A Role for Saccharomyces cerevisiae Cul8 Ubiquitin Ligase in Proper Anaphase Progression*

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We have undertaken a study of the yeast cullin family members Cul3 and Cul8, as little is known about their biochemical and physiological functions. We demonstrate that these cullins are associated in vivo with ubiquitin ligase activity. We show that Cul3 and Cul8 are functionally distinct from Cdc53 and do not interact with ySkp1, suggesting that they target substrates by Skp1- and possibly F-box protein-independent mechanisms. Whereas null mutants of CUL3 appear normal, yeast cells lacking CUL8 have a slower growth rate and are delayed in their progress through anaphase. The anaphase delay phenotype can be complemented by ectopic expression of Cul8 but not by any other yeast or human cullins, nor by a cul8 mutant deficient in binding to RING finger protein Roc1. Deletion of the RAD9 gene suppressed the anaphase delay phenotype of cul8Δ, suggesting that loss of Cul8 function may compromise genomic integrity. These results indicate that in addition to the anaphase-promoting complex, mitotic progression may involve another E3 ubiquitin ligase mediated by Cul8 protein.

Ubiquitin-mediated proteolysis is a rapid and irreversible mechanism used by eukaryotic cells to ensure unidirectional cell cycle progression and proper cell division. This process requires a cascade of three enzymatic activities: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligase) enzymes (1–3). Unlike E1 and E2, the E3 ubiquitin ligase also to cell growth and animal development. Mutations in CDC53 caused a G1 cell cycle arrest in both budding and fission yeast and at least six genes in worm, fruit fly, and human (6, 7). The functional importance and conservation of cullins is underscored by the finding that a subunit of APC E3 ligase, APC2, contains limited sequence homology to cullins (8, 9). Ample genetic evidence establishes that cullin proteins perform various physiological functions critical not only to the cell cycle but also to cell growth and animal development. Mutations in C. elegans cul-2 gene did not affect embryonic and postembryonic somatic cell division, but inhibited the G1 to S transition and chromosome condensation at a later stage of development (14). Although a mouse model has yet to be established to determine the effects of CUL2 loss, human CUL2 has been linked to the von Hippel-Lindau tumor suppressor protein (VHL) and ubiquitination of hypoxia-inducible factor α (15–17). The pleiotropic effects seen with mutation of cullins, even of orthologues, can likely be attributed to the fact that each cullin-associated ligase may mediate the ubiquitination of multiple substrates.

The biochemical mechanisms underlying the function of most cullins remain essentially uncharacterized. The ubiquitin ligase activities of SCF and APC are dependent on small RING finger proteins: ROC1/Rbx1/Hrt1 that interacts with Cdc53/CUL1, and Apc11 that binds with Apc2 (18–22). ROC1 (RING of cullins, formerly regulator of cullins) can associate with all human cullins (18), suggesting that most, if not all, cullins function as a subunit of ubiquitin ligases. Purified recombinant Apc11 protein can interact directly with E2-Ubc4 to promote...
E1-dependent polyubiquitin chain formation in vitro in the absence of Apc2 (23, 24). Similarly, purified recombinant ROC1 or its RING finger portion alone is capable of activating E2-UbcH5 to synthesize polyubiquitin chains in the presence of E1 (25). These results suggest that RING-E2, rather than cullin-RING, constitutes the catalytic core of the ubiquitin ligase. Consequently, one major function of the cullin subunits in various E3 ubiquitin ligases is to assemble the RING-E2 catalytic core and substrates together.

The targeting mechanisms utilized by cullins also are not well understood. Cdc53/CUL1 uses a highly conserved NH2-terminal domain to bind directly to an adapter protein, SKP1. Through SKP1, Cdc53/CUL1 associates with an F-box protein that in turn binds phosphorylated substrate (26–28). In a somewhat similar arrangement, human CUL2 binds directly to a SKP1-related protein, elongin C, and indirectly with VHL that in turn binds with substrate hypoxia-inducible factor α (15–17, 29–31). It is not known how other cullins target their substrates. In human cells, SKP1 only interacts with CUL1, not other cullins (32), suggesting the possibility that other cullins target their substrate via a SKP1- and possibly an F-box protein-independent mechanism. However, this interpretation is complicated by the fact that SKP1 has a related gene, elongin C, in mammalian cells and potentially large numbers of homologues in other eukaryotes including Drosophila, C. elegans, and Arabidopsis. It is conceivable that other SKP1 homologues may act as adaptors for other cullins.

In addition to CDC53 and APC2, the yeast Saccharomyces cerevisiae genome contains two other cullin genes, CUL3 and CUL8. Little is known, however, about the functional and biochemical properties of these two cullins. This investigation is directed toward these two issues.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—All S. cerevisiae strains were derived from YEF473 (α ura3-52 ade2-1 his3Δ200/200/200 trp1-63/63 trp5-2A leu2-3,112 his3Δ200/200/200 his3Δ200/200/200 trp1-63/63 his3Δ200/200/200 trp1-63/63). Mutant yeast strains were constructed using PCR-based gene deletion and modification by homologous recombination (33). cdc53-1 was a gift of M. Tyers (34). Primers for PCR products for all strains constructed were designed based on the sequences published in the database and contained 40 bp of sequence homologous to the gene-specific sequence and 20 bp homologous to the vector template. PCR was performed using the Expand Long Template PCR system (Roche Diagnostics) with the following protocol. Mix 1 (25 µl) containing 2.5 µl of Expand Buffer 1, 0.8 mM dNTPs, 10 µg of bovine serum albumin, and 2 µl each primer. Mix 2 (75 µl) contained 7.5 µl of Expand Buffer 1, 0.75 µl of Expand enzyme mixture, and 0.1 µg of template DNA. The two mixtures were added together (total 100 µl of reaction), mixed well, and immediately subjected to PCR: 20 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min/kb at 68 °C followed by a 10-min extension at 68 °C. PCR products from at least eight reactions were pooled, extracted once with phenol:chloroform, and ethanol precipitated. PCR products were transformed into the indicated strains (see below, Table I) using a standard protocol, plated onto rich medium (YPD plates), and incubated at 30 °C for 2 days. Plates were then replica plated onto appropriate selective medium for 2–3 days. Selected transformants were restreaked onto selectable medium twice. To identify transformants that had integrated by homologous recombination, PCR was performed on genomic DNA prepared by lyticase treatment using one primer that annealed to the integrated module and one primer that annealed to a region outside of that altered by the recombination. PCR products of the appropriate size confirmed homologous recombination. In addition, 2:2 segregation of the selectable marker also confirmed homologous recombination, where appropriate. Yeast strains are listed in Table I.

Yeast cullin and ROC cDNA sequences have been described previously (18). HIS-CDC53, HIS-CUL3, and HIS-CUL8 were inserted into the p14-ADH vector (CEN) and pEG3(JM)-ADH vector (2µ, modified from pEG-kt, (35)) by removing the GST tag and CYC1 promoter and replacing them with the ADH promoter for cdc53-1 rescue experiments. HA-CDC53, HA-CUL3, HA-CUL8, and HA-cul8-GST-c-wt were inserted into pRS313-ADH vector (CEN) for cul8Δ rescue experiments. yskp1Δ was amplified from genomic DNA by PCR, verified by DNA sequencing, and inserted in-frame into the PACT2 yeast two-hybrid vector. For Cul8 expression for the Cul8-Roc1 binding assay, either wild type Cul8 or cul8-GST-c-wt were subcloned into the pEG-kt expression vector to introduce a galactose-inducible promoter and NH2-terminal GST fusion tag. YEF473 wild type and cul8Δ yeast expressing tubulin-GFP were constructed by transforming the appropriate strain with linearized plasmid pAF5125 encoding GFP-TUB1-URA for integration at the URA3 locus (36, 37). The directed yeast two-hybrid assay was performed as described (38). Microscopic Analysis—For microscopic analysis, MATa cul8Δ:TRP1 yeast expressing the indicated proteins from a CEN-based plasmid were grown in selective medium to mid-log phase. Cells were fixed in culture medium with 3.7% formaldehyde, rinsed, and resuspended in mounting medium containing Hoechst 33258 dye or in water containing 4,6-diamidino-2-phenylindole to stain the DNA. Cells were examined using an Olympus IX70 microscope and Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) and categorized by the position of the nuclei within the cell (as indicated in Table II). Cells that did not contain organized nuclei, which were always large-budded, were counted as “other.” Numbers reflect percentages of the total population (38). For visualizing and measuring mitotic spindles, images of live cells were captured and analyzed as previously described (39) using a Nikon Optiphot microscope and Hamamatsu C4742-95 digital camera. Briefly, sets of five Z-series fluorescent images for each measurement were captured at 0.75-µm steps using MetaMorph software (Universal Imaging Corp., Westchester, PA). Images were then compiled and mitotic spindle lengths were measured using MetaMorph software.

Preparation of Yeast Lysates—Yeast was cultured at 30 °C unless otherwise indicated in YP medium or SD medium (lacking appropriate amino acids or uracil) containing 2% glucose or 2% raffinose plus 0.5–2% galactose, as appropriate. Cell lysates were generated as described (18). Briefly, yeast pellets were subjected to one freeze-thaw and resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 25 µg/ml leupeptin, 25 µg/ml aprotonin, 1 mM benzamidine, and 10 µg/ml trypsin inhibitor. Cells were mechanically lysed using acid-washed glass beads by vortexing 8 × 30 s with at
least 30 s on ice between each vortex. The suspension was cleared by centrifugation at 13,200 rpm at 4 °C for 30 min. Protein concentrations of whole cell extracts were measured using the Bradford assay reagent (Bio-Rad).

**Ubiquitin Ligase Activity Assay—** Yeast E1 was purchased from Affinity (UK). Expression vectors for His-tagged yeast Cdc34(2C) containing a deletion of the COOH-terminal domain that was not essential for its in vitro activity and His-tagged ubiquitin containing a protein kinase A recognition site (LRRASV) were gifts from Dr. Z-Q. Pan. Both proteins were purified with nickel beads. Purified ubiquitin was labeled with [γ-32P]ATP by incubating with cAMP kinase (Sigma) at 37 °C for 30 min. For ubiquitination assays, immunocomplexes immobilized on protein A-agarose beads were added to a ubiquitin ligation reaction mixture (30 μl) that contained 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2 mM NaF, 10 mM sodium acetate, 2 mM ATP, 0.6 mM dithiothreitol, and boiled for 3 min prior to 12.5% SDS-PAGE analysis. Yeast cullin ligases were obtained by immunoprecipitating yeast lysates (described above) with a monoclonal anti-HA antibody (12CA5).

**UV Irradiation—** From each mid-log phase culture, 500 cells were plated onto YPD plates and allowed to dry. Plates were exposed to 0, 25, 40, or 75 J/m² of UV irradiation using a UV Stratalinker 2400 (Stratagene) and then incubated with both E1 and Cdc34 as a negative control (Fig. 2D). As shown in Fig. 2D, ubiquitinated proteins were detected in a dose-dependent manner. The results from three experiments were averaged. Standard error bars are shown.

**RESULTS**

The budding yeast *S. cerevisiae* Genome Contains Four Cullin Genes—CDC53, CUL3, CUL5 (ORF YGR003w), CUL8 (ORF YJL047c), and a more distant relative, CUL1 in higher eukaryotes, identified at least four mammalian cullins. Combined analysis of yeast Cdc53 and its orthologue, CUL1 in higher eukaryotes, identified at least four distinct domains in Cdc53/CUL1. These include 1) an NH₂-terminal Skp1-binding domain, 2) a COOH-terminal domain that contains both a Rb ubiquitination site and a sequence required for CUL1 nuclear accumulation, 3) a Roc1-binding region, and 4) an uncharacterized region approximately between residues 205 and 570 implicated in E2 binding (32, 34, 40, 41). We considered each of these domains when characterizing the functions of Cull3 and Cull8.

**Yeast Cull3 and Cull8 Complexes Contain Ubiquitin Ligase Activity—** Yeast Cdc53 controls the G₁/S transition by mediating the specific ubiquitination of phosphorylated substrates. The ubiquitin ligase activity of the Cdc53 complex is dependent on its association with Roc1 (18, 20, 22). Like Cdc53, both Cull3 and Cull8 also interact with Roc1 (Ref. 18, and also see below Fig. 3B), suggesting that these two cullin complexes may also contain ubiquitin ligase activity. To test this directly, yeast strains were generated by PCR homologous recombination to individually overexpress from the GAL promoter HA-Cdc53, HA-Cul3, or HA-Cul8 in the background of GAL-driven Roc1 overexpression. Total cell lysates were immunoprecipitated with anti-HA antibody and subjected to a substrate-independent ubiquitin ligase assay in the presence and absence of E1 and E2 Cdc34 (Fig. 1E). An anti-HA immunoprecipitate derived from ROC1 overexpressing YEF473 cell lysate was incubated with both E1 and Cdc34 as a negative control (lane 10). This assay measures the ability of individual immunocomplexes to activate E2 Cdc34 to synthesize polyubiquitin chains via isopeptide bonds, some of which are linked to either cullins or Roc1 (data not shown). Whether these cullin- and Roc1-linked polyubiquitin chains correspond to an intermediate during ubiquitin transfer from an E2 to a substrate is not known. Because the isopeptide bond linking two ubiquitins is chemically the same as that linking the substrate and ubiquitin, it is believed that the synthesis of polyubiquitin chains in this substrate-independent assay represents genuine ubiquitin ligase activity. HA-Cdc53 immunoprecipitates contained robust levels of ligase activity dependent upon the presence of E1 and E2 as evidenced by a high molecular weight smear (lane 1). HA-Cul3 and HA-Cul8 immunocomplexes contained low but reproducible ubiquitin ligase activity (lanes 4 and 7) in the presence of E1 and E2. Densitometric quantitation confirmed that Cdc53 immunocomplexes activated Cdc34 to synthesize polyubiquitin chain formation 10.9 times the level of background (lane 10), whereas Cul3 and Cul8 immunocomplexes activated Cdc34-mediated polyubiquitination at 2.1 and 1.8 times background levels, respectively. Two possibilities could account for the relatively lower Cul3 and Cul8 activities. First, both Cull3 and Cull8 proteins are consistently expressed at lower levels than Cdc53 as determined by Western analysis using the same anti-HA antibody as a detection reagent (see below Fig. 2C). Second, Cdc53 may interact with or utilize Cdc34 more efficiently than Cul3 and Cul8. In light of the relatively modest ubiquitination observed in this assay from the Cul3 and Cul8 immunocomplexes, and in the subsequent Cul8 assay displayed in Fig. 2E, we consider it likely that both Cul8 and Cul3 could preferentially employ an E2 enzyme other than Cdc34.

*Cul8ΔRoc1* Has Impaired Binding to Roc1p—Based on our previous mutational analysis of human CUL1-ROC1 binding (41), we deleted seven residues in the COOH-terminal region of yeast Cull8 (residues 680–686, see Fig. 1C) to disrupt its binding with Roc1. Plasmids containing either GST-CUL8 or GST-CUL8Δ680–686 under the control of a galactose-inducible promoter were transformed into yeast containing an integrated HA₃-tagged ROC1, also under the control of a galactose-inducible promoter. After induction of the GAL promoter, cells were lysed and equal amounts of protein were immunoprecipitated with anti-HA antibody and resolved by SDS-PAGE. Cul8-Roc1 interaction was examined by immunoprecipitation-Western blots (Fig. 2A). *Lane 1* shows that HA₃-tagged Roc1p is capable of coimmunoprecipitating GST-Cull8, whereas a similar immunoprecipitation pulls down undetectable amounts of GST-Cul8Δ680–686 (*lane 2*). Separately, in a directed yeast two-hybrid assay, we used pGAD-Roc1 as the prey and either pGBKTS-CUL8 or pGBKTS-cul8Δ680–686 as bait. The mutant cul8 protein was severely impaired in activation of the histidine reporter gene relative to wild type Cul8, confirming that residues 680–686 in Cul8 are necessary for efficient binding with Roc1 (Fig. 2B). We therefore will refer to cul8Δ680–686 hereafter as cul8ΔRoc1.

**Roc1 Binding Is Necessary for Rub1 Modification and Ubiquitin Ligase Activity of Cul8—** All cullin proteins examined to date, not including APC2, are modified by the ubiquitin-like protein Nedd8/Rub1 (40, 42–44). Immunoblot analysis of yeast Cdc53, Cull3, and Cull8 revealed a doublet for all three yeast cullins (Fig. 2C). Judging by the mobility, the upper band most likely represents the Rub1/Nedd8-modified form. To confirm this we introduced a mutation, Lys791 to Ala, in CUL8. Lys 791 in yeast Cull8 is situated in the highly conserved COOH-terminal region. It corresponds to Lys720 and Lys689 in human CUL1 and CUL2 (41, 45, 46), and Lys711 and Lys686 in fission yeast Peu1 and Peu4 (47, 48) whose mutations abolished Nedd8/Rub1 conjugation (Fig. 1D). As shown in Fig. 2D, substitution of Lys791 with an alanine residue completely abolished the slow migrating form of Cul8 without affecting the fast...
FIG. 1. Yeast Cul3 and Cul8 immunocomplexes contain ubiquitin ligase activity. A, schematic diagram of *S. cerevisiae* Cdc53, Cul3, Cul8, and Apc2 with homologous regions shown in like colors. B, sequence conservation at the NH2-terminal domain of cullin 1 and cullin 3 proteins from human (*Hs*), fly (*Dm*), nematode (*Ce*), plant (*At*), fission yeast (*Sp*), and budding yeast (*Sc*). Identical residues are highlighted in blue. C, sequence comparison of the ROC1-binding domain of *S. cerevisiae* cullin and Apc2 proteins. Identical residues are highlighted in red. Boxed residues in Cul8 indicate a deletion mutant (*Hs*800–806) used in this study. D, sequence comparison of Rub1/NEDD8 modification sites (boxed) and flanking region of *S. cerevisiae* cullin proteins. Identical residues are highlighted in green. E, ubiquitin ligase assay of yeast cullin complexes. Lysates from yeast strains individually overexpressing the indicated HA-tagged cullins and Roc1 were generated. 1.5 mg of total protein from each strain was immunoprecipitated with 100 μl of monoclonal anti-HA antibody and immobilized on protein A beads. Beads were washed and then mixed with purified E1, yeast E2 Cdc34ΔC, 32P-labeled ubiquitin, and ATP. After incubation, the reactions were terminated by boiling the sample in Laemmli loading buffer and resolved by SDS-PAGE. The asterisk indicates a band that most likely corresponds to residual ubiquitin-Cdc34 thioester conjugate.
A Mitotic Function for Yeast Cullin 8 Ubiquitin Ligase

Fig. 2. Roc1 binding is essential for Rub-1 modification and ubiquitin ligase activity of Cul8. A, yeast expressing GST-Cul8 and HA–Roc1 under the control of galactose-inducible promoters were grown in mid-log phase and lysed. Lysates were immunoprecipitated with anti-HA antibody and blotted with anti-GST and anti-HA antibodies. HA-tagged Roc1 is able to coimmunoprecipitate GST-Cul8 (lane 1), but not GST-cul8Δroc1 (lane 2). B, directed yeast two-hybrid assay shows impaired Roc1 interaction with cul8Δroc1 relative to Roc1 interaction with wild type Cul8, as shown by activation of the histidine reporter gene. C, total cell lysates were prepared from yeast cells expressing HA-tagged culins under the control of the inducible GAL promoter, resolved by SDS-PAGE and immunoblotted with α-HA antibody. Each protein appeared as a doublet. The upper band corresponds to the Rub1-modified culin, and the lower band is unmodified culin. D, an internal deletion (Δ680–686) and a point mutation (K791A) were introduced into yeast Cul8 to disrupt its interaction with Roc1 and its conjugation by Rub1, respectively. HA-tagged wild type and mutant Cul8 were expressed in cul8Δ yeast from an ADH promoter. The total cell lysates were sequentially immunoprecipitated and immunoblotted with antibody to the HA epitope. Note that the upper Rub1-modified band was abolished by both mutations, indicating that Roc1 binding is necessary for Rub-1 modification of Cul8 protein in vivo. E, ubiquitin ligase assay of Cul8 immunocomplex. Lysates from cul8Δ yeast strains ectopically expressing the indicated HA-tagged wild type and mutants Cul8 were immunoprecipitated with 30 μl of monoclonal anti-HA antibody and immobilized on protein A beads. Beads were washed and then mixed with purified E1, yeast E2 Cdc34(ΔC), 32P-labeled ubiquitin, and ATP. After incubation, the reactions were terminated by boiling the sample in Laemmli loading buffer and resolved by SDS-PAGE.

We were interested in determining whether high level expression of either culin 3 or culin 8 could rescue the G1 cell cycle arrest caused by Cdc53 mutations (7, 50). We tested this by determining whether overexpression of Cul3 or Cul8 could complement the cdc53-1 temperature-sensitive mutation (Fig. 3A). cdc53-1 yeast cells were transformed with plasmids directed to ectopically express empty vector, HIS-CDC53, HIS-CUL3, or HIS-CUL8 from either a CEN-based plasmid or a 2μ-based plasmid. At the permissive temperature (25 °C) all yeast strains were viable. At the restrictive temperature (37 °C) only those yeast expressing Cdc53, but not Cul3 or Cul8, could complement the defect in the cdc53-1 strain. The ability of yeast culins 3 and 8 to complement cdc53-1, even when expressed from high copy number plasmids, provides evidence that these two culins are functionally distinct from Cdc53.

We showed previously that human CUL1 was the only culin family member capable of interacting with SKP1 (32). This suggests the possibility that other culins may target their substrate via SKP1- and F-box protein-independent mechanisms. However, this interpretation is complicated by the fact that SKP1 belongs to a multigene family in higher eukaryotes. It has been shown that culin 2 can interact with a SKP1-like protein, Elongin C (29, 30), to target substrate via an SCF-like mechanism. The yeast genome contains a single SKP1 gene, offering the opportunity to address more definitively whether other culins interact with Skp1. We determined whether yeast culins 3 and 8 could interact with ySkp1 using the yeast two-hybrid assay. Yeast culins were fused in-frame to the GAL4-binding domain and cotransformed into yeast with plasmids expressing either empty vector, yRoc1, or ySkp1 fused in-frame to the GAL4 activation domain. Only Cdc53 was capable of interacting with ySkp1 as determined by the activation of the histidine reporter gene (Fig. 3B). The expression of both Cul3 and Cul8 was confirmed by their positive interaction with yRoc1. The inability of Cul3 and Cul8 to interact with ySkp1 is consistent with the low degree of sequence conservation between the NH2-terminal regions of these two culins and the Skp1-binding domain of Cdc53. Within this region, there are 11 and 16 amino acid residues that are invariably conserved among CUL1 and CUL3 from different organisms, respectively, and only five are shared between these two culins (Fig. 1B). Thus, yeast culins 3 and 8 utilize a Skp1- and possibly a F-box-independent mechanism to target their substrates.

Yeast Deleted for Cul8 Have a Slower Growth Rate—To understand more about the function of Cul3 and Cul8 in yeast, we deleted each gene by PCR homologous recombination. The CUL3 ORF YGR003w was replaced with a kanMX6 selection cassette, and the CUL8 ORF YJL047c was replaced with a mutations disrupting Roc1 binding and Rub1 modification. Total cell lysates were prepared from cul8Δ yeast cells ectopically expressing HA-tagged CUL8 wild type, Roc1-binding deficient (Δ680–686), and Rub1-modification deficient (K791A) proteins. Lysates were immunoprecipitated with α-HA antibody and the ubiquitin ligase activity of wild type and mutant Cul8 immunocomplexes was determined. Whereas wild type Cul8 immunocomplex activated Cdc34 to synthesize polyubiquitin chains at 2.1 times the level of background, mutation at the Rub1 modification site reduced the ligase activity of Cul8 by 50% and deletion of the Roc1-binding region almost completely eliminated ubiquitin ligase activity of Cul8 (Fig. 2E). These results demonstrate that the Cul8 complex contains ubiquitin ligase activity that is dependent on its binding with ROC1 and is enhanced by the Rub1 modification.

Cullin 3 and Cullin 8 Are Functionally Distinct from Cdc53—Yeast Cdc53 provides an essential function for the cell. The inability of yeast culins 3 and 8 to complement cdc53-1 strain. The inability of yeast culins 3 and 8 to complement cdc53-1, even when expressed from high copy number plasmids, provides evidence that these two culins are functionally distinct from Cdc53.
were categorized as unbudded, small budded (bud smaller than 1/2 the size of the mother), or large budded (bud larger than 1/2 the size of the mother). Deletion of CUL8 resulted in an accumulation of large budded cells, increasing from 25.5% in the wild type population to 37.2% in cul8Δ cells, with a concomitant decrease of unbudded cells (44.3 to 33.1%). The proportion of small budded cells remained relatively unchanged (Fig. 4B, and Table II). These results suggest that loss of Cul8 function causes a defect in progression through the G2 to M transition, resulting in a slower growth rate.

Deletion of CUL8 Gene Delays Anaphase Progression—To analyze cul3Δ and cul8Δ yeast further, cultures were grown in complete medium to mid-log phase, formaldehyde fixed, stained with Hoechst or 4,6-diamidino-2-phenylindole, and inspected microscopically. Yeast deleted for CUL3 appeared similar to wild type cells, exhibiting a normal distribution of DNA in interphase and mitosis (data not shown). A fraction of large budded cul8Δ cells, on the other hand, displayed the DNA midway in the neck of the bud (Fig. 4B and Table II), indicative of anaphase delay. Quantification of more than 350 cells showed that while no cells from the wild type population accumulated DNA positioned midway in the neck of the bud, 14% of the cul8Δ cells were observed with this phenotype (Table II). In addition, some large budded cul8Δ cells did not appear to have organized, normally shaped nuclei, but instead their DNA was dispersed throughout the cell (classified as other in Table II, also see below for mitotic spindle analysis). It is not clear whether these cells represent a distinct phenotype resulting from the loss of Cul8 function, or a subsequent stage after a prolonged trapping of nuclei at the neck.

We wanted to confirm that the mid-anaphase defect was indeed because of the lack of Cul8 function. To examine this possibility, cul8Δ yeast were transformed with CEN-based plasmids that ectopically expressed empty vector or Cul8. Transformants were grown in minimal medium, formaldehyde fixed, and Hoechst stained. Cells were counted to quantitate the number of cells delayed at anaphase (Table II). cul8Δ yeast cells expressing empty vector displayed 7.2% of cells with nuclei caught at the neck of the bud. Note that this percentage is lower than that observed in untransformed cells grown in complete medium, and it is most likely because of culturing cells in minimal media to retain the plasmid. Expression of Cul8 significantly reduced the anaphase delay, confirming the authenticity of the phenotype. In contrast, neither Cul3 nor Cdc53 could compensate for Cul8 function when they were ectopically expressed in cul8Δ yeast on either CEN- (Table II) or 2μ-based plasmids (data not shown), confirming the functional distinction between the three cullins. Additionally, expression of human culins 2, 3, mouse 4A, and human 5 from a 2μ-based plasmid could not functionally complement the cul8Δ defect (data not shown). This is in contrast to human culin 1, which can functionally complement the cdc53-1 deficiency (51).

We next examined the mitotic spindle dynamics in live wild type and cul8Δ yeast cells after integrating a plasmid expressing tubulin-GFP fusion protein. Large budded cells, where the bud was nearly the same size as the mother cell, were randomly chosen from the asynchronous populations of both genotypes for microscopic examination. Cells were examined by fluorescent microscopy to determine the status of the microtubules, and were then categorized as metaphase spindles (mitotic spindle < 2 μm, Fig. 4C), anaphase spindles (mitotic spindle > 2 μm), or telophase (two distinct spindle pole bodies (52)). This analysis showed that while 61.7% of large budded wild type cells had progressed to telophase (Fig. 4C, open boxes), deletion of Cul8 resulted in a significant delay in progressing through anaphase. Only 17.9% of large budded cul8Δ cells had reached
telophase (Fig. 4C, solid boxes), whereas 66.7% of the cul8Δ cells were still in anaphase. The average length of mitotic spindles of metaphase and anaphase cells was 6.18 μm (S.D. 3.16 μm; n = 81) for wild type cells and 3.49 μm (S.D. 2.25 μm; n = 168) for cul8Δ cells, further indicating a delay in progressing through anaphase caused by the loss of Cul8 function. In addition to the spindles that appeared normal in the cul8Δ yeast, a portion of large budded cells displayed aberrant microtubules (Fig. 4D). Whether these abnormal structures were the result of a separate spindle defect from the anaphase delay, or were the result of catastrophic spindle breakdown following prolonged anaphase delay, is yet to be determined.

**Ectopic Expression of cul8ΔRoc1 Is Unable to Rescue the Anaphase Delay Phenotype in cul8Δ Yeast**—When wild type CUL8 was reintroduced into cul8Δ yeast, the percentage of cells in anaphase dropped to 42.3% compared with 66.7% in the cul8Δ cells; 35.9% of the cells examined had progressed to telophase (n = 78, Fig. 4C, striped boxes), indicating a partial rescue of cul8Δ deficiency. We interpret this incomplete rescue as the result of Cul8 expression at different (probably higher) levels than the endogenous gene, resulting in too high levels of Cul8 activity or titrating away factors (e.g. Roc1) that are required for the proper function of the other two cullins. Ectopic expression of cul8ΔRoc1, on the other hand, displayed a distribution of cells among metaphase, anaphase, and telophase nearly identical to that of cul8Δ cells; 70.7% of cells were observed in anaphase, and only 19.5% of the cells had progressed to telophase (n = 41, Fig. 4C, cross-hatched boxes). This result sup-

![Fig. 4. Deletion of CUL8 from yeast results in a slower growth rate and mitotic defects.](http://www.jbc.org/)

A, overnight cultures of wild type, cul3Δ, and cul8Δ yeast cells were inoculated into complete medium at a density of 6 × 10⁶ cells/ml. Samples were taken every 2 h to determine cell count. The y axis indicates the fold increase in cell number. This was determined by dividing the cell number from each time point by the number of cells initially inoculated. The figure represents the average of three experiments. B, wild type and culΔ mutant cells were grown to mid-log phase in complete medium, fixed in culture medium with 3.7% formaldehyde, rinsed, and resuspended in mounting medium containing 4,6-diamidino-2-phenylindole to stain the DNA. Arrows indicate DNA positioned midway in the neck of the bud. Quantification of cell morphological examination and DNA staining are presented in Table II. C, dynamics of mitotic spindles. 81 large budded wild type (open boxes) and 168 cul8Δ cells (solid boxes) expressing a GFP-tubulin fusion protein were randomly chosen from asynchronous populations and examined by fluorescence microscopy. Cells were categorized as metaphase (mitotic spindle < 2 μm), anaphase (mitotic spindle > 2 μm), or telophase (two distinct spindle pole bodies). In addition, cul8Δ cells expressing GFP-TUB1 were transformed with plasmids expressing HA-cul8 (striped boxes) or HA-cul8ΔRoc1 (cross-hatched boxes) to test for rescue of the anaphase delay phenotype. Ectopically expressed wild type Cul8 was partially able to complement the loss of endogenous Cul8 (striped boxes), but cul8ΔRoc1 had a negligible effect on rescuing the phenotype (compare cross-hatched boxes to solid boxes), as determined by categorizing mitotic spindle length. The average size of a typical yeast cell is ~5 μm. The transmission light and fluorescence images of a representative cell of each stage are shown. D, abnormality of spindle formation in cul8Δ cells. In addition to anaphase delay shown in C, a fraction of large budded cul8Δ cells displayed aberrant microtubules that cannot be categorized into a recognizable mitotic stage.
ports the hypothesis that the association of Cul8 with Roc1, and hence a ubiquitin ligase activity, is necessary for proper mitotic progression. Average length of metaphase and anaphase mitotic spindles in the two populations of large budded yeast was not appreciably different (3.60 μm in cells ectopically expressing Cul8 compared with 3.63 μm in cells ectopically expressing cul8<sup>Δ</sup>roc<sup>1</sup>); however, the variation in the lengths (S.D. of CUL8 cells = 3.42 μm compared with 1.91 μm in the cul8<sup>Δ</sup>roc<sup>1</sup> cells) suggests that the cells ectopically expressing wild type Cul8 have more dynamic spindles.

**Table II**

| Budding morphology distribution | 25.5% | 37.2% | 44.3% |
|---------------------------------|-------|-------|-------|
| wt                              | 30.2% |       |       |
| cul8<sup>Δ</sup>                |       | 33.1% |       |

~2500 cells counted for each genotype

**Deletion of RAD9 Suppresses the Mid-anaphase Delay Observed in cul8<sup>Δ</sup> Yeast**—A similar mid-anaphase delay phenotype was observed previously in studies using dicentric chromosomes that can be kept dormant under galactose conditions but activated under glucose conditions. Cells with two active centromeres were found to delay their cell cycle with one-third to one-half of the cell population observed as large budded cells with nuclear DNA spanning the neck (53, 54). This mid-anaphase delay was dependent upon the presence of the DNA damage checkpoint gene RAD9 (54) that is involved in recognizing and processing DNA lesions (55–57). Null mutants of rad9 are viable, but are prone to increased rates of spontaneous chromosome loss, sensitive to X and UV irradiation, and cannot arrest the cell cycle in G<sub>2</sub> after DNA damage (58). To determine whether the mid-anaphase delay caused by cul8<sup>Δ</sup> was also dependent upon Rad9, we generated a yeast strain lacking both CUL8 and RAD9 genes. cul8<sup>Δ</sup>/rad9<sup>Δ</sup> double mutant cells were grown in complete medium to mid-log phase, formaldehyde-fixed, Hoechst-stained, and microscopically inspected (Fig. 5A). Single rad9<sup>Δ</sup> mutants did not delay at mid-anaphase and displayed a wild type distribution of DNA (Table II). Furthermore, deletion of RAD9 was sufficient to suppress the anaphase delay caused by cul8<sup>Δ</sup>. Quantification of the cells (Table II) confirmed a significant reduction of cul8<sup>Δ</sup>-induced anaphase delay by the loss of Rad9 function. Only 1.4% of cul8<sup>Δ</sup>/rad9<sup>Δ</sup> cells were found in anaphase as compared with 14% in the cul8<sup>Δ</sup> single mutant, amounting to a 10-fold difference. Notably, some large

![Fig. 5](http://www.jbc.org/)

**Fig. 5. Deletion of RAD9 suppresses the anaphase delay phenotype observed in cul8<sup>Δ</sup> yeast.** A, cul8<sup>Δ</sup>/rad9<sup>Δ</sup> cells were grown to mid-log phase in complete medium, fixed in culture medium with 3.7% formaldehyde, rinsed, and resuspended in mounting medium containing Hoechst 33258 dye to stain the DNA. Quantitation of cells in mid-anaphase and late anaphase can be found in Table II. B, wild-type, cul8<sup>Δ</sup>, rad9<sup>Δ</sup>, and cul8<sup>Δ</sup>/rad9<sup>Δ</sup> yeast cells were grown to mid-log phase in complete medium. From each culture, 500 cells were plated onto four YPD plates, allowed to dry, and subjected to four different doses of UV irradiation (as indicated). Plates were incubated at 30 °C for 2 days at which time colonies were counted. Percent survivability was determined individually for each strain by dividing the number of colonies on each dosage plate (25, 50, and 75 J/m<sup>2</sup>) by the number of colonies on the respective mock treated plate (0 J/m<sup>2</sup>). The data plotted are the average of three experiments. Bars represent the standard error.
budded cells with unorganized nuclei persisted in the double mutant cells (indicated as Other in Table II). This may indicate that either only a portion of Cul8-mediated function is related to the Rad9 pathway or that these cells were past the point of recovery.

cul8Δ/ rad9Δ Double Mutants Remain Sensitive to UV Irradiation—Because co-deletion of RAD9 rescued the mid-anaphase phenotype of cul8Δ yeast, we decided to test whether co-deletion of CUL8 reciprocally rescued rad9Δ sensitivity to UV irradiation. To do this, we performed UV survival curves for wild-type, cul8Δ, rad9Δ, and cul8Δ/ rad9Δ yeast (Fig. 5B). Individual cultures were grown in complete medium until mid-log phase. From each culture, 500 cells were plated onto each of four YPD plates and allowed to dry. Plates were subjected to four different doses of UV irradiation (0, 25, 50, and 75 J/m²) and grown for 2 days at 30 °C at which time colony sizes were counted. Both wild type and cul8Δ yeast displayed high rates of survivability, indicating that the cul8Δ single mutant was not sensitive to UV irradiation. rad9Δ and cul8Δ/ rad9Δ yeast demonstrated equal sensitivities to the dosages administered, with 50% survival falling at about 45 and 40 J/m², respectively. This indicates that although the cul8Δ-induced mid-anaphase delay was suppressed by co-deletion of rad9Δ, UV sensitivity caused by rad9Δ was not reciprocally affected by CUL8 loss.

DISCUSSION

We provide evidence in this paper that three yeast cullins are functionally and mechanistically distinct. Neither CUL3 nor CUL8 could functionally complement the G₁ cell cycle arrest of cdc53-1 cells. This is despite the fact that human CUL1 can fully complement this defect (51). Moreover, none of the four mammalian cullins or two yeast cullins that we examined ameliorated the cul8Δ-induced mid-anaphase delay. These findings support the idea that each yeast cullin functions in a distinct pathway. These functional distinctions appear to be related, at least in part, to the mechanistic differences between these cullins in substrate recruiting. As in the case for mammalian cullins where SKP1 only interacts with CUL1 (32), ySKP1 interacts only with Cdc53, not with Cul3 or Cul8. The presence of only a single SKP1 gene in the yeast genome argues that Cul3 and Cul8 must utilize a Skp1- and possibly F-box protein-independent mechanism to target substrates. A question that remains to be addressed is whether additional sequences in the cullins contribute to their functional distinction. It would be interesting to determine whether a chimeric Cul3 or Cul8 engineered to contain the Skp1-binding domain from Cdc53 could rescue the cdc53-1 cell cycle defect.

A major finding reported in this study is that Cul8 may be part of a second E3 ubiquitin ligase that plays a role, albeit not essential, in mitotic control in budding yeast. Several lines of evidence support this notion. First, it has been established that cullin ligase activity requires the association of a RING-H2 family member. We previously showed that Cul8 interacts strongly with Roc1 and weakly, but reproducibly, with Apc11 in a yeast two-hybrid assay (18). The current study furthers that assumption by showing that Roc1 binds to Cul8, and by demonstrating that Cul8 immunocomplexes possess low, but reproducible, ubiquitin ligase activity in the presence of Roc1. Second, it is conceivable that Cul8 may assemble two separate E3 ligases with Roc1 and Apc11. Because there is no evidence suggesting the involvement of any cullin or Roc protein in the APC ligase, we believe that the Cul8 ligase is distinct from, rather than a component of, the APC ligase. Third and most dramatically, deletion of the CUL8 gene resulted in viable haploid cells that exhibited an anaphase delay (Fig. 4 and Table II). Whereas cells do not require CUL8 for mitotic division, there is clearly a role for Cul8 in proper timing of mitotic progression. It would be interesting to determine how many cell divisions a cul8Δ cell undergoes before transforming into a large budded cell with a disorganized nucleus and broken spindle.

The finding that mitotic progression involves an additional E3 ubiquitin ligase activity is unexpected. Thus far, the APC has been the only major E3 ubiquitin ligase linked to mitotic control. Two major classes of mitotic regulatory proteins that are degraded during mitosis, anaphase inhibitors and mitotic cyclins, are substrates of the APC. There are, however, data suggesting the existence of APC-independent ubiquitin-nation during mitosis. Deletion of the C. elegans cul-2 gene inhibited the G₁ to S transition as well as chromosome condensation at a later stage of development (14), suggesting a possible involvement of CUL-2 function in both G₁/S and G₂/M transition. Several F-box proteins, including Grr1, Cdc4, and Ctf13, are unstable during mitosis and are degraded by the ubiquitin-proteasome pathway (59–62). The degradation of these F-box proteins is not restricted to mitosis, however, and can occur throughout the cell cycle, probably by an SCF-de-pendent pathway. The more convincing evidence concerns the degradation kinetics of F-box protein Emi1, which binds to APC activator Cdc20 and inhibits Cdc20-APC ligase activity. Even though Emi1 is destroyed specifically during mitosis, depletion or blockage of APC activity does not affect the kinetics of its degradation, suggesting that Emi1 is degraded by an APC-independent pathway (63).

Previous studies identified a mid-anaphase checkpoint by the observation that anaphase progression contains discrete kinetic and morphological transitions (52). Introduction of double-stranded DNA breaks into a chromosome at mitosis by the activation of an artificial, dicentric chromosome caused a RAD9-dependent mid-anaphase delay. This delay is characterized by large budded cells with nuclear DNA spanning the neck and a partially elongated spindle (53, 54). A portion of cul8Δ cells exhibit a phenotype that is morphologically similar to the mid-anaphase delay caused by double-stranded DNA breaks (Fig. 4), and it is similarly suppressed by simultaneous deletion of RAD9 (Fig. 5). These findings suggest that loss of CUL8 may compromise genome integrity, activating the Rad9-dependent anaphase checkpoint. In a simple linear pathway this result would place Cul8 upstream of Rad9. However, we favor a model where Rad9 protein detects Cul8 loss-induced DNA or chromati-damage and causes a delay during mitosis, as opposed to being a direct substrate of the Cul8 ligase. For example, Cul8 activity could be involved in resolving or preventing topical chromosomal abnormality, or it might coordinate a process like sister chromatid separation. Loss of CUL8 function could cause chromatid tension or double-stranded DNA breaks that are subsequently detected by a RAD9-dependent checkpoint pathway. That cul8Δ yeast cells are not sensitive to UV irradiation also argues against the possibility that CUL8 functions as a component in the UV-induced DNA damage pathway. Thus, while the mechanistic details of the function of Cul8 remain in question, there is a clear link established between Cul8 function and proper anaphase progression based upon the Rad9 and Cul8 genetic interaction.

The substrate(s) of Cul8 ubiquitin ligase is not known and could include proteins that contribute to mitotic processes such as spindle assembly and kinetochore structure and function. In addition to Roc1, a genome-wide yeast two-hybrid screen identified three potential Cul8 interacting proteins: ORF YHR114W encoding Bzz1, ORF YFL059W encoding Smz3, and ORF YJR091C encoding Jsn1 (64). All three putative Cul8 interacting proteins remain molecularly and functionally un-characterized, although none is essential for cell viability.
triguingly, overexpression of Jsn1 was found to suppress an allele-specific mutation in tubulin that increases the stability of microtubules and causes a defect for spindle elongation, implicating a role for Jsn1 during anaphase (65). Whether Jsn1 or a protein in its functional pathway is a potential substrate of Cul8 ligase is an interesting possibility. Clearly, the challenge now is to identify the substrate(s) of the Cul8 ligase and to elucidate its mechanism of substrate targeting. Not only will this shed light on the role that Cul8 plays in mitosis, but it also could help in elucidating the mechanism(s) by which other cullins target their substrates.

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A Role for *Saccharomyces cerevisiae* Cul8 Ubiquitin Ligase in Proper Anaphase Progression

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