SCF (Skp1–cullin/Cdc53–F-box protein) ubiquitin ligases bind substrates via the variable F-box protein and, in conjunction with the RING domain protein Rbx1 and the ubiquitin–conjugating enzyme Ubc3/Cdc34, catalyze substrate ubiquitination. The cullin subunit can be modified covalently by conjugation of the ubiquitin-like protein Rub1/NEDD8 (neddylation) or bound noncovalently by the protein CAND1 (cullin-associated, neddylation-dissociated). Expression of the Candida albicans CAND1 gene homolog CaTIP120 in Saccharomyces cerevisiae is toxic only in the presence of CaCdc53, consistent with a specific interaction between CaTip120 and CaCdc53. To genetically analyze this system in C. albicans, we deleted the homologs of RUB1/NEDD8, TIP120/CAND1, and the deneddylation gene JAB1, and we also generated a temperature-sensitive allele of the essential CaCDC53 gene by knock-in site-directed mutagenesis. Deletion of CaRUB1 and CaTIP120 caused morphological, growth, and protein degradation phenotypes consistent with a reduction in SCF ubiquitin ligase activity. Furthermore, the double Carub1−/−CaTip120−/− mutant was more defective in SCF activity than either individual deletion mutant. These results indicate that CAND1 stimulates SCF ubiquitin ligase activity and that it does so independently of neddylation. Our data do not support a role for CAND1 in the protection of either the F-box protein or cullin from degradation but are consistent with the suggested role of CAND1 in SCF complex remodeling.

Cullin-RING ligases (CRLs), the largest family of ubiquitin ligases, mediate the specific degradation of a variety of cellular proteins. The largest and most versatile subset of CRLs contains the SCF (Skp1-cullin-F box) ubiquitin ligases, consisting of four subunits: Skp1, Cul1 (Cdc53 in Saccharomyces cerevisiae), the RING finger protein Rbx1 (also called Roc1 or Hrt1), and one of several F-box proteins (FBPs) (8, 54, 66). The crystal structure of the SCF complex reveals that Cul1 is an elongated protein that forms a scaffold on which the ubiquitin-conjugating enzyme (Ubc), substrate, and other subunits are positioned (49, 70). Rbx1 binds near the C terminus of Cul1 and recruits the Ubc. Skp1 binds near the N terminus of Cul1 and in turn recruits the different FBPs via the N-terminal F-box domain (49, 70). The C-terminal domain of the FBP binds to the substrate and tethers it near the catalytic center of the core complex (49, 61, 70). Accordingly, the substrate specificity of the complex is determined by the identity of the FBP (53). For S. cerevisiae, sequence analysis identified some 21 putative FBPs (66), the vast majority of which were found experimentally to function in degradation of specific proteins (2, 40).

The ubiquitination activity of the SCF complex can be modulated by reversible conjugation of the ubiquitin-related protein NEDD8/Rub1 to the cullin subunit, at a single lysine residue located in the C-terminal domain, in a process called neddylation (51). Nedlylation is mechanistically similar to ubiquitination, in that NEDD8/Rub1 is activated by E1 (Uba3/Ub1) and E2 (Ubc12) enzymes (37, 41) and requires Rbx1 (30) and an E3-like protein, Dcn1 (34, 35). Deneddylation of cullins is promoted by Csn5/Jab1, a subunit of another conserved multiprotein complex, the COP9 signalosome (CSN) (9, 15, 27). Nedlylation is essential in mammalian cells (10, 26), Caenorhabditis elegans (29), Arabidopsis thaliana (4), and fission yeast (Schizosaccharomyces pombe) (50), whereas deletion of its pathway has minimal discernible phenotypes in budding yeast (S. cerevisiae) (37, 41). Biochemical data suggest that neddylation increases SCF activity by increasing its affinity for the Ubc (31, 55). Recently, neddylation was also shown to induce significant conformational flexibility in the SCF complex, possibly facilitating contact between the E2 enzyme and the acceptor lysines on the substrate and on the elongating ubiquitin chain (18, 55).

The role of neddylation has also been linked functionally to CAND1 (cullin-associated, Nedd8-dissociated), a protein that binds unnedlylated cullin and competes with Skp1-FBP binding (43, 69). CAND1 has two binding sites, on either end of the cullin: one overlaps with the SKP1 binding site, and one overlaps with the neddylation site (24). Whereas CAND1 suppresses SCF ubiquitination activity in vitro (43, 47, 69), mutant analyses of both Arabidopsis (11, 14, 20) and, more recently, C. elegans (5) are consistent with CAND1 promoting the activity of the SCF and of additional CRLs in vivo. Interestingly, the deneddylation complex CSN also exhibits a similar inconsistency between its negative activity on CRL function in vitro and its promotion of CRL function in vivo. To explain these discrepancies, it was proposed that both CSN and CAND1 function to protect FBPs (12, 16, 28, 63) or cullin (32, 68) from autocalytic ubiquitination and degradation.
Like most fungal genomes, the probe the functional relationships between these regulators of the mutants, as well as genetic epistasis analysis, enabled us to the core SCF component Cdc53 (CUL1) and of its modifiers Rub1 in the opportunistic pathogen Candida albicans, which is unable to grow at 37°C. The orf19.1674/CaCDC53 plasmid is the functional homolog of C. albicans CDC53, able to switch between two distinct growth forms: a yeast form and a hyphal (or mold) form (65). The switch to hyphal mechanisms. SCF-mediated protein degradation also plays a role in yeast and hyphal growth (65). The opportunistic pathogen Candida albicans is a dimorphic fungus, able to switch between two distinct growth forms: a yeast form and a hyphal (or mold) form (65). The switch to hyphal growth occurs in response to various extracellular stimuli and involves transcriptional (42) and posttranscriptional (45, 71) mechanisms. SCF-mediated protein degradation also plays a role in C. albicans morphogenesis, as evidenced by the hyphal phenotype of the Cao94+ mutation (2, 59) and the pseudohyphal phenotype of the Cagtr1- mutant (7, 40) and of CaCdc53 depletion (62). Like most fungal genomes, the C. albicans genome also contains easily discernible homologs of the NEDD8, CAND1, and CSN5 genes. In order to analyze the functions of CAND1 and neddylation in SCF activity in C. albicans, we generated mutants of the core SCF component Cdc53 (CUL1) and of its modifiers Rub1 (NEDD8), Jab1 (CSN5), and Tip120 (CAND1). The phenotypes of these mutants, as well as genetic epistasis analysis, enabled us to probe the functional relationships between these regulators of the SCF complex in vivo.

MATERIALS AND METHODS

Plasmids and strains. The C. albicans and S. cerevisiae strains used in this study are listed in Tables 1 and 2. Plasmid and strain construction is described in the supplemental material.

Protein analysis. Protein levels were assayed by Western blotting using the following monoclonal antibodies: 9E10 to detect the Myc epitope, anti-FLAG (Sigma) to detect the FLAG epitope, and HA.11 (Covance) to detect the hemagglutinin epitope. Proteins were extracted by the quantitative NaOH–2-mercaptoethanol method, as described previously (64). To compare steady-state protein levels, equal protein amounts were loaded; to monitor protein disappearance after promoter shutoff, equal culture volume equivalents were loaded. Loading and transfer were monitored by Ponceau staining of the membrane and by actin quantitation using an anti-β-actin antibody (abb2242; Abcam). Enhanced chemiluminescence (ECL) signals were quantitated with a Bio-Rad Chemidoc apparatus. Pulse-chase analysis of proteins in C. albicans was performed as described in reference 33. For FLAG-CaCdc53 immunoprecipitation, exponentially growing cultures induced for 1 h with galactose and then shifted to glucose for 2 h were spun down, resuspended in 1 ml lysis buffer (0.5% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonfonyl fluoride [PMSF], and a 1:500 dilution of antiprotease cocktail containing chymostatin, pepstatin A, leupeptin, and antipain [10 mg/ml of each in dimethyl sulfoxide]), and broken with glass beads (0.5 mm) in a BeadBeater apparatus for 3 pulses of 1.5 min each. The extracts were cleared from nonspecific interactions by a 15-min incubation with protein A Sepharose beads prior to immunoprecipitation with M2 FLAG Sepharose beads (Sigma). Beads were washed 3 times in lysis buffer, and proteins were eluted in gel loading buffer.

Microscopy and flow cytometry. Cells were fixed in 70% ethanol and visualized with a Zeiss Axioslager M1 microscope equipped with differential interference contrast (DIC) optics, using a 40× or 100× objective. Colonies were visualized with a Zeiss Stemi 2000C binocular microscope. For flow cytometry, cells were prepared as described previously (25), with some modifications. A total of 10^6 to 10^7 cells were fixed in 70% ethanol for 1 h to overnight, washed with 0.2 M Tris, pH 7.5, and then incubated overnight in a shaker at 37°C in 0.2 M Tris, pH 7.5, 10 mM EDTA, 1 mg/ml RNase A. The cells were then spun down, resuspended in 50 mM HCl with 5 mg/ml pepsin, incubated for another 2 h at 37°C, and washed with 0.2 M Tris, pH 7.5. Approximately 5 × 10^6 cells were then incubated for at least 15 min at 30°C while shaking in 0.3 ml 0.2 M Tris, pH 7.5, with 2 μM SYTOX (Molecular Probes, Inc.). The cells were diluted 1:10 in 0.2 M Tris, pH 7.5, just before injection into the flow cytometer (Beckton-Dickinson FACScalibur). A total of 20,000 un gated events were recorded for each run.

RESULTS

Generation of a C. albicans CDC53 is allele. We identified, by homology searching, C. albicans orf19.1674 as the closest bidirectional homolog of S. cerevisiae CDC53. In order to experimentally validate this assignment, orf19.1674 was cloned into an S. cerevisiae vector and introduced into the S. cerevisiae cdc53-1 mutant, which is unable to grow at 37°C. The orf19.1674/CaCdc53 plasmid suppressed the temperature sensitivity of that strain (data not shown); furthermore, we subsequently found that substitution of the ScCdc53 coding sequence with the CaCdc53 coding se-

### TABLE 1 C. albicans strains used in this study

| Strain   | Genotype                           | Source or reference |
|----------|------------------------------------|---------------------|
| KC2 (CAF3) | ura3Δ::yihm4344/ura3Δ::yihm434 | This work           |
| KC190    | cdc53Δ::KANr                         | This work           |
| KC208    | cdc53Δ::KANr                         | This work           |
| KC209    | cdc53Δ::KANr                         | This work           |
| KC218    | cdc53Δ::KANr                         | This work           |
| KC219    | cdc53Δ::KANr                         | This work           |
| KC228    | cdc53Δ::KANr                         | This work           |
| KC229    | cdc53Δ::KANr                         | This work           |
| KC235    | cdc53Δ::KANr                         | This work           |
| KC236    | cdc53Δ::KANr                         | This work           |
| KC237    | cdc53Δ::KANr                         | This work           |
| KC238    | cdc53Δ::KANr                         | This work           |
| KC239    | cdc53Δ::KANr                         | This work           |
| KC242    | cdc53Δ::KANr                         | This work           |
| KC247    | cdc53Δ::KANr                         | This work           |
| KC471    | Ura3                                 | This work           |

### TABLE 2 S. cerevisiae strains used in this study

| Strain   | Genotype                           | Source or reference |
|----------|------------------------------------|---------------------|
| W303-1A  | MATa ura3-1 leu2-3-112 trp1-1 ade2-1 his3-11,15 | R. Rothstein       |
| M7474    | W303-1A cdc53-1                      | M. Tyers            |
| BY4742   | ura3Δ::KANr                         | EUROSCARF           |
| KY1293   | ura3Δ::KANr                         | This work           |
| KY1294   | ura3Δ::KANr                         | This work           |
| KY1306   | ura3Δ::KANr                         | This work           |

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sequence yielded a viable strain (see below and Fig. 3), thereby confirming that CaCDC53 is a functional homolog of ScCDC53. While in S. cerevisiae the core SCF subunit genes CDC53, SKP1, and RBX1 and the cell cycle-related F-box protein gene CDC4 are all essential, for C. albicans, a mutant with deletion of the CDC4 homolog is viable (2). Assuming that in C. albicans the core SCF complex may, like CaCDC4, be nonessential, we initially attempted to delete both alleles of CaCDC53. Although the first allele was readily deleted, we repeatedly failed to obtain a deletion of the second allele, suggesting that CaCDC53 may in fact be essential. In order to study its function, we proceeded to create a temperature-sensitive (ts) allele in C. albicans, modeled on the S. cerevisiae cdc53-1 allele. The cdc53-1 mutation causes an arginine-to-cysteine change at position 488 (52), in a region of the protein highly conserved between ScCdc53 and CaCdc53. The homologous arginine residue encoded by CaCDC53, R462, was mutated to cysteine. The remaining allele of the CaCDC53/Cadc53Δ::LEU2 strain was then replaced with CaCDC53R462C (see Materials and Methods). The resulting strain was unable to grow at 37°C, but this growth defect was complemented by reintroducing a wild-type CaCDC53 copy into the strain, indicating that the CaCDC53 mutation was responsible for the temperature sensitivity of the strain (Fig. 1A). At 24°C and 30°C, the CaCDC53R462C strain grew as well as the wild-type strain; however, even at the growth-permissive temperature of 30°C, the mutant already showed a clear morphological phenotype, consisting of an elongated cell shape (Fig. 1B). When shifted from 24°C to 37°C, the mutant strain rapidly ceased to proliferate, and microscopic examination of the cells after the shift to 37°C indicated a rapid induction of germ tube-like extensions (Fig. 1C). Even at the semipermissive temperature of 30°C, Ccn1 and CaCln3 (both substrates of SCFCaGRR1) (40), Sol1 (a substrate of SCFCaCDC4) (2), and CaGcn4 (a rapidly degraded homolog of the S. cerevisiae SCFCDC4 substrate Gcn4) (23) were all significantly stabilized (Fig. 1D). In all cases, slower-migrating forms of the proteins accumulated in the mutant. These bands may correspond to phosphorylated forms of the proteins that are normally recognized by the SCF complexes and rapidly degraded in the wild-type strain.

Identification of C. albicans RUB1 and JAB1/CSN5. A homology search of the C. albicans genomic database identified orf19.330.1 as the closest bidirectional homolog of S. cerevisiae RUB1. Sequence comparison between CaRub1, ScRub1, and human NEDD8 (Fig. 2A) indicated that NEDD8 is equally related to either fungal protein (about 60% identity), and surprisingly, the
The upper band is undetectable in the CaCdc53 wild-type strain, CaCdc53-3xHA did in fact migrate as two bands; deleted for both alleles of CaCdc53, the CaCdc53 is undeddylation in the CaCdc53 wild-type strain in liquid. On agar plates, CaCdc53 is undeddylation in the CaCdc53 wild-type strain versus the CaCdc53 mutant and was in fact more filamentous than that of the Carub1-/- strain (Fig. 2D), possibly due to the added haploinsufficiency of CaCdc53 in the CaCdc53RRR-/- strain versus the Carub1-/- strain. These results are consistent with the lack of CaCdc53 neddylation being responsible for the morphogenetic phenotype of the Carub1-/- mutant. 

Identification of the C. albicans CAND1 homolog CaTip120. It is striking that prevention of CaCdc53 neddylation shows a clear morphogenetic phenotype in C. albicans, suggesting that neddylation is required for optimal SCF function in this organism, whereas deletion of the neddylation pathway in wild-type S. cerevisiae causes no morphological or substrate degradation de-
fects (37, 41; our unpublished results). Comparison of the gen-
ome of the two organisms suggested a possible reason for this
difference: C. albicans contains an open reading frame with signif-
ificant (P = 10^{-12}) homology to human CAND1/TIP120, whereas
in the S. cerevisiae genome, no obvious CAND1 ortholog could be
detected (recently, Lag2 was identified as an S. cerevisiae protein
able to bind Cdc53 [44, 60], but it is not clear yet whether Lag2 fulfills
a similar function to that of CAND1 in other organisms). CAND1 (cullin-associated, Nedd8-dissociated) was shown to in-
hbit SCF activity in vitro by binding to cullin; this binding is
counteracted by neddylation of cullin (43, 69). Thus, this raises
the possibility that in C. albicans, CaCdc53 modification by CaRub1 is required to counteract the inhibitory activity of
CAND1. Conversely, expressing CAND1 in S. cerevisiae may ren-
cullin neddylation essential in this organism (in keeping with
standard yeast gene nomenclature, the C. albicans CAND1/TIP120
gene homolog, orf19.6729, was named CaTIP120).

To test whether orf19.6729 in fact expresses a functional
CAND1 homolog with respect to interaction with Cul1/Cdc53, we
expressed it in S. cerevisiae under the control of the inducible
GAL1 promoter. Protein gel electrophoresis suggested that the
expression in an ened version of orf19.6729 as the Ca
CAND1/TIP120 human homolog, orf19.6729, as the Ca
suggested (recently, Lag2 was identified as an S. cerevisiae protein
able to bind Cdc53 [44, 60], but it is not clear yet whether Lag2 fulfills
a similar function to that of CAND1 in other organisms). CAND1 (cullin-associated, Nedd8-dissociated) was shown to in-
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gene homolog, orf19.6729, was named CaTIP120).

To test whether orf19.6729 in fact expresses a functional
CAND1 homolog with respect to interaction with Cul1/Cdc53, we
expressed it in S. cerevisiae under the control of the inducible
GAL1 promoter. Protein gel electrophoresis suggested that the
translation start site of orf19.6729 is misannotated and that the
actual start site is 139 nucleotides (nt) downstream (see Fig. S1 in
the supplemental material). We accordingly expressed the short-
ened version of orf19.6729 as the CaTIP120 homolog for further
analyses in S. cerevisiae.

If CaTIP120 inhibits SCF activity by binding to the cullin, and
since SCF activity is essential, overexpression of CaTIP120 should
be toxic. However, expression of the GAL1-CaTIP120 construct
had no effect on growth of wild-type S. cerevisiae (Fig. 3A, upper
row). We surmised that this might be due to an inability of C.
albicans Tip120 to interact with the S. cerevisiae cullin, Cdc53.
Therefore, an S. cerevisiae strain was built in which the resident
CDC53 gene was replaced with CaCDC53, either wild type or
CaCDC53^{RRR}, under the control of the ScCDC53 promoter. The
viability of those strains confirmed that CaCdc53 was able to stand
in functionally for ScCdc53 and to interact with the basic compo-
ents of the essential SCF complex. Notably, however, the S.
cerevisiae cdc53Δ:CaCDC53 strain was unable to grow at 37°C,
whereas a cdc53Δ strain carrying the CaCDC53 gene with its native
promoter on a plasmid grew well at that temperature (not shown).
This suggests either that CaCDC53 is not expressed efficiently
from the ScCDC53 promoter or that S. cerevisiae SCF function is
not optimal with CaCdc53.

Induction of GAL1-CaTIP120 in the CaCDC53 strain was
toxic, as expected if CaTIP120 was a negative regulator of CaCdc53
(Fig. 3A). In addition, as expected if neddylation was able to par-
tially counteract CaTIP120 binding to CaCdc53, the CaCDC53^{RRR}
strain, carrying the presumably unneddylatable allele of CaCDC53,
was even more sensitive to CaTIP120 expression (Fig. 3A). Similarly,
S. cerevisiae CaCDC53 strains deleted for RUB1 or for the Rub1 liga-
gene DCNI were also hypersensitive to expression of CaTIP120 (Fig.
3A). In contrast, the JAB1 deletion strain was not more sensitive
and may be marginally more resistant to CaTIP120 expression. Taken
together, these data are consistent with the toxicity of CaTIP120 on
CaCdc53 being counteracted by neddylation of CaCdc53. Since SCF
function is required for progression through the cell cycle, specifically
at the G1-to-S transition (19), we used fluorescence-activated cell
sorter (FACS) analysis to assay the effect of CaTIP120 expression on
cell cycle distribution. The CaTIP120-inhibited cultures accumulated
in the G1 phase of the cell cycle (Fig. 3B), consistent with defects in
SCF complex activity.

Since the S. cerevisiae CaCDC53 background may exhibit re-
duced SCF activity, based on its temperature sensitivity at 37°C,
the toxicity of CaTIP120 expression in this background could al-
ternatively have been explained as an indirect synthetic effect of
the reduction in SCF activity, not an effect of direct interaction
with CaCdc53. We therefore checked that the same GAL1p-
CaTIP120 construct, expressed in the S. cerevisiae cdc53Δ-1 mutant
at the semipermissive temperature of 30°C, had no effect on cell
growth (see Fig. S2 in the supplemental material). Thus, the pheno-
type resulting from the expression of CaTIP120 in S. cerevisiae is
consistent with it being a negative regulator of the SCF complex by
direct binding with its cognate cullin, CaCdc53.

Finally, to obtain more direct evidence for a physical inter-
action between CaCdc53 and CaTIP120, FLAG-tagged CaCdc53 was

![FIG 3 CaTIP120 expression in S. cerevisiae. (A) The CaTIP120-expressing plasmid KB1958 was transformed into strains KY1068 (wild type; ScCDC53), KY1293 (Sccdc3Δ::CaCDC53), KY1294 (Scscdc53Δ::CaCDC53^{RRR}), KY1310 (Scscdc53Δ::CaCDC53 rub1Δ), KY1309 (Scscdc53Δ::CaCDC53 jdb1Δ), and KY1306 (Scscdc53Δ::CaCDC53 don1Δ). Serial 5-fold dilutions of overnight cultures were spotted onto SC plates without uracil and with the indicated carbon source and were incubated at 30°C for 3 days (galactose) or 2 days (glucose). (B) FACS analysis of strains KY1068, KY1293, and KY1294 harboring plasmid KB1586 at the indicated times of galactose induction. (C) Coimmunoprecipitation of CaTIP120 with CaCdc53. Plasmid KB1960, expressing Myc-CaTIP120 under the control of the GAL1 promoter (+), or a vector plasmid (−) was transformed into strain KY1352 expressing FLAG-tagged CaCdc53 under the control of the ScCDC53 promoter (+) or expressing untagged CaCdc53 (−). (Top) Whole-cell extracts (WCE) were analyzed for Myc-
CaTIP120 expression by Western blotting (W.B.). FLAG-CaCdc53 was immu-
noprecipitated (i.p.) from the extracts, and the precipitate was analyzed by Western blotting for the presence of Myc-CaTIP120 (middle) or FLAG-
CaCdc53 (bottom). The asterisk indicates a band cross-reacting with the anti-
FLAG antibody. Note that CaCdc53 runs as two bands, representing the ned-
dyalted and unneddylated species.](ec.asm.org/FIG_3.jpg)
immunoprecipitated from cells expressing Myc-tagged CaTip120. As shown in Fig. 3C, Myc-CaTip120 immunoprecipitated specifically with FLAG-CaCdc53, confirming that these proteins physically interact.

**Phenotype of C. albicans TIP120 mutant.** If an important role of neddylation is to counteract the inhibitory activity of CAND1, then the clear phenotypic effect of deletion of the NEDD8 homolog RUB1 in C. albicans, as opposed to the scant phenotypic effect of the homologous deletion in S. cerevisiae, may be linked to the absence of a CAND1 homolog in the latter organism. A straightforward prediction of this model is that deleting CaTIP120 should suppress the phenotype of the CaRUB1 deletion. To test this prediction, we deleted CaTIP120 in both the wild-type background and the Carub1−/− background. The CaTIP120−/− mutant by itself displayed no apparent morphological phenotypes (Fig. 4A; see Fig. S3 in the supplemental material). However, contrary to our prediction, the Carub1−/− CaTIP120−/− double mutant, far from suppressing the cell elongation phenotype of Carub1−/−, exacerbated it (Fig. 4A; see Fig. S3).

Since the CaTIP120−/− mutant displayed no apparent morphology or growth phenotype under normal growth conditions (yeast extract-peptone-dextrose [YPD] medium, 30°C or 37°C), we also assayed for growth effects under stress conditions. The Carub1−/− and CaTIP120−/− single and double mutants, as well as the Carab1−/− mutant, were grown in the presence of 0.03% H2O2. The Carub1−/− mutant displayed markedly reduced growth under these conditions (Fig. 4B), in excess of the relative growth reduction of the Carub1−/− versus wild-type strains observed under standard growth conditions. The CaTIP120−/− mutant also showed markedly reduced growth in 0.03% H2O2. The double mutant showed further growth reduction compared to each mutant separately. The Carub1−/− mutant did not show any growth defect under these conditions. We also tested reversion of the H2O2 sensitivity phenotype by expressing the shortened CaTIP120 ORF (starting at nt +139) (see Fig. S1 in the supplemental material) or an epitope-tagged version of CaTIP120 (Fig. 4C). This confirmed both that the H2O2 sensitivity phenotype was due to the loss of Tip120 and that the shorter ORF is functional in *C. albicans*.

We next tested the effects of CaRub1 and CaTip120 on SCF activity more directly, by assaying degradation of a known SCF substrate, CaCln3, which depends on the SCFCaGRR1 complex for its degradation (40). The epitope-tagged substrate was expressed from the repressible MAL2 promoter. Decay of the steady-state protein bands was followed after promoter repression with glucose. In the wild-type strain, CaCln3 exhibited initial fast decay followed by persistence of a small residual protein population (Fig. 4D). The decay in the CaTIP120−/− mutant was similar, except that the initial decay was somewhat retarded. A similar kinetics was detected in the CaRub1−/− mutant. In the CaRub1−/− mutant, a significant stabilization was detected, accompanied by accumulation of a slower-migrating species of CaCln3. Finally, in the double CaRub1−/− CaTIP120−/− mutant, the protein was further stabilized, but the migration pattern, a mixture of slower- and faster-migrating bands, was different from that of the CaRub1−/− mutant and suggested the persistence of several phosphospecies of CaCln3 (Fig. 4D). Taken together, these data indicate that in vivo, CaRub1 and CaTip120 are not antagonistic but rather act synergistically on SCF function.

**Effects of neddylation and CaTIP120 on stability of the F-box protein CaGrr1.** One possible role of the neddylation/deneddylation cycle is that it is required to protect SCF complex components
Therefore, we constructed a CaCdc53 allele tagged N-terminally with the Myc epitope tag. This allele expressed in S. cerevisiae was able to complement the CDC53 deletion (see Fig. S4).

The expression of CaCdc53 under the control of the heterologous CUP1 promoter enabled us first to test the simple hypothesis that the Carub1<sup>−/−</sup> and Catip120<sup>−/−</sup> mutants were defective in SCF activity due to reduced CaCdc53 levels, because if this were the case, then the Carub1<sup>−/−</sup> and Carub1<sup>−/−</sup> Catip120<sup>−/−</sup> morphological phenotypes might be suppressed by CaCDC53 overexpression from the extra CaCDC53 copy under control of the CUP1 promoter. As shown in Fig. 6A, no such suppression of the morphological phenotype was detected in the presence of the added CUP1p-Myc-CaCDC53 allelle. On the other hand, Western blot analysis of Myc-CaCdc53 did reveal some differences in steady-state levels of the protein in the mutants (Fig. 6B). Since the variations in Myc-CaCdc53 levels could be attributed to either variations in cullin stability or indirect effects on CUP1 promoter activity, we performed two additional experiments. In the first, we analyzed the steady-state levels of the CaCdc53-3xHA construct, which is under regulation of the native CaCDC53 promoter and is thus expected to more faithfully represent the levels of native CaCdc53. Normalized to β-actin, the differences between the various strains did indicate a 2.5-fold reduction in CaCdc53 level in the Carub1<sup>−/−</sup> mutants (Fig. 6C). However, when we directly asayed the stability of Myc-CaCdc53 by pulse-chase analysis with radioactive methionine, the protein appeared largely stable in all strains tested (Fig. 6D), suggesting that reduced cullin stability cannot account for the phenotypes of the Carub1<sup>−/−</sup> and Catip120<sup>−/−</sup> mutants.

Western blot analysis of CUP1p-Myc-CaCDC53 further revealed an increased fraction of neddylated Myc-CaCdc53 in the Cjab1<sup>−/−</sup> deneddylation mutant, as expected, but also, unexpectedly, a slight decrease in the fraction of neddylated Myc-CaCdc53 in the Catip120<sup>−/−</sup> mutant (38% versus 51% in the wild type) (Fig. 6B). In the strain set expressing the CaCdc53-3xHA allele, overall levels of neddylation were lower, and in the Catip120<sup>−/−</sup> strain in particular, neddylation was barely detectable (Fig. 6C).

Whereas Western blotting revealed the steady-state distribution of neddylated versus unneddylated CaCdc53, the pulse-chase experiment shown in Fig. 6D enabled us to follow the kinetics of neddylation of newly synthesized Myc-CaCdc53. The fraction of neddylated cullin took some 60 min to approach a steady-state level of 50% in the wild-type background (Fig. 6E). In the Cjab1<sup>−/−</sup> mutant, as expected, neddylation occurred faster and reached a higher level before reaching a plateau at over 80%. The kinetics of neddylation in the Catip120<sup>−/−</sup> mutant, however, was more complex. The initial fraction of neddylated Myc-CaCdc53 observable right after labeling was slightly but reproducibly (in 2 independent experiments) higher in the Catip120<sup>−/−</sup> strain. Subsequently, neddylation in the Catip120<sup>−/−</sup> strain proceeded more slowly and eventually reached a plateau at a level just below that of the wild-type strain (Fig. 6E), in accordance with the lower steady-state fraction of neddylated CaCdc53 detected by Western blotting in the Catip120<sup>−/−</sup> strain (Fig. 6B).

**DISCUSSION**

*C. albicans* as a model system for studying the SCF ubiquitin ligase. SCF complexes represent the most versatile class of ubiquitin ligases. In spite of the availability of crystal structures which provide high-resolution pictures of the cullin in association with the other SCF components (49, 70) and with CAND1 (24), as well...
as the effects of cullin neddylation (18), questions remain regarding the mechanics of SCF ubiquitin ligase activity and, notably, the interrelated roles of the SCF modifiers Nedd8 and CAND1. It is likely that the roles of neddylation, deneddylation, and CAND1 are related to the dynamics of SCF function in the cellular context, and these roles may thus be studied usefully in a genetic model system. Here we identified and analyzed the functions with an analysis of the expression in vivo and CAND1 binding that was characterized in vitro with the homologous human proteins (24, 43, 69). However, genetic analysis in C. albicans indicated that CaTip120 and neddylation (CaRub1) are both synergistic with SCF function (62), a prediction that was borne out by our experiment (Fig. 1C).

**Dissociation of CAND1 from cullin in the absence of neddylation.** Expression of the C. albicans CDC53 sequence ortholog in S. cerevisiae enabled us to confirm its functional homology. Furthermore, by revealing the genetic interaction between CaCdc53 and CaTip120 in S. cerevisiae, this ectopic reconstitution indicated that these proteins interact specifically in vivo (Fig. 3). The relation of CaTip120 toxicity to neddylation in S. cerevisiae, i.e., the hypersensitivity of the strain to CaTip120 in the absence of neddylation, further correlated with the antagonism between cullin neddylation and CAND1 binding that was characterized in vitro with the homologous human proteins (24, 43, 69). However, genetic analysis in C. albicans indicated that CaTip120 and neddylation (CaRub1) are both synergistic with SCF function in vivo, rather than antagonistic, similar to observations in other genetic systems (5, 14). It is possible that in S. cerevisiae, the high levels of CaTip120 expression under the control of the GAL1 promoter turn CaTip120 into a repressor rather than an activator of SCF activity; however, we noted that ectopic overexpression of CaTip120 in C. albicans is not deleterious (our unpublished observations). Furthermore, expression of CaTip120 from promot-
ers that are considerably weaker than that of GAL1 did not relieve its toxicity in the S. cerevisiae CaCDC53 rub1Δ strain, let alone transform it into an SCF activator (our unpublished data). Thus, an alternative possibility is that an additional C. albicans factor(s) that is missing from S. cerevisiae turns CaTip120 into an activator, e.g., by allowing its dissociation from CaCdc53.

Indeed, recent reports show that in mammalian cell culture, CAND1 binds weakly to Cul1 in spite of low levels of neddylation (13), and inhibition of neddylation does not significantly increase the fraction of CAND1-bound cullin (3, 39), suggesting that neddylation cannot constitute the only factor limiting CAND1-cullin association. Similarly, our observation that CAND1 promotes SCF activity in C. albicans even in the Carub1Δ/Δ mutant background implies that removal of neddylation does not immediately lead to sequestration of the cullin in an inactive cullin-CAND1 complex. Thus, any model of SCF function should accommodate a mechanism for CAND1 association with and dissociation from the cullin in the absence of neddylation.

CAND1 promotes SCF activity in the absence of neddylation. The Catip120Δ/Δ mutant phenotype matches the phenotypes of mutants in Arabidopsis thaliana CAND1/ETA2/HVE, in the sense that the phenotypes of the plant mutants are consistent with a decrease in SCF activity and thus that, in plants as well, CAND1 appears to promote SCF activity in vivo (1, 11, 14, 20). Among their phenotypes, mutants in AtCAND1 show reduced auxin signaling and stabilization of the SCFTR1 substrate Axr2. Regarding the interaction between neddylation and CAND1, Chuang et al. (14) notably showed by double mutant analysis that the axr1Δ-12 mutant, a hypomorphic mutant of the RuB1 activation enzyme, was essentially epistatic to etal-1. The conclusion of this study was that the role of neddylation cannot be solely to counteract sequestration of the cullin subunit by CAND1. Whether CAND1 has a role in the absence of neddylation could not be addressed in these studies, because neddylation is essential in plants. We could address this question rigorously in C. albicans by deleting CaTIP120 in the Carub1Δ/Δ background. The increased severity of the double mutant phenotype, at the levels of both morphology and H2O2 sensitivity, suggests that Tip120Δ/CAND1 in fact stimulates SCF activity independently of neddylation.

The complexity of the interaction between neddylation and CAND1 is underscored by the observation that CaCdc53 neddylation levels are reduced, rather than induced, in the absence of CaTIP120 (Fig. 6). This effect is exacerbated with the CaCdc53-3xHA allele. In this construct, a 33-amino-acid extension is added to the C terminus of Cul1. We noticed that the C terminus of Cul1, which is highly conserved among all orthologs, is adjacent (<1 nm) to the neddylation site, according to the Cul1 crystal structure (24). Thus, the addition of the epitope tag sequence to the C terminus may partially hinder neddylation, although we cannot explain why CaCdc53-3xHA neddylation would be specifically sensitive to the loss of CaTIP120.

Role of CAND1 in promoting SCF activity. Neddylation was found to promote ubiquitination activity of the SCF complex (51). Deneddylation would thus be expected to reduce SCF activity; paradoxically, however, similar to the CAND1 mutants, mutants of the CSN complex showed reduced SCF activity in vivo (15, 57). Two alternative models were suggested to resolve the so-called “CSN/CAND1 paradox.” One is that deneddylation and cullin sequestration by CAND1 protect SCF components such as the cullin and F-box proteins from adventitious ubiquitination and degradation in the absence of substrate (16, 63, 67, 68). The second is that the neddylation/deneddylation cycle, together with CAND1, promotes the exchange of F-boxes on the cullin platform (6, 15, 56).

We did not see evidence that either the cullin or the FBP was destabilized in any of our C. albicans mutants. To the contrary, the stability of CaGrr1, the specific receptor for CaCln3, was increased in all mutants tested. Thus, protection of the F-box protein by CAND1 cannot be invoked to explain the Catip120Δ/Δ phenotype, at least in the case of CaGrr1. However, our data are consistent with a role for neddylation and CAND1 in coordinating disassembly of SCF complexes and reassembly of alternative complexes, enabling exchange of the F-box protein component on the cullin. In the wild-type background, SCF activity is responsible for the basal level of CaGrr1 turnover, as evidenced by its stabilization in the Cadcc53Δ/Δ mutant, i.e., some level of CaGrr1 attrition may be a natural consequence of its incorporation into active ubiquitination complexes. Stabilization of CaGrr1 in the Carub1Δ/Δ and Catip120Δ/Δ mutants may thus reflect a defect in incorporation into the SCF complex, which would also explain stabilization of the substrate CaCln3.

The kinetics of CaCdc53 neddylation could similarly be explained by a role for CAND1 in SCF complex assembly. In the wild-type strain, the slow kinetics of RuB1-CaCdc53 conjugate formation suggests that cullin neddylation may depend—at least in part—on a rate-limiting event, such as, e.g., incorporation of the cullin into an active SCF complex. The more complex kinetics of CaCdc53 neddylation in the Catip120Δ/Δ mutant, with a higher initial neddylation rate followed by a slower second step of neddylation, may reflect two different modes of cullin neddylation: (i) initial neddylation of newly synthesized, uncomplexed cullin, which may normally be held in check by CaTip120/CAND1 binding and, in the Catip120Δ/Δ mutant, may directly reflect the balance of neddylation and deneddylation activity on the free cullin; and (ii) neddylation of the cullin as a consequence of incorporation into active SCF complexes, which in the Catip120Δ/Δ strain may be partially inhibited due to slower FBP exchange and SCF complex formation. The proposed role of CaTIP120/CAND1 in SCF complex remodeling may also explain the H2O2 sensitivity of the Catip120Δ/Δ strain: to the extent that FBP exchange may acquire added importance under changing external conditions, the sensitivity of the Catip120Δ/Δ strain to H2O2 stress might be due to a failure to assemble specific SCF complexes required under these circumstances.

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