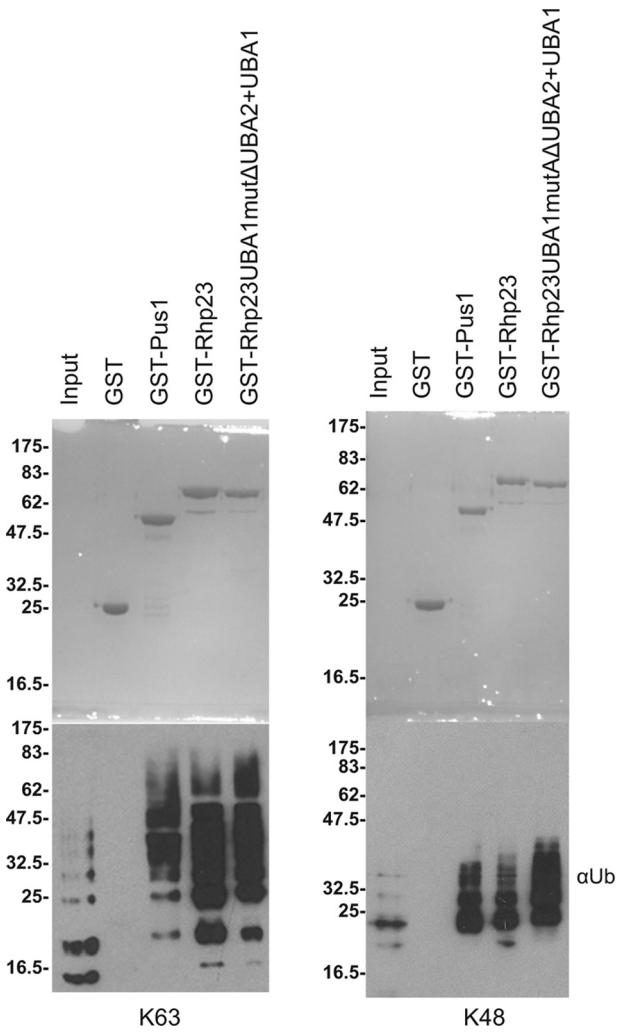

FIGURE 6. **UBA1 domain binding of Ub chains is independent of its position within the Rhp23 sequence.** GST fusion proteins of full-length Pus1, Rhp23, and Rhp23 carrying point mutations in the UBA1 and in which the UBA2 was replaced with a copy of the authentic UBA1 domain (Rhp23UBA1mutΔUBA2+UBA1) were incubated with Lys^{48}-linked Ub_{2-7} and Lys^{63}-linked Ub_{3-7}. Proteins were separated by 10% SDS-PAGE (*top panel*), and blots were probed with Ub antibody (*bottom panel*). The chimeric RhpUBA1mutΔUBA2+UBA1 protein can bind both Lys^{48} and Lys^{63} chains at a level similar to WT Rhp23, suggesting that the UBA1 domain is responsible for the majority of Rhp23 Ub binding ability regardless of its position within the protein.

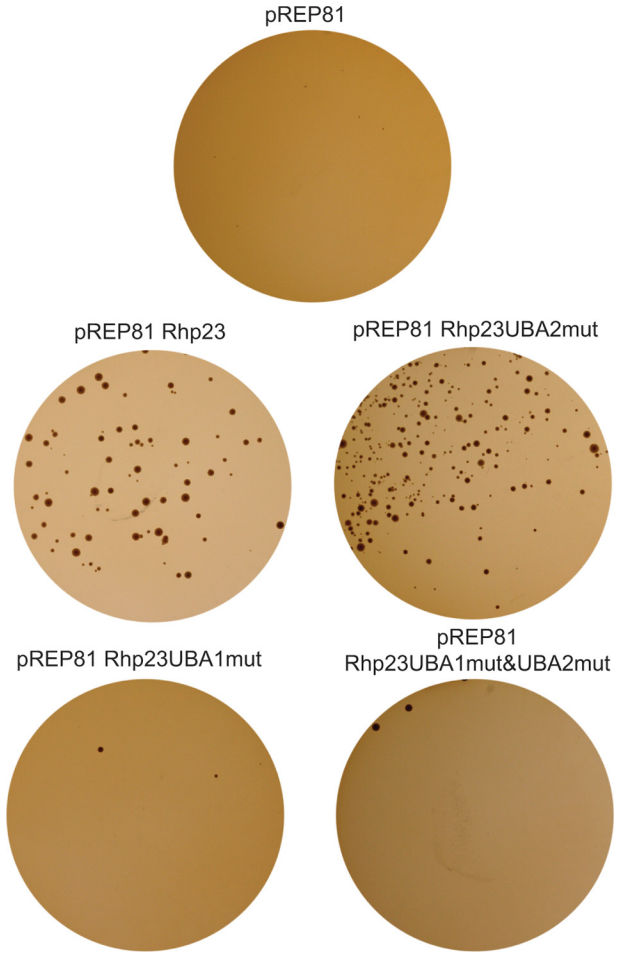

FIGURE 7. ***In vivo* rescue of the *rhp23Δpus1Δ ts* phenotype.** pREP81 constructs were stably integrated into an *rhp23:ura* strain and then crossed to *pus1::natR*. Spores were plated on PMG-Leu-Ura+NAT to select for double mutants expressing the pREP81 construct and incubated at 36 °C to put stress on the UPS. The double mutants expressing empty vector, Rhp23UBA1mut, or Rhp23UBA1&UBA2mut were not viable under these conditions. The expression of WT Rhp23 and Rhp23UBA2mut rescued the temperature-sensitive phenotype. Taken together, these results demonstrate that *S. pombe* only requires Rhp23 with an active UBA1 domain to transfer ubiquitinated substrates to the proteasome.

UBA1 is responsible for the majority of the Ub binding capability of *S. cerevisiae* Rad23 (28).

The chimeric proteins shed further light on the role of the UBA1 domain as the major Ub binding factor of Rhp23. We have shown that the Rhp23 UBA1 domain can restore *in vitro* Ub binding to a form of Pus1 from which the UIM has been deleted (39, 40). Furthermore, we demonstrated that Ub binding can be restored to a version of Rhp23 in which UBA1 is functionally compromised by mutation by replacing UBA2 with the authentic UBA1 sequence.

Although UBA1 makes the most substantial contribution to the observed affinity and selectivity of Rhp23 for Ub chains, chain binding to the full-length protein both *in vitro* and *in vivo* is enhanced when UBA2 is present. UBA2 may not only slightly increase the observed affinity but may also tune the discrimination between Lys^{48} and Lys^{63} chains. Consistent with this model, whereas isolated UBA1 shows a 7-fold selectivity for Lys^{48}-Ub_2 against mono-Ub and a 3-fold selectivity against Lys^{63}-Ub_2, the equivalent selectivities for WT Rhp23 are 20- and 15-fold, respectively. Raasi *et al.* (22) have reported a similar phenomenon whereby isolated Rad23 UBA1 bound preferentially to Lys^{63} chains, but the preference of a ubiquitin-like domain-UBA1 fragment protein was switched to Lys^{48} chains.

We conclude from these studies that the functionally significant binding properties of Ub binding domains can only be fully appreciated when studied in their authentic context. Our studies also suggest that although the UBA1 domain of Rhp23 is sufficient for its Ub binding function in the UPS the UBA2 contributes to discrimination between Lys^{48} and Lys^{63} chains. This function may be in addition to previously suggested roles of the UBA2 in protecting the protein from being degraded (24, 25) or facilitating Rhp23 dissociation from the proteasome (41).

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