The HECT E3 ubiquitin ligase Rsp5 is important for ubiquitin homeostasis in yeast

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Abstract The HECT E3 ubiquitin ligase Rsp5, a yeast member of the Nedd4 family, has been implicated in many different aspects of cell physiology. Here, we present evidence that Rsp5 function is important for ubiquitin homeostasis. Several observations suggest that ubiquitin is limiting in the *rsp5-1* mutant. Reduced synthesis of ubiquitin appears to contribute to ubiquitin depletion. A transient inhibition of general protein synthesis is observed in a wildtype strain upon heat-shock. While the wildtype cells quickly recover from this transient arrest, the *rsp5-1* cells remain arrested. This suggests that Rsp5 is important for recovery from heat-induced protein synthesis arrest. Our results suggest that *rsp5* phenotypes should be interpreted with caution, since some of the phenotypes could be simply the result of ubiquitin limitation.

Keywords: Heat-shock; Stress response; *UBI4*; Protein turnover

1. Introduction

The Rsp5 protein of the yeast *Saccharomyces cerevisiae* is a member of the HECT (homology to E6-AP carboxyl terminus) E3 family of ubiquitin ligases [1]. It is an essential protein under standard growth conditions. The essential function of Rsp5 at ambient growth temperature is related to the regulation of fatty acid synthesis [2]. In addition, Rsp5 has been implicated in many other aspects of cell physiology. Several reports suggest that Rsp5 is involved in transcriptional regulation [1]. Consistent with a role in transcription, it has been demonstrated that Rsp5 binds to the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Rbp1) [3] and mediates its ubiquitination [4]. Recent findings suggest that Rsp5 is required for mRNA export from the nucleus further point to a global role of Rsp5 in gene expression [5,6].

Besides its nuclear roles, several cytoplasmic functions have been ascribed to Rsp5. It has been reported that Rsp5 is important for mitochondrial inheritance [7]. Also, Rsp5 appears to play a role in endocytosis of plasma membrane proteins [8]. In addition, Rsp5 appears to be involved in the ubiquitination of biosynthetic cargo that enters the endocytic pathway at the level of endosomes [9,10]. Furthermore, Rsp5 appears to exert a function at endosomes that is independent of cargo ubiquitination. Rsp5 could affect endocytosis by interfering with the organization of the actin cytoskeleton. A link between Rsp5 and the actin cytoskeleton is suggested by genetic interactions between *rsp5* and several mutants affected in genes encoding actin cytoskeletal components and by the demonstration of direct physical interactions between Rsp5 and components of the actin cytoskeleton [11,12].

Here, the stress response in the *rsp5-1* mutant was examined more closely. We obtained evidence that ubiquitin is limiting in the *rsp5-1* mutant under stress conditions. Reduced ubiquitin synthesis appears to contribute to the ubiquitin deficiency in the *rsp5-1* mutant. Our results suggest that one has to be cautious about the interpretation of *rsp5* phenotypes, since some phenotypes could simply be the result of ubiquitin limitation.

2. Materials and methods

2.1. Strains, plasmids and materials

Standard techniques and media were used for the growth of yeast strains [13]. The yeast strains used are listed in Table 1. The *rsp5-1* allele was obtained from FW1808 (originally from Fred Winston, Harvard Medical School). To construct the *UBI4p-lacZ* plasmid pRK969, a *UBI4* promoter fragment (710 bp upstream of *ATG* codon) was amplified by PCR and inserted into the *lacZ* fusion vector YEp356 [14] cut with *Sal*I and *Hin*dIII. In plasmid pRK813, a 3.7 kb PCR-fragment containing the *RSP5* ORF was inserted into YCplac33 cut with *Eco*RI and *Hin*dIII. Plasmid YEp96 [15] contains a ubiquitin gene under the control of the *CUP1* promoter. Ubiquitin expression was induced by the addition of 0.5 mM $CuSO_4$ to the growth medium 3 h before extract preparation. For growth on solid medium, 0.5 mM $CuSO_4$ was added to the agar plate. As vector controls, plasmids YEplac112 and YEplac195 [16] were used. The monoclonal anti-ubiquitin antibody (P4D1) was obtained from Covance.

2.2. LacZ assay

Cells were grown overnight in SD/CAS medium at 25 °C to exponential phase. 10 OD_{600} units (5×10^8 cells) were harvested and resuspended in fresh medium to a density of $OD_{600} = 0.5$. Then, the cells were shifted to 37 °C. At time intervals, 2 OD_{600} aliquots of cells were harvested and washed once in ice-cold Z-buffer (0.1 M Na-phosphate, 10 mM KCl, and 1 mM $MgSO_4$, pH 7.0). The cells were resuspended in 100 μl Z-buffer and lysed by agitation with 400 mg of glass beads for 3 min. After addition of another 100 μl Z-buffer, the cell extract was removed from the glass beads and spun for 2 min at $13\,000 \times g$ to remove cell debris. A suitable amount of the cleared cell extract (10–50 μl) was added to 1 ml of Z-buffer containing 1 mg/ml ONPG (2-nitrophenyl-β-D-galactopyranoside) and incubated at 30 °C until a yellow color developed. The reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3 and the absorbance was measured at 420 nm. Protein concentration was determined by a Bradford assay.

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Table 1
Yeast strains

Strain	Genotype	Reference
FW1808	*MATα his4-912ΔR5 lys2-128 Δura3-52 rsp5-1*	F. Winston
JD52	*MATa his3-Δ200 leu2-3 112 lys2-801 trp1-Δ63 ura3-52*	J. Dohmen
JD116	*MATa* his3-*Δ200 leu2-3 112 lys2-801 trp1-Δ63 ura3-52 Δdoa4::LEU2*	J. Dohmen
RKY1724	*MATα his3-Δ200 lys2 trp1-Δ63 ura3-52 rsp5-1*	This study
RKY1734	*MATa* his3-*Δ200 leu2-3 112 lys2 trp1-Δ63 ura3-52 rsp5-1*	This study

2.3. Metabolic labeling

Cells were grown overnight at 25 °C to exponential phase in minimal medium (SD) with required nutrients. 6 OD_{600} units (3×10^8 cells) were harvested and resuspended in fresh medium to a density of $OD_{600} = 1$. The cells were shifted to 37 °C and after different time periods at 37 °C, 1 ml aliquots of cells were labeled with 7 μCi of Tran ^{35}S-label (ICN) for 10 min. Then, the cells were harvested, washed once in 1 ml cold 10 mM NaN_3, to remove unincorporated label, and lysed by agitation with 400 mg glass beads for 3 min in 110 μl of lysis buffer (0.3 M sorbitol, 50 mM MOPS, pH 7.5, and 10 mM NaN_3 + protease inhibitors). To determine the total amount of label taken up by the cells, a 10 μl aliquot was removed from the cell extract and analyzed by liquid szintillation counting. To determine the amount of label incorporated into protein, another 10 μl aliquot was added to 1 ml of 10% TCA and kept on ice for 1–2 h. Then, the samples were collected by vacuum filtration over glass fiber filters and washed twice with 10 ml 10% TCA. Finally, the filters were analyzed by liquid szintillation counting.

3. Results

3.1. Lack of accumulation of high-molecular weight ubiquitin-conjugates in the rsp5-1 mutant upon heat-shock

In the course of our studies on the role of Rsp5 in protein trafficking, we examined the effect of the conditional *rsp5-1* mutant on the overall pattern of ubiquitinated proteins in yeast cell extracts. Yeast cells carrying the *rsp5-1* mutation grow fairly normal at low temperature (25 °C), but stop growth after transfer to high temperature (37 °C). When wildtype cells, grown at 25 °C, were heat-shocked by a shift to 37 °C, an accumulation of high-molecular weight ubiquitin-conjugates in the range of 80–200 kDa was observed by Western blotting with anti-ubiquitin antibodies (Fig. 1A). The amount of ubiquitin-conjugates steadily increased with time, reached its maximum at about 1 h and declined to pre-shift levels at later time points (not shown). Strikingly, this accumulation of ubiquitin-conjugates was not observed in the *rsp5-1* mutant. When the same experiment was performed with strains overexpressing ubiquitin from the plasmid YEp96, accumulation of conjugates could partially be restored in the *rsp5-1* mutant. The wildtype pattern was restored completely by transformation of the *rsp5-1* strain with a plasmid carrying the wildtype *RSP5* gene (Fig. 1B).

These experiments suggest that the lack of accumulation of high-molecular weight ubiquitin-conjugates in the *rsp5-1* mutant after heat-shock could be the result of an ubiquitin deficiency. This ubiquitin-deficiency could be linked to the temperature-sensitivity of the *rsp5-1* mutant. We, therefore, tested whether overexpression of ubiquitin is able to rescue the temperature-sensitivity of the *rsp5-1* mutant. As can be seen in Fig. 2, *rsp5-1* cells overexpressing ubiquitin from the multicopy plasmid YEp96 were indeed able to grow at high temperature, albeit more slowly than wildtype. The control strain carrying an empty vector was not able to grow at 37 °C. This is in line

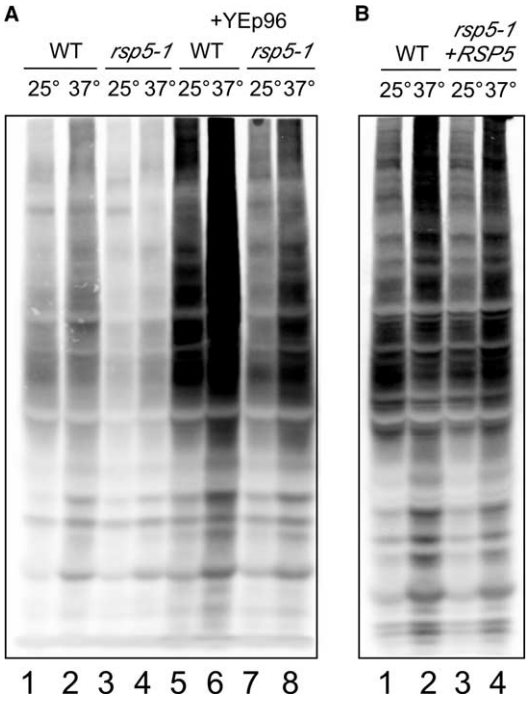

Fig. 1. Accumulation of high-molecular weight ubiquitin-conjugates after heat-shock. Cells were grown in SD/CAS medium to exponential phase at 25 °C. Cell extracts were prepared from cells grown at 25 °C (lanes 1, 3, 5, and 7) or from cells shifted to 37 °C for 1 h before extract preparation (lanes 2, 4, 6, and 8). Equal amounts of protein were loaded in each lane. Cell extracts were analyzed by Western blotting for the presence of ubiquitin with anti-ubiquitin antibodies. (A) JD52 (wt, lanes 1, 2, 5, and 6) and RKY1734 (*rsp5-1*, lanes 3, 4, 7, and 8) transformed with the vector plasmid YEplac112 (lanes 1–4) or the ubiquitin overexpression plasmid YEp96 (lanes 5–8); (B) JD52 (wt, lanes 1 and 2) transformed with the vector plasmid YEplac195 and RKY1734 (*rsp5-1*, lanes 3 and 4) transformed with pRK813 (*RSP5*).

with a previous report demonstrating, suppression of another *RSP5* mutation (*mdp1-1*) by overexpression of ubiquitin [17]. The defect of the *rsp5-1* strain is caused by a single amino acid exchange in the HECT-domain of Rsp5 (L733S) [18]. We were interested to know whether overexpression of ubiquitin can also compensate for the complete loss of the *RSP5* gene. Since *RSP5* deletion strains are non-viable at all temperatures on standard growth media, this issue was investigated by tetrad analysis. A diploid yeast strain heterozygous for the *RSP5* deletion was transformed with the ubiquitin overexpressing plasmid YEp96. After sporulation, tetrads were dissected and examined for growth on rich medium. A 2:2 segregation of viable to non-viable spores was observed (not shown). Analysis of the genetic markers demonstrated that all viable spores carried the wildtype *RSP5* allele. No viable Δ*rsp5* spores were recovered. Since some of the Δ*rsp5* spores should have carried

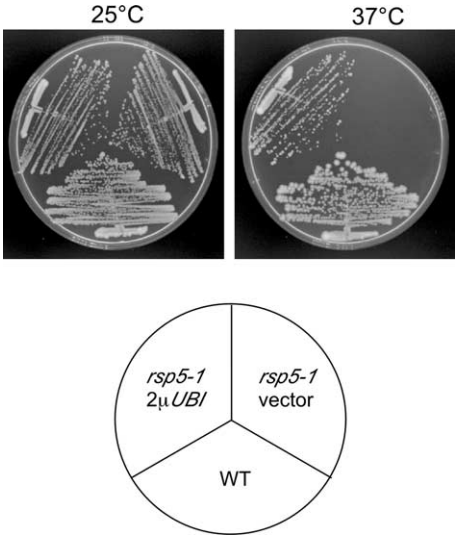

Fig. 2. Suppression of *rsp5-1* ts phenotype by ubiquitin overexpression. The wildtype strain JD52 or the *rsp5-1* strain RKY1734 transformed with the 2μ-*UBI* plasmid YEp96 or with the vector YEplac112 was plated on SD/CAS plates with 0.5 mM $CuSO_4$ and incubated at 25 or 37 °C for 3 days.

the ubiquitin plasmid, this demonstrates that overexpression of ubiquitin is not able to compensate for a complete loss of *RSP5*. Thus, apparently the protein encoded by the *rsp5-1* allele retains some residual function.

3.2. Altered ubiquitin homeostasis in the rsp5-1 mutant

Our experiments suggest that ubiquitin is limiting in the *rsp5-1* mutant at high temperature. There are two possible explanations for this ubiquitin deficiency: either there is an enhanced demand for ubiquitin in the *rsp5-1* strain, e.g., to compensate for a partially defective ubiquitin ligase activity of the Rsp5-1 protein, or the free ubiquitin level is lower than normal. To distinguish between these possibilities, free ubiquitin levels were examined in *rsp5-1* and wildtype strains. Cell extracts were separated on Tricine gels [19] and the level of free ubiquitin was determined by Western blotting with anti-ubiquitin antibodies (Fig. 3). At 25 °C, the free ubiquitin level in the *rsp5-1* mutant was comparable to wildtype. However, in the cells that were shifted to 37 °C for 1 h before extract

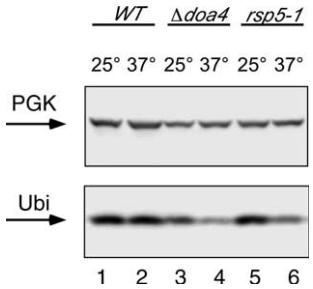

Fig. 3. Lowered free ubiquitin levels in *rsp5-1*. The strains JD52 (wildtype), JD116 (Δ*doa*4) and RKY1734 (*rsp5-1*) were grown at 25 °C to exponential phase in YPD medium. Cell extracts, prepared from cells grown at 25 °C (lanes 1, 3, and 5) and from cells shifted to 37 °C for 1 h (lanes 2, 4, and 6), were separated on a Tricine gel [19] and analyzed by Western blotting with anti-PGK antibodies (upper panel) or anti-ubiquitin antibodies (lower panel).

preparation, the ubiquitin level was reduced to about half of the wildtype level. For comparison, the Δ*doa*4 mutant was included. It has been reported previously that mutants defective for the deubiquitinating enzyme Doa4 display lower free ubiquitin levels presumably because ubiquitin is degraded along with its substrate proteins [20]. As can be seen in Fig. 3, the *rsp5-1* and Δ*doa*4 mutants show a similar lowered ubiquitin level after a 1 h shift to 37 °C. As a loading control, the blots were probed with antibodies against the glycolytic enzyme phosphoglycerate kinase (PGK). The intensity of the PGK bands was comparable in all lanes.

3.3. Defective recovery of protein synthesis after heat-shock in the rsp5-1 mutant

Under stress-conditions, like heat-shock, the *UBI4* gene, which contains five repeats of the ubiquitin-coding region, is upregulated [21]. Failure to respond properly to stress conditions could contribute to the observed ubiquitin deficiency of the *rsp5-1* mutant. We, therefore, examined whether the *UBI4* promoter is properly regulated upon heat-shock in the *rsp5-1* mutant. To facilitate detection of *UBI4* promoter activity, the *UBI4* promoter was cloned in front of the β-galactosidase (*lacZ*) gene of *Escherichia coli* on a single copy plasmid. This plasmid was introduced into wildtype and *rsp5-1* strains. Cells were pregrown at 25 °C to exponential phase and shifted to 37 °C. At different time points after the shift to 37 °C, cell extracts were prepared from a constant culture volume and the LacZ activity was determined from these cell extracts. Although the specific activity (U/mg protein) was somewhat lower in the *rsp5-1* strain compared to wildtype, the *UBI4* promoter was induced about 2-fold upon heat-shock in both strains (Fig. 4A). This indicates that heat-shock signaling is not affected in the *rsp5-1* mutant and that the *UBI4* promoter responds normally to heat-shock. A similar result was obtained with another stress-induced promoter, the *HSP26* promoter (not shown). However, a different picture was obtained when the volume activities (U/ml extract) were compared (Fig. 4B). Here, the LacZ activity increases about 4-fold in the wildtype strain during the time course of the experiment (120 min), while the activity of the *rsp5-1* mutant stays nearly constant. It becomes clear from Fig. 4C that this discrepancy is due to a lack of protein accumulation in the *rsp5-1* strain. While the protein concentration (mg/ml extract) continuously increases in the wildtype strain as the cells grow, it stays nearly constant in the *rsp5-1* mutant. This indicates that protein synthesis is inhibited in the *rsp5-1* mutant after shift to 37 °C. This block in protein synthesis does not appear to be a secondary consequence of the *rsp5-1* defect, since it occurs immediately after shift to 37 °C. Also, the *rsp5-1* cells stay viable during the time course of the experiment. When the cells were plated at 25 °C after 120 min at 37 °C, no difference in viability could be detected between wildtype and *rsp5-1* cells (not shown).

The capacity to synthesize proteins at 37 °C was examined more closely by metabolic labeling. Cells were incubated at 37 °C for varying time periods and were subsequently labeled with [^{35}S]methionine for 10 min. To determine incorporation of radioactive methionine into protein, proteins were TCA-precipitated from cell extracts, collected onto glass-fiber filters and counted in a szintillation counter. When the experiment was performed with the wildtype strain, a drop in protein synthesis rate was observed after 20 min at 37 °C, down to 50–70% of the initial rate (Fig. 5). After 40–60 min at 37 °C, the

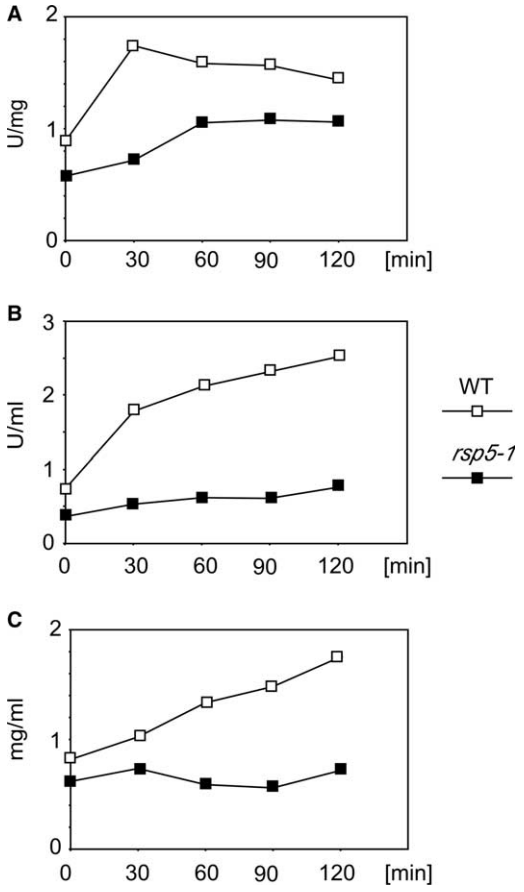

Fig. 4. Heat-induction of the *UBI4* promoter. JD52 (wildtype, open squares) and RKY1734 (*rsp5-1*, filled squares) transformed with the *UBI4p-lacZ* plasmid pRK969 were pre-grown at 25 °C in SD/CAS medium and shifted to 37 °C (t_0). At time intervals after shift to 37 °C, LacZ activity and protein concentration were determined from cell extracts prepared from constant culture volumes. (A) Specific LacZ activity (U/mg), (B) volume acitivity (U/ml), (C) protein concentration (mg/ml).

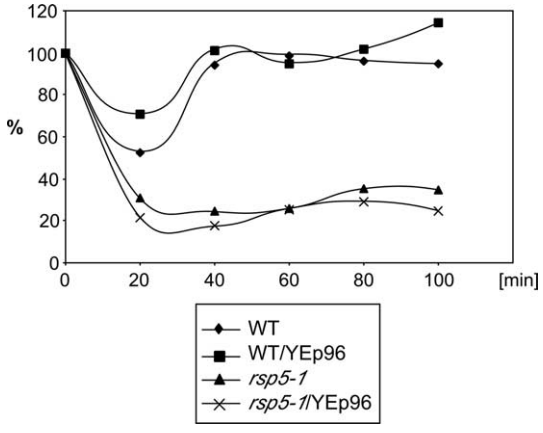

Fig. 5. Heat-induced protein synthesis arrest. JD52 (wildtype) and RKY1734 (*rsp5-1*) transformed with YEp96 (2μ-*UBI*) or with the vector YEplac112 were pre-grown at 25 °C in minimal medium and shifted to 37 °C (t_0). At different time intervals after shift to 37 °C, cells were labeled with Tran ^{35}S-label for 10 min. The amount of label incorporated into protein normalized to the total amount of label taken up by the cells is plotted against the time of preincubation at 37 °C. The protein synthesis rate at t_0 was set to 100%.

wildtype completely recovered from this transient protein synthesis arrest. With the *rsp5-1* strain, a drop in protein synthesis rate was observed as well, however, this drop was more pronounced than in wildtype (down to 10–30% of the initial rate). Furthermore, the cells did not recover from this protein synthesis arrest during the time course of the experiment. Overexpression of ubiquitin from the plasmid YEp96 could not restore protein synthesis in the *rsp5-1* mutant. This experiment suggests that the *rsp5-1* mutant is defective in recovery from the heat-induced protein synthesis arrest. These effects can be observed within 20 min after shift to 37 °C and are thus most likely an immediate consequence of the *rsp5-1* defect.

4. Discussion

We present evidence that the HECT E3 ubiquitin ligase Rsp5 is important for ubiquitin homeostasis. Analysis of the phenotypes of the *rsp5-1* mutant suggests that ubiquitin is limiting in this mutant. Reduced synthesis of ubiquitin seems to contribute to the reduction of free ubiquitin levels in the *rsp5-1* mutant at high temperature. In a wildtype strain, a transient decrease in protein synthesis was observed upon heat-shock. In contrast to wildtype, the *rsp5-1* mutant did not recover from this transient protein synthesis arrest during the time course of the experiment. Under stress conditions, like heat-shock, the cells seem to have a higher demand for ubiquitin, presumably because a higher load of misfolded proteins has to be disposed off by ubiquitin-mediated degradation. This higher demand is met by upregulation of the *UBI4* gene [21]. Although, heat-shock signaling at the *UBI4* promoter appears to be unaffected, the amounts of ubiquitin synthesized after heat-shock are probably insufficient due to the prolonged protein synthesis arrest in the *rsp5-1* mutant.

What could be the reason for this protein synthesis arrest in the *rsp5-1* mutant? Ubiquitination by Rsp5 appears to play a role in DNA damage-induced degradation of Rbp1 [22]. Upon UV-irradiation, or nucleotide starvation, RNA polymerase complexes may become stalled on DNA. It has been proposed that ubiquitination of the large subunit of RNA polymerase II facilitates displacement of stalled RNA polymerase complexes from DNA [23,24]. Perhaps, RNA polymerase complexes are also stalled on DNA upon heat-shock. This would offer an explanation for the requirement of Rsp5 in recovery from heat-induced protein synthesis arrest. But, of course other explanations are possible, like, e.g., direct control of RNA polymerase activity by ubiquitination. Furthermore, it has been reported recently that *rsp5* mutants are defective in the nuclear export of mRNA [5,6]. But, since transcription elongation and nuclear export are tightly coupled [25], it is difficult to say whether the export defect is an immediate or indirect consequence of the *rsp5* defect. In any case, a severe defect in protein synthesis may be anticipated from these findings.

Rsp5 has been implicated in the ubiquitination of many proteins [8]. In many cases, the evidence for an involvement of Rsp5 is solely based on the lack of ubiquitination after prolonged incubation at 37 °C. Here, we show that ubiquitination of at least some proteins can simply be prevented by depletion of ubiquitin, as it occurs in the *rsp5-1* mutant. This illustrates that lack of ubiquitination at 37 °C in the *rsp5-1* mutant cannot be the only criterion to prove a specific role of Rsp5 in the ubiq-

uitination of a given protein. For several proteins, a direct physical interaction with Rsp5 has been demonstrated. In these cases, it is safe to conclude that these are true Rsp5 substrates.

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