The Deubiquitinating Enzyme Ubp2 Modulates Rsp5-dependent Lys\(^{63}\)-linked Polyubiquitin Conjugates in Saccharomyces cerevisiae*1

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The functions of Lys\(^{63}\)-linked polyubiquitin chains are poorly understood, as are the enzymes that specifically generate Lys\(^{63}\)-linked conjugates. Rsp5 is a HECT (homologous to E6AP C terminus) ubiquitin ligase involved in numerous processes, and an associated deubiquitinating enzyme, Ubp2, modulates its activity. A dramatic increase in Lys\(^{63}\)-linked conjugates was observed in ubp2Δ cells. The formation of these was Rsp5-dependent, and ubp2Δ phenotypes could be suppressed by prevention of formation of Lys\(^{63}\)-linked conjugates. Cell wall integrity was impaired in rsp5-1 cells and in cells defective in Lys\(^{63}\)-polyubiquitination, as assayed by calcofluor white sensitivity, and ubp2Δ and rsp5Δ mutants suppressed the calcofluor white sensitivity of rsp5-1. A large fraction of the Lys\(^{63}\)-conjugates in ubp2Δ cells bound to Rsp5, and a proteomics approach was used to identify Rsp5 substrates subject to Ubp2 regulation. Two closely related proteins, Csr2 and Ecm21, were among the identified proteins. Both were efficiently Lys\(^{63}\)-polyubiquitinated by Rsp5 and deubiquitinated by Ubp2. Together, these results indicate that Ubp2 modulates Lys\(^{63}\)-polyubiquitination of Rsp5 substrates in vivo, including ubiquitination of two newly identified Rsp5 substrates.

Ubiquitination of target proteins is catalyzed by a cascade of at least three classes of enzymes, known as E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-protein isopeptide ligases) (1). There are two main classes of E3 ubiquitin ligases, known as HECT and RING E3s. HECT E3s participate directly in the chemistry of ubiquitin conjugation by forming a covalent thioester intermediate with ubiquitin at an active site cysteine residue within the HECT domain (2), whereas RING E3s appear to function as docking surfaces for activated E2s and substrates. Rsp5 is the best-characterized HECT E3 in Saccharomyces cerevisiae, and direct homologs of Rsp5 (the Nedd4 family of HECT E3s) exist in all animals (3). Rsp5 is the only essential gene among the five HECT E3s in budding yeast (4), and the minimal essential function of Rsp5 is the ubiquitination of the Spt23 transcription factor, leading to a proteasome-catalyzed processing event that is required for its activation (5). Rsp5 has been reported to function in a variety of other cellular processes including ubiquitin-mediated endocytosis of plasma membrane proteins such as Gap1 (6), Fur4 (7), and Ste2 (8) and the delivery of biosynthetic cargo such as Cps1 into the endosomal lumen (9). Rsp5 has also been implicated in RNA export (10), ubiquitination of the large subunit of RNA polymerase II (11), mitochondrial inheritance (12), and cell wall biogenesis (13), indicating that Rsp5 is a multifunctional protein capable of ubiquitinating many substrates in many different locations.

Ubiquitination is a reversible process in that the isopeptide bond between ubiquitin and a substrate protein, as well as isopeptide bonds between ubiquitin molecules in a polyubiquitin chain, can be cleaved by deubiquitinating enzymes (DUBs). There are at least 18 DUBs characterized in S. cerevisiae to date, including 16 ubiquitin specific proteases (UBPs), an ubiquitin C-terminal hydrolase, and a JAMM (JAB1/MPN/Mov34 metalloenzyme) motif metalloprotease. The best-characterized function of DUBs is to facilitate the rescue of ubiquitin monomers from proteolytic degradation. For example, Doa4/Ubp4 is an endosomal membrane-associated protease that cleaves ubiquitin molecules from endocytic cargo prior to vacuolar degradation, thus maintaining total cellular ubiquitin pools (14, 15), and Ubp6 is a proteosome-associated enzyme that is required for ubiquitin homeostasis and thought to rescue ubiquitin molecules prior to proteasomal degradation of substrates (16). Other roles of the DUBs include processing of ubiquitin precursors to mature forms and reversing ubiquitination of substrates (17). There have been several reports of E3s that are physically associated with DUBs (18–20), and in these cases the DUBs reverse autoubiquitination of the E3s, thus increasing the stability of the E3 by protecting them from proteosomal degradation. Recently, Rsp5 has been shown to be physically associated with Ubp2 (21). Autoubiquitination of Rsp5 in vivo has not been previously reported, and the half-life of Rsp5 is unaffected by its catalytic activity, suggesting that Ubp2 does not regulate the stability of Rsp5. Rather, genetic and
biochemical evidence indicated that Ubp2 antagonizes Rsp5-mediated ubiquitination of target proteins (21). The interaction of Rsp5 and Ubp2 is indirect and is mediated by a third protein, Rup1. Rup1 contains ubiquitin-associated domain and no other characterized functional domains or motifs.

Ubiquitin contains seven lysine residues that can potentially serve as acceptor sites for additional ubiquitin molecules to form polyubiquitin chains. Lys63-linked polyubiquitin chains are the primary signals for targeting to the 26 S proteasome, whereas monoubiquitination and some other forms of polyubiquitination appear to mediate alternative functions (22–24). Lys63-linked chains serve non-proteolytic functions in DNA repair pathways (25–27), kinase activation (28), and receptor endocytosis (6, 7), although they may also be capable of proteasome targeting (29). Lys64-linked chains have been shown to be involved in a DNA repair pathway (31). We showed previously that Rsp5 preferentially catalyzes Lys64-linked polyubiquitination of substrates in vitro, whereas Ubp2 preferentially disassembles Lys63-chains in vitro (21). Here we show that Rsp5 and Ubp2 modulate Lys63-linked polyubiquitination in vivo, and the essential function of Rsp5 at elevated temperature requires Lys63 polyubiquitination and identify two new targets of Rsp5 and Ubp2 that are subject to Lys63 ubiquitination.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Plasmids—A list of yeast strains is shown in Table 1. SUB492 and SUB493 strains express wild type and K63R ubiquitin as a sole source of ubiquitin, respectively, as previously described (25; see Table 1). ubp2Δ mutants were introduced into both SUB492 and SUB493 strains using kanamycin selection, as described previously (21), generating YK018 and YK019, respectively. csr2Δ and ecm21Δ mutants were introduced into FW1808 strain using the same method to generate YK028 and YK029, respectively. The rup1Δ mutation was introduced into YK009 strain using HIS3 selection, generating YK30. pUB39 is a Lys3-marked plasmid that expresses wild type ubiquitin under the CUP1 promoter. pUB115, pUB119, pUB195, and pUB197 are identical to pUB39, except for the K48R, K6R, K29R, and K63R mutations, respectively (25). Plasmids for overexpressing UBP2 and ubp2C745S were described previously (21). CSR2 and ECM21 open reading frames were PCR amplified from genomic DNA and cloned into the pYES2 vector (Invitrogen) encoding an N-terminal hemagglutinin epitope for in vitro and in vivo expression. For the expression of N-terminally tandem affinity purification-tagged ubiquitin, the UBI4 open reading frame is subcloned into the pYES2-NTAP vector (21). The C-terminal codons of UBI4 encoding GG altered to AA codons for expression of NTAP-Ub-AA75–76. For the AD CB-containing medium, ADB (Sigma) was dissolved in water and added to synthetic minimal medium as final concentrations of 100 or 200 µg/ml where indicated. For the calcofluor white (CFW)-containing medium, CFW (Sigma) was dissolved in water and added to yeast extract/peptone/dextrose medium as final concentrations of 5 or 7 µg/ml where indicated.

Table 1

| Strain           | Genotype                                      | Reference          |
|------------------|-----------------------------------------------|--------------------|
| FY56             | MATα his4-912 Δhis-1 Δlys2-128Δ ara3-52       | Huberget et al. (11) |
| FW1808           | MATα his4-912 Δhis-1 Δlys2-128Δ ara3-52       | Huberget et al. (11) |
| BY4743           | MATα his3 leu2Δ met15 ura3                     | Open Biosys.       |
| YK003            | MATα rtp-1 ubp2Δ:KanMX6 his4-912 Δhis-1 Δlys2-128Δ ara3-52 | Open Biosys.       |
| YK004            | MATα rtp-1 rup1Δ:KanMX6 his4-912 Δlys2-128Δ ara3-52 | Open Biosys.       |
| YK009            | MATα ubp2Δ:KanMX6 his3 leu2Δ met15 ura3       | Open Biosys.       |
| SUB492           | MATα lys2-801 leu2Δ, 112 ura3-52 his3Δ 200 trp1-1[am] ubi1Δ ubi2Δ:TRPl ubi3Δ ura3 Δ ub-2 ub4Δ 2Δ:LEI2 [pUB39][pUB100] | Spence et al. (25) |
| SUB493           | Isogenic to SUB492 except for pUB197 instead of pUB39 | This study         |
| YK018            | MATα ubp2Δ:KanMX6 his3 leu2Δ his3Δ 200 trp1-1[am] ubi1Δ ubi2Δ:TRPl ubi3Δ ura3 Δ ub-2 ub4Δ 2Δ:LEI2 [pUB39][pUB100] | This study         |
| YK019            | MATα ubp2Δ:KanMX6 his3 leu2Δ his3Δ 200 trp1-1[am] ubi1Δ ubi2Δ:TRPl ubi3Δ ura3 Δ ub-2 ub4Δ 2Δ:LEI2 [pUB39][pUB100] | This study         |
| YK028            | MATα rtp-1 csr2Δ:KanMX6 his4-912 Δhis-1 Δlys2-128Δ ara3-52 | This study         |
| YK029            | MATα rtp-1 ecm21Δ:KanMX6 his4-912 Δlys2-128Δ ara3-52 | This study         |
| YK030            | MATα ubp2Δ:KanMX6 his3 leu2Δ his3Δ 200 trp1-1[am] ubi1Δ ubi2Δ:TRPl ubi3Δ ura3 Δ ub-2 ub4Δ 2Δ:LEI2 [pUB39][pUB100] | This study         |
| YK032            | MATα ubp3Δ:KanMX6 his3 leu2Δ met15 ura3       | Open Biosys.       |
| YK033            | MATα ubp4Δ:KanMX6 his3 leu2Δ met15 ura3       | Open Biosys.       |

Table 1: Yeast strains used in this study
cleavage reaction was done for 2 h at room temperature on a rotator. The eluted ubiquitinated proteins were purified on GST-Rsp5C777A immobilized on glutathione-Sepharose by incubation for 2 h at 4 °C. Sepharose was washed two times with 1% Nonidet P-40 buffer followed by 0.1% Nonidet P-40 buffer as a final wash before they were analyzed on a 10% SDS-PAGE and stained with Coomassie Blue. Bands were excised from a Coomassie Blue stained gel and subjected to in-gel tryptic digestion. The fragmented peptides were analyzed by liquid chromatography-mass spectrometry.

In Vitro Ubiquitination/Deubiquitination Assays—In vitro ubiquitination and deubiquitination assays were performed in the presence of 10 mM Tris, pH 7.5, 50 mM NaCl, 5 mM ATP, 5 mM MgCl₂, 0.1 mM dithiothreitol, and 50 μg/ml ubiquitin (Sigma). Bacterially expressed Rsp5, Rup1, and Ubp2 were purified on glutathione-Sepharose, and GST was removed by cleavage with PreScission protease (Amersham Biosciences). Purification of baculovirus-expressed human E1 and UbcHis⁷ were performed as described previously (32). In vitro translated ⁳⁵S-labeled Csr2 or Ecm21, synthesized using rabbit reticulocyte lysate, were used as substrates. The ubiquitination reactions were carried out for 30 min at room temperature followed by an additional 30 min for deubiquitination by Rup1/Ubp2. For the assays using Lys⁰, Lys⁴⁸-only, and Lys⁶³-only ubiquitin (Boston Biochem), the in vitro translated substrates were partially purified by DEAE anion exchange column to remove endogenous ubiquitin. The reactions were performed as describe above and stopped by addition of SDS-PAGE loading buffer, and the samples were analyzed on 8% SDS-PAGE followed by autoradiography.

RESULTS

Modulation of Lys⁶³ Polyubiquitin Conjugates by Rsp5 and Ubp2—We previously reported that Ubp2 preferentially disassembles Lys⁶³-linked polyubiquitin chains relative to Lys⁴⁸-linked chains in vitro (21). To further analyze Ubp2 activity in vivo, we compared the amount of overall ubiquitin conjugates in total cell extracts from wild type UBP2 and ubp2Δ cells. A strong increase in total ubiquitin conjugates was seen in ubp2Δ cells, as determined by immunoblotting with anti-ubiquitin antibody (Fig. 1A). Reintroduction of a plasmid-based wild type Ubp2 gene under GAL1 promoter control reduced the conjugate level below that seen in the wild type UBP2 strain, whereas reintroduction of the active site cysteine-to-serine ubp2 mutant (C745S, C-S) did not suppress conjugate accumulation (Fig. 1B).

To determine whether the increased ubiquitin conjugates in the ubp2Δ cells represented an accumulation of Lys⁶³-linked polyubiquitin chains, we took advantage of strains in which all four endogenous ubiquitin genes were eliminated and replaced by a plasmid-borne wild type ubiquitin gene or a mutated ubiquitin gene encoding K63R ubiquitin (25). The ubp2Δ mutation was introduced into both of these strains. As expected, the ubp2Δ mutation led to an increase in ubiquitin conjugates in the strain expressing wild type ubiquitin (Fig. 1C), whereas the ubp2Δ mutation in the K63R strain resulted in only a slight accumulation of conjugates, suggesting that the vast majority of

FIGURE 1. Ubp2 modulates Lys⁶³-linked conjugates in S. cerevisiae. A, total ubiquitin conjugates were compared in total extracts of UBP2 (BY4741) and ubp2Δ (YK009) cells by immunoblotting with anti-ubiquitin antibody. An anti-Rpa1 immunoblot (anti-70K) is shown of the same extracts as a loading control. B, total ubiquitin conjugates in the ubp2Δ mutant were examined as in A upon overexpression of wild type Ubp2 or the catalytically inactive Ubp2-C745S (C-S) proteins. C, total ubiquitin conjugates in cell extracts from wild type ubiquitin (SUB492), wild type ubiquitin/ubp2Δ (YK018), K63R ubiquitin (SUB493), and K63R ubiquitin/ubp2Δ (YK019) strains were compared by anti-ubiquitin immunoblotting.
the elevated ubiquitin conjugates in the ubp2Δ mutant represented Lys63-linked chains. The small but consistently increased level of ubiquitin conjugates observed in the ubp2Δ/K63R strain suggests that Ubp2 may have a limited capacity to recognize ubiquitin linkages other than Lys63.

The ubp2Δ mutation conferred increased sensitivity to the toxic proline analogue L-azetidine-2-carboxylic acid3 (ADCB; 30 °C for 4 days. This result strongly suggests that the majority of proteins that accumulate are the result of Rsp5 ubiquitination activity, we analyzed the effect of the ubp2 mutant on the accumulation of ubiquitin conjugates observed in the ubp2Δ/K63R strain, further indicating that Lys63-linked polyubiquitination of one or more proteins led to ADCB hypersensitivity. Consistent with this, the ubp2Δ mutant did not display increased ADCB sensitivity in cells that expressed K63R-ubiquitin as the sole source of ubiquitin (Fig. 2A, upper left panel). Even in a wild type UBP2 background, cells expressing K63R ubiquitin as the sole source of the ubiquitin were resistant to ADCB compared with the wild type ubiquitin strain, further indicating that Lys63-linked polyubiquitination was required to confer sensitivity to ADCB (Fig. 2A, lower left panel). GAL1 promoter-driven expression of UBP2 in the ubp2Δ mutant conferred ADCB resistance beyond that of wild type UBP2 cells, whereas overexpression of the C-S mutant did not suppress ADCB sensitivity (Fig. 2B).

Together, the results shown in Figs. 1 and 2 indicate that the ubp2Δ mutation leads to an overall accumulation of Lys63-linked polyubiquitin conjugates. To determine whether the conjugates that accumulate are the result of Rsp5 ubiquitination activity, we analyzed the effect of the rsp5-1 hypomorphic temperature-sensitive mutation on the accumulation of conjugates. A strong reduction in overall conjugates was observed in the rsp5-1/ubp2Δ mutant compared with RSP5/ubp2Δ cells (Fig. 3A), both at normal and elevated growth temperatures. This result strongly suggests that the majority of ubiquitin conjugates that accumulate in the ubp2Δ strain are the result of Rsp5-dependent ubiquitin conjugation, consistent with our previous demonstration that Rsp5 preferentially catalyzes conjugation of Lys63-linked chains in vitro (21).

To demonstrate that Rsp5 catalyzes Lys63 chain formation in vivo, wild type ubiquitin, K63R ubiquitin, or K48R ubiquitin was overexpressed in the rsp5-1 mutant (Fig. 3B, top). Overexpression of wild type ubiquitin rescued the growth defect of the rsp5-1 mutant at elevated temperature, as reported previously (33). Overexpression of K48R ubiquitin also rescued growth, whereas overexpression of K63R ubiquitin did not. K6R ubiquitin also rescued rsp5-1 temperature sensitivity, whereas rescue by K29R ubiquitin was partial compared with wild type ubiquitin (not shown). Overexpression of K63R ubiquitin had no effect on the growth of wild type RSP5 cells (not shown). These results demonstrate that the ability of Rsp5 to form Lys63-linked conjugates is linked to its essential function at elevated temperature, although they do not rule out that Rsp5 might synthesize other types of polyubiquitin chains in vivo, particularly Lys29-linked chains.

Physical Association of Lys63-conjugated Proteins with Rsp5 and Ubp2—Rsp5 has been shown to interact directly with several of its substrates, generally mediated by the WW domains of Rsp5.
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Rsp5 and proline-containing motifs in the substrate proteins (4). Because the Lys^{63} conjugates that accumulate in the ubp2Δ mutant are the result of Rsp5 ubiquitination activity, we predicted that a significant fraction of the conjugates in extract from ubp2Δ cells might stably interact with purified GST-Rsp5 in vitro. To test this, extracts were made from UBP2 wild type cells, ubp2Δ, ubp3Δ, and ubp4Δ mutant cells. Although ubp3Δ and ubp4Δ cells also accumulated ubiquitin conjugates (Fig. 4A), there was no known relationship between Rsp5 and either Ubp3 or Ubp4, and we therefore did not expect ubiquitinated proteins in these extracts to interact with GST-Rsp5. As shown in Fig. 4A, GST–Rsp5 bound a large fraction of the input conjugates from the ubp2Δ cell extract, but not from any of the control extracts. This further substantiates that the conjugates that accumulated in the ubp2Δ mutant were likely to be a direct result of Rsp5-catalyzed ubiquitination.

Interestingly, GST–Ubp2 also bound to a significant fraction of conjugates in the extract from ubp2Δ cells (Fig. 4, B and C), whereas GST–Ubp6 or GST–E6AP (a human HECT E3) did not (Fig. 4C). Because GST–Ubp2 can bind to Rup1 and Rsp5 that are present in cell extract (21), it is possible that the binding of GST–Ubp2 to conjugates was indirect and through Rup1 and Rsp5. This appears likely because association of the conjugates with GST–Ubp2 significantly decreased in the ubp2Δ/rup1Δ mutant (Fig. 4B). However, a fraction of the conjugates was still bound in the absence of Rup1, suggesting that Ubp2 may be able to recognize some of its targets directly. This is consistent with the fact that Ubp2 can deubiquitinate Rsp5 substrates in vitro in the absence of Rup1, albeit with reduced efficiency (21).

Rsp5 and Ubp2 Regulate Cell Wall Homeostasis—The temperature sensitivity of rps5 mutants can be partially rescued by sorbitol, an osmotic stabilizer (34, 21), and a recent report also indicated that Rsp5 affects cell wall integrity (13). An assay that reflects the effect of rps5 mutations on cell wall integrity is sensitivity to CFW, a chitin-binding molecule. As shown in Fig. 5A, the rps5-1 mutant was sensitive to CFW at 30 °C. According to the model that Rup1 and Ubp2 functionally antagonize Rsp5, we predicted that ubp2 or rup1 mutations would lead to increased Rsp5 activity and therefore rescue CFW sensitivity of the rps5-1 mutant. This was the case (Fig. 5A) indicating that Rup1/Ubp2 activity and Rsp5 activities are balanced, in part, to achieve cell wall homeostasis. As with the ADCB sensitivity, CFW sensitivity of the rps5-1 strain was also linked to the formation of Lys^{63}-polyubiquitin conjugates. Overexpression of wild type ubiquitin suppressed the CFW sensitivity, whereas overexpression of K63R ubiquitin did not and in fact led to increased CFW sensitivity (Fig. 5B). The K63R-only ubiquitin strain (K63R ubiquitin as the sole source of ubiquitin) itself was also hypersensitive to CFW compared with the equivalent wild type ubiquitin strain (Fig. 5C), further indicating that formation of Lys^{63}-polyubiquitin conjugates by Rsp5 was required for cell wall biogenesis and/or integrity.

Identification of Two Substrates Regulated by Rsp5 and Ubp2—It is not known whether all targets of Rsp5, or only a subset, are subject to potential regulation by Ubp2. The results shown in Fig. 4 suggested that we could identify the Ubp2-responsive substrates of Rsp5 by mass spectrometry-based identification of the proteins in the GST–Rsp5 pulldown from ubp2Δ cell extract. For this purpose, we expressed amino-terminally TAP-tagged ubiquitin in ubp2Δ cells (35). Total TAP-ubiquitin conjugates were isolated on IgG-Sepharose and analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody. B, cell extracts were prepared from the indicated strains, and binding of conjugates to GST–Ubp2 was analyzed as in A. C, cell extracts from ubp2Δ cells (YK009) were incubated with GST–Rsp5, GST–Ubp2, GST–E6AP, and GST–Ubp6 proteins immobilized on glutathione–Sepharose and analyzed as in A.
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**FIGURE 5.** Ubp2/Rup1 modulates effects of Rsp5 on cell wall homeostasis. 
A, deletion of ubp2 or rup1 rescues CFW sensitivity of the rsp5-1 strain. RSP5 (FY36), rsp5-1 (FW1808), rsp5-1/ubp2Δ (YK003), and rsp5-1/rup1Δ (YK004) cells were 10-fold serially diluted and plated on either YPD or YPD plates containing 5 μg/ml CFW. Cells were grown at 33 °C for 4 days. B, overexpression of wild type ubiquitin, but not K63R ubiquitin, rescues CFW sensitivity of SUB493 were 10-fold serially diluted and plated on YPD plates with or without 5 μg/ml CFW and grown at 30 °C for 2 days. C, Lys$^{63}$-linked polyubiquitination is required for resistance to CFW. Wild type ubiquitin (SUB492) and K63R-only ubiquitin (SUB493) were 10-fold serially diluted and plated on either YPD or YPD containing 7 μg/ml CFW and grown at 30 °C for 3 days.

ubiquitin conjugates could be efficiently purified on IgG-Sepharose (supplemental data). To exclude the possibility that TAP-ubiquitin was merely serving as an acceptor for endogenous ubiquitin, thereby forming free polyubiquitin conjugates, TAP-ubiquitin was expressed in which the terminal diglycine residues were altered to alanine residues (TAP-Ub- AA$_{75-76}$). No significant accumulation or purification of ubiquitin conjugates was detected with the AA75–76 mutant, (FY56), rsp5-1/rup1Δ (YK004) cells were 6-fold serially diluted and plated on either YPD or YPD plates containing 5 μg/ml CFW. Cells were grown at 33 °C for 4 days. A, deletion of ubp2 or rup1 rescues CFW sensitivity of the rsp5-1 strain. RSP5 (FY36), rsp5-1 (FW1808), rsp5-1/ubp2Δ (YK003), and rsp5-1/rup1Δ (YK004) cells were 10-fold serially diluted and plated on either YPD or YPD plates containing 5 μg/ml CFW. Cells were grown at 33 °C for 4 days. B, overexpression of wild type ubiquitin, but not K63R ubiquitin, rescues CFW sensitivity of SUB493 were 10-fold serially diluted and plated on YPD plates with or without 5 μg/ml CFW and grown at 30 °C for 2 days. C, Lys$^{63}$-linked polyubiquitination is required for resistance to CFW. Wild type ubiquitin (SUB492) and K63R-only ubiquitin (SUB493) were 10-fold serially diluted and plated on either YPD or YPD containing 7 μg/ml CFW and grown at 30 °C for 3 days.

Rsp5 and Ubp2 Modulate Lys$^{63}$-Ubiquitin Conjugates

**FIGURE 6.** Csr2 and Ecm21 are substrates of Rsp5 and Ubp2. A, in vitro translated $^{35}$S-labeled Csr2 or Ecm21 was incubated with GST-Rsp5 immobilized on glutathione-Sepharose. Bound proteins were detected by SDS-PAGE and autoradiography. In vitro translated p53 was used as a negative control. B, in vitro translated $^{35}$S-labeled Csr2 or Ecm21 was incubated with E1, E2, Ub, and ATP in the presence or absence of Rsp5 as described previously (21). Reactions were stopped after 30 min, and the products were analyzed on SDS-PAGE and autoradiography. C, Rsp5-catalyzed ubiquitination reactions were carried out as in B, except that the endogenous ubiquitin present in the translated reactions was first removed by anion exchange chromatography. The reactions were then performed in the absence of added ubiquitin (lane 2) or the presence of wild type ubiquitin, Lys$^{63}$ ubiquitin, Lys$^{48}$-only ubiquitin, and Lys$^{63}$-only ubiquitin (lanes 3–6). Products were analyzed as in B. D, Csr2 and Ecm21 were ubiquitinated by Rsp5 as in B, and after 30 min, Ubp2 and Rup1 were added to the reactions followed by an additional 30-min incubation.
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might be unable to cleave the most proximal ubiquitin moieties from these substrates.

**DISCUSSION**

We have shown here that Ubp2 modulates Rsp5-dependent Lys\(^{63}\)-linked polyubiquitination in vitro. To our knowledge, the only *S. cerevisiae* enzymes other than Rsp5 that are known to be dedicated to the generation of Lys\(^{63}\)-linked polyubiquitin chains in *S. cerevisiae* are the Mms2/Ubc13 E2 enzyme complex, which function in the Rad6-dependent DNA damage tolerance pathway (26). Whereas other DUBs in yeast are likely to have activity against Lys\(^{63}\)-linked chains, Ubp2 is the only yeast deubiquitinating enzyme that is known to have a strong preference for disassembly of Lys\(^{63}\)-linked conjugates over Lys\(^{48}\)-linked conjugates, both in vitro and in vivo. Although the substrate selectivity of Mms2/Ubc13 appears to be very restricted, the dramatic effect of the ubp2Δ mutation on accumulation of total Lys\(^{63}\)-linked conjugates, along with the fact that Rsp5 has a broad range of target proteins, suggests that Rsp5 and Ubp2 may modulate a significant fraction of total Lys\(^{63}\) conjugation activity in yeast. Although Rsp5 is the only HECT E3 shown to preferentially catalyze Lys\(^{63}\) polyubiquitin linkages, the determinants that confer this specificity, relative to Lys\(^{48}\)-specific HECT E3s (e.g. human E6AP), remain to be identified. The strong similarity of Rsp5 to the Nedd4 family of mammalian HECT E3 suggests that these enzymes might also preferentially catalyze Lys\(^{63}\)-linked polyubiquitination.

A previous report showed that an *rsp5* mutant exhibits increased cell wall chitin levels and increased sensitivity to CFW (13) and the *rsp5-1* mutant is shown here to be hypersensitive to CFW. A reflection of a potential cell wall defect of the *rsp5-1* strain was our previous observation that the temperature sensitivity of the *rsp5-1* strain could be rescued by sorbitol, an osmotic stabilizer (21). The fact that both *ubp2* and *rup1* mutations rescued the CFW sensitivity of the *rsp5-1* mutant is consistent with our previous report that the Rup1-Ubp2 complex antagonizes the function of Rsp5. We isolated two closely related but largely uncharacterized proteins, Csr2 and Ecm21, as substrates of Rsp5, both of which appear related to cell wall integrity. Csr2 is isolated as a multiplicity suppressor of a *chsgspa2* mutant, which exhibits aberrant cell wall structure and a defect in polarized cell growth (39). The *ecm21Δ* mutant is shown to be synthetically lethal with *chs1Δ* and *chs5Δ* (40), which are essential for chitin synthesis, suggesting roles for Csr2 and Ecm21 in cell wall integrity. Neither *ecm21Δ* or *csr2Δ* mutations lead to either enhanced sensitivity or resistance to CFW, although deletion of either partially rescued the CFW sensitivity of *rsp5-1* (not shown), suggesting possible roles of these proteins in Rsp5-mediated cell wall homeostasis.

Although both Csr2 and Ecm21 are previously shown to be ubiquitinated in vivo in large-scale ubiquitin proteomic projects (36–38), specific E3 enzymes responsible for their modification are not known. Csr2 and Ecm21 were both efficiently ubiquitinated via Lys\(^{63}\) linkages by Rsp5 in vitro, consistent with the fact that 1) the CFW-sensitive phenotype of *rsp5-1* strain was rescued by overexpressing wild type ubiquitin, but not K63R ubiquitin, and 2) a strain expressing K63R-only ubiquitin as the sole source of ubiquitin was also sensitive to CFW.

Therefore, we propose that Rsp5-catalyzed Lys\(^{63}\)-ubiquitination of specific target proteins, including but perhaps not limited to Csr2 and Ecm21, promotes cell wall biogenesis and/or stability. The precise biochemical function of Csr2 and Ecm21 and how this affects cell wall homeostasis is so far unknown.

Lys\(^{63}\) linkages are known to be involved in at least some Rsp5-mediated processes, including endocytosis of plasma membrane proteins such as Gap1 and Fur4 (6, 7) and regulation of mitochondrial distribution (12). There is no direct evidence that Lys\(^{63}\) linkages are involved in posttranslational modification of target proteins. It was previously proposed that Spt23 is monoubiquitinylated by Rsp5 (41). Although the vast majority of polyubiquitin chains that accumulated in the *ubp2Δ* cells were Lys\(^{63}\)-linked, a small increase in total conjugates seen in the *ubp2Δ*/*K63R* suggested that Ubp2 might be capable of catalyzing deconjugation of other types of chains or possibly removing monoubiquitinylated linkages from target proteins. With regard to the latter, Ubp2 can reverse the in vitro ubiquitination of certain Rsp5 substrates completely, generating the unmodified target protein (21). In other cases, as with Csr2 and Ecm21, Ubp2 appears to trim back the chain nearly completely, but the fully unmodified target protein is not regenerated. This suggests a possible cooperative function of Ubp2 in promoting monoubiquitination by Rsp5 for some substrates, where Rsp5 first catalyzes polyubiquitination of a substrate, with Ubp2 generating a monoubiquitinated form of the protein by disassembly of the chain but leaving the proximal ubiquitin in place. This might explain how Rsp5, which appears to be a very processive enzyme in vitro, can generate monoubiquitinylated proteins in vivo such as monoubiquitinylated Spt23, Rvs167, or Vps9 (41–43).

Unlike the well characterized role of Lys\(^{48}\) linkages in targeting proteins to the 26 S proteasome, the biochemical function of the Lys\(^{63}\) linkages is unclear. The function is generally considered to be non-proteolytic, with the best-characterized examples being in the NF\(\kappa\)B signaling pathway, DNA repair pathway, and ubiquitin-mediated endocytosis. It has been suggested that Lys\(^{63}\)-linked conjugation of certain substrate can be recognized by the proteasome in vitro (29); however, the fact that Lys\(^{63}\) conjugates accumulate in *ubp2Δ* cells suggests that at least the bulk of Lys\(^{63}\)-conjugated Rsp5 substrates are not shunted to the proteasome. The structure of Lys\(^{63}\)-linked polyubiquitin chains is likely to be distinct from Lys\(^{48}\) chains (44), consistent with distinct functional roles of the chain types. The identification of Rsp5 and Rup1/Ubp2 as a group of enzymes that specifically modulate Lys\(^{63}\) chain formation in vivo will allow further exploration of the precise biochemical function of this polymeric protein modifier.

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