Rub1p Processing by Yuh1p Is Required for Wild-Type Levels of Rub1p Conjugation to Cdc53p

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NOTE

Rub1p, like ubiquitin, is conjugated to proteins. Before protein conjugation, the carboxyl-terminal asparagine residue of Rub1p is removed. Rub1p conjugation is dependent on the carboxyl-terminal processing enzyme Yuh1p, whereas Rub1p lacking the asparagine residue is conjugated without Yuh1p. Thus, Yuh1p is the major processing enzyme for Rub1p.

Rub1p is a ubiquitin-like protein conjugated to the cullin Cdc53p in the yeast Saccharomyces cerevisiae (7, 9, 11). Cdc53p is a subunit of the ubiquitin ligases (E3s) known as SCFs (SKP1–Cullin–F-box complexes) (12, 23). SCFs are RING finger-containing complexes necessary for the ubiquitylation of cell cycle regulators and transcription factors (4, 18–20). The SCFs target these substrates for degradation via the 26S proteasome (4, 18–20). Although the function of Rub1p conjugation remains elusive, altered expression of SCF proteins can be lethal in the absence of Rub1p conjugation to Cdc53p (9).

Rub1p and ubiquitin conjugation are enzymatically related. Rub1p is activated by an E1 enzyme composed of a heterodimer of Enr2/Ula1p and Uba3p. Enr2/Ula1p and Uba3p resemble the amino-terminal and carboxyl-terminal portions of the ubiquitin-activating enzyme Uba1p, respectively (9, 11). Rub1p forms a thioester linkage with Uba3p (11). Next, Rub1p is transferred to the Rub1p-conjugating enzyme Ubc12p, a homolog of the ubiquitin-conjugating enzymes (11). Enr2/Ula1p, Uba3p, and Ubc12p are necessary for Rub1p modification of Cdc53p. Yeast cells containing a conditional allele of SKP1 are also deficient for Rub1p conjugation to Cdc53p, even under permissive conditions (9). The Rub1p attachment site on Cdc53p is likely to be lysine 760 based on the attachment site in cullin-2 of NEDD8, the mammalian homolog of Rub1p (21). Removal of a portion of Cdc53p, including lysine 760, does not cause inviability in yeast cells; however, these cells resemble mutants lacking enzymes of the Rub1p pathway (9).

Rub1p is 53% similar to ubiquitin (9). Therefore, we reasoned that the enzymes known as ubiquitin hydrolases might also act on Rub1p. In S. cerevisiae, there are 16 genes that encode such proteins, termed UBP1 through UBP16 (1). Deletion mutants of these genes, except for UBP6 and UBP10, were purchased from Research Genetics (Table 1) and screened for the ability to conjugate Rub1p to Cdc53p. Yeast cells were grown at 30°C on yeast extract-peptone-dextrose medium by standard procedures (2), and cell extracts were prepared as previously described (5, 12). Extracts from cells with mutations in the ubiquitin hydrolases contained Rub1p conjugated to Cdc53p (Fig. 1A and B). Rub1p-Cdc53p conjugates were also detected in cells with mutations in UBP6 (B. Linghu and M. G. Goebl, unpublished observations). Yeast cells also contain Yuh1p, a homolog of the ubiquitin carboxyl-terminal hydrolases which remove small adducts from ubiquitin (10). In cells with mutations in YUH1, Rub1p-Cdc53p conjugates were virtually absent (Fig. 1B). To verify that the loss of Rub1p conjugation to Cdc53p is due to the loss of YUH1, a yuh1Δ strain (R16911) in which YUH1 is replaced by a kanamycin resistance gene was mated to FY23, the YUH1 wild-type strain. This diploid was sporulated, and tetrads were dissected and examined for kanamycin resistance (G418 concentration, 200 µg/ml) and Rub1p conjugation to Cdc53p. Kanamycin-resistant cells derived from isolated spores failed to conjugate Rub1p to Cdc53p (Fig. 1C). These results indicate that the YUH1 gene is critical for Rub1p conjugation to Cdc53p.

In all known ubiquitin-like conjugations, the carboxyl-terminal glycine is attached via an isopeptide linkage to a lysine on a substrate protein (6, 16). However, the initial gene product made from each ubiquitin gene and RUB1 has a carboxyl-terminal extension (14, 15) (Fig. 2). Therefore, removal of these extensions is necessary before ubiquitin or Rub1p is suitable for substrate conjugation.

If the function of Yuh1p were to remove the carboxyl-terminal asparagine of Rub1p, then expression of a RUB1 gene encoding a Rub1p lacking the carboxyl-terminal asparagine would suppress the loss of Rub1p conjugation to Cdc53p in yuh1Δ strains. Therefore, we transformed a yuh1Δ rub1Δ strain with two plasmids, pRub1RGG, which expresses a RUB1 gene encoding a mutant Rub1p lacking the carboxyl-terminal asparagine, and pRub1RGGN, which
expresses wild-type *RUB1* (details of plasmid construction are available upon request) (17). These plasmids are derived from p426ADH and PCR DNA fragments of *RUB1* (13). The *RUB1* gene is expressed from the yeast *ADH1* promoter. Cell extracts were obtained from these transformants and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using anti-HA. Whereas HA-Rub1p-Cdc53p conjugates lacking asparagine were detected regardless of the presence of Yuh1p, wild-type HA-Rub1p was only found conjugated to Cdc53p in the presence of Yuh1p (Fig. 3). While a search was conducted for synthetic interactions between *YUH1* and the SCF genes, none were detected. However, since our initial report of this phenomenon, we have subsequently found the synthetic interactions to vary greatly among strains (M. Goebel and J. Callis, unpublished observations).

A mammalian homolog of Yuh1p, UCH-L3, processes NEDD8 in vitro (22). Our results suggest that a function of UCH-L3 has severe neurological disorders that lead to early death (8). We therefore propose that NEDD8 processing and UCH-L3 have severe neurological disorders that lead to early death (8). We therefore propose that NEDD8 processing

To verify that Rub1p lacking asparagine is attached to Cdc53p in the absence of Yuh1p, we transformed the rub1Δ and rub1Δyuh1Δ strains with plasmids p3HA-Rub1-RGG and p3HA-Rub1-RGGN. These plasmids, derived from p426ADH, again provided the *ADH1* promoter to express *RUB1* encoding a fusion protein of three hemagglutinin (HA) epitopes at the amino terminus of mutant Rub1p lacking asparagine or wild-type Rub1p, respectively. Cell extracts were prepared from the transformants and subjected to SDS-PAGE and Western blot analysis using anti-HA. Whereas HA-Rub1p-Cdc53p conjugates lacking asparagine were detected regardless of the presence of Yuh1p, wild-type HA-Rub1p was only found conjugated to Cdc53p in the presence of Yuh1p (Fig. 3). While a search was conducted for synthetic interactions between *YUH1* and the SCF genes, none were detected. However, since our initial report of this phenomenon, we have subsequently found the synthetic interactions to vary greatly among strains (M. Goebel and J. Callis, unpublished observations).

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| Strain  | Genotype                        | Source                  |
|---------|---------------------------------|-------------------------|
| FY23    | MATa ura3-52 trp1Δδ3 leu2Δ1     | Fred Winston            |
| FY24    | MATa ura3-52 trp1Δδ3 leu2Δ1     | Fred Winston            |
| FY78    | MATa his3ΔΔ200                   | Fred Winston            |
| BL2     | MATα                             | Segregant of FY24 × FY78|
| BY4741  | MATα his3ΔΔ1 leu2Δ0 met15Δ0 ura3Δ0 | Research Genetics       |
| BY4742  | MATα his3ΔΔ1 leu2Δ0 his2Δ0 ura3Δ0 | Research Genetics       |
| BY4743  | BY4741 × BY4742                  | Research Genetics       |
| R4073   | rub1::Km in BY4741               | Research Genetics       |
| R2825   | ura3::Km in BY4741               | Research Genetics       |
| R5214   | ubc12::Km in BY4741              | Research Genetics       |
| R5485   | uba3::Km in BY4741               | Research Genetics       |
| YPH172  | MATa ura3-52 his2-801 ade2-101 trp1Δδ3 his3ΔΔ200 leu2Δ1 skpΔ1::TRP1 skp1-3::LEU2 CFIII(CEN3.L. YPH983) HIS3 SUP11 | Phillip Hieter          |

**TABLE 1. Yeast strains used in this study**

| Strain  | Genotype                        | Source                  |
|---------|---------------------------------|-------------------------|
| R16911  | yuh1::Km in BY4742              | Research Genetics       |
| R33819  | urbp1::Km/urbp1::Km in BY4743   | Research Genetics       |
| R32380  | urbp2::Km/urbp2::Km in BY4743   | Research Genetics       |
| R36148  | urbp3::Km/urbp3::Km in BY4743   | Research Genetics       |
| R34044  | doe4::Km/doe4::Km in BY4743     | Research Genetics       |
| R35842  | urbp5::Km/urbp5::Km in BY4743   | Research Genetics       |
| R32315  | urbp7::Km/urbp7::Km in BY4743   | Research Genetics       |
| R30809  | urbp8::Km/urbp8::Km in BY4743   | Research Genetics       |
| R36404  | urbp9::Km/urbp9::Km in BY4743   | Research Genetics       |
| R36014  | urbp11::Km/urbp11::Km in BY4743 | Research Genetics       |
| R31228  | urbp12::Km/urbp12::Km in BY4743 | Research Genetics       |
| R33093  | urbp13::Km/urbp13::Km in BY4743 | Research Genetics       |
| R33195  | urbp14::Km/urbp14::Km in BY4743 | Research Genetics       |
| R30892  | urbp15::Km/urbp15::Km in BY4743 | Research Genetics       |
| R32755  | urbp16::Km/urbp16::Km in BY4743 | Research Genetics       |
| JL268   | MATα ade2 leu2 his3 ura3 trp1 rub1::TRP1 | Judy Callis          |
| BLA1    | MATα his3 trp1 ura3 leu2 his2   | Segregant of R16911 × FY23 |
| BLA2    | MATα yuh1::Km ura3 leu2 his2   | Segregant of R16911 × FY23 |
| BLA3    | MATα trp1 ura3 leu2             | Segregant of R16911 × FY23 |
| BLA4    | MATα yuh1::Km his3 ura3 leu2   | Segregant of R16911 × FY23 |
| BLB1    | MATα trp1 ura3 leu2 his2        | Segregant of R16911 × FY23 |
| BLB2    | MATα yuh1::Km his3 ura3 leu2   | Segregant of R16911 × FY23 |
| BLB3    | MATα yuh1::Km his3 ura3 leu2 his2 | Segregant of R16911 × FY23 |
| BLB4    | MATα his3 trp1 ura3 leu2        | Segregant of R16911 × FY23 |
| BLC1    | MATα his3 trp1 ura3 leu2 his2   | Segregant of R16911 × FY23 |
| BLC2    | MATα yuh1::Km trp1 ura3 leu2   | Segregant of R16911 × FY23 |
| BLC3    | MATα yuh1::Km ura3 leu2 his2   | Segregant of R16911 × FY23 |
| BLC4    | MATα his3 ura3 leu2             | Segregant of R16911 × FY23 |
| BLF1    | MATα yuh1::Km rub1::TRP1 trp1 ura3 leu2 | Segregant of BLC2 × JL268 |
| BLF7    | MATα yuh1::Km rub1::TRP1 ade2 trp1 ura3 leu2 | Segregant of BLC2 × JL268 |
and cullin modification are critical for the development and function of neural tissue.

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REFERENCES

1. Amerik, A. Y., S. J. Li, and M. Hochstrasser. 2000. Analysis of the deubiquitinating enzymes of the yeast Saccharomyces cerevisiae. Biol. Chem. 381: 981-992.

2. Burke, D., D. Dawson, and T. Stearns. 2000. Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
3. Day, I. N., L. J. Hinks, and R. J. Thompson. 1990. The structure of the human gene encoding protein gene product 9.5 (PGP9.5), a neuron-specific ubiquitin C-terminal hydrolyase. Biochem. J. 268:521–524.

4. Feldman, R. M. R., C. C. Correll, K. B. Kaplan, and R. J. Deshaies. 1997. A complex of Cdc5p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell 91:221–230.

5. Goehl, M. G., L. Goetsch, and B. Byers. 1994. The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. Mol. Cell. Biol. 14:3022–3029.

6. Herskho, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425–479.

7. Hochstrasser, M. 1998. There’s the Rub: a novel ubiquitin-like modification linked to cell cycle regulation. Genes Dev. 12:901–907.

8. Kuribara, L. J., T. Kikuchi, K. Wada, and S. M. Tilghman. 2001. Loss of UCH-L1 and UCH-L3 leads to neurodegeneration, posterior paralysis, and dysphagia. Hum. Mol. Genet. 10:1963–1970.

9. Lauber, D., N. Mathias, J. M. Laplaza, W. Jiang, Y. Liu, J. Callis, M. G. Goehl, and M. Estelle. 1998. Modification of yeast Cdc53p by the ubiquitin-related protein Rub1p affects function of the SCF complex. Genes Dev. 12:914–926.

10. Larsen, C. N., B. A. Krantz, and K. D. Wilkinson. 1998. Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry 37:3358–3368.

11. Liakopoulos, D., G. Doenges, K. Matuschewski, and S. Jentsch. 1998. A novel protein modification pathway related to the ubiquitin system. EMBO J. 17:2208–2214.

12. Mathias, N., S. L. Johnson, M. Winey, A. E. M. Adams, L. Goetsch, J. R. Pringle, B. Byers, and M. G. Goehl. 1996. Cdc53p acts in concert with Cdc1p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins. Mol. Cell. Biol. 16:6634–6643.

13. Mumberg, D., R. Müller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122.

14. Okaynak, E., D. Finley, and A. Varshavsky. 1984. The ubiquitin gene: head-to-tail repeats encoding polyubiquitin precursor protein. Nature 312:663–666.

15. Okaynak, E., D. Finley, M. J. Solomon, and A. Varshavsky. 1987. The yeast ubiquitin genes: a family of natural gene fusions. EMBO J. 6:1429–1439.

16. Pickart, C. M. 2001. Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 70:503–533.

17. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

18. Seol, J. H., R. M. R. Feldman, W. Zachariae, A. Shevchenko, C. C. Correll, S. Lyapina, Y. Chi, M. Galova, J. Claypool, S. Sandmeyer, K. Nasmyth, A. Shevchenko, and R. J. Deshaies. 1999. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. 13:1614–1626.

19. Skowyra, D., K. L. Craig, M. Tyers, S. J. Elledge, and J. W. Harper. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91:209–219.

20. Skowyra, D., D. M. Koepp, T. Kamura, M. N. Conrad, R. C. Conaway, J. W. Conaway, S. J. Elledge, and J. W. Harper. 1999. Reconstitution of G1 cyclin ubiquitination with complexes containing SCF$^{	ext{Erst}}$ and Rbx1. Science 284: 662–665.

21. Wada, H., E. T. H. Yeh, and T. Kamitani. 1999. Identification of NEDD8-conjugation site in human culin 2. Biochem. Biophys. Res. Commun. 257: 100–105.

22. Wada, H., K. Kito, L. S. Caskey, E. T. H. Yeh, and T. Kamitani. 1998. Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochem. Biophys Res. Commun. 251:688–692.

23. Willems, A. R., S. Lanker, E. E. Patton, K. L. Craig, T. F. Nason, N. Mathias, R. Kohayashi, C. Wittenberg, and M. Tyers. 1996. Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell 86:453–463.