Novel Interaction between Apc5p and Rsp5p in an Intracellular Signaling Pathway in *Saccharomyces cerevisiae*

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The ubiquitin-targeting pathway is evolutionarily conserved and critical for many cellular functions. Recently, we discovered a role for two ubiquitin-protein ligases (E3s), Rsp5p and the Apc5p subunit of the anaphase-promoting complex (APC), in mitotic chromatin assembly in *Saccharomyces cerevisiae*. In the present study, we investigated whether Rsp5p and Apc5p interact in an intracellular pathway regulating chromatin remodeling. Our genetic studies strongly suggest that Rsp5p and Apc5p do interact and that Rsp5p acts upstream of Apc5p. Since E3 enzymes typically require the action of a ubiquitin-conjugating enzyme (E2), we screened E2 mutants for chromatin assembly defects, which resulted in the identification of Cdc34p and Ubc7p. Cdc34p is the E2 component of the SCF (Skp1p/Cdc53p/F-box protein). Therefore, we analyzed additional SCF mutants for chromatin assembly defects. Defective chromatin assembly extracts generated from strains harboring a mutation in the Cdc34p SCF subunit or a nondegradable SCF target, Sic1p 

The ubiquitin-targeting pathway is integral to many cellular activities (45). Ubiquitin is covalently attached to target proteins, thus marking them for degradation or modifying their activity. Ubiquitin contains seven lysines upon which polyubiquitin chains can potentially be built (1, 44). The particular lysine linkages utilized determine the fate of a ubiquitinated protein. For example, polyubiquitin chains built through K29 and K48 target proteins for proteasomal degradation (8, 40), whereas polyubiquitin K63 chains are associated with stress resistance, DNA repair, signal transduction, and degradation via the vacuole (1, 12, 16, 51, 52, 58). Three classes of proteins are required for ubiquitination of target proteins (23). First, a ubiquitin-activating enzyme (E1) passes ubiquitin from the E1 active-site cysteine to an active-site cysteine within a ubiquitin-conjugating enzyme (E2) via a transthiolation reaction. Next, the E2 transfers ubiquitin either directly to a lysine residue within a target protein or to the active-site cysteine within a ubiquitin-protein ligase (E3). Finally, the E3 modifies the target protein with ubiquitin. There are multiple E2 and E3 activities within a cell, enabling the control of many cell processes through ubiquitin signaling.

The E2 family consists of at least 13 members in *Saccharomyces cerevisiae* and over 50 in humans (45). They share a core region of homology, which encompasses the active-site cysteine (30). E2s play a role in diverse cellular processes (2, 32, 36, 46), and some E2s have overlapping functions. For example, Ubc4p and Ubc5p are functionally redundant (49) and Ubc6p and Ubc7p physically and functionally interact in endoplasmic reticulum (ER)-associated protein degradation (ERAD) (5, 13). Ubc7p may interact with an additional E2, as Cdc34p and Ubc7p are both involved in resistance to heavy metals (14, 31). However, Cdc34p has no apparent role in ERAD, but rather is the E2 component of the E3 SCF (Skp1p/Cdc53p/F-box protein) (42). Thus, the wide variety of E2 family members ensures that the ubiquitin-targeting pathway can respond to multiple stimuli.

The E3 family of proteins is as structurally diverse as it is functionally (reviewed in references 22, 28, and 55). Typically, E3 enzymes fall into two general classes that are differentiated based on the presence of either a RING (really interesting new gene) finger or HECT (homology to the E6AP C terminus) domain. The RING finger class is broad, as some RING finger enzymes are part of large multisubunit complexes while others function as monomers. The RING finger E3s are found in multiple cellular compartments, and the larger RING E3 complexes, the APC (anaphase-promoting complex) and the SCF, are critical for cell cycle progression. The APC, presumably localized to the nucleus (54), is a 13-subunit complex in *S. cerevisiae* that targets proteins that inhibit sister chromatin separation (Pds1p) and exit from mitosis (Cib2p) for degradation (22). Although much is known regarding APC activity, little is known regarding the function of the individual subunits. Apc2, a conserved member of the Cullin family, and Apc1p, the RING finger component of the APC complex, have been shown to form the catalytic core of the APC in vitro (18, 38, 53). There is also evidence that yeast Apc5p and Apc10p are required for chromatin assembly and...
extended life span (19, 21) and that human Apc5 is associated with ribosomes in an APC-independent manner (34). Furthermore, additional work with multiple model systems has found the likely existence of APC subcomplexes, the significance of which remains largely unknown (4, 9, 25, 57).

The SCF complex was originally described as an E3 for the cyclin-dependent kinase inhibitor Sic1p (50). Progression through the G1/S transition requires the phosphorylation, ubiquitination, and proteasome-dependent degradation of Sic1p (11). The evolutionarily conserved SCF complex contains the Cullin Cdc53p, the RING protein Hrt1p, Skp1p, and an F-box protein, in addition to Cdc34p (55). Within the yeast cell, multiple forms of the SCF exist, defined by the specifically bound F-box protein, that provide a wide diversity of functions in vivo (37).

Progression through the cell cycle requires precise and timely interactions between the APC and the SCF (39). For example, the APC plays a role in blocking SCF activity in G1 by targeting the SCF-associated F-box protein, Skp2, for degradation (3, 61). The APC also targets Hsl1p, a kinase that promotes SCF dependency of Swe1p, for degradation (7). Furthermore, the APC and SCF are linked through RAS/PKA signaling; RAS/PKA inhibits APC activity, while it promotes SCF activity (29). It appears that a complex network of interactions involving multiple E3 activities and signaling pathways is required for successful navigation through the yeast cell cycle.

In contrast to large RING finger complexes, HECT-domain enzymes are generally monomeric (28). In S. cerevisiae, five HECT-domain proteins have been described, with Rsp5p the only one essential for viability (26, 59). Rsp5p encodes an N-terminal C2 domain required for lipid interactions and internal WW domains required for substrate interactions in addition to the catalytic C-terminal HECT domain (24, 48). Rsp5p is involved in many cellular activities ranging from plasma membrane protein turnover and organelle biogenesis to various nuclear functions (24, 48), despite being localized to the plasma membrane and adjacent to vacuoles (15, 60). However, mechanisms involved in promoting Rsp5p-dependent nuclear activity remain largely unknown.

Here, we investigate how different components of the ubiquitin-targeting pathway interact. We demonstrate that Ubc7p, Cdc34p/SCF, the APC, and Rsp5p interact in a signaling pathway that potentially links extracellular signals with chromatin remodeling.

### MATERIALS AND METHODS

#### Yeast strains and methods.

Table 1 lists the yeast strains and plasmids used in this study. The nonphosphorylatable SIC1 mutant strain was generated by gene replacement of wild-type endogenous SIC1 with a linear DNA fragment encoding a SIC1 allele mutated at residues 2, 5, 33, and 76. The SIC1 allele was under the control of the GAL promoter (Pgal) and URA3 was used as a selectable marker in transformations. The plasmid pRS316-gal-Mt-sic (generously provided by D. Stuart, University of Alberta) was digested with EcoRV, the linear DNA fragment encoding pBgalSIC1, and URA3 was gel purified and transformed into wild-type W303 strains. Correct integrations were identified by replica plating transplants that grew on glucose onto galactose-supplemented media to drive the expression of PgalSIC1, causing a G1 arrest. The Cdc34-2 Δubc7::LEU2 strain (YTH1102) was constructed by transforming YTH448 with a PCR fragment generated by using primers 500 bp upstream and downstream of the UBC7 start and stop codons and UBC445 genomic DNA as a template. The Δubc7::LEU2 PCR fragment was also transformed into YTH1 (YTH1099) to construct conjugal partner strains. Correct integrations were confirmed by PCR analysis. Double mutants of apc5C4 and rps5C4 with Δubc7::kanMX6 were generated by crossing YTH1155 and YTH520, respectively, with YTH1504. All other strains were generated as indicated.

Yeast cells were grown in YPD (yeast extract, peptone, dextrose), YAPD (YPD supplemented with 50 μg of adenine/ml), complete medium, SD (synthetic dextrose), SD lacking uracil (SD–Ura), and SD lacking leucine (SD–Leu) according to standard protocols (47). Galactose was supplemented at 2% in place of glucose. Copper sulfate was added at a final concentration of 0.5 mM to induce genes under the control of the CUP1 promoter. Cadmium chloride was used at the concentrations shown to inhibit the growth of sensitive cells. Benomyl was resuspended in 1% ethanol to a final concentration of 20 mg/ml and used at a final concentration of 12.5 μM in vitro chromatin assembly assays were performed according to previously published protocols (19, 20). Spot dilutions were conducted by first concentrating fresh overnight yeast cultures to 2 × 108 cells/ml. A 10-fold serial dilution series was then prepared, and 5 μl of each dilution was spotted onto the appropriate media. The plates were placed in the appropriate incubator for 3 to 8 days. The plates were then scanned with an Epson Perfection 1650 scanner.

#### Genetic techniques.

Standard genetic techniques were used as described previously (47). Double mutants were generated by crossing the appropriate strains, and the correct mutant combinations were determined by scoring the segregation of selectable markers and phenotypes.

#### Standard DNA manipulations.

Escherichia coli strains JM109 and DH10B were used to propagate DNA plasmids. DNA manipulations such as restriction enzyme digestions, DNA minipreps, yeast and E. coli transformations, yeast genomic DNA preparation, and Southern analysis have been described previously (47).

#### Immunoprecipitations and Western analysis.

For immunoprecipitation experiments, cell extracts were prepared from 50 ml of yeast cultures grown to an optical density at 600 nm (OD600) of approximately 0.75, according to published protocols (17). Briefly, extracts were prepared from wild-type cells cotransformed with R12-Myc and either pYEX4T-1 or pYEX-UBC7. A 0.5 mM concentration of Cu(SO4)2 was added 2 h prior to cell harvesting to induce both the R12-Myc and the UBC7-pYEX-based constructs. The cell pellets were resuspended in a 1× volume of N150 buffer (50 mM HEPES [pH 7.8], 5 mM magnesium acetate, 10% glycerol, 0.08 M NaCl, and 60 mM potassium acetate with 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine-HCl, 25 μg of tosylsulfonyl phenylalanyl chloromethyl ketone/ml, 5 μg of leupeptin/ml, 3.5 μg of pepstatin/ml, and 10 μg of aprotonin/ml added fresh). Beads (3 mm) were added, and the suspension was beard beaten once for 20 s at 4°C. The suspension was then centrifuged for 20 min in a cold microcentrifuge, and the pellet was discarded. Glutathione-Sepharose beads were added to 1 mg of extract protein and incubated for 1 h at 4°C. Following the incubation, the beads were pelleted and washed twice in N150. The supernatant served as the input control. Loading buffer was then added to the samples, which were boiled for 5 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with antibodies against the glutathione S-transferase (GST) or Myc epitope according to previously published procedures (17). The primary antibodies were detected by secondary antibodies conjugated to horseradish peroxidase and visualized by ECL. Other Western blotting was performed as previously described (19).

#### RESULTS

The apc5C4 and rps5C4 mutations compromise the ubiquitin pathway. Previously, we demonstrated that increased expression of ubiquitin suppressed apc5C4 and rps5C4 (chromatin assembly) phenotypes (19). To understand how ubiquitin influenced rps5C4 phenotypes, we prepared extracts from wild-type and rps5C4 cells. Ubiquitin protein content was then analyzed using an antibody against ubiquitin (Fig. 1A). The results show that rps5C4 cells grown at the permissive temperature have reduced ubiquitin protein content compared to that of the wild type. Thus, it appears likely that the growth and chromatin assembly defects observed in rps5C4 cells (19) result from depletion of ubiquitin protein content.
TABLE 1. Yeast strains and plasmids used in this study

| Yeast strain or plasmid | Yeast genotype or plasmid markers | Source or reference |
|-------------------------|-----------------------------------|---------------------|
| **Yeast strains**        |                                   |                     |
| S288c background         |                                   |                     |
| YTH5                    | MATa ade2 his3Δ200 lys2Δ201 ura3-52 | 19                  |
| YTH520                  | MATa ade2 his3Δ200 leu2-3,112 ura3-52 lys2 rsp5Δ-5 (8th generation) | 19                  |
| YTH1155                 | MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 (7) ura3-52 apa5Δ::PA::His5* | 19                  |
| YTH1504 (BY4741)        | MATa his3ΔD1 leu2Δ20 Ose1ΔD0 Ura3ΔD0 Δubc7::kanMX6 | W. Xiao              |
| YTH1574                 | MATa ade2 his3Δleu2 lys2Δ201 (7) ura3 apa5Δ::PA::His5* Δubc7::kanMX6 | This study           |
| YTH1603                 | MATa ade2 his3Δleu2 lys2Δ201 (7) ura3 apa5Δ::PA::His5* Δubc7::kanMX6 | This study           |
| YTH1604                 | MATa ade2 his3Δleu2 lys2Δ201 (7) ura3 apa5Δ::PA::His5* Δubc7::kanMX6 | This study           |
| YTH1636                 | MATa ade2 his3Δ200 lys2Δ201 ura3-52 | This study           |
| YTH1637                 | MATa ade2 his3Δ200 lys2Δ201 ura3-52 apa5Δ::PA::His5* | This study           |
| YTH1653                 | MATa ade2 his3Δleu2 lys2Δ201 ura3 Δubc7::kanMX6 | This study           |
| YTH1682                 | MATa ade2 his3Δleu2 lys2Δ201 ura3 Δubc7::kanMX6 | This study           |
| YTH1683                 | MATa ade2 his3Δleu2 lys2Δ201 ura3 apa5Δ::PA::His5* | This study           |
| YTH1684                 | MATa ade2 his3Δleu2 lys2Δ201 ura3 | This study           |
| YTH1685                 | MATa ade2 his3Δleu2 lys2Δ201 ura3 apa5Δ::PA::His5* Δcde3-2 | This study           |
| YTH1696                 | MATa ade2 his3Δ200 lys2Δ201 ura3-52 apa5Δ::PA::His5* | This study           |
| YTH1697                 | MATa ade2 his3Δ200 lys2Δ201 ura3-52 apa5Δ::PA::His5* | This study           |
| **W303 background**     |                                   |                     |
| YTH1                   | MATa ade2 his3 leu2 trp1 ura3 | 19                  |
| YTH448 (DSY 1096)       | MATa bar1 ade1 his2 leu2 trp1 ura3 cdc34-2 | D. Stuart           |
| YTH449 (DSY 1097)       | MATa bar1 ade1 his2 leu2 trp1 ura3 cdc34-2 | D. Stuart           |
| YTH519                 | MATa ade2 his3 leu2 trp1 ura3 apa5Δ::PA::His5* | 19                  |
| YTH601                 | YTH1 pGAL SIC1Δ4mms | This study           |
| YTH606                 | MATa ade2 his3 leu2 trp1 ura3 cdc3-4 (5 backcrosses with YTH1) | This study           |
| YTH1082                | MATa ade2 his3 leu2 trp1 ura3 apa5Δ::PA::His5* cdc3-4 | This study           |
| YTH1096 (DSY1098)       | MATa bar1 ade1 his2 leu2 trp1 ura3 cdc3-4-1 | D. Stuart           |
| YTH1097 (DSY1099)       | MATa bar1 ade1 his2 leu2 trp1 ura3 cdc3-4-1 | D. Stuart           |
| YTH1099                | MATa ade2 his3 leu2 trp1 ura3 Δubc7::LEU2 | This study           |
| YTH1102                | MATa bar1 ade1 his2 leu2 trp1 ura3 cdc3-4-2 Δubc7::LEU2 | This study           |
| YTH1146                | MATa ade2 his3 leu2 trp1 ura3 cdc3-4-2 Δubc7::LEU2 | This study           |
| YTH1468                | MATa ade2 his3 leu2 trp1 ura3 cdc3-4-2 Δubc7::LEU2 | This study           |
| **DF5α background**     |                                   |                     |
| YTH444 (MHY501)         | MATa gol2 his3Δ200 leu2-3,112 lys2-801 trpl-1 ura3-52 | M. Ellison          |
| YTH445 (MHY907)         | MATa his3Δ200 leu2-3,112 lys2-801 trpl-1 ura3-52 Δubc7::LEU2 | M. Ellison          |
| YTH446 (MHY508)         | MATa his3Δ200 leu2-3,112 lys2-801 trpl-1 ura3-52 Δubc4::HIS3 Δubc5::LEU2 | M. Ellison          |
| YTH447 (MHY509)         | MATa his3Δ200 leu2-3,112 lys2-801 trpl-1 ura3-52 Δubc4::HIS3 Δubc5::LEU2 | M. Ellison          |
| MHY601                 | MATa ade2-1 can1-100 his3-11 leu2-3,112 lys2-801 trpl-1 ura3-1 Δubc8::URA3 | M. Ellison          |
| YKE40                  | MATa ade2-1 his3-832 trpl-1 trp1-289 ura3-52 rad5::URA3 (URA3 lost on FOA) | M. Ellison          |
| YL10                  | MATa his3Δa leu2Δ201 trp1Δ63 ura 3-52 cdc34-2 | M. Ellison          |
| BF264-15Du background   |                                   |                     |
| DSY841 (YTH602)         | MATa bar1 ade1 his2 leu2 trp1 ura3ΔΔns cdc4-3 | D. Stuart           |
| DSY842 (YTH603)         | MATa bar1 ade1 his2 leu2 trp1 ura3ΔΔns cdc4-3 | D. Stuart           |
| **Plasmids**            |                                   |                     |
| YC50                   | URA3 CEN-ARS | 19                  |
| pYE4X5-1               | 2μm leu2Δ URA3 CUP1 GST | Clontech            |
| pTH54                 | APC5 (500 bp 5’ and 69 bp 3’ deleted) CEN ARS URA3 | 19                  |
| pES7                  | URA3 2μm CUP1prom UB-MYC | M. Ellison          |
| pTER20                | URA3 2μm CUP1prom UB-MYC (K29R, K48R, K63R) | 1                   |
| pTER46                | URA3 2μm CUP1prom UB-MYC (K29, all other Ks are R) | 1                   |
| pTER48                | URA3 2μm CUP1prom UB-MYC (K48, all other Ks are R) | 1                   |
| pTER49                | URA3 2μm CUP1prom UB-MYC (K63, all other Ks are R) | 1                   |
| pRS306-gal-Mt-SIC1     | PgalSIC1 mutated at residues 2, 5, 33, and 76 (nonphosphorylatable) | D. Stuart           |
| pYE4X4-TCDC34         | GST-DCDC34 | Exclone library (ResGen) |
| pYE4X4-UBC7           | GST-UBC7 | Exclone library (ResGen) |
| R12-Myc               | TRP1 2μm CUP1prom Myc N-terminal fusion | This study           |

*APA, protein A; FOA, fluoroorotic acid.

Next, to elucidate the relationship between apa5Δ4 and ubiquitin, we asked whether specific lysine residues in ubiquitin are required for APC function. For this analysis, the apa5Δ4 strain was transformed with the wild type and mutant ubiquitin constructs where all seven lysines were mutated to arginine, except for one: K29 (KRR), K48 (RRK), or K63 (RRK), which are the three most thoroughly documented polyubiquitin linkages used in S. cerevisiae. As a control, a mutant version of ubiquitin was used that expressed all lysines except K29, K48, and K63 (RRR) (all ubiquitin constructs were kindly supplied by M.
Ellison). The results shown in Fig. 1B illustrate that while wild-type ubiquitin restored growth to apc5CA cells grown at 37°C, apc5CA cells remained temperature sensitive (ts) when expressing the RRR mutant or the empty vector. Thus, the remaining four lysines, K6, K11, K27, and K33, do not contribute to polyubiquitin chain building by the APC. Constructs expressing only K29 were unable to suppress the apc5CA growth defect, whereas K63 was capable of partial growth suppression. The construct expressing only K48 (RKR), on the other hand, restored growth of apc5CA cells at 37°C to the level of wild-type ubiquitin (Fig. 1B). The use of K48 by the APC is therefore necessary and sufficient and is consistent with a role for targeting of proteins for proteasome degradation by the APC (for a review, see reference 43). The ability of K63 linkages to partially restore the apc5CA defect could reflect a role for the APC in functions other than proteasome-dependent degradation.

Rsp5p functions upstream of Apc5p in the same pathway. The combined observations that Rsp5p and Apc5p are both critical for mitotic chromatin assembly and that apc5CA and rsp5CA mutants both compromise ubiquitin targeting prompted us to ask whether Rsp5p and Apc5p do in fact function together. To gather evidence in support of this hypothesis, we generated apc5CA rsp5CA mutants by crossing isogenic apc5CA and rsp5CA strains and surveyed them for defects in various assays.

First, we assayed chromatin assembly in extracts prepared from the double mutant. The assembly efficiency was no worse than that of either single mutant (data not shown). One interpretation of this observation is that Apc5p and Rsp5p function together. To gather evidence in support of this hypothesis, we generated apc5CA rsp5CA mutants by crossing isogenic apc5CA and rsp5CA strains and surveyed them for defects in various assays.

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**FIG. 1.** Defective ubiquitin targeting is a key component of the apc5CA and rsp5CA phenotypes. (A) rsp5CA cells have reduced ubiquitin protein levels. Wild-type (WT) (YTH5) and rsp5CA (YTH520) cells were grown to mid-log phase in YPD medium at 23°C. The cells were harvested, and protein extracts were prepared. Western blotting assays using antibodies against ubiquitin (UB) and actin were performed. (B) The apc5CA ts phenotype is suppressed by increased expression of ubiquitin containing only a single lysine at position 48. apc5CA (YTH1155) cells were transformed with the plasmids shown and grown on SD–Ura plates at the temperatures indicated. Ubiquitin encodes seven lysines that were mutated in different combinations in the plasmids used. Ub, wild-type ubiquitin; RRR, R29, R48, and R63, with all other lysines wild type; K29 (29), arginine substituted at all positions except K29; K48 (48), arginine substituted at all positions except K48; K63 (63), arginine substituted at all positions except K63. YCp50 is the empty vector control, and APC5 is wild-type APC5 cloned into the YCp50 vector. der., derivative. The ubiquitin variants were under the control of the CUP1 promoter, and copper was required to observe the effects shown.
mitotic defect. We determined the cell cycle profiles of \textit{apc5}^{C4}, \textit{rsp5}^{C4}, and \textit{apc5}^{C4} \textit{rsp5}^{C4} cells by fluorescence-activated cell sorting (FACS) analysis of unsynchronized cells grown to early log phase at room temperature or 37°C (Fig. 2A). As previously observed, \textit{apc5}^{C4} cells accumulate with replicated DNA at 37°C (19). On the other hand, \textit{rsp5}^{C4} cells accumulate with unreplicated DNA at 37°C. The combination of the \textit{apc5}^{C4} and \textit{rsp5}^{C4} alleles also resulted in an accumulation of cells with unreplicated DNA at 37°C. Thus, the \textit{rsp5}^{C4} mutation masks the expression of \textit{apc5}^{C4} phenotypes, suggesting that \textit{rsp5}^{C4} creates a block in the pathway prior to reaching Apc5p. The epistatic interaction shown in Fig. 2A is consistent with the idea that Rsp5p functions upstream in a pathway involving Apc5p.

Lastly, we tested the growth characteristics of the strains shown in Fig. 2A in the presence of the microtubule-destabilizing drug benomyl. Growth in the presence of benomyl causes a mitotic delay, as microtubules undergo a dramatic reorganization upon entrance into mitosis, but it was not known how benomyl would influence \textit{apc5}^{C4} and \textit{rsp5}^{C4} phenotypes. The \textit{apc5}^{C4} mutation conferred increased resistance to benomyl (Fig. 2B). Unassembled spindles resulting from destabilized microtubules are recognized by the spindle checkpoint machinery (27). When activated, the spindle checkpoint inhibits APC activity, halting mitotic progression until spindles are properly assembled. Thus, \textit{apc5}^{C4} could confer increased resistance to benomyl by increasing microtubule stability or, indirectly, the increased time spent in G2/M by \textit{apc5}^{C4} mutants is sufficient to overcome the effects of benomyl.

Since we propose that Rsp5p and Apc5p function in the same pathway, we asked whether \textit{rsp5}^{C4} cells also experience increased resistance to benomyl and whether the \textit{rsp5}^{C4} mutation influences the effect of benomyl on the \textit{apc5}^{C4} mutation. Our results demonstrate that \textit{rsp5}^{C4} cells are unaffected by benomyl and that \textit{rsp5}^{C4} suppresses the increased resistance of \textit{apc5}^{C4} cells (Fig. 2B). Thus, a second epistatic interaction of \textit{rsp5}^{C4} over \textit{apc5}^{C4} adds strength to the idea that Rsp5p acts upstream of Apc5p.

**The E2 proteins Ubc7p and Cdc34p are required for in vitro chromatin assembly.** E3 proteins require the prior activity of an E2 protein. Thus, in order to identify E2 proteins required for APC and/or Rsp5p function, we used the in vitro chromatin assembly assay to identify E2 proteins involved in chromatin assembly by screening a bank of known E2 mutants for chromatin assembly defects. Superciling assays performed on extracts prepared from \textit{	extit{ubc8}}/H9004, \textit{	extit{ubc6}}/H9004, and \textit{	extit{ubc8}}/H9004 cells showed competence for chromatin assembly (data not shown). The results for \textit{	extit{ubc8}} suggest that its previously elucidated roles in histone H2B ubiquitination, telomeric gene silencing, and DNA repair (6, 46) do not impinge on chromatin assembly. Likewise, the role Ubc8p plays in histone acetylation (36) does not influence mitotic chromatin assembly. Of the E2 mutants that genetically interact with \textit{rsp5} mutants (\textit{ubc1}, \textit{ubc4}, and \textit{ubc5} mutants; reviewed in references 24 and 48), only the \textit{ubc1} mutant was slightly defective for in vitro chromatin assembly, whereas the strain containing the double \textit{ubc4} \textit{ubc5} disruption was unaffected (Fig. 3A). In contrast, extracts prepared from \textit{ubc7} and \textit{cdc34} cells were defective for in vitro chromatin assembly (Fig. 3A). The results of these experiments confirm and extend the involvement of the ubiquitin system in chromatin assembly and also add regulatory specificity, as only two of the tested E2s were significantly defective.

**Cdc34p acts in the Rsp5p/APC pathway through the SCF.** The chromatin assembly defect in \textit{cdc34} cells is intriguing but is complicated by the fact that Cdc34p is the E2 component of the SCF ubiquitin ligase (2). It is therefore possible that the \textit{cdc34} compromised chromatin assembly reflects a defect associated with the SCF, not with Cdc34p. Thus, it may be that we have discovered a third E3 involved in the Rsp5p/Apc5p pathway and not, in fact, an E2 utilized by the APC or Rsp5p. To clarify this possibility, we prepared extracts from a strain harboring the \textit{cdc53-1} allele, as Cdc53p is the Cullin subunit of
The SCF (42). Extracts prepared from cdc53-1 cells were indeed defective for in vitro chromatin assembly (Fig. 3B), suggesting the involvement of the SCF, rather than an uncharacterized function of Cdc34p.

To confirm that the cdc34-2 and cdc53-1 defects reflect a role for SCF in the Rsp5p/APC pathway, we tested whether degradation of Sic1p, a target of SCF that inhibits cell cycle progression into G1 (10), is required for proper chromatin assembly. Sic1p must be phosphorylated before it can be ubiquitinated and degraded (41). Therefore, we generated a non-phosphorylatable version of endogenous Sic1p that was under control of the SCF (42). Extracts prepared from cdc53-1 cells were indeed defective for in vitro chromatin assembly (Fig. 3B), suggesting the involvement of the SCF, rather than an uncharacterized function of Cdc34p.

The SCF and Rsp5p have antagonistic functions. Our observations suggest that the mitotic chromatin assembly pathway is regulated as early as G1, as the rsp5<sup>C4</sup>, cdc34-2, and cdc53-1 mutants accumulate or arrest in G1 at elevated temperatures. We therefore asked whether rsp5<sup>C4</sup> and SCF mutants genetically interact. We crossed rsp5<sup>C4</sup> into cdc34-2 and cdc53-1 backgrounds to generate double mutants. We then compared the single and double mutants under cdc34-2, cdc53-1, and rsp5<sup>C4</sup> restrictive conditions, such as occur at 14 to 16°C, which is restrictive for cdc34-2 and cdc53-1, and at 37°C, restrictive for all three mutants. At 37°C, the single and double cdc34-1 (Fig. 4A) and cdc34-2 (data not shown) mutants failed to grow. Under cold-sensitive (cs) conditions, however, the rps5<sup>C4</sup> allele was capable of suppressing the cdc34-2 and cdc53-1 cs defects (Fig. 4A). This indicates that Rsp5p activity is inhibitory to SCF function.

Based on the observations that the SCF and Rsp5p function antagonistically, we predicted that cdc34-2 would suppress an rps5<sup>C4</sup> phenotype. Cells expressing a ts allele of rps5 are sensitive to low doses of CdCl<sub>2</sub> (33). We therefore grew single and double cdc34-2 and rps5<sup>C4</sup> mutants on increasing concentrations of CdCl<sub>2</sub> to assess their sensitivity. The cdc34-2 mutant is not sensitive to increasing concentrations of CdCl<sub>2</sub>, and it partially alleviates the CdCl<sub>2</sub> sensitivity conferred by the rps5<sup>C4</sup> allele (Fig. 4B). This is consistent with the hypothesis that Rsp5p and Cdc34p (and most likely the SCF) interact.
antagonistically. At present we have not defined the relationship between sensitivity to CdCl₂ and chromatin assembly. However, it is worth noting that cellular responses to a changing environment require chromatin and transcriptional remodeling.

UbC7p and Rsp5p physically interact. Our experiments suggest that Cdc34p, rather than functioning as an E2 for Rsp5p or the APC, functions instead in the pathway defined by Apc5p/Rsp5p within the confines of the SCF. In comparison, based on localization and phenotype experiments, we propose

FIG. 4. Rsp5p and the SCF act antagonistically. (A) Expression of the *rsp5*<sup>CA</sup> allele in cells defective for SCF function suppresses an SCF cs phenotype. *rsp5*<sup>CA</sup> cells (YTH521) were crossed with *cdc34-2* (YTH448) and *cdc53-1* (YTH1096) cells to generate double mutants. The different double and single mutants were struck onto YPD plates and incubated at the temperatures indicated. After 3 to 10 days, depending on the temperature, the plates were scanned. WT, wild type. (B) The *cdc34-2* allele partially suppresses *rsp5*<sup>CA</sup> CdCl₂ sensitivity. The *cdc34-2* *rsp5*<sup>CA</sup> double mutant was spot diluted onto YPD medium supplemented with increasing concentrations of CdCl₂ and compared to wild-type and single mutant cells. The plates were grown at 23°C for 3 to 4 days, after which they were scanned.
that Ubc7p may function as an E2 for Rsp5p. For example, Ubc7p colocalizes to the ER membrane with Ubc6p (5), Rsp5p is found adjacent to vacuoles (15, 60), and both $\Delta$ubc7 and $\Delta$rsp5 mutations are sensitive to cadmium chloride (31, 33; this work).

To test the hypothesis that Ubc7p acts as an E2 for Rsp5p, we first asked whether Ubc7p and Rsp5p physically interact. Coimmunoprecipitation experiments utilizing GST-tagged Ubc7p and Myc-tagged Rsp5p were performed. The Myc epitope was fused to the N terminus of an Rsp5p version lacking the C2 domain, while maintaining the WW and HECT domains (R1/2-Myc). R1/2-Myc was used as it was hypothesized to bind substrate tighter than full-length protein (59). Consistent with previous experiments, R1/2-Myc complemented the $\Delta$ubc7 strain (data not shown; 59). Wild-type cells were cotransformed with R1/2-Myc and either GST or GST-Ubc7p. The genes were induced with 0.5 mM Cu(SO4)2 for 2 h prior to extract preparation, as all genes were driven by the CUP1 promoter. Following induction, protein extracts were prepared by using a bead-beat procedure. GST pull downs were performed, and the supernatants (sup) and pellets (pel) were analyzed by Western blotting with antibodies against Myc and GST. Figure 5A illustrates that both GST and GST-Ubc7p were separately recovered in the pellet fraction in GST pull-down experiments. On the other hand, R1/2-Myc was recovered only in the pellet fraction when GST-Ubc7p was included in the extract. This experiment indicates that Rsp5p and Ubc7p interact in vivo, providing the first evidence that Ubc7p can act as an E2 for Rsp5p.

$\Delta$ubc7 and $\Delta$rsp5 mutations synthetically interact. To investigate the Rsp5p-Ubc7p interaction further, we asked whether $\Delta$ubc7 and $\Delta$rsp5 mutations genetically interact. We constructed an $\Delta$ubc7 $\Delta$rsp5 double mutant and compared the growth of the different mutants on increasing amounts of CdCl2. Our results show that the double mutant is approximately 100 times more sensitive to CdCl2 than $\Delta$rsp5 cells (Fig. 5B) yet has characteristics similar to those of the $\Delta$rsp5 mutant under all other conditions tested (data not shown). This could indicate that Rsp5p and Ubc7p interact only to modulate CdCl2 intake. Nonetheless, the observed synthetic interaction between $\Delta$ubc7 and $\Delta$rsp5 is consistent with Rsp5p and Ubc7p having overlapping functions. However, since $\Delta$ubc7 cells still function in the absence of UBC7, it is clear that

![Image](image_url)
Rsp5p has E2 partners other than Ubc7p for its myriad of functions, as previous data suggest (24, 48).

To obtain further evidence supporting our hypothesis that Rsp5p, Cdc34p, and Ubc7p functionally interact within the constraints of a signaling pathway, we overexpressed CDC34 and UBC7 individually in wild-type (Fig. 6B) and rsp5CA (Fig. 5C) cells. As CDC34 and UBC7 were under the control of the CUP1 promoter, the transformants were assayed for growth at various temperatures in the presence (induced) and absence (uninduced) of copper. When uninduced, the levels of Ubc7p and Cdc34p derived from the CUP1 promoter are greater than endogenous levels (data not shown). At permissive temperatures, induction of expression of CDC34 and UBC7 in rsp5CA cells had no effect (Fig. 5C). However, at 31°C, which is partially restrictive for rsp5CA, induced and uninduced expression of CDC34 severely exacerbated the rsp5CA phenotype. This supports our hypothesis that Cdc34p interacts antagonistically with Rsp5p. On the other hand, only induced UBC7 expression inhibited rsp5CA growth (Fig. 5C). Since induced expression of UBC7 had no effect on wild-type cells (Fig. 6B), we propose that wild-type RSP5 is required in the presence of induced UBC7. The observation that overexpression of UBC7 negatively impacts the rsp5CA growth phenotype, together with our finding that Rsp5p and Ubc7p physically interact, suggests that excess Ubc7p sequesters ts Rsp5p protein in an inactive complex that is inaccessible to other interacting partners.

**Ubch7p and the SCF components Cdc34p and Cdc53p positively interact.** An indirect approach to understanding how Rsp5p and Ubc7p function together is to ask whether Δubc7 interacts with SCF mutants in a manner similar to Δrsp5CA. Thus, we asked whether Δubc7 suppressed cdc34-2 growth phenotypes, as observed with Δrsp5CA, under cs conditions. Interestingly, Δubc7 did not suppress cdc34-2 phenotypes (as rsp5CA did at 16°C) but accentuated them (Fig. 6A). For example, the cdc34-2 Δubc7 growth defect was more severe at 37 and 16°C than those of either of the single mutants. Thus, this observation suggests that Cdc34p and Ubc7p function together. To gain support for our conclusion that Cdc34p and Ubc7p interact positively, we overexpressed CDC34 and CDC34 in the cdc34-2 SCF mutant. CDC34 expression complemented the cdc34-2 mutant (Fig. 6B). In support of our observation that Ubc7p functions together with the SCF, UBC7 suppressed the cdc34-2 ts phenotype. The effects were observed in the presence and absence of copper. UBC7 and CDC34 also increased the growth rate of cdc53-1 cells at 30°C and 37°C (data not shown). Taken together, our data support a model where Rsp5p and Ubc7p physically and functionally interact but have opposing effects on the SCF.
rps5C4 and Δubc7 have opposite effects on apc5C4. Our initial experiments suggested that Rsp5p and Apc5p function together in a similar pathway (Fig. 2). However, although Rsp5p and Ubc7p physically and functionally interact (Fig. 5), Rsp5p and Ubc7p have opposite effects on the SCF (compare Fig. 4 with Fig. 6). To understand whether Rsp5p and Ubc7p also act antagonistically with Apc5p, we constructed an apc5C4 Δubc7 double mutant and tested growth at elevated temperatures. At elevated temperatures, deletion of UBC7 suppressed the apc5C4 ts phenotype (Fig. 7A), indicating that Ubc7p has a negative influence on Apc5p. To confirm our observation that Ubc7p and Apc5p antagonistically interact, we overexpressed UBC7 in apc5C4 cells. Increased expression of UBC7 further impaired, albeit only weakly, the growth of apc5C4 cells at 37°C (Fig. 7B). Together, our results suggest that Ubc7p activity has a negative influence on Apc5p. Therefore, Rsp5p and Ubc7p have opposite effects on Cdc34p, Cdc53p, and Apc5p.

apc5C4 and cdc34-2 have a weak additive effect. Previous observations showed that mutations to the SCF (cdc4-1 and cdc34-2) suppressed the ts phenotypes of APC mutations (cdc16 and cdc27) (29). Since cdc34-2 partially suppresses the rps5C4 CdCl2 sensitivity (Fig. 4B), we addressed the question of whether apc5C4 and cdc34-2 interact through an analysis of apc5C4 cdc34-2 double-mutant phenotypes. We found that while CdCl2 did not affect the growth of single cdc34-2 and apc5C4 mutants, apc5C4 cdc34-2 cells were approximately 100 times more sensitive than either single mutant (Fig. 7C). We also observed that cdc34-2 weakly exacerbated the apc5C4 ts growth defect (data not shown). Together, these observations suggest that Cdc34p and Apc5p have a weak positive interaction. However, as shown in Fig. 7B, overexpression of CDC34 had little impact on apc5C4 cells. Thus, it is likely that Apc5p and Cdc34p impinge on similar activities, but the interaction is weak, indicating that the mutations do not significantly interact genetically.

The results presented here support a model where three E3 enzymes, the SCF, the APC, and Rsp5p, interact in a complex pathway involving the E2 Ubc7p. This report describes the novel finding that Ubc7p likely acts as an E2 for Rsp5p. The opposite effects of Rsp5p and Ubc7p with the SCF and the APC can be explained by the differential interaction of Rsp5p with Ubc7p and with other E2 enzymes, such as Ubc1p, Ubc4p, and Ubc5p. Thus, our work suggests that the role of the various ubiquitin-targeting components is highly regulated and that the interplay between the components is highly dynamic.

DISCUSSION

In our previous work, we identified two E3s required for mitotic chromatin assembly, the Apc5p subunit of the APC and Rsp5p (19, 20). In this study, we asked whether the involvement of Apc5p and Rsp5p in chromatin assembly reflects an interrelationship between the two proteins. It is clear that defective ubiquitin targeting is a key determinant of both apc5C4 and rps5C4 phenotypes, as (i) rps5C4 cells grown at 30°C have reduced ubiquitin protein levels (Fig. 1A), and (ii) overexpression of a ubiquitin molecule expressing lysine at only position 48 was sufficient to suppress the apc5C4 ts defect (Fig. 1B). The APC targets proteins for degradation in a proteasome-dependent manner, and lysine 48 in ubiquitin is utilized to build polyubiquitin chains for proteasome-dependent degradation. On the other hand, Rsp5p has been shown to utilize K63 linkages (24). Thus, in order to determine if Apc5p and Rsp5p act together or as opposing balances, we characterized genetic interactions between the apc5C4 and rps5C4 mu-
tations. We observed that the *rsp5<sup>CA</sup>* allele masked all *apc5<sup>CA</sup>* phenotypes studied, such as growth at various temperatures on different carbon sources, plasmid loss, response to benomyl, and DNA content (data not shown; Fig. 2). These observations suggest that the *rsp5<sup>CA</sup>* mutation blocks the pathway upstream of Apc5p, so that the *apc5<sup>CA</sup>* allele is not expressed. Taken together, our data strongly suggest that APC activity is compromised in *apc5<sup>CA</sup>* cells and that Rsp5p acts upstream of the APC in the same pathway.

To extend our analysis, we sought to discover E2 components acting within the Rsp5p/Apc5p pathway by screening E2 mutants for chromatin assembly defects. This resulted in the identification of the E2 enzymes Cdc34p and Ubc7p (Fig. 3). Cdc34p is the E2 component of the SCF E3 complex. To determine whether Cdc34p has an uncharacterized function as an E2 for either Rsp5p or the APC, we characterized the chromatin assembly profiles of the SCF Cullin subunit, Cdc53p, and of an SCF target protein, Sic1p. Extracts prepared from a *cdc53-1* mutant and from cells expressing a nondegradable version of Sic1p were also chromatin assembly defective. Thus, the *cdc34-2* defect reflects a role for the SCF in mitotic chromatin assembly rather than a novel function for Cdc34p as an E2 for Rsp5p or the APC. In support of this conclusion, we were unable to pull down Myc-tagged Rsp5p with GST-tagged Cdc34p (data not shown).

Our experiments suggest the novel function for Ubc7p as an E2 for Rsp5p (Fig. 5). First, Ubc7p and Rsp5p physically interact, which is consistent with the colocalization of both Rsp5p and Ubc7p to cytoplasmic structures (5, 15, 60). Second, disruption of *UBC7* in *rsp5<sup>CA</sup>* cells exacerbates *rsp5<sup>CA</sup>* CdCl<sub>2</sub> sensitivity, suggesting that Rsp5p and Ubc7p may function together to regulate CdCl<sub>2</sub> intake. Third, overexpression of *UBC7* in *rsp5<sup>CA</sup>* cells impairs growth at semirestrictive temperatures. One explanation for this observation is that enhanced binding of Ubc7p to Rsp5<sup>CA</sup> protein may sequester the mutant protein so that it is no longer accessible to other binding partners. This could occur if the Rsp5<sup>CA</sup> protein has a higher affinity for Ubc7p than wild-type protein or reduced activity of the mutant protein lowers the functional threshold of unbound protein below acceptable levels. Alternatively, overexpressed Ubc7p may outcompete other E2s, such as Ubc1p, Ubc4p, and Ubc5p, mutants of which have been genetically linked to *rsp5* (24, 48), for Rsp5p binding. Thus, reduced binding of other E2s to mutant Rsp5 when Ubc7p is overexpressed could explain the decreased growth of *rsp5<sup>CA</sup>* cells at semipermissive temperatures. Our future work will be designed to address this hypothesis.

A genetic analysis indicates that the components identified in this study exhibit a complex interaction profile. Since *rsp5<sup>CA</sup>* mutations suppressed *cdc34-2* and *cdc53-1* cp phenotypes and the *cdc34-2* mutant partially suppressed the *rsp5<sup>CA</sup>* CdCl<sub>2</sub> sensitivity (Fig. 4), we conclude that Rsp5p negatively interacts with the SCF. The observation that *rsp5<sup>CA</sup>* cells accumulate with unreplicated DNA at restrictive temperatures is consistent with Rsp5p playing a role in progression through G<sub>1</sub>/S (Fig. 2A). Our results linking Rsp5p in a negative manner with the SCF (Fig. 4) and positively with Apc5p (Fig. 1) are consistent with results of a previous study showing that the APC and the SCF act antagonistically (29). In that study, *cdc34-2* mutants suppressed *cdc27-1* mutants. It was therefore proposed that SCF and APC activities are temporally regulated so that SCF activity, which is required for the G<sub>1</sub>/S transition, is restricted until the mitosis-specific APC activity is complete. In our hands, however, the *cdc34-2* mutation did not suppress the *apc5<sup>CA</sup>* mutation (data not shown). Perhaps the APC subunits, Cdc27p and Apc5p, have independent functions in regards to Cdc34p. Nonetheless, by controlling the activity of the SCF, Rsp5p is indirectly controlling the activity of the APC.

We also observed opposing modes of SCF and APC regulation through ∆*ubc7* interactions. However, in this case, the interactions were opposed to that observed for the *rsp5<sup>CA</sup>* mutant, as our results indicate that Ubc7p acts positively with Cdc34p but negatively with Apc5p (Fig. 6, 7A, and 7B). Since we predict that Rsp5p and Ubc7p physically and functionally interact in a positive manner, we found the opposite manner of interaction with the SCF and the APC to be intriguing. We speculate that this difference could reflect Rsp5p interactions with various E2 enzymes. For example, perhaps the negative regulation of the SCF by Rsp5p is mediated through interactions with Ubc1p, Ubc4p, or Ubc5p rather than Ubc7p. We will also test the possibility that cell cycle position or various external stimuli contribute to the Rsp5p-E2 interaction.

The observations presented in this report provide insight into the breadth of the regulatory network defined by the ubiquitin-protein ligases Apc5p and Rsp5p. The position of Rsp5p on the cytosolic face of the plasma membrane places it in an ideal location to receive extracellular signals. Rsp5p can then respond to these signals by setting a signaling cascade in motion. Glucose signaling antagonistically controls the APC (inhibits [35]) and the SCF (activates [29]). Thus, Rsp5p could, in part, mediate the response of the APC and the SCF to glucose, as glucose triggers the Rsp5p-dependent turnover of some proteins (24, 48). The complex phenotypic spectrum exhibited by *rsp5* mutants could be explained by the potentially elaborate interaction of Rsp5p with multiple E2s. Here, we show that the interaction of Rsp5p with Ubc7p may be specific for mitotic chromatin assembly. Finally, this study raises many interesting and exciting questions. For example, what is the nature of the Rsp5p-SCF interaction? Furthermore, how does the signal received by Rsp5p reach the APC? This question invokes the involvement of a protein capable of shuttling across the nuclear membrane.

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